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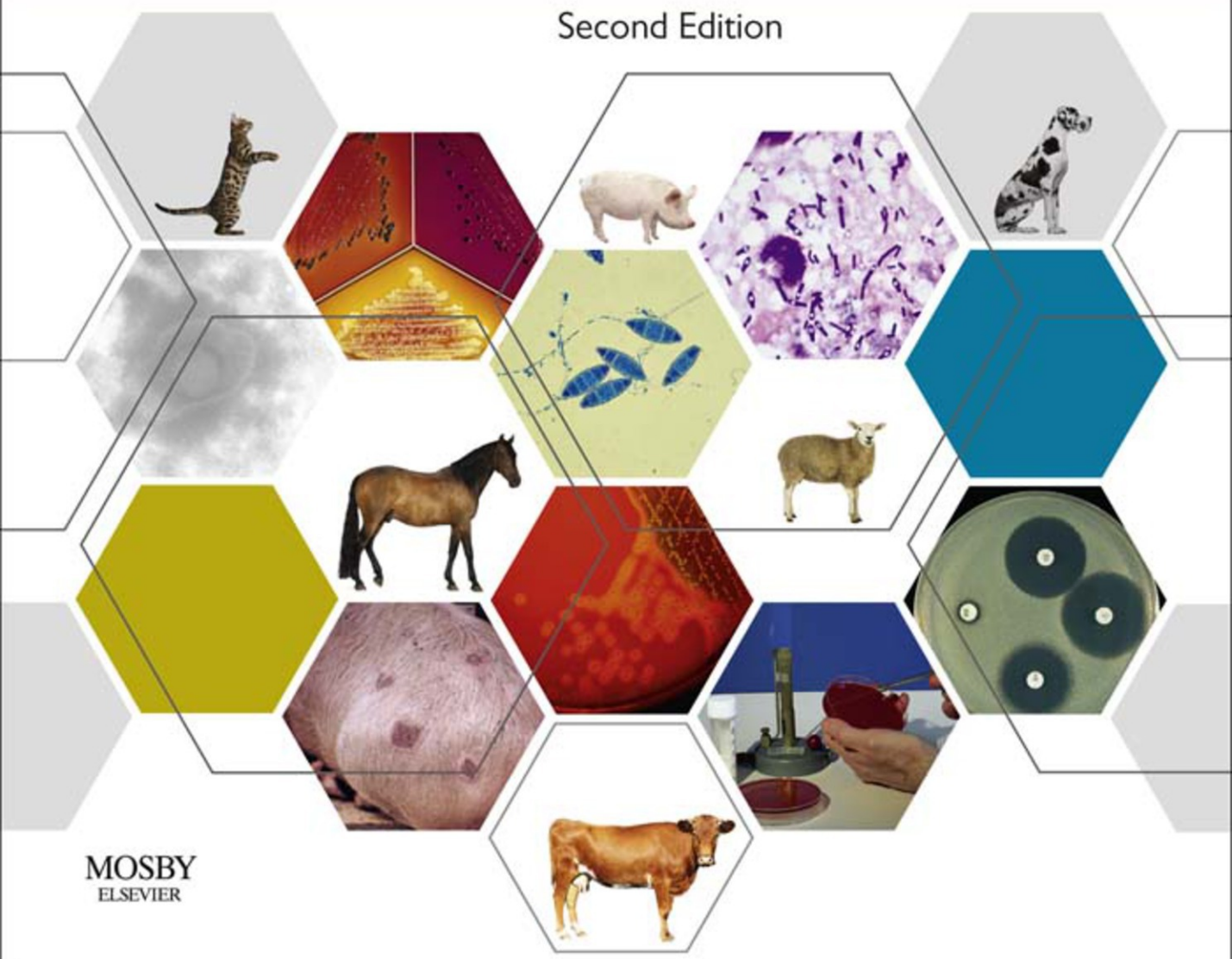
CLINICAL VETERINARY MICROBIOLOGY

Second Edition



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Clinical Veterinary Microbiology

Second edition

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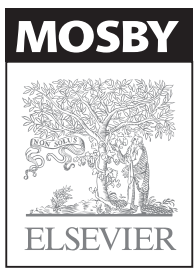

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Dedication

This book is dedicated to the memory of
Margery E Carter

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Preface

A lot of water has passed under the bridge since the popular first edition of this book. Needless to say the writing of textbooks is not a full-time occupation for any of the authors but rather a complementary activity to teaching, research and diagnostic service. This project was driven by a love of books. On more than one occasion the writing faltered due to other more pressing commitments and the vicissitudes of life in general. We have tried to retain the essential structure and character of the first edition while updating and adding new material as necessary. In particular, the virology section has been greatly expanded with the addition of a chapter for each of the families containing viruses of veterinary importance. For taxonomy we have relied heavily on resources such as the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.cict.fr/), the virus list of the International Committee on Taxonomy of Viruses (ictvonline.org/index.asp) and Index Fungorum (www.indexfungorum.org/). For information on the diagnosis of many of the more important infectious diseases of farm animals we have frequently consulted the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals on the website (www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/) of the OIE (Office International des Epizooties – World Organisation for Animal Health). We hope we have succeeded in producing a book that is useful, informative and appealing.

Dublin, 2013

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Acknowledgements

Assembling material for a book requires forward planning, attention to detail and the means to put in place the knowledge and ideas of the authors. We were fortunate to have the resources of the first edition to draw upon in bringing this project to fruition. We are indebted to Professor Emeritus Joe Quinn for being our mentor and greatest supporter in this endeavour.

Veterinary microbiology lends itself to illustration and we have tried to include suitable illustrative material in the form of colour images and line drawings. A number of colleagues supplied images which we would like to acknowledge: Professor KP Baker (38.7), Centres for Disease Control (31.13 and 34.2), Dr SJ Cook and Professor CJ Issel (3.3, 3.16 and 3.22), Dr DO Cordes (11.14, 42.7, 42.8, 42.9 and 44.7), Professor FWG Hill (29.1), Dr L Hoffmann (31.12), Dr G Joseph (18.7, 18.8 and 22.1), Professor JF Kazda (11.5, 11.6 and 11.7), Mr Aonghus Lane (36.21 and 36.22) and Dr N Seiranganathan (35.8).

We also wish to acknowledge our predecessors in the Dublin Vet School, particularly Mr BT Whitty, Mr MA Gallaher, Professor PJ Quinn and Dr Margery Carter who were prominent in our formative training and who left for posterity a wealth of illustrative material and stained smears, many of which we have used to illustrate individual chapters. We would also like to record our appreciation to colleagues who furnished material for photography: Dr HF Bassett (10.17), Mr MJ Casey (68.4), Mr RP Cooney (11.12), Mr P Costigan (5.4, 5.5, 51.1 and 51.2), Dr WJC Donnelly (12.2, 12.3 and 67.2), Mr E Fitzpatrick (41.2), Dr HA Larkin (35.2), Dr GHK Lawson (33.4), Mr JB Power (31.8 and 31.10), Dr GR Scott (34.1 and 35.1) and Professor Emeritus BJ Sheahan (39.2).

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Section 1

General procedures in microbiology

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Chapter 1

Collection and submission of diagnostic specimens

Choosing and Working with a Laboratory

In choosing a laboratory clinicians must consider the cost of the service and the ease of access. However, it is equally important to determine if a laboratory is accredited, has a specialization in the relevant species, offers a service at weekends and holidays and has staff with the necessary expertise and training to assist with disease investigations. The quality of the dialogue between the veterinary practice and the laboratory has an enormous influence on the effectiveness of the service. Good communication is essential. The clinician and the microbiologist must establish and maintain contact with each other. An accurate diagnosis is based on the interpretation of both clinical and laboratory data.

If the clinician is not absolutely certain of the correct sample to collect they should seek advice from the laboratory prior to collecting the samples. The samples must be appropriate for the purpose required which may be diagnosis, certification, surveillance or the monitoring of vaccine efficacy or the response to antimicrobial therapy. A clinical diagnosis cannot be confirmed by the examination of inappropriate samples. In disease control situations samples must be collected systematically for a definite purpose, for example, the lifting of movement restrictions or the commencement of breeding. Laboratory tests can be both expensive and time-consuming. It is important for all concerned that resources are not wasted in the generation of irrelevant information.

Both the veterinary practitioner and the laboratory have an essential role in the isolation of new agents and the detection of emerging disease patterns. In influenza outbreaks, for example, the submission of samples to the laboratory for virus isolation is necessary to identify new strains of viruses. This facilitates the updating of vaccines with epidemiologically relevant viruses.

Some laboratories are involved in contract research and perform safety and efficacy studies for the regulatory authorities. All diagnostic laboratories have a role to play in the monitoring of the efficacy of existing products. Clinicians have a responsibility to inform the microbiologist of the vaccination and/or therapeutic history of the animal to facilitate independent assessment of different products and treatment regimens. Microbiologists in turn have a responsibility to advise clinicians with regard to their findings.

General Principles for Sample Collection

- Samples should be taken from the affected site(s) as early as possible following the onset of clinical signs. This is particularly important in viral diseases as shedding of virus is usually maximal early in the infection. This is also true of enteric bacterial pathogens.
- It is useful to collect samples from clinical cases and in-contact animals, particularly if there has been an outbreak of disease. In-contact animals may be at an earlier stage in the infection with a greater chance of them shedding substantial numbers of microorganisms.
- Samples should be obtained from the edge of lesions and some macroscopically normal tissue included. Microbial replication will be most active at the lesion's edge.
- It is important to collect specimens as aseptically as possible, otherwise the relevant pathogen may be overgrown by numerous contaminating bacteria

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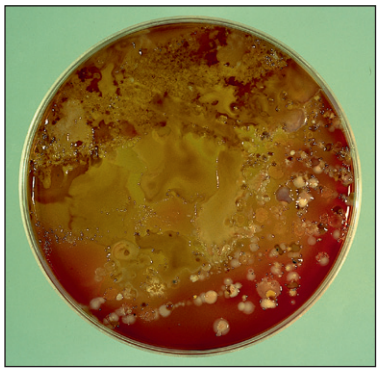

Figure 1.1 Grossly contaminated blood agar plate demonstrating the need to collect specimens as carefully as possible.

Figure 1.2 Sterile disposable containers and swabs for specimen collection.

(Fig. 1.1). In certain circumstances a guarded swab should be used to bypass an area with a large population of normal flora.

- The laboratory should be informed if treatment has commenced in order that counteractive measures may be taken to increase the possibility of isolating bacteria or that an alternative method of detection such as polymerase chain reaction (PCR) may be employed.
- When possible a generous amount of sample should be taken and submitted, such as blocks of tissue (approximately 2 cm^3), biopsy material, or several millilitres of pus, exudate or faeces. For serology, at least 5 mL of blood should be obtained to allow a number of tests to be carried out if necessary and to allow the sample to be stored and tested with subsequent samples.
- Cross-contamination between samples must be avoided. This is essential where a highly sensitive amplification technique such as PCR is to be used for the detection of the aetiological agent.
- Precautions must be taken to avoid human infection where a zoonotic condition is suspected.

Tissue

Postmortem material should be collected as soon as possible after death. However it is sometimes worthwhile submitting old and even partially decomposed samples if that is all that is available. In the outbreak of African horse sickness in the Iberian Peninsula in the late 1980s virus was isolated from the bone marrow of a horse that had been buried for eight days. An organism does not have to be viable or even entire for its genome to be detected by polymerase chain reaction. Thus it may be possible to obtain a diagnosis by PCR from a sample that is totally unsuitable for histopathological examination or agent isolation.

Tissues from outside the body cavities should be collected first followed by tissues from the thorax and then the abdomen. Sterile instruments should be used to collect tissue samples of at least 1 cm^3 which should then be placed in separate sterile screw-capped jars (Fig. 1.2). If the laboratory is some distance away tissue may be forwarded in virus or bacterial transport medium. It is important to remember that virus transport medium usually contains antibiotics thus rendering the sample unsuitable for bacterial examination. Tissue for histopathological examination should be placed in at least 10 times its volume of neutral buffered 10% formalin.

In cases of abortion the whole foetus and placenta should be submitted. If this is not possible then samples of tissue, a piece of affected placenta, foetal abomasal contents (ruminants) and uterine discharge (if applicable) should be forwarded to the laboratory. A clotted blood sample from the dam for serological examination may yield additional information.

Swabs and discharges

Fluids are preferable to swabs as the greater sample volume increases the likelihood of detecting the causal organism. Samples for agent isolation should be placed in sterile containers. Viruses and many bacteria are susceptible to desiccation especially if collected on a dry swab. Formulae for suitable transport media for viruses, chlamydia and other organisms are given in Appendix 2. Whenever possible the sample should be collected from the specific site of infection. The usual short cotton wool swabs are generally unsatisfactory for obtaining nasopharyngeal specimens of epithelial cells and mucus for the investigation of respiratory disease of large animals. Guarded swabs are necessary for certain bacteriological examinations where misleading results could be generated due to contaminants from adjacent sites that are colonized with bacterial flora. Similarly, fungal organisms from the environment

readily contaminate the nasal passages and upper trachea. The diagnostic laboratory should be consulted before collecting samples for the isolation of specific pathogens that require specialized media or culture conditions, for example, *Taylorella equigenitalis*, *Chlamydophila psittaci* or *Mycoplasma* species. The laboratory will either supply specialist swabs and transport media or recommend a reputable source, as appropriate.

Samples from skin lesions

If intact pustules or vesicles are present, the surface should be disinfected with 70% ethyl alcohol, allowed to dry, and material aspirated from the lesion with a sterile syringe and fine needle. A swab may be taken from the raw surface of ulcers. A biopsy of wound tissue should be collected after the superficial area has been cleaned and debrided.

In cases where ringworm is suspected, hair should be plucked from the lesion and the edge of the lesion scraped with a blunt scalpel blade until blood begins to ooze. Plucked hair, skin scrapings (including the scalpel blade itself) and any scab material that is present should be submitted. These specimens will also allow detection of mange or a bacterial infection, if present. In cases of orf the crust and scrapings from the edge of the lesion should be collected. In birds feather follicle skin is useful in the diagnosis of Marek's disease.

Blood

Blood should be withdrawn using an aseptic technique into a dry syringe or vacutainer. If collected in a syringe, care must be taken not to cause haemolysis. The needle should be removed prior to expelling the sample carefully into a sterile dry tube. It is not acceptable to submit blood to the laboratory in a syringe. Glass tubes are fragile. However, blood clots often retract poorly in plastic bottles making it difficult to separate the serum. Whole blood should never be frozen prior to submission to a laboratory as the ensuing lysis of red blood cells makes it impossible to perform many serological assays.

Serological tests are usually performed on the serum harvested from clotted samples. Paired samples are frequently required to make a definitive diagnosis on the basis of serology. It is advisable to confirm the most appropriate sampling interval with the microbiologist who should also advise as to usefulness of a single blood sample.

Blood for the isolation of viruses or bacteria should be prevented from clotting. The laboratory should advise on the most appropriate anticoagulant. As a bacteraemia can be intermittent, it is advisable to take more than one sample within a 24-hour period. The blood should be added aseptically and without delay to one of the special commercial blood-culture bottles. Blood samples for culture should be kept cool and submitted to the laboratory as quickly as possible.

Faeces

A faeces sample freshly voided or collected from the rectum is preferable to a rectal swab which often does not have enough faecal matter for agent detection. A faeces sample (about the size of the end of a thumb) may be forwarded to the laboratory without transport medium. Faecal swabs should be placed in medium such as buffered glycerol saline to avoid desiccation. Some organisms are shed intermittently and samples may need to be collected over several days.

Urine samples

Urine samples may be submitted for urinalysis, bacterial microscopy and culture or for a bacterial viable count to establish whether clinical bacteriuria is present. For bacteriological procedures the preferred methods of collection are by cystocentesis, by catheter or mid-stream urine sample.

Abscesses

If possible about 3 mL of pus should be collected together with scrapings from the wall of the abscess. Pus at the centre of an abscess is often sterile. Pus from recently formed abscesses will yield the best cultural results. Anaerobic bacteria can often be cultured from abscesses.

Eye

A conjunctival swab may be taken gently holding the palpebrae apart. Scrapings may also be taken with a fine sterile spatula. The cells should be washed carefully into transport medium.

Bovine mastitic milk samples

Milk samples should be collected from cows as soon as possible after the mastitis is first noticed and not from animals treated with either intramammary or systemic antibiotics. The udder should not be rinsed with water unless very dirty. If the udder and teats are washed, they should be dried thoroughly with a paper towel. Usually it is sufficient to wipe the teats vigorously, using 70% ethyl alcohol on cotton wool, paying special attention to the teat sphincters. Antiseptics should be avoided. The two teats furthest from the operator are wiped first and then the two nearest teats. The sterile narrow-necked collection bottle must be held almost horizontally. The first squirt of milk from each teat is discarded and then, for a composite sample, a little milk from each quarter is

directed into the bottle. The milk should be collected from the two near teats first and then from the two far teats, so that one's arm is less likely to accidentally brush against a cleaned teat.

Specimens for anaerobic culture

A good collection method is essential because many anaerobes do not survive frank exposure to the oxygen in the air for more than 20 minutes. It is important not to contaminate the samples by contact with adjacent mucosal surfaces as these have a resident anaerobic flora. Specimens from animals that have been dead for more than four hours are usually unsuitable because of the rapid postmortem invasion of the animal body by anaerobes from the intestinal tract. Bone marrow is a good specimen to collect for the diagnosis of blackleg or malignant oedema as bone marrow appears to be one of the last tissues to be invaded by contaminating bacteria. A piece of rib stripped of the periosteum could be submitted to the laboratory for the extraction of bone marrow. Any specimens for the attempted isolation of anaerobes must arrive at the laboratory as soon as possible after collection. Collection of samples for anaerobe culture on ordinary swabs is usually of no value. Acceptable samples include blocks of tissue (4 cm^3) or several millilitres of fluid placed in a sterile closed container. Anaerobic transport medium is essential for swabs.

In suspected enterotoxaemia cases, where the demonstration of a specific toxin is required, at least 20 mL of ileal contents should be submitted. A loop of ileum with contents, tied off at each end, is acceptable or the ileal contents drained into a secure sterile container.

Sample Submission

Laboratories usually supply a variety of sample containers and transport media. The laboratory should supply sample submission forms. These forms must be completed by the veterinary practitioner and must give specific details of the tests required as well as clinical history, differential diagnoses, vaccination history, therapy, age of animal, etc. The latter will enable the microbiologist to choose the most appropriate tests and to avoid unnecessary expenditure. A complete history will also allow the laboratory to identify a particularly urgent situation and expedite the processing of the sample. In certain instances owners may be concerned that information in relation to their animals is kept confidential. In such circumstances the clinician can assign a code name or number to the animal and the owner but should never omit clinical detail that will facilitate the selection of the appropriate tests and the interpretation of the results. The laboratory must be notified if the differential diagnosis includes a fungal infection or any agent that is potentially infectious for people.

Many laboratories only set up certain tests on particular days or at a designated time each day. Clinicians need to familiarize themselves with the laboratory timetable in order for them to provide an efficient service to their own clients. An awareness of the time it takes to perform certain assays is essential. Some assays may take weeks to complete. Where certification is required, samples should be submitted in good time and allowance should be made for repeat testing and/or the collection of a second sample if necessary.

The clinician should contact the laboratory in advance if they are not a regular client or if the tests required are not routine. It is essential to give the laboratory adequate time to prepare for the receipt of a sample which requires specialist testing. It is inadvisable, for example, to submit a sample that needs to be passaged on a cell line that the laboratory does not use on a regular basis without prior discussion. In such circumstances the sample may have to be stored for days if not weeks, while cells are resuscitated from the freezer. In certain cases it may be necessary to forward the sample to a specialist laboratory. If samples are being submitted to a laboratory in another country an import licence may be required.

Samples should be collected and delivered to the laboratory as early in the day as possible so that processing can commence on the same day. If a result is required urgently this must be indicated on the request form and the head of the laboratory should be notified in advance of the arrival of the sample. Prompt delivery to the laboratory will maximize the possibility of obtaining a diagnosis. Viruses only replicate in living cells and a delay in sample submission may result in loss of viability. The transit time needs to be minimized. Samples should be transported in contact with cold packs or wet ice. If transportation to the laboratory is delayed, most samples should be held in the refrigerator at +4 °C rather than frozen. Serum harvested from clotted blood samples can be stored frozen for extended periods.

The labelling of samples must be clear and unambiguous. Samples must be submitted individually in separate leak-proof containers that are clearly marked, indicating the identity of the sample (tissue, exudate, etc.), animal identification and the date of collection (Fig. 1.3). Container caps should be screwed on tightly and taped, if necessary, to avoid leakage. All samples sent in the post must be labelled and packed in accordance with the regulations of the postal authorities. Glass tubes and other fragile containers must be adequately packaged to ensure they are not broken in the mail. Samples must always be surrounded by sufficient absorbent material to soak up the entire sample in the case of breakage or leakage. In general, triple packaging of diagnostic samples is required. The secondary or outer packaging must be a rigid container. The package should be clearly labelled with the words 'biological substances'.

Figure 1.3 Sturdy, leak-proof containers for transporting microbiological specimens.

Interpretation of Diagnostic Results

There must be full co-operation between the laboratory and the veterinary practice for the benefit of the patient and owner. It is the responsibility of the clinician to collect and submit the appropriate samples accompanied by specific requests or adequate history. It is the responsibility of the microbiologist to interpret the results in relation to the information supplied. The quality of the diagnostic activity undertaken by the laboratory depends to a large degree on the clinical and epidemiological information supplied by the veterinary clinician.

The following points are pertinent when interpreting reports from a diagnostic microbiology laboratory:

- A negative diagnostic report does not necessarily mean that the suspected microorganism is not the aetiological agent of the condition. There may be many reasons for the failure of the laboratory to isolate and identify the pathogen, such as a bacterium being overgrown by contaminants, a virus or other fragile microorganism having died on the way to the laboratory or the animal may have stopped excreting the microorganisms before the sample was taken. Unexpected negative results should be discussed with the head of the laboratory who may decide to perform additional tests for the detection of the suspect agent. They may also be able to advise on the collection of alternative samples or suggest other possible causal agents.
- It must not always be assumed that the detection of a virus or bacteria establishes a diagnosis. The laboratory result must be interpreted in light of the clinical signs, vaccinal history, etc. If the sample is collected from a site normally colonized with bacteria, including species that have the potential to cause disease in certain circumstances, the culture results need to be interpreted cautiously. Some latent viruses may reactivate and bacteria proliferate in response to a condition which they have not caused. Apparently healthy animals can be subclinical shedders of microorganisms such as salmonellae, rotaviruses, enteroviruses or coronaviruses in faeces and leptospires in urine. Even if these potential pathogens are isolated and identified, the illness or death might have been due to another cause.
- The clinician should always discuss unexpected results with the laboratory. This may assist the head of the laboratory in the early detection of a problem with a particular technique. The microbiologist must be prepared at all times to act on information received and to critically appraise the procedures in the laboratory.
- It is important that the difficulties associated with some types of samples and the limitations of certain assays are flagged to the clinician. This will help to promote intelligent utilization of the laboratory and to avoid unrealistic expectations.
- Bacteria such as members of the *Enterobacteriaceae* are ubiquitous. Isolation of microorganisms may represent contamination of the sample by faeces or soil or their presence could be due to postmortem invasion.
- Some bacteria such as salmonellae, leptospires or *Mycobacterium avium* subsp. *paratuberculosis* may be shed intermittently. A repeat sample following a negative examination report might be worthwhile.
- *Escherichia coli* in diarrhoeal faecal samples from farm animals is usually only significant if the animal is under 10 days of age and if the *E. coli* isolate possesses the K88, K99, F41 or 987P fimbrial antigens, associated with enteropathogenicity. Pigs are the exception as they are also susceptible to colibacillosis soon after weaning and to oedema disease in the growing period.
- Diagnosis of a mycotic disease thought to be due to a ubiquitous fungus such as *Aspergillus fumigatus* should always be confirmed histopathologically. It is necessary to demonstrate the fungal hyphae actually invading the tissue and causing a tissue reaction.
- Herd or flock vaccination programmes will modify the interpretation of serological tests. Serological diagnosis of recent viral exposure usually requires the comparison of two samples collected approximately two weeks apart.
- Conflicting results may reflect the sensitivity of different assays. Thus a sample that is negative by enzyme-linked immunosorbent assay (ELISA) and by isolation may be positive by PCR.

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Chapter 2

Bacterial pathogens: Microscopy, culture and identification

MICROSCOPY

A good-quality microscope with a built-in light source (bright-field microscopy) as well as low-power, high-dry and oil-immersion objectives is required. A darkfield condenser is a useful addition necessary for the visualization of unstained preparations such as those of spirochaetes. Microscopes for microbiology require a higher degree of resolution than those used for haematology or histopathology. Figure 2.1 indicates the size of bacteria relative to that of an erythrocyte and to viruses. Bacteria are measured in micrometres (μm) which are 10^{-6} m while viruses are measured in nanometres (nm) which are 10^{-9} m. Using an oil-immersion objective lens on a light microsope, a magnification of 1000× can be achieved to visualize bacteria. On account of their small size viruses are visualized by using an electron microscope, which utilizes a beam of electrons to achieve magnifications of the order of 100,000×.

Stained Smears from Pathological Specimens

Stained smears made from lesions can yield a considerable amount of information inexpensively and quickly. Table 2.1 summarizes the information that can be gained from the various diagnostic staining techniques.

Preparing Bacterial Smears

Microscope slides are not always clean enough to use directly from the supplier. Rubbing with a clean, soft cloth and passing quickly through the Bunsen flame may be sufficient to remove a greasy film. If not, a mildly abrasive liquid cleaner can be used followed by rinsing the slide thoroughly and wiping it dry with a clean cloth.

A blunt scalpel and forceps should be kept in a container of 70% ethyl alcohol. The instruments are flamed and cooled before use. Afterwards they should be placed into a container of disinfectant. When making a smear from tissue lesions, the specimen is held firmly with the forceps and the scalpel is used to scrape deep into the material. A small amount of the scrapings is placed on the cleaned microscope slide. Another clean slide is used with a scissor action to prepare a thin smear. With liquid or semiliquid specimens, a little of the sample is placed on the slide with a sterile swab. The contents of the swab are smeared over the surface of the slide, with the aim of having thick and thin areas of specimen present. The smears are allowed to dry thoroughly before proceeding further.

Fixing the Smears

The reasons for fixing the smears include killing the vegetative bacteria, rendering them permeable to the stain and ensuring that the material is firmly fixed to the slide. Fixed and stained smears should be handled carefully as not all bacteria, especially endospores, may have been killed. After use, the stained smears should be autoclaved or soaked in a reliable disinfectant (24–48 hours) before discarding.

For routine staining the smears are fixed by passing the slide, smear side up, quickly through the Bunsen flame two or three times, taking care not to overheat the smear. This can be tested on the back of the hand; the slide should feel warm but not hot enough to burn.

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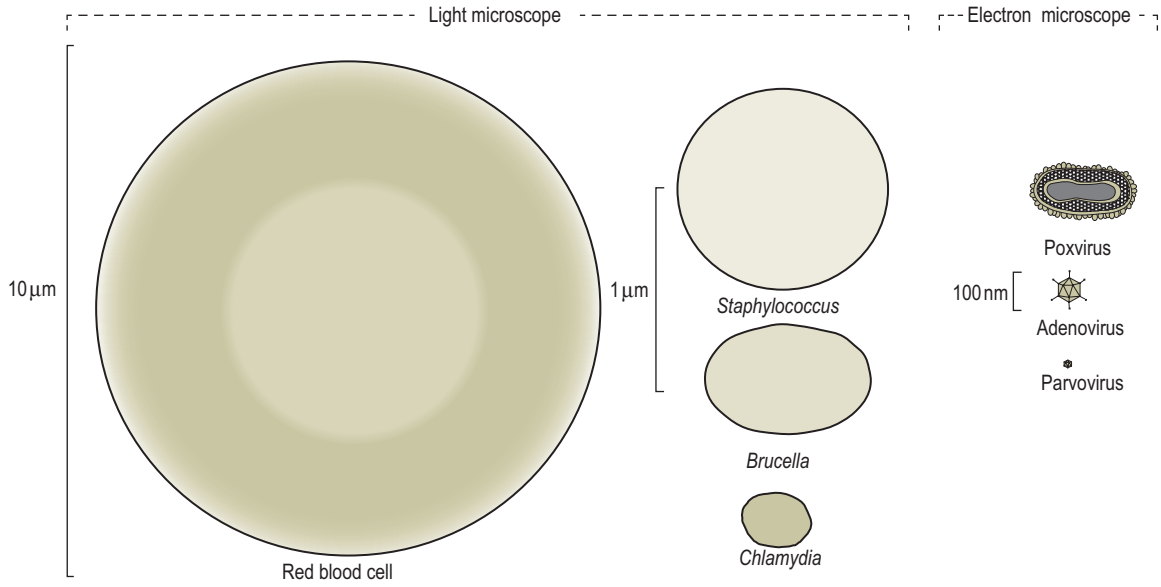

Figure 2.1 Comparison of the relative sizes of a red blood cell, bacteria and viruses.

Table 2.1 Diagnostic uses of stained smears

Disease and species affected	Specimen	Pathogens	Appearance in stained smears
Gram stain			
Abscesses or suppurative conditions	Pus or exudate	*Staphylococcus* species	Gram + cocci, often in clumps
Many animal species		*Streptococcus* species	Gram + cocci, usually in chains
		Trueperella pyogenes	Gram + rods, pleomorphic
		Corynebacterium pseudotuberculosis	Gram + rods
		Pseudomonas aeruginosa	Gram – rods
		Pasteurella multocida	Gram – rods
		Fusobacterium necrophorum	Gram –, long, slender filaments, often staining irregularly
Strangles Horses	Pus	*Streptococcus equi* subspecies *equi*	Gram + cocci, often in chains
Suppurative broncho-pneumonia or superficial abscesses Foals and young horses	Pus	*Rhodococcus equi*	Gram + rods with tendency to coccal forms

Table 2.1 Diagnostic uses of stained smears—cont'd

Disease and species affected	Specimen	Pathogens	Appearance in stained smears
Dermatophilosis (Streptothricosis) Many animals, mainly in sheep, cattle and horses	Scabs	*Dermatophilus congolensis*	Gram +, filamentous and branching with coccal zoospores arranged two or more across
Canine nocardiosis and canine actinomycosis	Pus or thoracic aspirates	*Nocardia asteroides* *Actinomyces viscosus*	Both Gram +, filamentous and branching, *Nocardia* spp. MZN +
Bovine actinobacillosis (wooden tongue)	Granules from pus	*Actinobacillus lignieresii*	Gram – rods
Bovine actinomycosis (lumpy jaw)		Actinomyces bovis	Gram +, filamentous and branching
Clostridial enterotoxaemia Sheep and calves mainly	Scrapings from small intestine of **recently** dead animal	*Clostridium perfringens*	Gram +, fat rods. Large numbers suggestive of enterotoxaemia
Urinary tract infections Dogs and cats	Fresh, carefully collected urine	*Escherichia coli, Proteus* species, *Staphylococcus* species and *Enterococcus faecalis*	Gram – rods Gram + cocci Gram + cocci
Dilute carbol fuchsin (simple) stain			
***Campylobacter* infections** Infertility in cattle. Abortion in sheep and cattle	Vaginal mucus or foetal stomach contents	*Campylobacter fetus*	Curved rods that can be in chains giving 'seagull' forms
Swine dysentery	Faeces or scrapings from colon	*Brachyspira hyodysenteriae*	Numerous long (7.0 μm) but finely spiralled bacteria
Foot rot in sheep	Exudate from hoof	*Dichelobacter nodosus*	Rods with a knob on one or both ends
		Fusobacterium necrophorum	Long, slender filaments, staining irregularly
Modified Ziehl–Neelsen (MZN) stain			
Brucellosis in cattle, sheep, pigs and dogs	Foetal stomach contents, vaginal discharge, placenta	*Brucella* spp.	Small, red coccobacilli in clumps
Nocardiosis Canine nocardiosis Bovine mastitis	Pus and aspirates Sediment from centrifuged milk	*Nocardia asteroides*	Long-branching filaments, many staining bright red
Chlamydial infections Sheep and cattle: abortion Lambs, calves and pigs: polyarthritis Cats: feline pneumonitis	 Cotyledons Joint fluid Conjunctival scrapings	 *Chlamydophila abortus* *Chlamydophila pecorum* *Chlamydophila felis*	Small, red coccobacilli in clumps. Similar to brucellae Small, red coccobacilli

Continued

Table 2.1 Diagnostic uses of stained smears—cont'd

Disease and species affected	Specimen	Pathogens	Appearance in stained smears
Ziehl–Neelson (acid-fast-) stain			
Tuberculosis Cattle and other species	Suspect lesions	*Mycobacterium bovis*	Long, thin, bright red rods, can appear beaded. Usually not numerous in smears
Poultry, other avian species and cervical lymph nodes of pigs	Suspect lesions	*Mycobacterium avium*	As above but larger numbers of acid-fast rods usually present
Feline leprosy	Scrapings from lesions	*Mycobacterium lepraemurium*	Large numbers of red-staining, acid-fast rods present
Paratuberculosis (Johne's disease) in cattle and sheep	Faeces, smear from ileocaecal valve area and mesenteric lymph nodes	*Mycobacterium avium* subspecies *paratuberculosis*	Fairly short, red, acid-fast rods in clumps
Giemsa stain			
Anthrax in cattle, sheep and pigs	Blood smear from ear vein or fluid from peritoneal cavity (pigs)	*Bacillus anthracis*	Purplish, square-ended rods in short chains surrounded by a reddish-mauve capsule
Feline infectious anaemia	Thin blood smear	*Mycoplasma haemofelis*	Small, dark-blue coccal forms on red blood cells
Avian spirochaetosis	Blood smear taken during febrile period	*Borrelia anserina*	Helical bacteria, 8–20 µm long and 0.2–0.5 µm wide with five to eight spirals
Dermatophilosis (Streptothricosis) Many animals, mainly sheep, cattle and horses	Scabs	*Dermatophilus congolensis*	Blue, filamentous and branching with coccal zoospores arranged two or more across
Polychrome methylene blue			
Anthrax in cattle, sheep and pigs	Thin blood smear from ear or tail vein. Fluid from peritoneal cavity (pigs)	*Bacillus anthracis*	Blue, square-ended rods in short chains surrounded by a pinkish-red capsule
Wet preparations			
Leptospirosis in many animal species	Centrifuged deposit of urine or kidney tissue	*Leptospira* serovars	Helical, approx.15 µm long and 0.15 µm wide. Appears beaded with hooked ends. Dark field microscopy
Ringworm in many animal species	Hair and skin scrapings in 20% KOH	*Microsporum* and *Trichophyton* spp.	Chains of refractile, round arthrospores on hairs. High-dry objective
Other suspected fungal infections in many animal species	Tissue, exudates, biopsies in 20% KOH	*Aspergillus fumigatus, Candida albicans* and others	Fungal mycelial elements or budding yeast cells. High-dry objective
Bovine trichomoniasis	Purulent uterine discharge in saline. Keep warm and examine within one hour of collection	*Trichomonas foetus*	Protozoan agent with three free flagella and an undulating membrane. Low/high-dry objectives

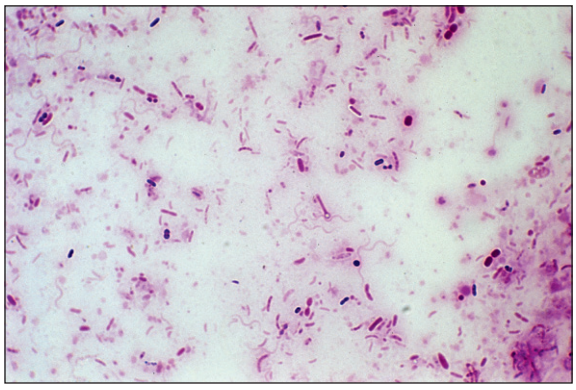

Figure 2.2 Gram-stained porcine faecal smear showing Gram-positive (blue) and Gram-negative (red) bacteria. Note range of morphological forms. (×1000)

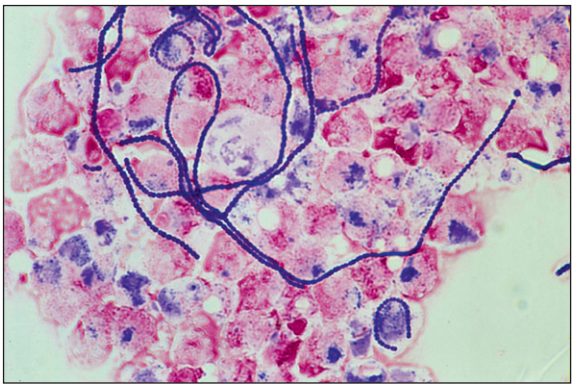

Figure 2.3 Gram-stained smear of mastitic milk (bovine) showing Gram-positive streptococci in chains and inflammatory cells. (×1000)

Dried smears to be stained by the Giemsa stain are first fixed in absolute methyl alcohol for three minutes and then dried.

Staining the Smears: Staining Techniques

The fixed smears are placed on a staining rack over a sink. The staining solutions are flooded over the entire smear and left on the slide for the appropriate time. Between each staining reagent the smear is washed under a gently running tap, excess water tipped off and the next reagent added. Finally the stained smear is washed and air-dried. The preparation method of each of the staining solutions is given in Appendix 1.

Gram stain

Crystal violet 60 seconds

Gram's iodine (mordant) 60 seconds

Gram's decolourizer 15 seconds

Counter-stain (dilute carbol fuchsin or safronin) 60 seconds

Gram-positive bacteria retain the crystal violet-iodine complex and stain purple-blue. Gram-negative bacteria are decolourized and are stained red by the counter-stain. Gram-stained smears are illustrated (Figs 2.2–2.6, inclusive). There can be slight differences in the composition of the reagents for the Gram stain. If using a commercial kit set for Gram staining always follow the manufacturer's directions.

Dilute carbol fuchsin (DCF):

A simple staining technique

Dilute carbol fuchsin four minutes

Wash and air-dry.

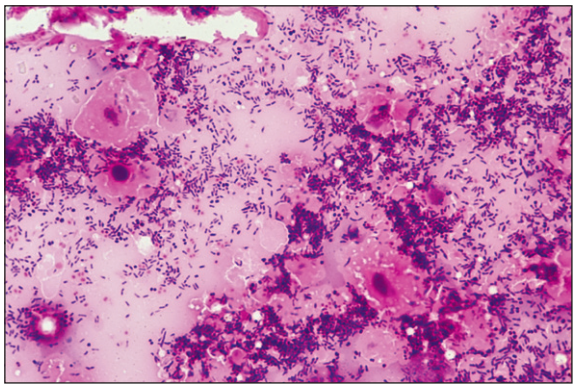

Figure 2.4 Gram-stained smear from an abscess with the Gram-positive, pleomorphic rods of *Trueperella* (*Arcanobacterium*) *pyogenes* predominating. (×1000)

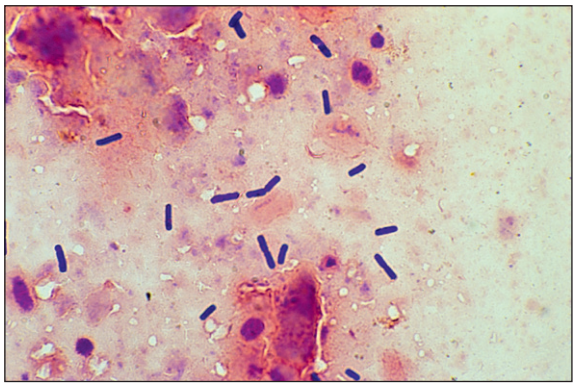

Figure 2.5 Gram-stained smear from the mucosa of the small intestine (lamb recently dead from enterotoxaemia): large Gram-positive rods of *Clostridium perfringens*. (×1000)

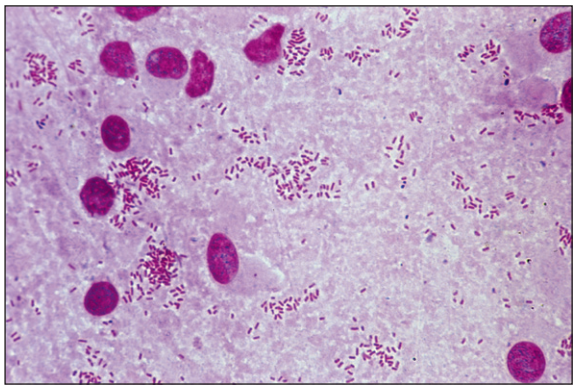

Figure 2.6 Gram-stained smear of urine (dog with cystitis): Gram-negative rods of *Escherichia coli.* (×1000)

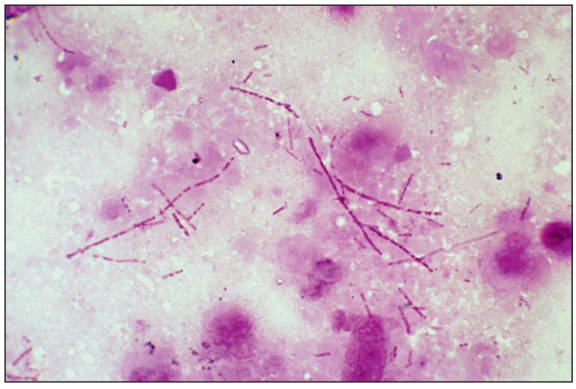

Figure 2.7 DCF-stained smear from a bovine abscess: red filaments of *Fusobacterium necrophorum* showing typical irregular staining. (×1000)

The stain is used for some Gram-negative bacteria such as *Campylobacter fetus*, *Brachyspira hyodysenteriae* or *Fusobacterium necrophorum* (Fig. 2.7) where a greater depth of stain aids microscopic visualization.

Modified Ziehl–Neelsen (MZN)

Dilute carbol fuchsin 15 minutes

Acetic acid (0.5%) 15 seconds

Methylene blue two minutes

Wash and air-dry.

MZN-positive bacteria such as *Nocarida asteroides* (Fig. 2.8), *Brucella* spp. (Fig. 2.9) and *Chlamydophila abortus* stain a bright red with the background and other bacteria staining blue.

Ziehl-Neelsen (ZN) or acid-fast stain

Strong carbol fuchsin 10 minutes with heat

Acid-alcohol decolourizer 15 minutes with several changes

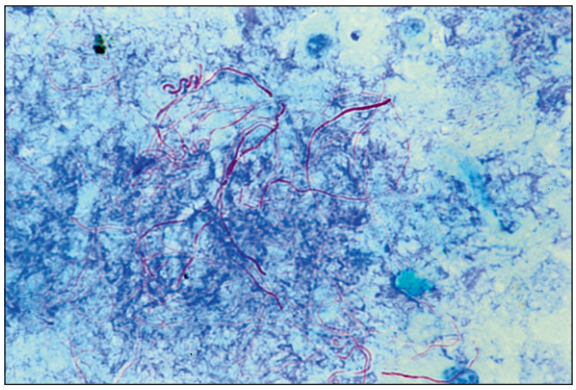

Figure 2.8 MZN-stained smear of a thoracic aspirate from a dog with a pleural effusion: MZN-positive branching filaments of *Nocardia asteroides*. (×1000)

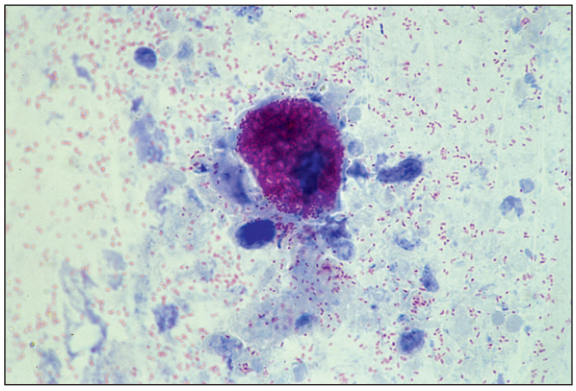

Figure 2.9 MZN-stained smear from a bovine placenta (an abortion case): red MZN-positive coccobacilli of *Brucella abortus* in clumps. (×1000)

Methylene blue 20 seconds

Wash and air-dry.

ZN-positive or acid-fast bacteria, such as the pathogenic *Mycobacterium* sp., stain bright red with the background and other bacteria counter-stained blue (Fig. 2.10). Heating the strong carbol fuchsin can be carried out in one of two ways:

- Strong carbol fuchsin is flooded onto the fixed smear with the slide on the rack over a sink. A cotton wool swab, on a metal rod, is dipped in alcohol and set alight. This is used to gently heat the smear and carbol fuchsin from below. The stain is allowed to steam for the 10 minutes but not to boil. The sink should be rinsed with water before starting the heating process in case any inflammable reagents are present.
- Heat the strong carbol fuchsin in a boiling tube to just below boiling point. Wear protective goggles

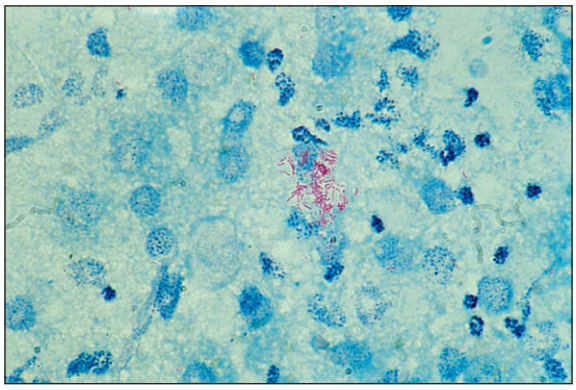

Figure 2.10 ZN-stained smear from a tuberculous lesion in a hen: red ZN-positive thin rods of *Mycobacterium avium*. (×1000)

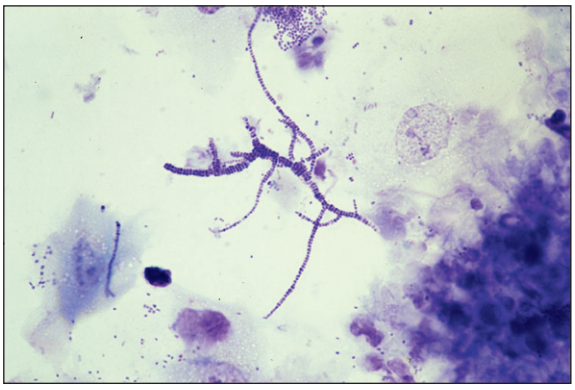

Figure 2.11 Giemsa-stained smear from ovine scab material showing branching filaments and zoospores of *Dermatophilus congolensis*. (×1000)

when carrying out this procedure and direct the tube away from you. Add the hot stain to the smear on the staining rack over the sink. Keep topping-up the smear with hot stain for the full 10 minutes.

Alternative method for the Ziehl–Neelsen stain

The advantage of the method using the brilliant green counter-stain is that it is almost impossible to over-do the counter-staining.

Strong carbol fuchsin three minutes

Wash in distilled water

Acid-alcohol decolourizer three minutes exactly

Several washes in distilled water

Alkaline brilliant green three minutes

Wash in distilled water and air-dry.

The ZN-positive or acid-fast pathogenic *Mycobacterium* spp. are stained a bright red with the background and other microorganisms stained green.

Giemsa stain

The dried smear is first fixed in absolute methyl alcohol for three minutes

l part Giemsa stain + 9 parts buffer 60 minutes

Wash with the buffer

Drain and air-dry.

The Giemsa stain is used to stain spirochaetes such as *Borrelia anserina*; to demonstrate the capsule of *Bacillus anthracis*; to stain organisms such as *Mycoplasma haemofelis*; and it can demonstrate the morphology of *Dermatophilus congolensis* more clearly than the Gram method (Fig. 2.11).

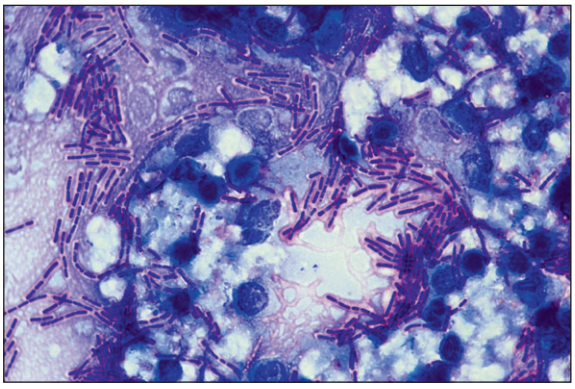

Figure 2.12 Polychrome methylene-blue-stained bovine blood smear showing capsulated *Bacillus anthracis*. Note the square-ended rods in short chains with pink capsule surrounding the blue cytoplasm. (×1000)

Polychrome methylene blue stain (M'Fadyean's Reaction)

Polychrome methylene blue is methylene blue solution that has been allowed to oxidize by storing it exposed to the air (loosely plugged) for several months. A thin blood or exudate smear taken from a suspect case of anthrax is air-dried, flame-fixed and flooded with the stain for two to three minutes. The stained smear is washed and dried. The rods of *B. anthracis* stain blue and the capsular material a pale pink colour (Fig. 2.12). Any suspect anthrax material should be handled with care and the stained slides autoclaved after use. Viable spores may be present on the slide after staining.

Wet preparations

Some microorganisms can be demonstrated microscopically without the use of staining techniques. Wet

preparations can be examined by phase contrast or dark-field microscopy and by the use of the high-dry objective of the light microscope, with the condenser slightly lowered. Fungal structures in tissues or skin scrapings can be placed in a few drops of 10–20% potassium hydroxide (KOH) on a microscope slide, under a cover slip, for two to four hours to allow clearing of the preparation to occur. The slide is then examined under the high-dry objective of the microscope. This is particularly useful to demonstrate the arthrospores of dermatophytes (Fig. 2.13). Methods for microscopic examination of specimens for fungal elements are given in more detail in the mycology section.

Figures 2.14 and 2.15 summarize the staining reactions and cellular morphology of some of the more commonly encountered Gram-positive and Gram-negative bacteria.

Figure 2.13 KOH wet preparation of bovine hairs infected with *Trichophyton verrucosum* showing arthrospores. (×400)

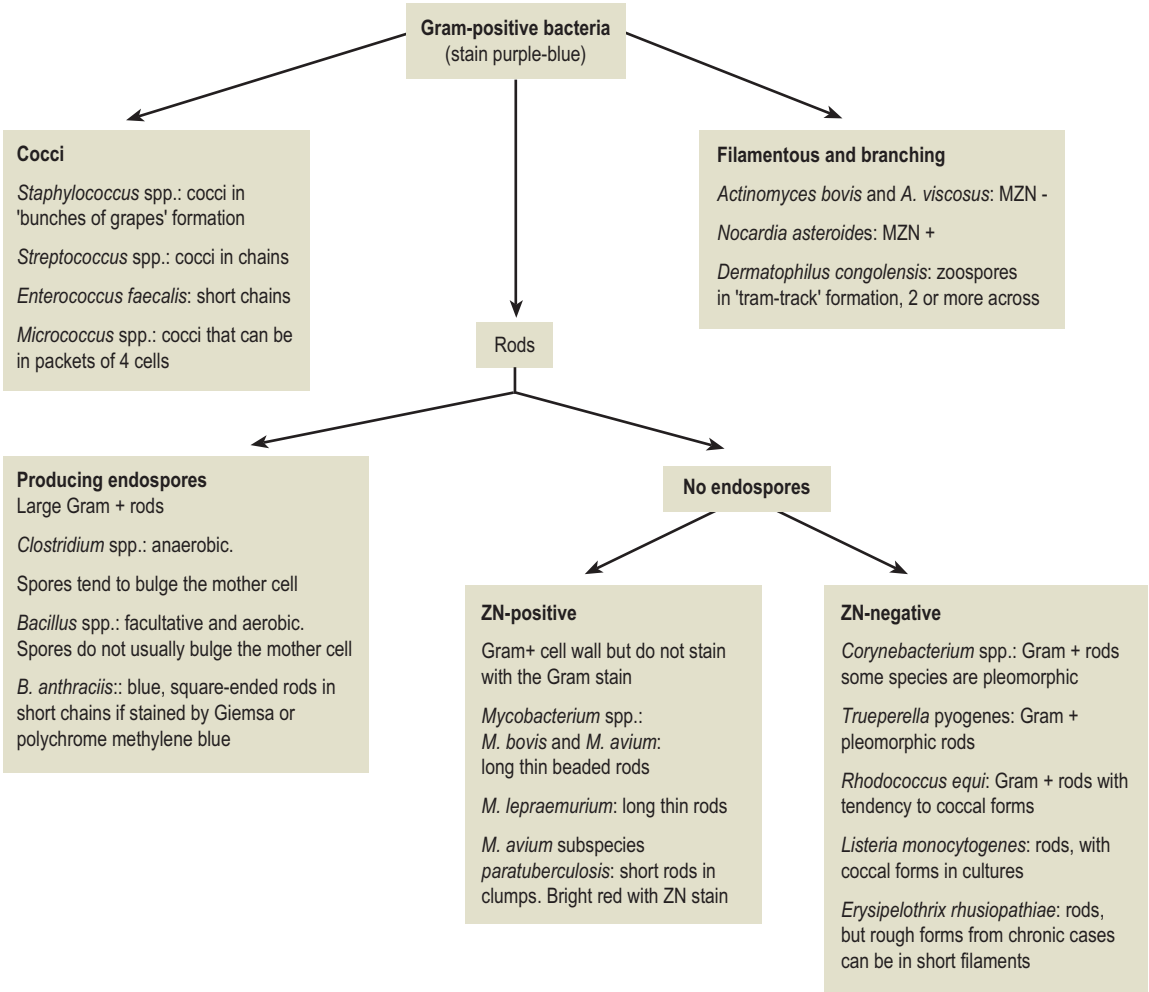

Figure 2.14 Summary of the staining reactions and cellular morphology of Gram-positive bacteria. (MZN = modified Ziehl–Neelsen stain, ZN = Ziehl–Neelsen stain)

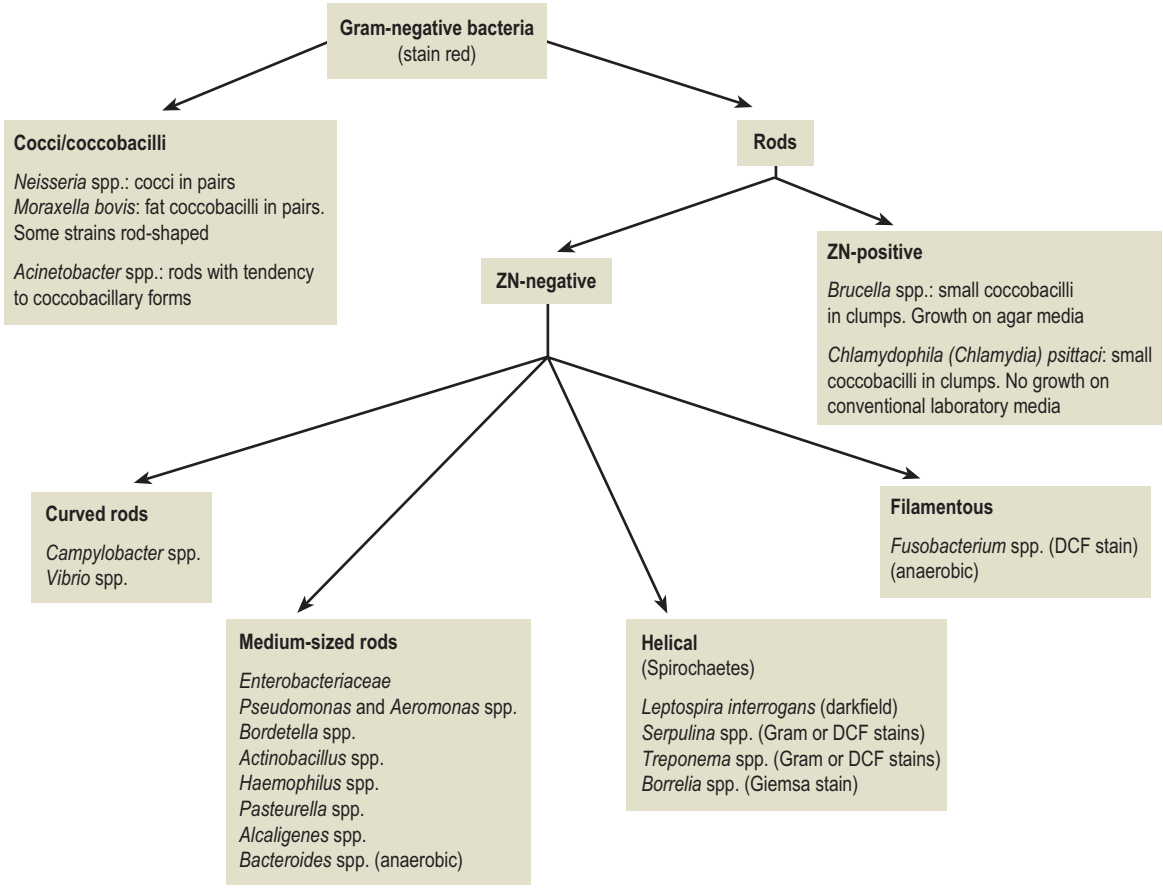

Figure 2.15 Summary of the staining reactions and cellular morphology of Gram-negative bacteria. (MZN = modified Ziehl–Neelsen stain, DCF = dilute carbol fuchsin)

BACTERIOLOGICAL MEDIA

Diagnostic bacteriological media can be divided into the following categories:

- **Chemically defined media:** in these, the exact amounts of each ingredient are known. They are mainly used for experimental purposes but citrate broth is an example of a chemically defined medium that is used in diagnostic bacteriology.
- **Basic nutritive media:** these are capable of sustaining growth of the less fastidious bacteria. Nutrient agar is an example.
- **Enrichment broths:** a liquid medium that permits the growth and detection of a particular bacterium, which may have made up only a small proportion of the bacteria in the original inoculum. An example is selenite broth for the selection of salmonellae.
- **Selective media:** these agar media have been made selective for the growth of a particular bacterium or group of bacteria and are used extensively in diagnostic bacteriology. They contain inhibitory substances that prevent the growth of unwanted bacterial species. Many selective media, such as brilliant green and MacConkey agars can also be described as indicator media.
- **Indicator media:** these are particularly useful in diagnostic bacteriology. They are designed to give a presumptive identification of bacterial colonies due to the biochemical reactions in the media. Indicator media often contain fermentable sugars plus a pH indicator that gives a colour change in the media (Table 2.2). MacConkey agar contains the fermentable sugar lactose and neutral red as the pH indicator. Bacteria such as *Escherichia coli* that ferment lactose produce acidic metabolites that cause the colonies and surrounding medium to appear

Table 2.2 pH indicators used in diagnostic media and biochemical tests

pH indicator	pH range Alkaline Acid	Colour change Alkaline Acid	Media and biochemical tests
Andrade's indicator	7.2–5.5	Colourless–pink	Peptone water sugars
Bromocresol purple	6.8–5.2	Purple–yellow	Purple agar base, lysine decarboxylase broth
Bromothymol blue	7.6–7.0–6.0	Blue–green–yellow	O-F medium, Simmons citrate, Smith-Baskerville medium (*Bordetella* spp.)
Litmus	8.3–4.5	Blue–red	Litmus milk medium
Methyl red	6.4–4.4	Orange–red	Methyl red test
Neutral red	8.0–6.8	Pale yellow–red	MacConkey agar, *Yersinia* selective medium
Phenol red	8.4–6.8	Red–yellow	XLD*, brilliant green and TSI** agar, peptone water sugars, Christensen urea agar

Figure 2.16 Indicator media: clockwise from top left, XLD, brilliant green, MacConkey and EMB agars.

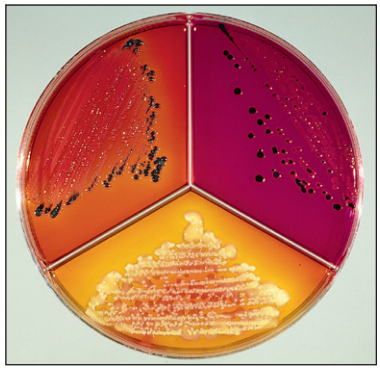

Figure 2.17 XLD medium with *Proteus* sp. (left), *Salmonella* sp. (right) and *Klebsiella* sp. (bottom).

pink. Salmonellae that cannot ferment lactose will use the peptones in the medium with the production of alkaline metabolic products. *Salmonella* colonies and surrounding medium are pale straw in colour. Other indicator media may be designed to show hydrogen sulphide production (xylose-lysine-deoxycholate, XLD – agar) or aesculin hydrolysis (Edwards medium). Blood agar, although an enriched medium may also be considered as an indicator medium as it shows the type of haemolysis of a particular bacterium.

Examples of media used in diagnostic bacteriology are given in Table 2.3 and illustrated (Figs 2.16 to 2.20, inclusive). They can be obtained commercially as dehydrated powders or can often be purchased as pre-poured plates.

Preparation of Culture Media

The manufacturer's instructions should always be followed but a few additional general points include:

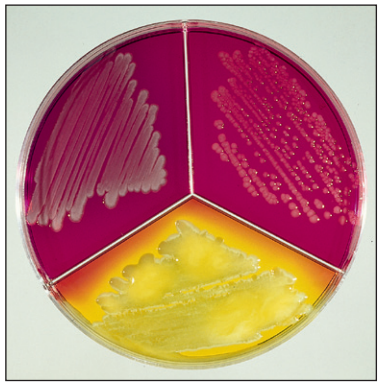

Figure 2.18 Brilliant green agar with *Pseudomonas aeruginosa* (left) *Salmonella* sp. (right) and *Klebsiella* sp. (bottom).

Table 2.3 Examples of media used in diagnostic bacteriology

Medium	Uses	Sugars and other substrates	pH indicator	Inhibitor(s)
Nutrient agar	Basic nutritive medium. Growth of less fastidious bacteria	_	_	_
Blood agar	Growth of most bacteria including many of the fastidious species	Red blood cell (shows haemolysis)		
MacConkey agar	Growth of the *Enterobacteriaceae* and some other Gram-negative bacteria	Lactose	Neutral red	Bile salts
Brilliant green agar	*Salmonella* isolation. A few other bacteria will grow on this medium	Lactose Sucrose	Phenol red	Brilliant green dye
XLD agar*	*Salmonella* isolation. Some other bacteria will grow on XLDagar*	Xylose, lactose sucrose, lysine, and H_2S detection	Phenol red	Bile salts
TSI agar**	*Salmonella* identification	Lactose, sucrose, dextrose, H_2S detection.	Phenol red	_
Selenite broth	Enrichment broth for isolation of salmonellae	_	_	Selenite
EMB agar***	Presumptive identification of *Escherichia coli*	Lactose. Saccharose	Eosin and methylene blue	_
Edwards medium	Selective for the streptococci. Indicates aesculin hydrolysis and haemolysis	Aesculin Red blood cells	_	Crystal violet and thallous sulphate
Purple base agar + 1% maltose	Presumptive differentiation of *Staphyloccus aureus* and *S. pseudintermedius*	1% maltose	Bromocresol purple	_
Chocolate agar	Enriched medium for *Haemophilus* species and *Taylorella* species	Lysed red cells. X and V factors released	_	_

* = Xylose-lysine-deoxycholate agar

** = Triple sugar iron agar

*** = Eosin-methylene blue agar (colonies of *E. coli* have a metallic sheen)

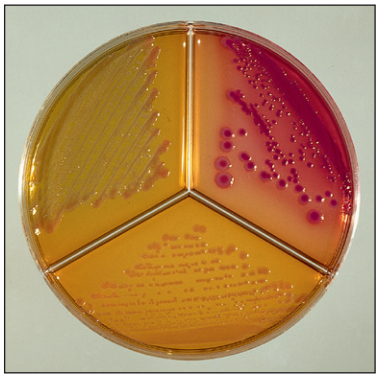

Figure 2.19 MacConkey agar with *Pseudomonas aeruginosa* (left), *Klebsiella* sp. (right) and *Salmonella* sp. (bottom).

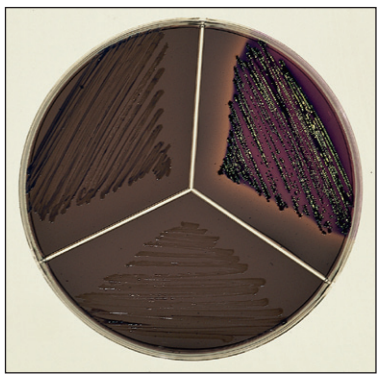

Figure 2.20 Eosin methylene blue agar with *Proteus* sp. (left), *Escherichia coli* (right) and *Salmonella* sp. (bottom).

- Clean glassware that has been rinsed free from detergents and other chemicals should be used.
- The glassware need not be sterile unless sterilized medium is being decanted into it.
- The appropriate amount of dehydrated medium is weighed out, placed in a flask and distilled water added to it. Glass-distilled water must be used, because this is free from chloride and heavy metal ions that can be inhibitory to bacteria.

The medium is prepared in a flask with a capacity of about twice the final volume of the medium as this will allow for adequate mixing and the frothing of the medium during heating. Media not containing agar can usually be dissolved with gentle agitation, but dehydrated media containing agar is best dissolved by bringing to the boil with continuous stirring, using a glass rod or a hot plate that incorporates a magnetic stirrer system. Dehydrated media once dissolved are usually sterilized in an autoclave at 121°C for a holding time of 15 minutes. Some media, such as Edwards, contain ingredients that cannot tolerate this high temperature and they can be autoclaved at 115°C for a holding time of 20 minutes or according to the manufacturer's instructions. Certain media such as brilliant green agar are inhibitory to many bacteria and these media are brought to the boil only and not autoclaved. Media containing agar should be cooled after autoclaving in a water bath at 50–54°C before the medium is poured into Petri dishes. Agar solidifies at about 42°C. The standard (90 mm) Petri dish should contain about 15 mL of agar medium, about one third full. Thus, one litre of medium should yield between 60–70 plates. Some additives to the medium, such as serum or red blood cells, will not tolerate high temperatures and are added as sterile solutions or suspensions once the medium has cooled to 50–54°C. After the poured plates are set, they are allowed to dry thoroughly at room temperature or for a few hours in the incubator at 37°C. For use, the surface of the agar must not contain obvious moisture. The plates are stored, agar side upwards, in a refrigerator at 4°C. Commercially available systems for the preparation and pouring of large numbers of plates are available.

Preparation of blood agar plates

The blood agar base is prepared from dehydrated powder, sterilized and cooled to 50–54°C in the usual manner. Sterile blood at the rate of 5–10% vol/vol is added to the cooled agar base and mixed well before the plates are poured. If bubbles form on the surface of the poured plates, a low Bunsen flame is quickly passed across the surface of the agar before the agar sets. If the sterile blood has been stored in the refrigerator, it should be warmed to 37°C before being added to the agar medium to avoid thermal shock to the red cells.

Figure 2.21 Sterile apparatus for the collection of blood (left) and items for blood agar preparation: blood agar base, sheep blood, Petri dishes and two poured plates.

Collecting sterile blood

Bovine, equine or ovine blood is most suitable for veterinary bacteriology. Sterile blood can be obtained commercially or it can be collected from a young animal that has no evidence of antibodies to the major veterinary pathogens and has not been treated with antibacterial agents. A strict aseptic technique must be used. The area over the jugular vein is clipped. The area is then saturated with 70 per cent ethyl alcohol, wiped and allowed to dry thoroughly before venepuncture. To prevent the blood from clotting one of the following methods could be used:

- Collection into a purchased human blood-donor kit.
- Collection into a pre-sterilized apparatus consisting of a tube leading into a conical flask containing glass beads (3 mm) (Fig. 2.21). The flask is agitated continuously during collection and for at least five minutes after obtaining the blood. This defibrinated blood can be decanted into sterile bottles for storage in a refrigerator. The glass beads can be recovered from the fibrin clot and reused.

A sterile anticoagulant solution, such as 0.2% sodium citrate, can be used either in the flask, in the place of the glass beads or blood can be collected in a sterile syringe and immediately added to the anticoagulant.

Choice of culture media

For routine isolation of bacteria, blood agar and MacConkey agar is used. The blood agar will support the growth of most of the pathogenic bacteria and many of the Gram-negative bacteria will grow on the MacConkey agar. A comparison of the growth on the two media can indicate the types of bacteria that have been isolated. Any special media used for specific pathogens are mentioned under the appropriate group of bacteria in Section 2.

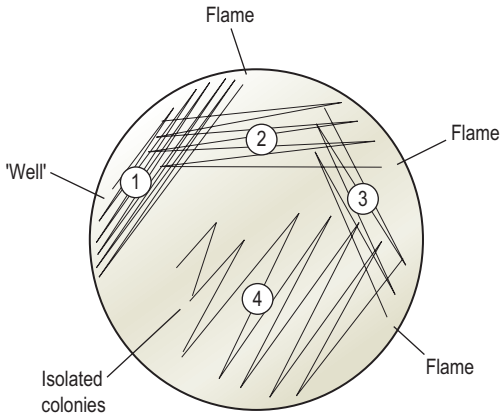

Figure 2.22 Quadrant streaking method for obtaining isolated bacterial colonies on agar media. The loop should be flamed before and after streaking.

Inoculation of Culture Media

If the specimen is a piece of tissue, it is easier to manipulate if it is first placed in an empty sterile Petri dish. Forceps and scalpel, previously held in 70% ethyl alcohol are flamed and allowed to cool. The tissue is scraped, paying particular attention to the edge of an observable lesion. A little of the tissue scrapings is placed at the edge or 'well' of each culture plate to be inoculated. Some of the tissue could be used to make a smear on a microscope slide for staining.

When the specimen is liquid or semiliquid it is best applied to the well of the plate using a sterile swab, first using the swab to mix the sample uniformly. Before discarding the swab a smear could be made for microscopy. When either inoculating or streaking culture media plates it is preferable to start with the non-inhibitory medium, such as blood agar, and then inoculate or streak any inhibitory or selective medium such as MacConkey agar.

Streaking the agar plates

The object of plate streaking is to obtain isolated bacterial colonies that are required for observing colonial morphology, antibiotic sensitivity testing and for biochemical identification. The quadrant streak method, using the whole plate, is usually employed for most diagnostic specimens (Fig. 2.22).

The plate is first labelled, on the agar side, with the type of specimen, date of inoculation and reference number. A waterproof marker pen is used and the writing should be kept as near to the edge of the plate as possible so that after incubation the bacterial colonies are not obscured. Two inoculating loops are used so that one can be cooling while the other is in use. The loops should be flamed (Fig. 2.23) before starting and then after streaks 1, 2 and 3. The number of times the loops are flamed will depend on the

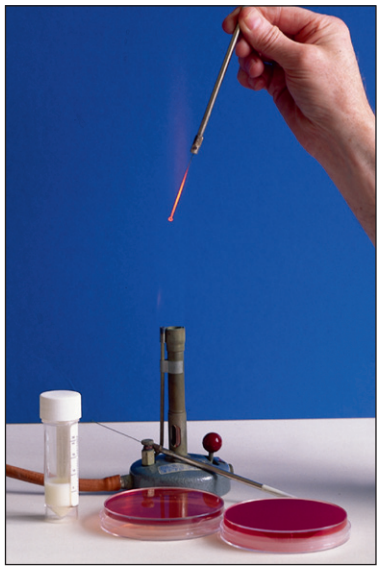

Figure 2.23 Correct technique for flaming an inoculating loop.

Figure 2.24 Plate inoculation technique: streaking culture media.

estimated number of bacteria in the original inoculum. The loop must always be flamed after streak 4 before putting it down. The inoculating loop should be kept as near to parallel to the agar surface as possible to prevent the loop from digging into the agar (Fig. 2.24). Alternatively, disposable plastic loops can be used, a new loop being used after each streak. Streaks 1, 2 and 3 should be kept as close to the edge of the plate as is practical, thus leaving plenty of room for streak 4 where it is hoped to

obtain isolated colonies (Fig. 2.25). This last area of the plate should be fully utilized, keeping the streak lines as close together as possible.

Half-plating or quarter-plating (Fig. 2.26) can be employed where the initial inoculum is judged to contain only a few bacterial cells such as with bovine mastitic milk samples.

Incubation of the Inoculated Culture Plates

As well as the selection of the media, temperature and time of incubation must be considered together with the gaseous atmosphere under which the culture plates should be incubated. These will depend upon the bacterial pathogens that are being sought. The following incubation temperatures, times and atmospheric conditions are offered as general guidelines.

Incubation atmosphere

- Normal atmosphere (aerobic) for most of the pathogenic bacteria and all the fungi

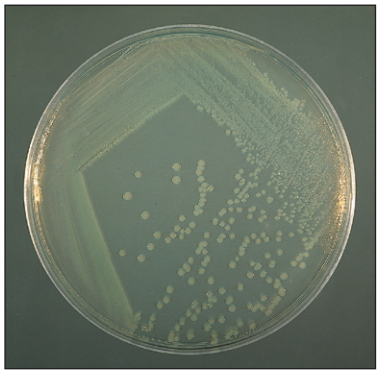

Figure 2.25 MacConkey agar streaked with *Enterobacter aerogenes* showing isolated colonies (see area 4, Figure 2.22).

- 5–10% CO_2:
  - *Actinobacillus pleuropneumoniae*
  - *Actinomyces viscosus*
  - *Brucella* species
  - *Campylobacter jejuni* and *C. fetus* (optimally 6% O_2, 10% CO_2, 84% N_2)
  - *Dermatophilus congolensis* (primary isolation)
  - *Francisella tularensis*
  - *Haemophilus* species
  - *Histophilus* species
  - *Taylorella equigenitalis*
- Anaerobic:
  - *Actinomyces bovis*
  - *Bacteroides* species
  - *Clostridium* species
  - *Actinobaculum suis*
  - *Fusobacterium* species
  - *Peptoniphilus* species
  - *Brachyspira hyodysenteriae.*

Incubation temperature

- 37°C: Most of the microorganisms pathogenic for animals including the mycoplasmas and fungi that cause systemic mycoses
- 42°C:
  - *Campylobacter jejuni*
  - *Brachyspira hyodysenteriae*
- 28°C–30°C *Leptospira* serovars
- 25°C: The dermatophytes except *Trichophyton verrucosum* that tolerates 37°C
- 4°C: For cold enrichment of *Listeria monocytogenes* (from brain samples) and *Yersinia enterocolitica* and *Y. pseudotuberculosis* (from faecal samples).

Incubation time

- 24–48 hours: most of the rapidly growing bacteria
- 48–72 hours: the rapidly growing bacteria when plated on selective media

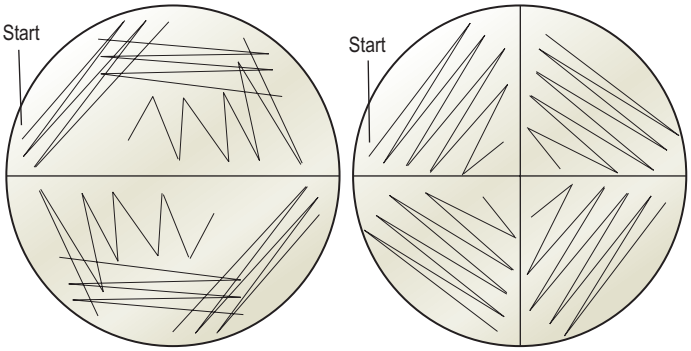

Figure 2.26 Half-plating and quarter-plating on agar media. These techniques should be used only if the sample is likely to contain small numbers of bacteria or a single bacterial species, such as mastitic milk samples.

- 4–6 days:
  - *Brucella* species
  - *Campylobacter* species
  - *Nocardia asteroides*
  - *Mycoplasma* species
  - Atypical mycobacteria
  - The relatively fast-growing fungi.
- 2–3 weeks: most of the dermatophytes (*T. verrucosum* up to five weeks) and *Mycobacterium avium*
- 3–8 weeks: *Mycobacterium bovis*
- 4–16 weeks: *Mycobacterium avium* subspecies *paratuberculosis*.

Bacteria not yet grown on conventional agar media

These include the rickettsiae, the haemotropic mycoplasmas, *Chlamydophila* species, *Clostridium piliforme, Lawsonia intracellularis* and some mycobacteria. Many of these microorganisms can be cultured in the yolk sac of fertile hen's eggs and/or in selected cell cultures.

Table 2.4 gives the features of some commonly isolated bacteria on routinely used media (blood and MacConkey agars) and their appearance in a Gram-stained smear from the cultures.

Disposal of Culture Plates and Pathological Materials

An important consideration, when setting up a microbiology laboratory, is the safe disposal of culture plates and pathological specimens that may pose a human health hazard. Methods of destroying the pathogens that the materials contain include:

- Placing the materials in an autoclavable bag and sterilizing in an autoclave
- Enclosing the materials in a strong leak-proof plastic bag and burning in an incinerator
- The least satisfactory option is to soak the culture plates and other pathological material in a suitable disinfectant for at least 48 hours before the contents can be safely discarded.

Materials containing pathogenic microorganisms should not leave the laboratory until they have been sterilized.

IDENTIFICATION OF BACTERIAL PATHOGENS

Identification of the bacterial pathogen(s) involved in animal disease essentially depends upon:

- Knowledge of the animal species, clinical signs of disease and/or the type of pathological lesion.
- Examination of stained smears made directly from specimens.
- The growth and colonial characteristics of the bacterial pathogen on:
  - Blood agar:
    - size of the colony
    - morphology of the colony, for example whether it is in a rough or smooth form
    - pigment production (Fig. 2.27)
    - whether haemolytic, and if so the type of haemolysis (Fig. 2.28).
  - Selective/indicator media: these will also demonstrate some biochemical reactions of the bacterium.

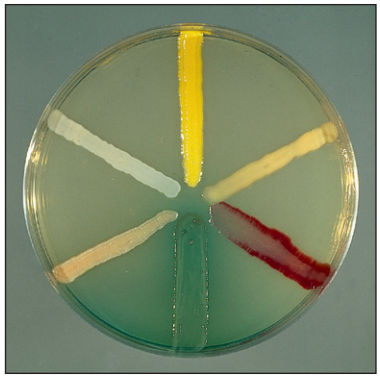

Figure 2.27 Chromogenic bacteria on nutrient agar: clockwise from top, *Micrococcus* sp. (yellow), *Rhodococcus equi, Serratia rubidaea, Pseudomonas aeruginosa* (diffusion of pigment), *Micrococcus roseus* and *Staphylococcus pseudintermedius* (no pigment).

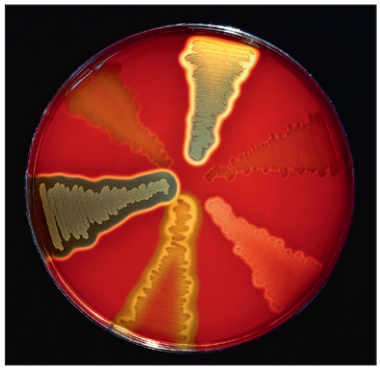

Figure 2.28 Patterns of haemolysis produced by streptococci on sheep blood agar: clockwise from top, beta-haemolytic Group A, *S. uberis* (alpha), *S. agalactiae* (beta), *Enterococcus faecalis* (alpha), beta-haemolytic Group C, *S. pneumoniae* (alpha).

Table 2.4 Features of some commonly isolated bacteria on routine media

Bacterium	Blood agar		MacConkey agar		Gram stain		General comment
	Colony*	Haemolysis	Growth	LF/NLF	Reaction	Shape	
Trueperella (Arcanbacterium) pyogenes	Translucent, pin-point 0.5 mm	+ (hazy)	–		+	R(C)	Hazy haemolysis along streak line often before small colonies can be seen. Very pleomorphic in the Gram-stained smear. Catalase –
Beta-haemolytic streptococci	Translucent, glistening and round 0.5–1.0 mm	+	–		+	C	The size of the clear zone of haemolysis varies with the Lancefield group and species. Catalase –
Listeria monocytogenes	White, smooth and round 0.5–1.0 mm	+	–		+	R(C)	Colonies very similar to the beta-haemolytic streptococci. Young colonies yield many coccal cells. Catalase +
Moraxella bovis	White, smooth and round 0.5–1.5 mm	+ (–)	–		–	R(C)	Colonies very similar to the above two bacteria. Gram – and cells in pairs as rods or fat cocci. Some variants are non-haemolytic
Mannheimia haemolytica	White/grey, smooth and round 0.5–1.5 mm	+	+ (Pin-point)	LF	–	R	Colonies similar to the above three bacteria but are Gram – rods and will tolerate MacConkey agar. Some strains are haemolytic only under the colonies
Enterococcus faecalis	White, smooth and round 0.5–1.0 mm	+ (alpha)	+ (Pin-point)	LF	+	C	Greenish (alpha) haemolysis. Red pinpoint colonies on MacConkey agar, although Gram +
Alpha-haemolytic streptococci	White, smooth and round 0.5–1.0 mm	+ (alpha)	–		+	C	Greenish or partial haemolysis. Not usually pathogenic, can be part of the normal flora
Erysipelothrix rhusiopathiae	White, smooth and round Some strains rough 0.5–1.5 mm	+ (alpha at 48 hours)	–		+	R	Non-haemolytic at 24 hours' incubation but alpha-haemolysis under the colonies occurs at 48 hours. Rough, dry colonies especially from the chronic forms of the disease. Catalase –

Table 2.4 Features of some commonly isolated bacteria on routine media—cont'd

Bacterium	Blood agar		MacConkey agar		Gram stain		General comment
	Colony*	Haemolysis	Growth	LF/NLF	Reaction	Shape	
Staphylococcus aureus, S. pseudintermedius or S. intermedius	White or yellow, smooth round and shiny 2.0–3.0 mm	+ (may be double)	–		+	C	Human and bovine strains of *S. aureus* have a golden–yellow pigment. Hold plate to a bright light to see characteristic double-haemolysis of some strains. Catalase +
Clostridium perfringens	Grey, flat and often irregular edge 2.0–3.0 mm	+ (double)	–		+	R	Anaerobic with double or target haemolysis. Colonies tend to have irregular edges
Escherichia coli	Grey, smooth, shiny and round 2.0–3.0 mm	V	+	LF	–	R	Characteristic 'coliform' smell. Bright pink colonies on MacConkey agar about the same size as on blood agar
Aeromonas hydrophila	Grey, flat, round and shiny 2.0–3.0 mm	+	+	V	–	R	Foul smell is characteristic but differs from that of *E. coli.* Variable lactose fermentation but good growth on MacConkey agar
Pseudomonas aeruginosa	Blue-green, flat, round. Some have metallic sheen 2.5–4.0 mm	+	+	NLF	–	R	Amount of pyocyanin (blue-green pigment) varies between strains. Characteristic fruity-musty smell
Bacillus species	Grey, dry, granular with irregular edges 3.0–5.0 mm	+ (–)	–		+	R (spores)	Many haemolytic (exception *B. anthracis*). Dry colonies as no capsular material produced on lab media. Rhizoid (*B. mycoides*) and other unusual colony-types
Corynebacterium pseudo-tuberculosis	Opaque, dry, crumbling 0.5–1.0 mm	V	–		+	R	Haemolysis variable. The cells tend to be less pleomorphic than *Trueperella pyogenes*
Corynebacterium renale	Grey-white, round and moist 0.5–1.0 mm	–	–		+	R	Small, moist colonies at 24–48 hours' incubation, become drier later. Urease +

Continued

Table 2.4 Features of some commonly isolated bacteria on routine media—cont'd

Bacterium	Blood agar		MacConkey agar		Gram stain		General comment
	Colony*	Haemolysis	Growth	LF/NLF	Reaction	Shape	
Brucella species	Translucent, convex and round 0.5 mm	–	–		–	R(C)	Some brucellae require 10% CO_2 for growth. Colonies not visible until two to three days' incubation. MZN +
Campylobacter fetus	Small, delicate, round and opaque 0.5 mm	–	–		–	R curved	Small 'dew-drop' colonies after two to three days' incubation. Requires reduced oxygen tension for growth. Curved rods, if in pairs they have a 'seagull' appearance
Actinobacillus species	Grey to translucent, shiny and round 0.5–1.0 mm (not all species will grow on blood agar)	–	V	V	–	R	Small, round, non-haemolytic colonies. Variable growth on MacConkey agar
Pasteurella multocida	Translucent, smooth, round and shiny 1.0–2.0 mm	–	–		–	R	Translucent, round colonies that can appear pinkish on blood agar. Characteristic sweetish smell. Indole +
Bordetella bronchiseptica	Small, greyish-white and round 0.5–2.0 mm	–	+	NLF	–	R	Colonies small at 24 hours, becoming considerably larger later. Grows on MacConkey. Unreactive bacterium
Rhodococcus equi	Salmon pink and mucoid. Colonies coalesce 1.0–2.0 mm	–	–		+	R(C)	Pinkish-tan colonies, the colour becoming more definite with increased time. Mucoid colonies tend to merge
Staphylococcus epidermidis (coagulase –)	White, shiny, round and convex 2.0–3.0 mm	–	–		+	C	Colonies similar to coagulase + staphylococci but are non-haemolytic and always white
Micrococcus species	White, yellow tan or pink. Round, convex and shiny 2.0–3.0 mm	–	–		+	C	Shiny convex colonies, can be white but are often pigmented. Not considered pathogenic

Table 2.4 Features of some commonly isolated bacteria on routine media—cont'd

Bacterium	Blood agar		MacConkey agar		Gram stain		General comment
	Colony*	Haemolysis	Growth	LF/NLF	Reaction	Shape	
Salmonella species	Greyish, round and shiny 2.0–3.0 mm	–	+	NLF	–	R	Pale colonies on MacConkey agar. No smell (unlike most of the other members of the *Enterobacteriaceae*)
Yersinia species	Greyish, round and shiny 2.0–3.0 mm	–	+	NLF	–	R	Medium, round, smooth, non-haemolytic colonies. Non-lactose fermenter
Serratia marcescens and *S. rubidaea*	Red/orange, convex, round and shiny. Some strains white at 37°C 2.0–3.0 mm	–	+	NLF	–	R	Produce red pigment (prodigiosin). Some strains do not produce the pigment at 37°C
Klebsiella species	Grey, mucoid colonies that coalesce. 2.0–4.0 mm	–	+	LF	–	R	Colonies tend to be large, mucoid and pale pink on MacConkey agar. Non-motile
Enterobacter species	Grey, mucoid colonies that coalesce 2.0–4.0 mm	–	+	LF	–	R	Some isolates may appear very similar to colonies of *Klebsiella* species. Motile
Proteus species	Grey, swarming growth over the agar. Swarming can be in waves	–	+	NLF	–	R	Characteristic swarming growth on non-selective medium. Turns blood agar brown. Very foul smell. Colonies pale and discreet on MacConkey but edges may be irregular
Pseudomonas species other than *P. aeruginosa*	Grey or yellowish-green, flat and spreading. 2.5–4.0 mm	–	+	NLF	+	R	Large, flat colonies. Some may produce the yellowish-green pigment, pyoverdin

+ = Positive; – = Negative; V = Variable reaction; LF = Lactose-fermenter; NLF = Non-lactose fermenter; R = Rod; C = Coccus

* Size of colonies can be variable. Size is given and described after 48 hours' incubation, as even with fast-growing bacteria the colonies are more characteristic at this stage.

- The atmospheric conditions needed for growth can aid in the identification of the bacterium.
- Biochemical and other tests carried out on a pure culture of the suspected pathogen to confirm its identity.

Pure Culture Technique

Bacterial contaminants, representing those microorganisms from the normal flora, external environment or from post mortem invasion may be present on the plates together with the pathogen of interest. Subculture of the

bacterial colony type(s) considered most significant should be made to obtain pure cultures for use in identification tests. To obtain a pure culture, the top of two or three similar and isolated colonies should be touched with a sterile inoculating loop from the blood agar plate. Another blood agar plate and a MacConkey agar plate should be streaked out with the colonial growth. It is preferable to select colonies from a non-selective medium, such as blood agar because suppressed microcolonies of other bacteria on selective media could inadvertently be collected together with the colony of interest.

Primary Identification of Bacteria

Once a pure culture is obtained, the results from a few comparatively simple tests can often identify the bacterium to a generic level:

- A Gram-stained smear from the culture will establish:
  - the Gram reaction (Gram-positive or Gram-negative)
  - the colonial morphology (coccus or rod)
- Growth or absence of growth on MacConkey agar
- Catalase and oxidase tests
- Motility test
- An oxidation-fermentation (O-F) test in Hugh and Leifson's medium.

Gram reaction

Provided that the Gram-stained smear has been made from a pure culture, if any cells have retained the crystal violet–iodine complex then the bacterium is regarded as being Gram-positive. It is not unusual for some of the cells to decolourize, particularly older cells or those producing endospores. In some of the *Bacillus* species, such as *B. circulans*, all of the cells usually decolourize. Endospores in a Gram-stain are seen as unstained areas within the mother cell. The production of endospores could indicate that the bacterium is a *Bacillus* (aerobic) or a *Clostridium* (anaerobic) species.

Other tests to distinguish between Gram-positive and Gram-negative bacteria

If the results of the Gram stain are equivocal, other tests are available to aid in the differentiation of Gram-positive and Gram-negative bacteria.

LANA test

A swab is impregnated with L-alanine-4-nitroanilide (LANA). A colony is touched with the swab and the swab will turn yellow if the bacterium is Gram-negative (Fig. 2.29).

Figure 2.29 LANA test: Gram-positive (left) no reaction and Gram-negative (right) positive.

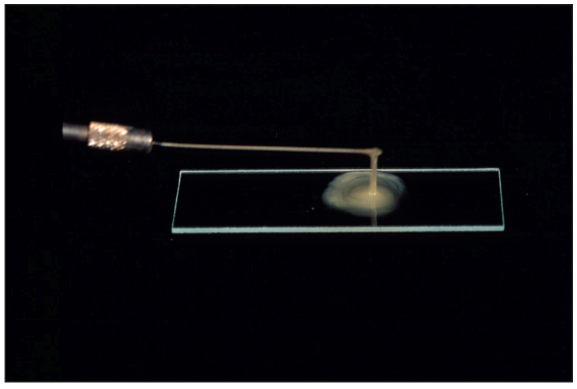

Figure 2.30 KOH test: showing a viscous gel formed only by Gram-negative bacteria.

KOH test

A loopful of the bacterium is taken from a non-selective medium (blood agar) and mixed with an equal amount of 3% potassium hydroxide (KOH) on a clean microscope slide. After thorough mixing the loop is lifted at intervals to see whether a gel is forming. If the bacterium is Gram-negative a viscous gel forms within 60 seconds while no gel is formed if the bacterium is Gram-positive (Fig. 2.30).

Susceptibility to vancomycin

The majority of Gram-positive bacteria (except for some lactobacilli and some enterococci) are susceptible to vancomycin whereas all Gram-negative bacteria are resistant (Fig. 2.31).

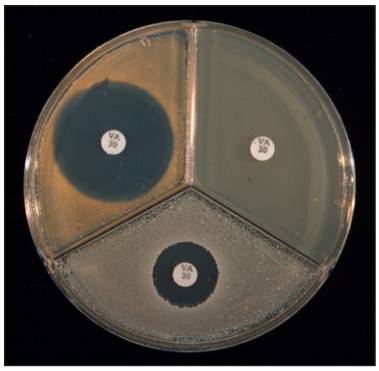

Figure 2.31 Differentiation of Gram-positive and Gram-negative bacteria using the susceptibility of most Gram-positive bacteria to vancomycin: *Rhodococcus equi* (left), *Escherichia coli* (right) and *Staphylococcus aureus* (bottom).

Cellular morphology (shape)

In this primary identification bacteria are regarded as being either a coccus or a rod, filamentous bacteria being viewed as elongated rods. Pleomorphic bacteria such as *Trueperella* (*Arcanobacterium*) *pyogenes* and *Rhodococcus equi* can appear coccal but there are usually a few rod-shaped cells present in the Gram-stained smear, and they are classified as rods for purposes of identification. *Listeria monocytogenes* also tends to produce many coccal forms especially from young cultures.

Growth or no-growth on McConkey agar

Commercial firms may prepare more than one type of MacConkey agar. These differ in the composition of the bile salts and as to whether or not crystal violet (anti-Gram-positive) is present. It is advisable to use a MacConkey agar that inhibits the majority of Gram-positive bacteria, will support the growth of all members of the *Enterobacteriaceae* but is selectively inhibitory to the other Gram-negative bacteria.

Catalase test

This test detects the enzyme catalase that converts hydrogen peroxide to water and gaseous oxygen. The reagent, 3% hydrogen peroxide, should be stored at +4 °C in a dark bottle. Extra care must be taken if the bacterium has been grown on blood agar, because the presence of red blood cells can lead to a false-positive reaction.

- **Method 1:** A loopful of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% hydrogen peroxide is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction (Fig. 2.32).

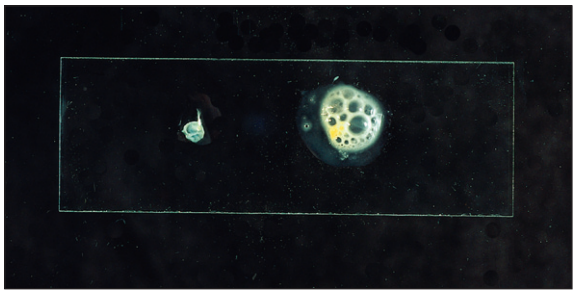

Figure 2.32 Catalase test on a slide: negative (left) and positive (right).

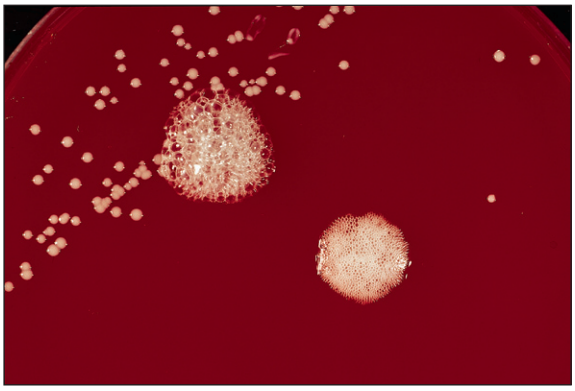

Figure 2.33 Catalase test on blood agar: strong positive reaction (left) from colonies and weaker reaction (right) from blood agar.

- **Method 2:** A drop of 3% hydrogen peroxide is added to a colony on the plate and another drop to an area of the blood agar plate without bacterial growth. Bubbles of oxygen gas will arise from the blood agar but greater gas production from the bacterial colony indicates a positive test (Fig. 2.33).

Oxidase test

This test depends on the presence of cytochrome c oxidase in a bacterial cell. Anaerobes are oxidase-negative. The reagents used in the tests should be colourless and be stored in a dark bottle at 4 °C. The solutions must not be used if they become dark blue. Auto-oxidation of the reagents may be retarded by the addition of 1% ascorbic acid. Other precautions include testing colonies on non-selective media that do not contain glucose or nitrate and the use of a glass rod, platinum loop or disposable plastic loop to pick up the bacterial growth. A conventional nichrome loop contains iron and if used, can result in a false-positive test. Several methods can be used for the oxidase test:

- **Method 1:** A piece of filter paper is moistened in a Petri dish with 1% aqueous solution of

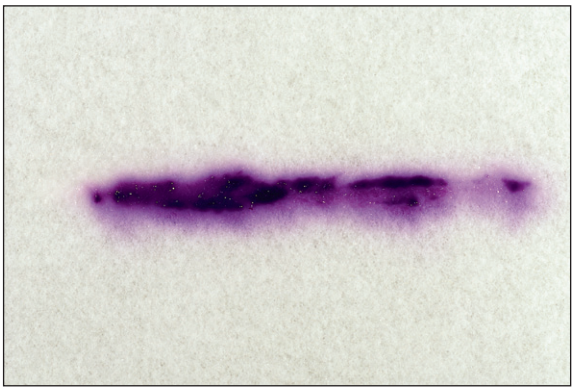

Figure 2.34 Oxidase test: filter paper method. A purple colour within 10 seconds indicates a positive reaction.

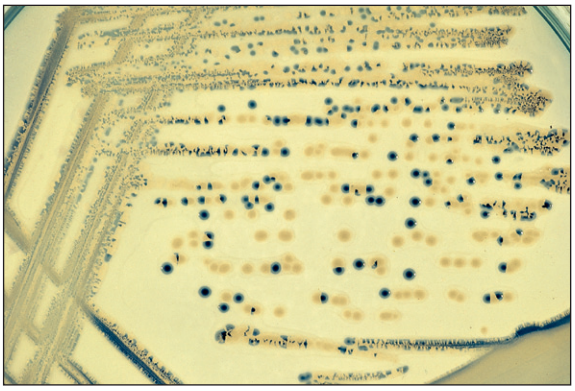

Figure 2.35 Oxidase test on agar medium: blue colonies are oxidase-positive.

tetramethyl-p-phenylenediamine dihydrochloride. The test bacterium is streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line within 10 seconds indicates a positive reaction (Fig. 2.34). *Pseudomonas aeruginosa* can be used as a positive control organism.

- **Method 2:** Equal volumes of 1% alpha-naphthol in 95% ethanol and 1% aqueous solution of p-aminodimethylaniline oxalate are mixed. A drop of the mixture is placed on the surface of a few colonies on a blood agar plate. Colonies developing a blue colour within 10–30 seconds is interpreted as a positive reaction (Fig. 2.35). Weak reactions occurring after two minutes are discounted. The colonies giving a positive reaction are viable, if subcultured within a few minutes of reading the test.
- **Method 3:** Commercial oxidase paper strips and oxidase sticks are available. The tests should be carried out according to the manufacturers' instructions.

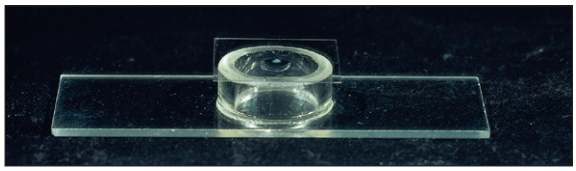

Figure 2.36 The hanging-drop method for detection of motility.

Motility tests

The majority of bacteria are motile by means of flagella. Motility can be temperature-dependent and some bacteria tend to be motile at ambient temperatures but not at 37°C. Two main methods are used to demonstrate motility:

- **Method 1:** Direct microscopy using a young broth culture (2–4 hours' incubation) of the bacterium incubated at room temperature. A 'hanging-drop' preparation is made by placing a drop of the broth culture in the centre of a clean cover slip and then inverting it over a plastic or glass ring (about 5 mm deep) fixed to a microscope slide (Fig. 2.36). The preparation is brought into focus under low-power and then examined with the high-power dry objective with reduced illumination. The bacterium is motile if individual cells are moving towards and away from other cells. Brownian movement is a constant and random jiggling of all bacterial cells and other small particles and must not be mistaken for true motility. If the bacterium appears to be non-motile by direct microscopy, then this negative result should be checked by the inoculation of motility media.
- **Method 2:** Semisolid motility media are available commercially. Tetrazolium salts can be added to these media to aid in the detection of motility. Before autoclaving the motility medium, 0.05 g of 2,3,5-triphenyltetrazolium chloride (TTC) is added per litre of medium. Tetrazolium salts are colourless but as the bacterium grows the dye is incorporated into the bacterial cells where it is reduced to an insoluble red pigment, formazan. The red colour forms only in the area of medium where the bacterium is growing (Fig. 2.37).
  - Motility media are prepared in test tubes. Two tubes of the medium are stab-inoculated using a straight wire. One tube is incubated at room temperature and the other at 37°C. The tubes are examined for motility after 24 and 48 hours. Motile bacteria migrate through the semisolid medium which becomes turbid. If TTC has been

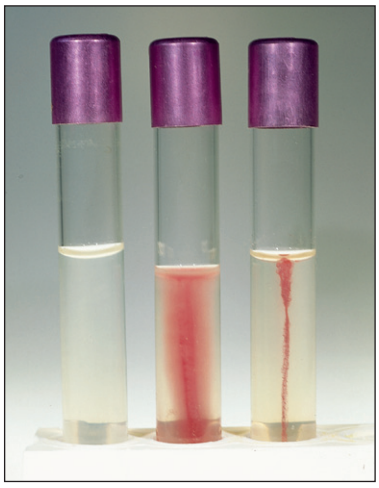

Figure 2.37 Motility medium (semisolid) with TTC indicator; from left, uninoculated, motile and non-motile bacteria.

incorporated into the medium, the motility is demonstrated by a red colour throughout the agar. The growth of a non-motile bacterium is confined to the stab line.

- Sulphide indole motility (SIM) medium can be used to detect motility and will also indicate indole and hydrogen sulphide production.

Oxidation-fermentation (O-F) test

This test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium. The medium is semisolid and usually contains glucose as the test sugar and bromothymol blue as the pH indicator. The uninoculated medium (pH 7.1) is green and if acid is produced by the bacterium, following metabolism of the glucose, the medium becomes yellow (pH 6.0). Bacteria that can metabolize glucose under either aerobic or anaerobic conditions are facultative anaerobes and in this test are said to be fermentative. The aerobes require atmospheric oxygen for growth and metabolism and are called oxidative. Some bacteria are unreactive in the conventional O-F medium, either because they are unable to grow in the basal medium or because they cannot utilize glucose.

Two tubes of the O-F medium are heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen. The tubes are then cooled rapidly under cold running water. Both tubes are stab inoculated with the bacterium. A layer of sterile paraffin oil is layered on top of one of the tubes (sealed tube) to a depth of about 1 cm. The inoculated tubes are incubated at 37°C and examined in 24 hours and then daily for up to 14 days (Fig. 2.38).

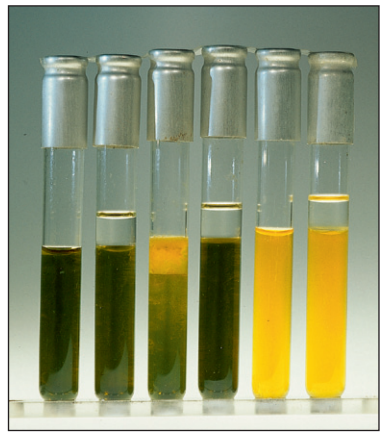

Figure 2.38 Oxidation-fermentation (O-F) test: results from left, unreactive (tubes 1 and 2); oxidation (tubes 3 and 4) and fermentation (tubes 5 and 6).

O-F test reactions

Results	Open tube	Sealed tube	Examples of bacteria
Oxidation	Yellow	Green	*Pseudomonas* spp.
Fermentation	Yellow	Yellow	*Aeromonas* spp.
Unreactive	Green	Green	*Bordetella* spp.

The conventional O-F medium is most suitable for non-fastidious Gram-negative bacteria. Modifications can be made to the medium to test for:

- Fastidious bacteria unable to grow in the medium. In this case the basal medium can be enriched with 2% serum and/or 0.1% yeast extract.
- Staphylococci and micrococci. The formula for the staphylococcal O-F test medium is:
  - Pancreatic digest of casein (tryptone) 10.0 g
  - Yeast extract 1.0 g
  - Agar 2.0 g
  - Bromocresol purple 0.001 g
  - Distilled water
  - 1000 mL

The ingredients are heated gently to dissolve them and autoclaved at 121°C for 15 minutes. The medium is cooled to 50°C and sterile solutions of glucose and mannitol are added to a final concentration of 1% for each sugar. Bromocresol purple is purple at pH 6.8 and yellow at pH 5.2.

Figures 2.39 and 2.40 give the primary identification of Gram-positive and Gram-negative bacteria, respectively, to a generic level on the basis of the previously described tests.

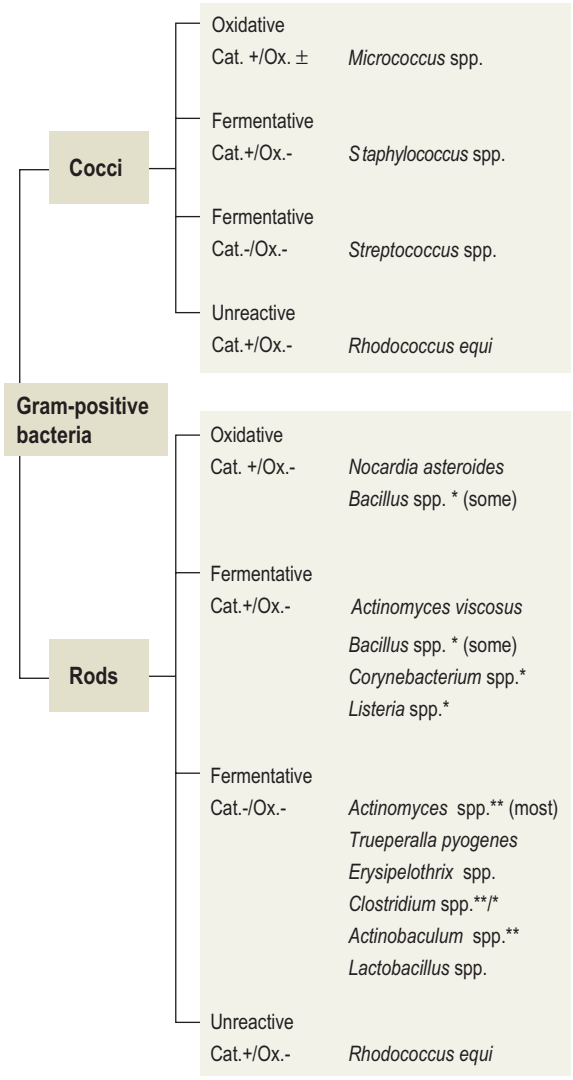

Figure 2.39 Primary identification of Gram-positive bacteria. (Cat. = catalase; Ox. = oxidase; + = positive reaction; – = negative reaction; ± variable; * = motile; ** = anaerobic)

Secondary Biochemical Tests for the Identification of Bacteria

Once the bacterium has been identified to a generic level, further tests can be carried out to identify the species of the bacterium. Secondary biochemical tests can be grouped in the following categories:

- Commercial medium, prepared in tubes, incorporating several biochemical tests.
- Conventional biochemical tests usually carried out in small bottles or tubes. These are summarized in Table 2.5 and additional methods of identification are given in Section 2 under the appropriate bacterial genus.
- Miniaturized methods that are available commercially and use small amounts of media in small chambers.
- Automated microbiology systems. These are beyond the scope of this book.

Commercial media incorporating several biochemical tests

- Kligler's iron agar contains two carbohydrates (sugars), glucose 0.1% and lactose 1.0% together with chemicals that indicate hydrogen sulphide production. The medium is similar to, and largely superseded by, triple sugar iron agar.
- Triple sugar iron (TSI) agar has three sugars, glucose 0.1%, lactose 1.0% and sucrose (occasionally saccharose) 1.0%. Phenol red is the pH indicator and ferrous sulphate or ferric ammonium citrate with sodium thiosulphate indicates hydrogen sulphide production. The medium is poured into test tubes and these are sloped before the agar sets to form slants. The medium is red (pH ≥ 7.3) if uninoculated or when an alkaline reaction occurs, and yellow (pH ≤ 6.8) if an acid reaction occurs (Fig. 2.41). TSI agar is essentially for the presumptive identification of *Salmonella* serotypes but is also useful for the differentiation of other members of the *Enterobacteriaceae*. This differentiation is enhanced if a tube of lysine decarboxylase broth (Fig. 2.42) is inoculated in parallel with the tube of TSI agar.
- **Lysine iron agar:** a solid medium, dispensed in tubes, for the detection of the decarboxylation of lysine and the production of hydrogen sulphide.
- **SIM medium:** a composite medium for the determination of hydrogen sulphide and indole production and motility (Fig. 2.43). It is used mainly for the *Enterobacteriaceae*.
- **MIO medium**: this medium incorporates tests for motility, indole production and the decarboxylation of ornithine by members of the *Enterobacteriaceae*.

Inoculation of TSI agar and lysine decarboxylase broth

One isolated colony characteristic for *Salmonella* species from the selective/indicator media (e.g. XLD, brilliant green or MacConkey agars) is touched with a straight inoculating wire. A tube of TSI agar is stab inoculated in the middle of the agar to within 5 mm from the bottom of the tube. On the withdrawal of the straight wire, the entire slant is streaked (right to the top). The wire will still have sufficient bacterial cells to inoculate the tube of lysine broth. Both tubes are incubated at 37°C for 18 hours with a loose cap on the TSI agar. This is essential for the correct

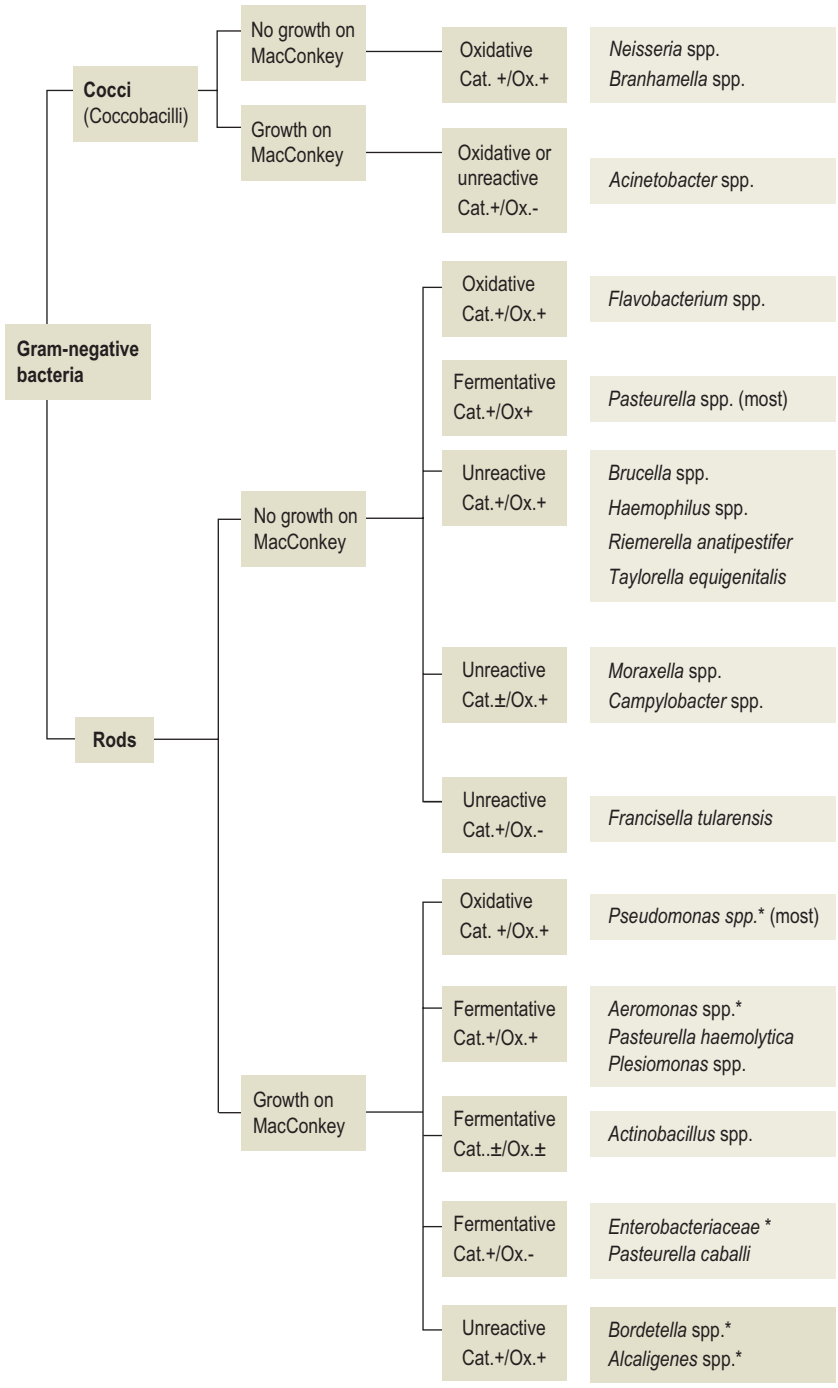

Figure 2.40 Primary identification of Gram-negative bacteria. (Cat. = catalase; Ox. = oxidase; + = positive reaction; – = negative reaction; ± variable; * = motile)

Table 2.5 Summary of some commonly used biochemical tests for the identification of bacteria

Test	Medium*	Incubation (aerobic)	Product tested for	Test reagent	Result	
					Negative (uninoculated)	**Positive**
Aesculin (esculin) hydrolysis	1. Aesculin broth: Aesculin 1 g, Peptone water 1000 mL, Ferric citrate 0.5 g. Autoclave at 115°C for 10 minutes (Fig. 2.44)	Up to 7 days at 37°C	Aesculin ↓↓ Aesculetin + iron salt ↓↓ Dark brown complex		Medium unaltered	Dark brown to black broth
	2. Aesculin agar e.g Edwards medium (Fig. 8.17)	2–3 days at 37°C		Wood's lamp (medium glows blue)	No browning of colonies (*Streptococcus agalactiae*)	Dark brown colonies and medium (*S. uberis*)
Carbohydrate fermentations				pH indicators:		Acid produced:
1. Peptone water sugars (non-fastidious bacteria)	1% sugar in peptone water (Fig. 2.45)	24–48 hours at 37°C	Enzymatic attack on sugar with acid and gas (Durham tube) production	Phenol red Andrade's	Red (pH 7.4) Pale yellow (pH 7.2)	Yellow (pH 6.0) Pink (pH 5.5)
2. CTA medium (fastidious bacteria)	Semisolid medium with crystine and trypticase. Carbohydrate discs placed in medium (Fig. 2.46)	18–48 hours at 37°C	Acid production and pH change	Phenol red	Red	Yellow
3. Solid agar medium such as purple agar base	1% sugar incorporated in solid agar medium (Fig 7.13)	24–48 hours at 37°C	Acid production and pH change	Bromocresol purple	Purple	Yellow colonies and medium
Citrate utilization	1. Koser citrate (liquid). Inoculate from broth with straight wire (Fig. 2.47)	Up to seven days at 37°C	Ability to use citrate as sole carbon source		No growth	Growth (turbidity)
	2. Simmons citrate. Inoculate solid agar slant (Fig. 2.48)	Up to seven days at 37°C	As above	Bromothymol blue	Green (pH 6.9) (*E. coli*)	Blue (pH 7.6) (*Salmonella* spp).
Coagulated serum slant (Loeffler)	Heavy inoculum of suspect *Trueperella pyogenes* as spot in centre of slant (Fig. 10.15)	One to three days at 37°C	Proteolytic action on coagulated serum		Slant unaltered (*Corynebacterium* spp).	Pitting of slant (*Trueperella pyogenes*)

Decarboxylase (lysine and ornithine) and dehydrolase (arginine) tests	*Broth base** with: 0.5% L-arginine or 0.5% L-lysine or 0.5% L-ornithine. + 0.1% glucose (Fig. 2.42)	Up to four days at 37°C	Arginine and ornithine to putrescine. Lysine to cadaverine. Products are alkaline	Bromocresol purple	Yellow (acid) Glucose only attacked. (*Proteus* spp.)	Purple (alkaline) (*Salmonella* spp. (most))
	Tubed agar media: Lysine iron agar. MIO medium (ornithine)		Many bacteria first ferment the glucose with acid (yellow). If they can then attack the amino acid, the medium reverts to alkaline (purple)			
Gelatin liquefaction	1. Nutrient gelatin. Stab inoculation (Fig. 2.49)	22°C for 30 days or 37°C for 14 days	Proteolytic activity (gelatinases) and gelatin liquefied		No liquefaction	Liquefaction. (Not solid at +4°C)
	2. Charcoal gelatin discs placed in a broth. (Discs do not liquefy at 37°C) (Fig. 2.50)	37°C for up to 14 days	As above	Charcoal particles are released when the gelatin is liquefied	No change	Free charcoal particles
	3. X-ray film method. Small strip in heavy inoculum of bacteria in trypticase soy broth (Fig. 2.51)	37°C for 48 hours	As above	Gelatin layer on X-ray film strip	No change (*Enterobacter* spp.)	Removal of gelatin layer leaving pale blue plastic film. (*Serratia marcescens*)
Hippurate hydrolysis	Sodium hippurate 10 g Heart infusion broth 1 litre Sterilize at 11.5°C for 20 minutes (Fig. 2.52)	24 hours at 37°C with an uninoculated control	Hippurate hydrolyzed to benzoic acid and glycine	Centrifuge test. Add 0.2 mL ferric chloride reagent to 0.8 mL of the supernatant*	No precipitate. (*Actinobacillus equuli* and *Streptococcus pyogenes*)	Permanent preciptate. (*Actinobacillus lignieresii, Streptococcus agalactiae*)
Hydrogen sulphide	1. Iron salts in media. e.g. TSI** and SIM*** agar (least sensitive test) (Figs 2.41, 2.43)	16 hours at 37°C	Hydrogen sulphide gas production		No change (*E. coli*)	Blackening of the medium. (*Salmonella* spp.)
	2. Bismuth sulphite agar (plate medium) (Fig. 2.54)	48 hours at 37°C	As above		No blackening	Blackening of colonies and media

Continued

Table 2.5 Summary of some commonly used biochemical tests for the identification of bacteria—cont'd

Test	Medium*	Incubation (aerobic)	Product tested for	Test reagent	Result	
					Negative (uninoculated)	**Positive**
Hydrogen sulphide *(continued)*	3. Lead acetate paper strip (most sensitive method).* Strip suspended over trypticase soy broth or serum glucose agar slants (*Brucella*) (Fig. 2.53)	35°C for up to seven days. Change lead acetate strip daily	Hydrogen sulphide gas production		No change on lead acetate strip	Blackening of the lead acetate strip. (*Brucella* spp.)
Indole test	1. Tryptone water (Fig. 2.55)	One to two days at 30°C	Tryptophan split to indole	Add 0.5 mL Kovac's* reagent to medium and shake. Read in 1 minute	Reagent layer yellow. (*Salmonella* spp.)	Reagent layer deep red. (*E. coli*)
	2. SIM medium in tubes (Fig. 2.43)	18–24 hours at 37°C	As above	a) Kovac's reagent (0.2ml) to tube. Stand for 10 mins b) Oxalic acid test paper* suspended over medium	No change in reagent colour No change in test strip	Reagent dark red Pink colour at lower end of paper
	3. Spot test for indole	24–48 hours at 37°C on blood agar or nutrient agar	As above	Filter paper saturated with Kovac's reagent. Rub colony over filter paper with a glass rod	No reaction. (*Mannheimia haemolytica*)	Blue colour on streak within 30 seconds. (*P. multocida*)
Malonate utilization	Malonate broth. (0.3% sodium malonate) (Fig. 2.56)	24 hours at 37°C	Utilization of malonate as sole source of carbon	Bromothymol blue	No change. (*Salmonella* spp. (most))	Growth and a deep blue colour. (*S. enterica* subsp. *arizonae*)

Methyl red (MR) test	Glucose phosphate peptone water (5 ml) (MR–VP broth) (Fig. 2.57)	Two days at 37°C or three to five days at 30°C	Small molecular weight acids such as formic and acetic	MR reagent*: 5 drops to medium	Yellowish (*Enterobacter cloacae*)	Red (acid) pH 4.4–6.0 (*E. coli*)
Nitrate reduction	1. Nitrate broth (0.1% KNO_3): 5 mL (Fig. 2.59)	24 hours at 37°C. (rarely up to five days)	Nitrate (NO_3) ↓↓ Nitrite (NO_2) ↓↓ Nitrogen gas (N_2)	Reagents for nitrite (A and B)* a. 0.5% alpha-naphthylamine in 5N acetic acid. b. 0.8% sulphanilic acid in 5N acetic acid. Add five drops of each reagent. Shake and wait one to two minutes	Colourless (no nitrite present) Add pinch of Zn dust. ↓↓ NO_3 converted to NO_2 ↓↓ Red (NO_3 not reduced)	Red (NO_3 to NO_2). Colourless (NO_3 reduced to N_2)
	2. KNO_3(40%) on filter paper*. Dry and sterilize. Place on blood agar and stab inoculate test bacterium 20 mm from paper strip. Use heavy inoculum and *E. coli* as a positive control (Fig. 2.60)	37°C and examine at four and 24 hours	Nitrate reduction		No reaction or very narrow zone of browning around inoculum	Wide zone of browning of medium between colony and strip. (*E. coli*)
ONPG test	Peptone water + 0.15% o-nitrophenyl-beta-D-galactopyranoside	24 hours at 37°C	The enzyme beta-galactosidase. Identifies potential lactose fermenters		Colourless after 24 hours. (*Salmonella* spp. (most))	Yellow colour. (*S. enterica* subsp. *arizonae*)
Phenylalanine deaminase test	Phenylalanine medium (BBL) (0.2% DL-phenylalanine) Tube with a slant. (Fig. 2.61)	Inoculate heavily. 35°C for four or 18–24 hours	Phenyl pyruvic acid formed. Acid reaction	10% aqueous ferric chloride. Add four to five drops direct to slant. Rotate and read in one to five minutes	Yellowish.	Green colour reaction in slant. (*Proteus, Morganella* and *Providencia* spp.)

Continued

Table 2.5 Summary of some commonly used biochemical tests for the identification of bacteria—cont'd

Test	Medium*	Incubation (aerobic)	Product tested for	Test reagent	Result	
					Negative (uninoculated)	Positive
Phosphatase test	Nutrient agar + 0.01% phenolphthalein diphosphate (Fig. 2.62)	18–24 hours at 37°C	Sufficient phosphatase to split phenolphthalein diphosphate	Ammonia vapour. Hold colonies on the agar plate over an open bottle of ammonia	Unchanged colonies. (Coagulase –ve staphylococci)	Colonies bright pink. (Coagulase +ve staphylococci)
Urease tests	1. Christensen media: a. Urea agar base + 2% urea (slant). b. Urea broth base + 2% urea. Use a heavy inoculum (Fig. 2.63)	Up to 24 hours at 37°C	Urease: splits urea with formation of ammonia (alkaline)	Phenol red	Yellow (*Salmonella* spp.)	Red (alkaline) (*Proteus* spp.)
	2. Spot test: moisten filter paper with a few drops of 10% urea agar base concentrate. Rub some culture onto the paper with a glass rod)		As above	As above	No change	Pink or red streak within two minutes
Voges–Proskauer (VP) test	5 mL glucose phosphate peptone water. (MR–VP broth) (Fig. 2.58)	Three to five days at 30°C	Acetoin derived from glucose	3 mL of 5% alphanaphthol in absolute ethyl alcohol and then 1 mL of 40% KOH. Shake and leave for five minutes	Colourless (*E. coli*)	Red (*Entero-bacter* spp. (most))

* = further details of medium or reagent given in Appendix 2.

** = Triple sugar iron agar

*** = Sulphide indole motility agar

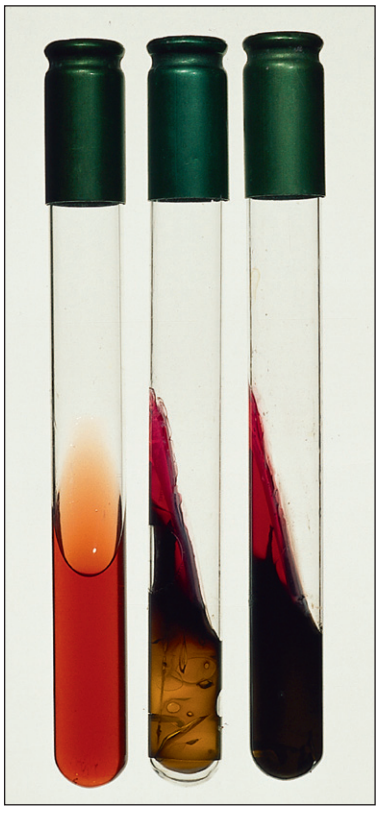

Figure 2.41 Triple sugar iron agar slope: from left, uninoculated, *Salmonella* sp. after 16 and 24 hours' incubation.

Figure 2.42 Lysine decarboxylase broth: from left, uninoculated, negative and positive reactions.

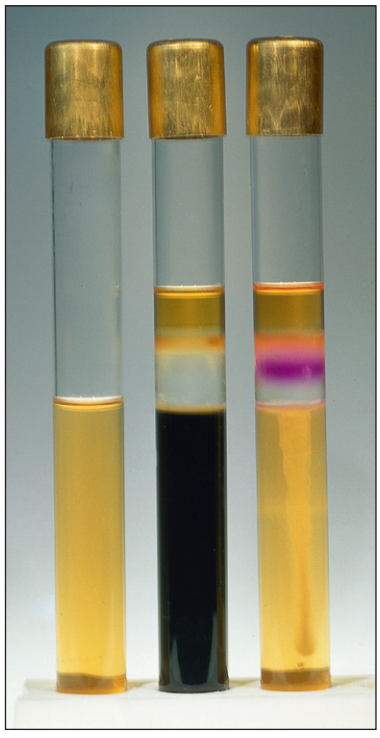

Figure 2.43 SIM (sulphide indole motility) agar: from left, uninoculated, H_2S+/Indole–/Motility+ and H_2S-/Indole+/motility–. This medium (like TSI) uses iron salts to detect H_2S production.

reaction to occur in the medium. The media are incubated at 37°C for 16 hours. If the incubation period is prolonged, the black colouration due to H_2S production tends to obscure the colour of the butt (Fig. 2.41). The biochemical reactions in lysine decarboxylase broth (Fig. 2.42) are summarized in Table 2.5. The biochemical reactions that occur in TSI agar are discussed in Chapter 17 (*Enterobacteriaceae*), the general interpretation of these reactions are as follows:

- Red (alkaline) slant and yellow (acid) butt: glucose fermentation only.
- Yellow (acid) slant and yellow (acid) butt: lactose and/or sucrose used as well as glucose.
- Blackening of the medium: hydrogen sulphide production.

Most *Salmonella* species give a red slant, yellow butt and produce H_2S when inoculated into TSI agar. The notation for these reactions is R/Y/H_2S+.

Conventional biochemical tests

A summary of some of the commonly used conventional biochemical tests is given in Table 2.5 and illustrated (Figs 2.44 to 2.63, inclusive). These tests are described and illustrated because although many laboratories commonly

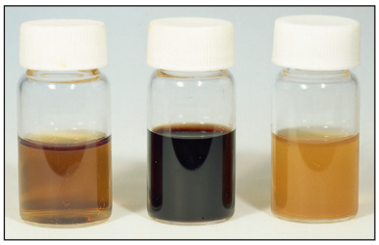

Figure 2.44 Aesculin hydrolysis: from left, uninoculated, positive and negative.

Figure 2.45 Peptone water 'sugars' (phenol red indicator) with a Durham tube to indicate gas production. From left, uninoculated, negative, positive fermentation/no gas production and positive fermentation/gas production.

Figure 2.46 CTA medium and carbohydrate disc for fastidious bacteria: from left, uninoculated, positive and negative.

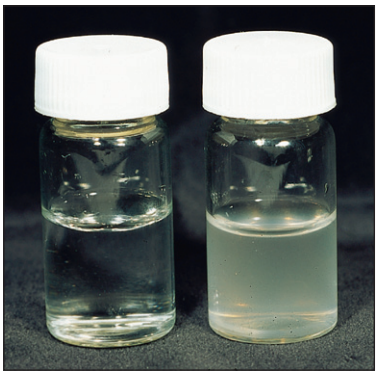

Figure 2.47 Koser citrate: uninoculated or negative (left), and positive (right).

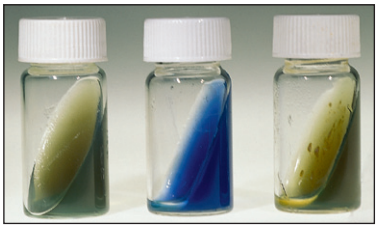

Figure 2.48 Simmons citrate: from left, uninoculated, positive and negative.

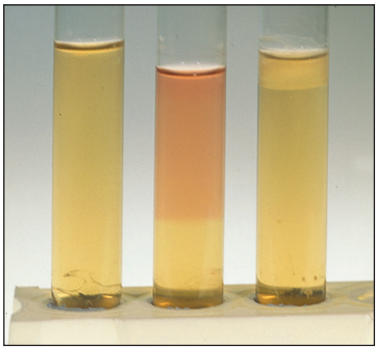

Figure 2.49 Gelatin liquefaction in nutrient gelatin (stab inoculation): from left, uninoculated or negative, *Serratia* sp. (positive), and *Proteus* sp. (positive).

employ miniaturized biochemical tests or molecular methods for identification of bacterial isolates, these systems are expensive. In certain circumstances, the laboratory may choose to carry out a limited number of biochemical identification tests using conventional methods.

Tests to determine the range of 'sugars' that a particular bacterium can catabolize are among the more commonly used biochemical tests. These 'sugars' are carbohydrates or alcohols, examples of these are:

- Monosaccharides: arabinose, fructose, galactose, glucose, mannose, ribose, ribulose and xylose
- Disaccharides: sucrose (glucose and fructose molecules), maltose (glucose×2), lactose (glucose and galactose)
- Trisaccharide: raffinose (glucose, fructose and galactose)
- Polysaccharide: inulin
- Alcohols: adonitol, dulcitol, mannitol and sorbitol.

Figure 2.50 Gelatin liquefaction using charcoal gelatin discs: uninoculated or negative (left) and positive (right).

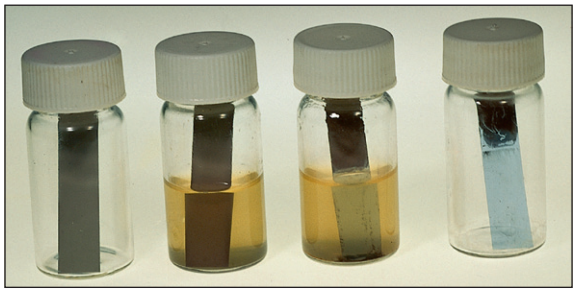

Figure 2.51 Gelatin liquefaction using the X-ray film method: from left, strips 1 and 2 negative, strips 3 and 4 positive.

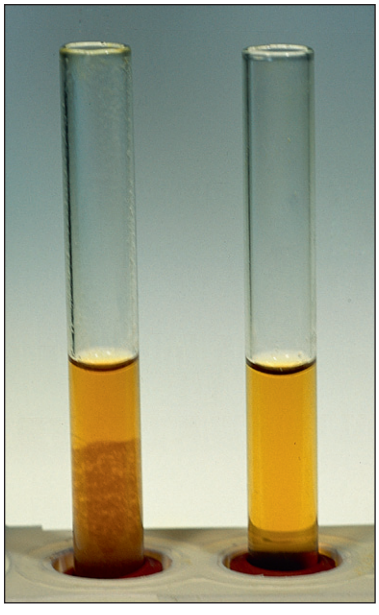

Figure 2.52 Hippurate hydrolysis: permanent precipitate, left (positive) and no precipitate, right (negative).

Figure 2.53 Lead acetate paper for detection of H_2S production: negative (left) and positive (right).

The fermentative or oxidative attack on the sugars by the bacterium produces acidic metabolites detected by a pH indicator. Gas may also be produced and in conventional peptone water sugars (1% sugar) the gas production is demonstrated by the inclusion of a Durham tube to trap the gas (Fig. 2.45). Cystine trypticase agar (CTA) medium can be used to test the more fastidious bacteria that are unable to perform in peptone water sugars. The sugar under test is added to the inoculated medium incorporated in a paper disc (Fig. 2.46). Another method to determine whether a bacterium can catabolize a certain sugar is to inoculate a solid agar medium to which the sugar has been added. An example of this type of test is purple agar with 1% maltose (pH indicator bromocresol purple), which can help to distinguish between *Staphylococcus aureus*, *S. intermedius* and *S. pseudintermedius* (Fig. 7.10).

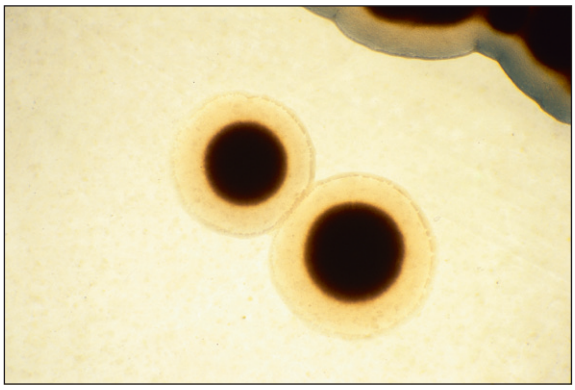

Figure 2.54 Close up of *Salmonella* Enteriditis colonies on bismuth sulphite medium, using bismuth salts to indicate H_2S production.

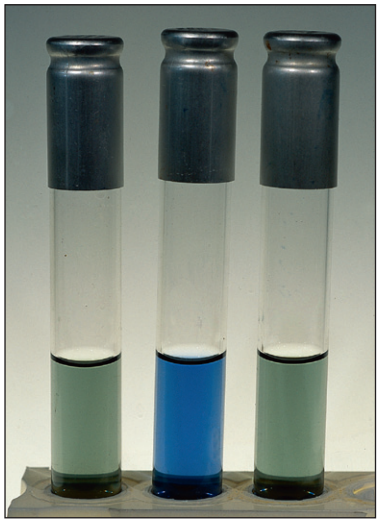

Figure 2.56 Malonate utilization: from left, uninoculated, positive and negative.

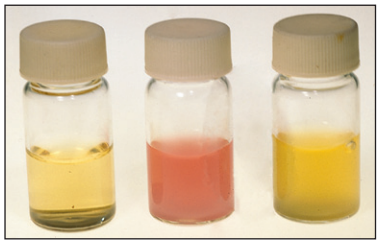

Figure 2.58 Voges-Proskauer (VP) test: from left, uninoculated, positive and negative.

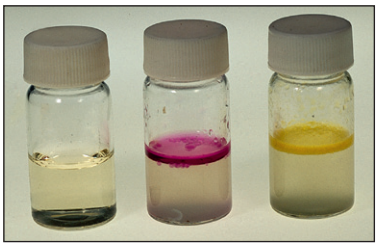

Figure 2.55 Indole test: from left, uninoculated, positive and negative.

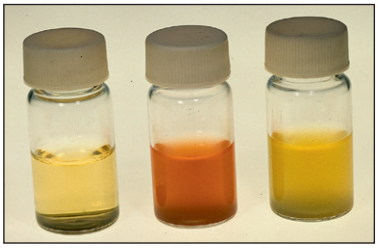

Figure 2.57 Methyl red (MR) test: from left, uninoculated, positive and negative.

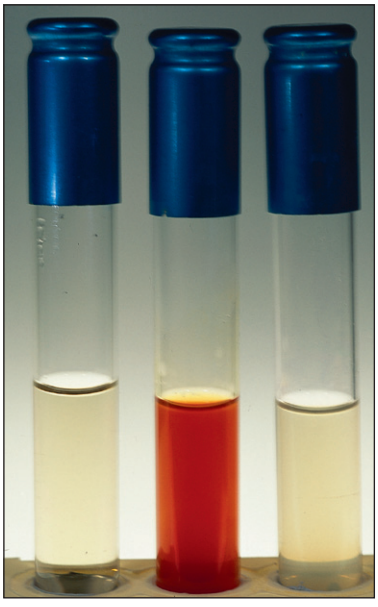

Figure 2.59 Nitrate reduction test (nitrate broth): from left, uninoculated, positive for nitrite and negative for nitrite.

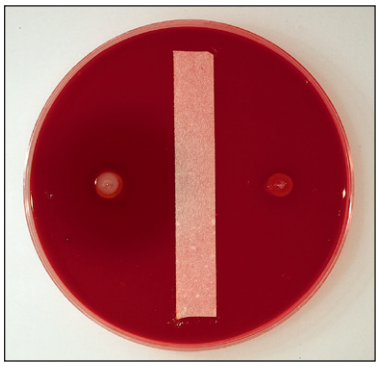

Figure 2.60 Nitrate reduction test (blood agar/nitrate strip): wide zone of browning around colony indicates a positive reaction (left) while absence of reaction (right) is negative.

Figure 2.61 Phenylalanine deaminase test: from left, uninoculated, positive and negative.

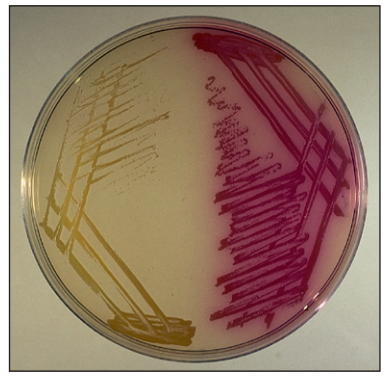

Figure 2.62 Phosphatase test: negative reaction left (coagulase-negative *Staphylococcus*) and positive reaction right (coagulase-positive *Staphylococcus*).

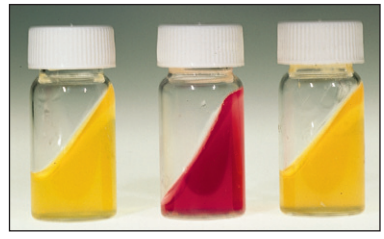

Figure 2.63 Urease test (Christensen medium): from left, uninoculated, positive and negative.

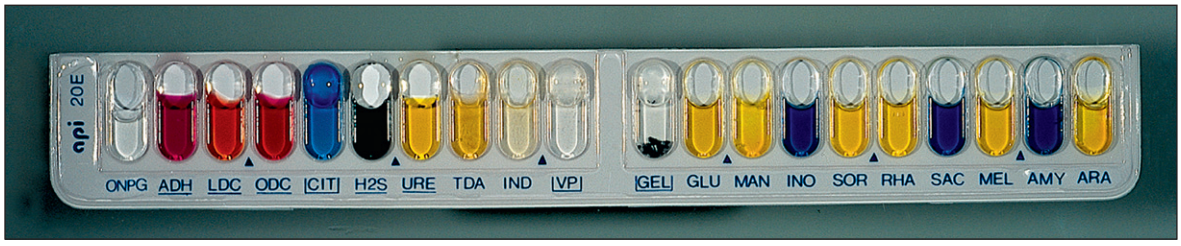

Figure 2.64 API 20E miniaturized identification system showing the reactions of a *Salmonella* sp.

Miniaturized methods for the identification of bacteria

These are available from several commercial companies. One of the most widely used systems is API (bioMérieux) and individual biochemical strips are available for the identification of the *Enterobacteriaceae* and other Gram-negative bacteria; anaerobes; non-fermenting bacteria; streptococci and some other Gram-positive bacteria; staphylococci and yeasts. The results of the miniaturized biochemical tests correlate well with those obtained by the conventional methods. The advantages of the miniaturized systems include the convenience when inoculating; the media and reagents are supplied ready for use and the results are easy and quick to interpret. An example of such a system is illustrated in Figure 2.64.

BACTERIAL CELL COUNTING TECHNIQUES

Sometimes it is necessary in diagnostic microbiology to enumerate bacterial cells in fluids such as autogenous

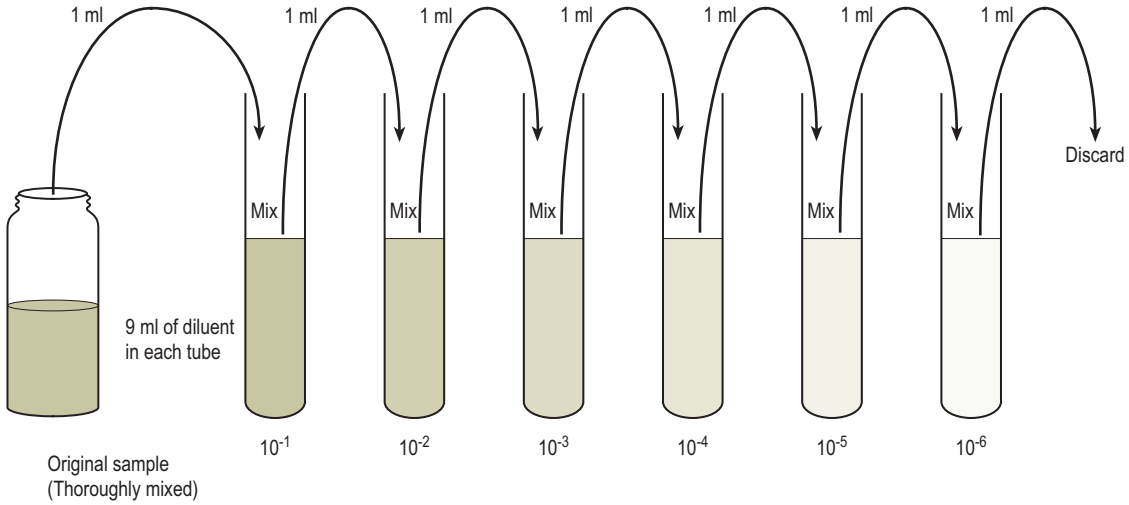

Figure 2.65 Preparation of ten-fold dilutions of a bacterial suspension before conducting a viable count to find the number of bacteria/mL in the original sample. The sample should be thoroughly mixed before sampling and a separate pipette should be used for each transfer step.

vaccines, water, milk or urine samples. Both viable and total counts can be carried out. Viable counting techniques are more commonly used in diagnostic and food hygiene procedures. Viable bacteria are capable of multiplication with the production of visible colonies on or in agar media. The assumption is made, in viable counting methods, that one well-spaced, bacterial cell gives rise to one colony. Bacterial colonies, rather than the bacterial cells, are counted in most of these methods. Total counts will enumerate both viable and non-viable bacterial cells. There are inherent errors in all the methods.

Viable Counting Methods

Serial 10-fold dilutions of the original fluid, containing bacteria, must first be made for each of the methods (Fig. 2.65). This must be carried out as accurately as possible to minimize avoidable errors and an aseptic technique should be used.

Spread plate method

A range of dilutions is used and an inoculum of 0.1 mL of each dilution is placed on the surface of an agar plate. The inoculum is spread rapidly over the entire agar surface using a thin, bent glass rod or a disposable plastic spreader. Plate count agar, nutrient agar or even MacConkey agar can be used if a viable count of *Escherichia coli* is required. At least two, and preferably four, plates should be inoculated per dilution. The plates are incubated for 24–48 hours at 25–37°C. The incubation temperature will depend on whether environmental or pathogenic bacteria are being sought. After incubation, plates inoculated with a sample dilution yielding between 30 and 300 colonies are read, for greatest accuracy (Fig. 2.66). The colony count should be an average of the two or four plates inoculated with the selected dilution. Various instruments are available to facilitate counting the colonies, including electronic counters (Fig. 2.67).

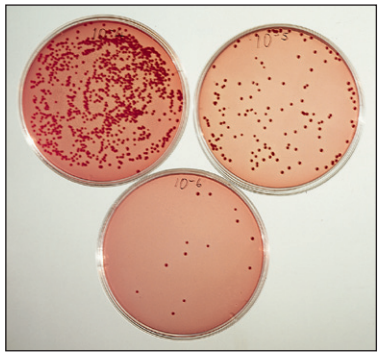

Figure 2.66 Colony counting, surface spread technique on MacConkey agar. The 10^{-5} dilution (top right) is suitable for counting.

Pour-plate method

This method is similar to the spread-plate technique except that the 0.1 mL inoculum is mixed thoroughly with molten agar, previously held in a water bath at 50°C. Two or four plates should be inoculated with each dilution. The agar is allowed to set and then incubated at 25–37°C for 24–48 hours. Plates inoculated with a sample dilution

Figure 2.67 Colony counting using an electronic counter.

that yields between 30 and 300 colonies per plate should be read. The colonies will be distributed throughout the agar as well as on the surface. The subsurface colonies assume a biconvex shape.

An example of a calculation for the spread- and pour-plate methods is given below.

- If there is a mean count of 250 colonies per plate at the 10^{-4} dilution and as the inoculum was 0.1 mL per plate:
- The number of bacteria/0.1 mL of original sample = 250×10^4
  Thus, the number of bacteria/1.0 mL of original sample = $250 \times 10^4 \times 10$
- $= 2.5 \times 10^7$

Miles–Misra technique

This method has the advantage of being economical with agar media. Lines can be drawn on the bottom of an agar plate with a waterproof marker, dividing it into 8 sectors. An inoculum of 0.02 mL, delivered as a drop, is placed on the agar in each sector. At least four drops per sample dilution should be tested. The inocula are allowed to dry and the plates incubated at 25–37°C for 24–48 hours. A sample dilution yielding about 30 colonies per drop should be selected (Fig. 2.68). The average colony count from at least four drops should be obtained. The calculation is similar to that for the two previous methods, but as the inoculum was 0.02 mL, the conversion factor will be 50 to obtain a figure for the bacteria/mL in the original sample.

Filtration method

This is a useful method for determining the number of bacteria in a water sample or other clear fluid where the bacterial number is low. A known volume of water is passed through a membrane filter of pore size 0.22 µm. The filter will retain the bacterial cells, and is aseptically placed, bacterial-side up, on the surface of an agar plate. The medium can be selective or non-selective, depending on the bacterial species being sought. Colonies will form on the surface of the filter after incubation and can be counted (Fig. 2.69). As the volume of the water or fluid is known, the bacteria/mL or per 100 mL of sample can be calculated.

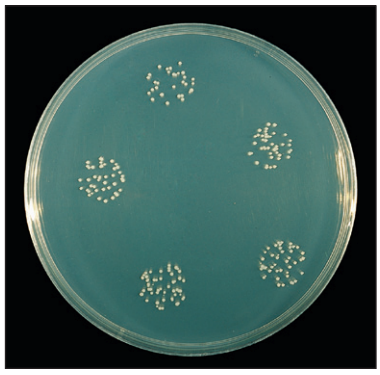

Figure 2.68 Miles–Misra technique: a viable bacterial counting method

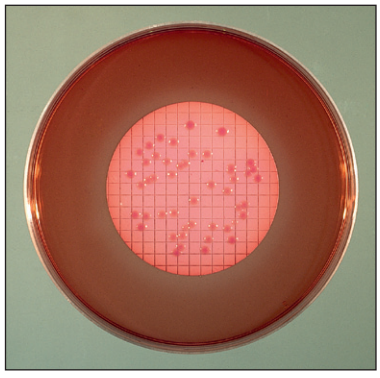

Figure 2.69 Filtration method for enumerating viable bacteria in water. After filtration, the membrane filter was placed on MacConkey agar with the subsequent growth of lactose-fermenting colonies.

Most probable number (MPN) techniques

These techniques are based on statistical probabilities and the assumption that there is a normal distribution of bacteria in liquid samples. If the liquid sample contains one viable bacterial cell, its growth and multiplication in a suitable broth can be detected by manifestations such as turbidity or acid and gas production. The methods can be used for most bacteria, but they are most commonly used for the detection of coliform bacteria in water supplies.

MacConkey broth with bromocresol purple as the pH indicator is often used for coliform counts. Acid production is indicated by a yellow colouration of the broth and gas is trapped by a Durham tube.

One recommended method is to take one 50 mL, five 10 mL and five 1 mL quantities of the water sample. The 50 mL and 10 mL volumes are each added to their own volume of double-strength broth, while the 1 mL samples are each added to 5 mL of single-strength broth. The inoculated tubes are incubated at 35 °C for 48 hours and then each is examined for acid and gas production. By referring to standard MPN probability tables (Anon 1982) the MPN of coliforms/100 mL of water sample can be determined. For example, if one each of the tubes inoculated with 50 mL, 10 mL and 1 mL samples of water, respectively, showed acid and gas production, then from the tables the MPN of coliforms/100 mL water would be 5. For the differential coliform count to specifically detect *Escherichia coli*, any tubes showing acid and gas production are subcultured into fresh MacConkey broth and incubated at 44 °C. Formation of acid and gas within 48 hours at this temperature is presumptive for *E. coli* and indicative of faecal pollution of the water.

Total Counts of Bacterial Cells

These methods do not distinguish between viable and non-viable cells and thus the bacterial count will include both living and dead cells.

Breed's direct smear method

A grease-free microscope slide is placed over a template 1 cm × 1 cm (area of 100 mm^2) and a 0.01 mL of sample is carefully spread over this area. The smear is allowed to air-dry, fixed by heat and stained with methylene blue for about one minute. After air-drying, the stained smear is examined under the oil-immersion objective. The bacterial cells should be counted in at least 50 fields throughout the area of the smear. An average bacterial cell count per field (N) should be obtained. The radius (r) for the particular microscope's oil-immersion field can be found (in mm) using a slide and eyepiece micrometer. The area of the field will be πr^2 or approximately $3.14 \times r^2$ square mm.

$$\text{Bacteria/mL in sample} = [N \times \text{Area of smear } (100\ mm^2) / \text{Area of one field } (3.14 \times r^2)] \times 100$$
$$= N \times 10^4 / 3.14 \times r^2$$

(where N = average bacterial count/field and r = radius of microscope's oil immersion field in mm).

The radius of the oil immersion field is usually about 0.08 mm so the area of the field will be 0.25 mm^2 and bacteria/mL = $N \times 4 \times 10^4$.

Counting chamber method

A Helber chamber or an improved Neubauer haemocytometer can be used for this technique. The technique for counting bacterial cells and the calculation of bacteria/mL in the liquid sample is similar to that for erythrocytes.

Turbidity standards

Brown's or McFarland's opacity tubes are available commercially. They consist of a series of 10 numbered, standard, thin glass tubes containing different dilutions of suspended barium chloride or barium sulphate, giving a range of opacities. The test bacterial suspension is placed in a 'blank' tube of similar dimensions to the standards. A visual comparison of opacity is made by rolling the test suspension across a printed page and matching it with a standard of comparable opacity. Tables are supplied with the opacity tubes that give the numerical equivalents (bacteria/mL) of the opacity standards for a certain range of bacteria. It is a convenient and simple method, but can only give an approximate total bacterial count.

Coulter counter

Coulter counters are automated, electronic counting instruments, usually used in haematology, but they can be adjusted to conduct total bacterial cell counts.

Surface Contact Plates

Special plastic plates are available to allow direct sampling of flat surfaces for bacteria. The technique can be used to detect a specific pathogen, such as *Salmonella*, when a selective medium would be appropriate, or to determine the degree of contamination of a surface using a non-selective medium. An exact quantity of agar must be used to fill these plates as the agar surface should project slightly above the rim of the plate. Surfaces are sampled by placing the agar gently on the area, the plate lifted carefully and the lid replaced. The plates are incubated at 30–37 °C for 24–48 hours and examined for colonial growth (Fig. 2.70). If required, the number of bacteria/cm^2 of surface could be calculated, as the plastic plates incorporate a grid on the base. These plates are widely used for monitoring hygiene standards in the food industry and are increasingly used in the healthcare industry also.

Molecular Methods of Bacterial Quantification

Real-time PCR techniques have been developed for the detection and quantification of many pathogenic bacteria. This technique is described in Chapter 3.

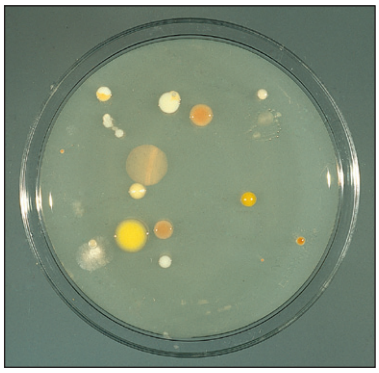

Figure 2.70 Surface contact plate for sampling surfaces: the agar projects slightly above the plastic rim to allow contact with a surface. This plate was used to sample a bench top and a mixed microbial flora was recovered.

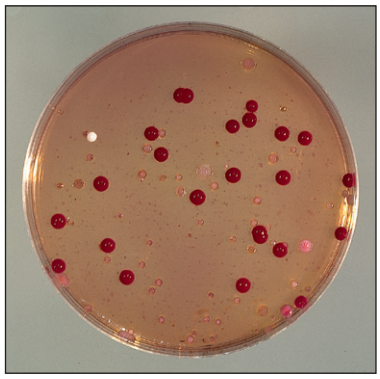

Figure 2.71 Demonstration of an aerosol of bacteria using *Serratia rubidaea* (red colonies) as a marker organism (MacConkey agar).

Use of Marker Bacteria

Occasionally investigations of infectious agents may require the use of a marker organism. *Serratia rubidaea* is ideal for the purpose as it is not considered to be pathogenic, is rarely isolated from animals or the environment and its colonies have a distinctive red pigmentation. Marker bacteria can be used for various purposes such as testing the efficiency of a disinfection programme, studying the dispersal of pathogenic microorganisms as an aerosol or the determination of the efficacy of sewage treatment. A MacConkey agar plate from an investigation to determine aerosol dispersal of bacteria is illustrated (Fig. 2.71).

REFERENCE

Anon, 1982. The bacteriological examination of drinking water supplies, Report No. 71. Departments of the Environment, Health, Social Security and Public Health Laboratory Service, HMSO, London.

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Chapter 3

Serological diagnosis

Serological testing is generally less expensive and easier to perform than the isolation or detection of an infectious agent, particularly virus. Clinicians often request serological screening as the first step in a disease investigation. Serology testing only requires clotted blood samples which are relatively easy to collect from large numbers of animals. Many of the classic serology techniques have been employed for decades and have a proven track record in the field. They are very reliable and the results are accepted internationally. Traditional serological diagnosis of infection is based upon identification of specific antibody. When an animal encounters an infectious agent or foreign substance for the first time, antibody to that agent or substance is usually (but not always) detectable in the serum within 10–14 days. The time required for antibody production is influenced by the nature, amount and route of exposure to the agent, the age and immune status of the animal and the sensitivity of the assay used to demonstrate circulating antibody. For some infectious organisms the production of detectable antibody may take considerably longer than 14 days, for example, antibodies to equine infectious anaemia virus may not be detected for more than 60 days post exposure. The first antibody response to antigen is referred to as the primary response. The interval between exposure and production of antibody is referred to as the latent period (or lag period). If exposure to the same antigen occurs again some time later, the animal responds with a stronger and more sustained response and the latent period is shorter due to the presence of memory B cells. This is termed the secondary response (Fig. 3.1). The memory response of an animal for an agent it has previously been exposed to is called the anamnestic response.

When blood sampling animals for serological testing for infectious diseases, two samples may be required to demonstrate an increasing level of circulating antibody. A fourfold or greater increase is considered significant in many serological tests and is known as seroconversion. This is indicative of recent exposure to the agent. The testing of paired samples collected 10 to 14 days apart frequently leads to a retrospective diagnosis which is of limited value in the management of acute illness. The presence of serum IgM antibody is generally accepted as evidence of a current or very recent infection but the detection of specific viral IgM antibody is rarely feasible for routine diagnosis in veterinary laboratories. However, an IgM ELISA is used extensively for the diagnosis of West Nile fever (WNF) in horses.

Serological examination of a single sample may be performed to determine if an animal has previously been exposed to an agent, to assist in the identification of a carrier or to evaluate the animal's immune status. However, the presence of antibody does not always correlate with protective immunity. Antibody-based immunity is the major defence system against extracellular bacteria and viruses as well as against bacterial exotoxins but cell-mediated immunity plays a more prominent role in host resistance to intracellular pathogens, for example, herpesviruses and *Salmonella* spp.

The majority of serological assays do not differentiate between exposure by natural infection and exposure by vaccination. Some serological assays do not allow the unequivocal differentiation of antigenically related organisms. Such assays may be used for screening purposes and the laboratory can revert to a type-specific assay when a positive result is obtained. In recent times the incorporation of highly purified recombinant antigens or synthetic peptides in some assays has improved their specificity and aided standardization of interpretation. However, in some instances this has led to a decrease in sensitivity. If a specific serological assay is not available then agent detection or isolation is a prerequisite for identification.

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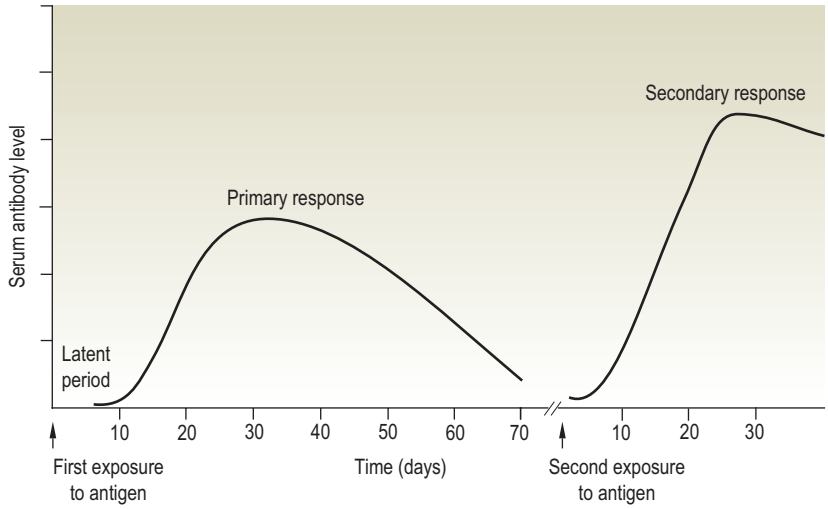


Figure 3.1 A comparison of specific antibody production during primary and secondary responses to infectious agents or to vaccination. Natural infection usually results in higher levels of antibody for a longer time period.

Serological assays depend on the amount of antibody present, the binding of this antibody to the antigen in the test and the detection of the antigen–antibody complexes. These complexes can be detected by a biological effect, for example, neutralization or a physical effect such as precipitation. Alternative detection systems use immunoglobulins labelled with a chromagen (immunohistochemistry, ELISA), a radioisotope (radioimmunoassay) or a fluorochrome (immunofluorescence). It is usual to dilute the serum of the test animal to determine the concentration of specific antibody present and this is expressed as the titre of that serum. Titre may be defined as the highest dilution of a serum which gives a demonstrable reaction in a defined test procedure.

International reference sera have been developed for many serological tests. Such sera are made available to national reference laboratories for the purpose of establishing national standards. Diagnostic laboratories should whenever possible use these national standards or prepare working standards against them for incorporation in the routine tests. Similarly, the use of reference strains for diagnostic antigen production promotes international harmonization of diagnostic testing.

Many of the immunological techniques described below can be used to detect antibody or antigen (see also Chapter 5).

Precipitation

When soluble antigen is added to homologous antibody at the correct ratio insoluble antigen–antibody complexes are formed. If the immune complexes are of sufficient size they precipitate out of solution. This form of immunological precipitation is termed the precipitin reaction. When diluted antigen is layered carefully over homologous antibody a band of precipitate forms where the ratio of the reagents is at optimal proportions. This method is termed the ring precipitin test (Fig. 3.2) and has found application in identifying streptococci and assigning them to their appropriate Lancefield groups. A limitation of this method is the requirement for high levels of antibody in the antiserum employed.

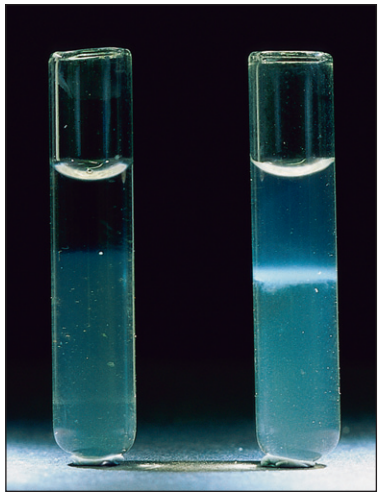


Figure 3.2 Ring precipitin test: negative (left) and positive (right).

Precipitation reactions can take place in semisolid media such as agar gels. When soluble antigen and antibodies are placed in wells cut in a gel, they diffuse towards each other and where they meet at or near optimal

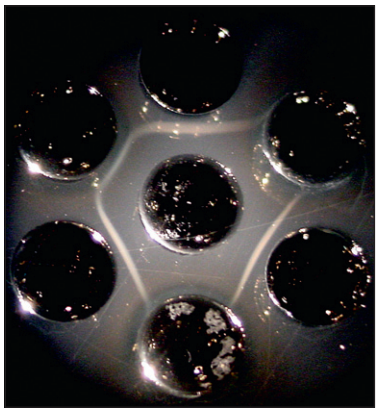


Figure 3.3 Coggins test. The precipitin line is formed in the agar gel when the antibodies in a positive serum sample in the test well and the equine infectious anaemia virus antigen in the centre well diffuse towards each other.

proportions, a precipitin line forms in the gel. Immunodiffusion in agar (Fig. 3.3) is applied in diagnostic tests for infectious diseases particularly in virology and the Coggins test for equine infectious anaemia is based on this method. The agar gel immunodiffusion test for caprine arthritis/encephalitis and for maedi-visna or ovine progressive pneumonia is a cost-effective method of identifying persistently infected carriers and is the prescribed test for international trade. It is also the traditional test for the detection of antibody to virus-infection-associated antigen (VIAA) of foot-and-mouth disease (FMD) virus and has been used extensively in FMD eradication in South America.

Agglutination

An agglutination reaction occurs when antibody reacts with particulate antigen and cross-links surface antigenic determinants. Bacteria, fungi, protozoa and red cells can be directly agglutinated by specific antibody. One drop of test serum is added to a standardized suspension of bacteria on a slide. After gentle rocking the result is read inside three minutes. In the presence of antibodies, the antigens are agglutinated (Fig. 3.4). Occasionally bacteria or other antigens may be deliberately coloured to facilitate interpretation of the result and, in addition, in the Rose Bengal agglutination test for *Brucella abortus*, the pH of the suspending fluid is dropped to a low level to eliminate non-specific agglutination and improve the reliability of the test (Fig. 3.5). The slide agglutination test is also used to demonstrate the presence of antibody in serum.

Quantitative agglutination can be performed in test tubes or microtitre plates by serially titrating the serum under test, usually in twofold dilutions. An equal volume of standardized antigen, such as bacteria, is added to each tube and a control tube with diluent. Following careful

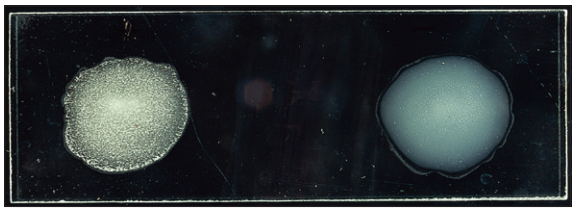


Figure 3.4 Slide agglutination test: positive reaction (left) and negative reaction (right).

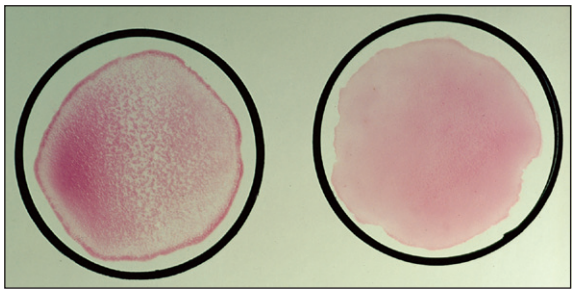


Figure 3.5 Rose Bengal plate agglutination test for *Brucella abortus* (coloured antigen): positive reaction (left) and negative reaction (right).

mixing the tubes are incubated, usually at 37°C. Results are recorded after a few hours and the highest dilution of the serum giving agglutination is called the antibody titre (Figs 3.6 and 3.7). For diagnostic purposes, two blood samples taken several weeks apart should be used to demonstrate a rising titre in infected animals.

One practical difficulty of importance in agglutination tests is the occasional absence of agglutination at low dilutions when high titred serum is being tested. This is referred to as the prozone phenomenon. The nature of this phenomenon is not entirely clear but is associated with the presence of non-agglutinating antibodies in some sera and probably with the inability of some antibodies to cross-link particulate antigen where epitopes are recessed deep in the cell wall or membranes of bacterial or mammalian cells. Because of the prozone phenonemon it is imperative that test sera be checked at several dilutions to avoid errors in reporting results.

Agglutination tests can be carried out with body fluids other than serum and in the diagnosis of *Brucella abortus* infections in cattle, milk is used. In the milk ring test, stained *Brucella* organisms are added to fresh milk, which is left to stand for a few hours. The stained bacteria remain dispersed throughout the milk if antibodies are absent but rise with the cream in a positive reaction (Fig. 3.8).

Passive agglutination is when a carrier particle such as latex is coated with the antigen and attaches to antibodies in the test serum to produce agglutination. A latex agglutination test is routinely used for the detection of contagious caprine pleuropneumonia in Africa. It is simple to

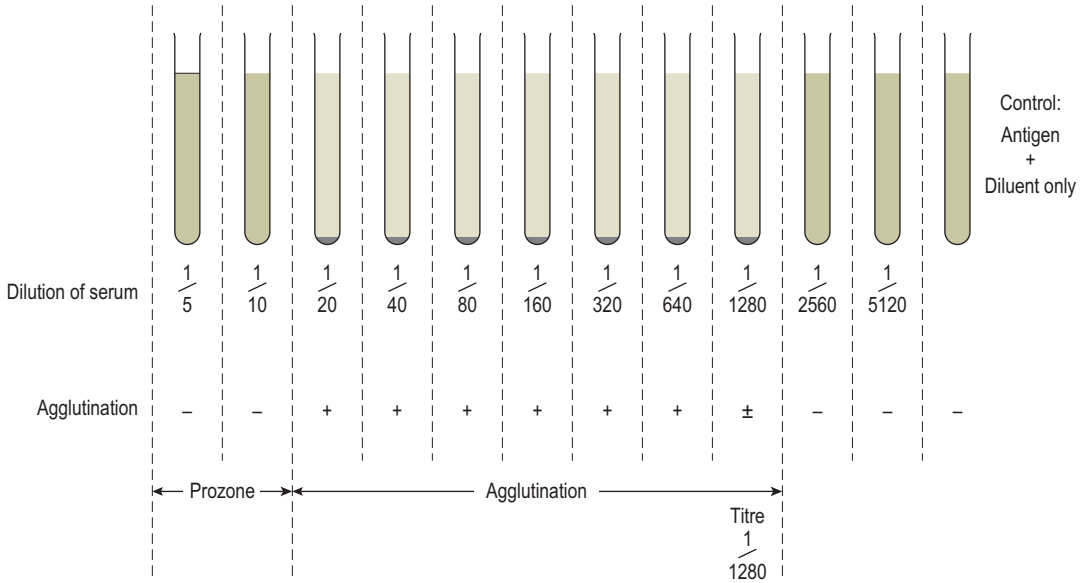


Figure 3.6 Interpretation of the tube agglutination test. Tubes with high levels of antibody, where agglutination does not occur, represent a prozone effect.

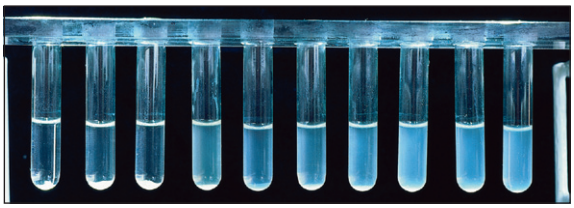


Figure 3.7 Tube agglutination test: doubling dilutions beginning at 1/10. The antibody titre is 1/40.

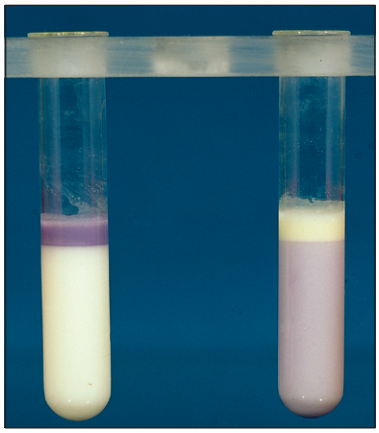


Figure 3.8 *Brucella* milk ring test (stained antigen): positive 3+ reaction (left) and negative (right).

perform and can be carried out in the field. A range of soluble antigens such as lipolysaccharides can be passively adsorbed by red blood cells while other antigens can be chemically coupled to erythrocytes. This method is referred to as passive or indirect haemagglutination and it is a sensitive method for measuring antibodies to soluble antigens attached to carrier red blood cells (Fig. 3.9). A number of other diagnostic procedures use the principle of agglutination. For detection of antibodies to leptospires, the microscopic agglutination test is carried out using a suspension of live microorganisms and a microscope equipped with a darkfield condenser.

Complement Fixation

The fact that antibody, once it combines with antigen activates complement, can be used for diagnostic purposes. The complement fixation test uses sheep red blood cells sensitized with rabbit antibody (haemolysin) as an indicator system. In the presence of complement, usually supplied by guinea pig serum, the sensitized cells are lysed. The test depends on a two-stage reaction system. The test serum is heated at 56°C for 30 minutes to destroy its complement activity before titration. Antigen is then added to the titrated serum and a precise amount of guinea pig complement is also added. After incubation at 37°C for 30 minutes, sensitized sheep red blood cells are added followed by a further incubation. Where complement is fixed by antibody in the test serum reacting with specific antigen, the sheep red cells do not lyse, but the sensitized sheep red blood cells will lyse if complement

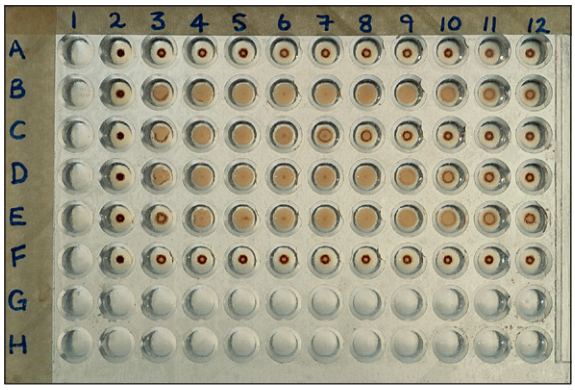


Figure 3.9 Indirect haemagglutination test (passive) for measuring antibody against soluble antigen (*Toxoplasma gondii* and sheep red blood cells). Column 2 downwards contains a control well for each serum and the carrier red cells. There are doubling dilutions of each serum starting at 1/8 in column 3. Interpretation: A (across), negative; B, positive 1/1024; C, positive 1/128; D, positive 1/512; E, positive 1/1024 and F, negative. U-type wells.

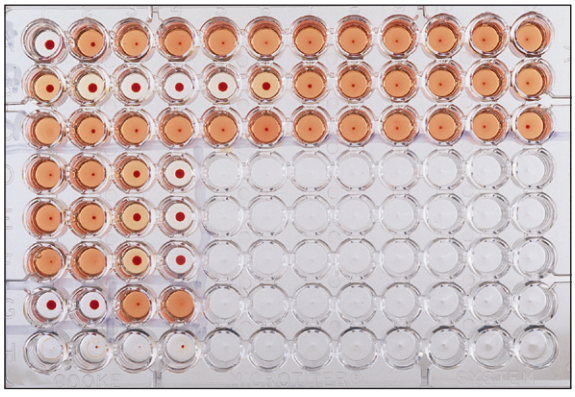


Figure 3.10 Complement fixation test (CFT) for enzootic abortion of ewes. Test sera in top three rows across with doubling dilutions starting at 1/8. Row 1, positive 1/8; row 2, positive 1/256; row 3, negative. Control wells below. Microtitre plate with V-type wells.

was not fixed because the test serum did not contain specific antibody (Fig. 3.10). Standardization of all the reagents used in the complement fixation test is essential and the assay takes 18–24 hours to complete. Although this assay can be used for the measurement of antibodies against most viruses, laboratories tend to opt for less complex techniques whenever possible. The complement fixation test is used routinely to diagnose equine herpesvirus 1 and 4 infections in horses and has been the standard test for paratuberculosis (Johne's disease) of cattle and for avian chlamydiosis for many years. A negative complement fixation result for paratuberculosis is required by many countries when importing cattle. The complement

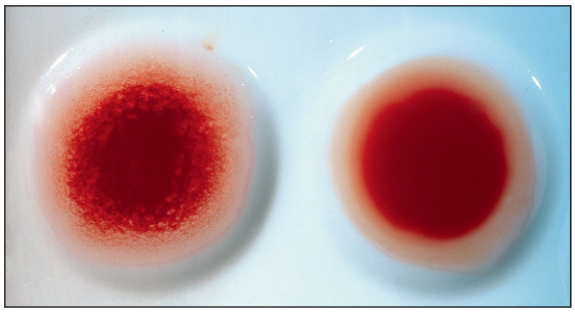


Figure 3.11 Screening virus haemagglutination test (equine influenza virus in allantoic fluid): positive reaction (left) and negative reaction (right) using human O red blood cells. The screening test was photographed before the red cells in the negative test had completely settled (formed a button). V-type wells.

fixation test is also widely used as a confirmatory test for bovine brucellosis.

Viral Haemagglutination and its Inhibition by Antibody

Some viruses such as influenza viruses, adenoviruses and arboviruses are capable of binding to red blood cells and agglutinating them. This is termed viral haemagglutination and antibodies specific for these viruses inhibit this haemagglutination by blocking their combining sites. It is usual to first screen the virus to determine its haemagglutinating activity for the red cells being used (Fig. 3.11). It is common to use four haemagglutinating units (HA units) with test sera. Virus is standardized and added to dilutions of test serum. After an appropriate interval, washed red blood cells are added to each well, including control wells, and the haemagglutination inhibition titre of the serum is recorded (Fig. 3.12). The test measures type-specific antibody and usually requires four to five hours to complete. Antibodies against influenza haemagglutinin correlate with protection and can be used to monitor vaccine efficacy.

Enzyme-linked immunosorbent assay (ELISA)

Enzymes such as alkaline phosphatase or peroxidase can be linked to antibody without interfering with the antibody's specificity or the enzyme's activity. The enzyme is detected by assaying for enzyme activity with its substrate (Fig. 3.13). Because of their simplicity and safety, ELISA methods have been widely used for the immunodiagnosis of bacterial, viral and parasitic infections. Enzyme immunoassays are extremely versatile and may be used to measure either antigen (see also Chapter 5) or antibody. The simplest format is the direct ELISA (Fig. 3.14) but the

indirect ELISA (Fig. 3.15) format is most commonly used to detect antibody in serum or body fluids. In carrying out the indirect ELISA for antibody, known antigens are fixed to wells in polystyrene plates, or suitable membranes, to which test serum dilutions and control sera are added. Following incubation, the wells are washed with buffered diluent and enzyme-conjugated anti-globulin raised in another species is added (there is a large selection of anti-species conjugates with different immunoglobulin specificities commercially available). After further incubation and washing, the specific substrate for the enzyme is added. The intensity of the colour reaction is measured in a spectrophotometer and is directly related to the amount of bound antibody (Fig. 3.16). Many commercial kits based on this format are available for the diagnosis of a large number of veterinary pathogens.

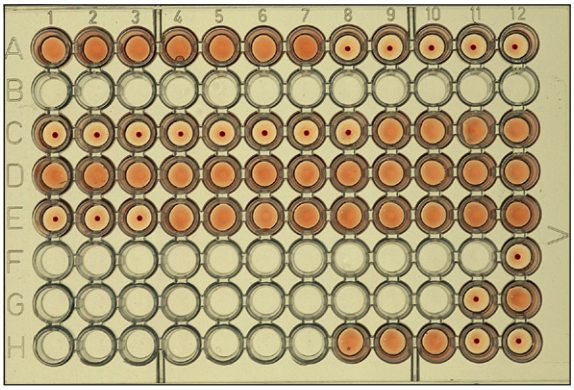


Figure 3.12 Haemagglutination-inhibition test (equine influenza virus and human O RBCs). Top row (A) titration of virus for haemagglutinating activity to determine 1 HA unit (well 7). Test sera are in rows C, D and E with doubling dilutions starting at 1/10. Interpretation: row C, positive 1/1280; D, negative; E, positive 1/40. Rows F, G and H contain reagent controls. V-type wells.

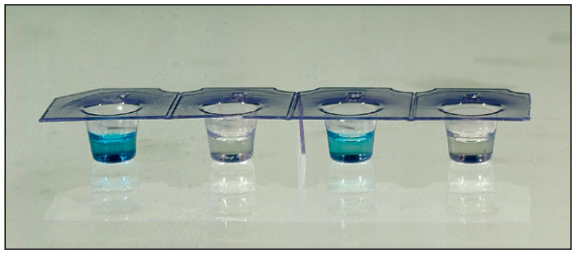
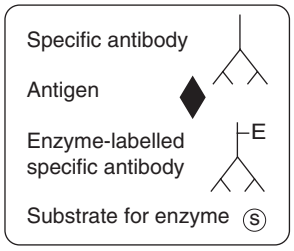


Figure 3.13 ELISA for the detection of feline leukaemia virus in serum: from left, positive control, negative control, positive test and negative test.



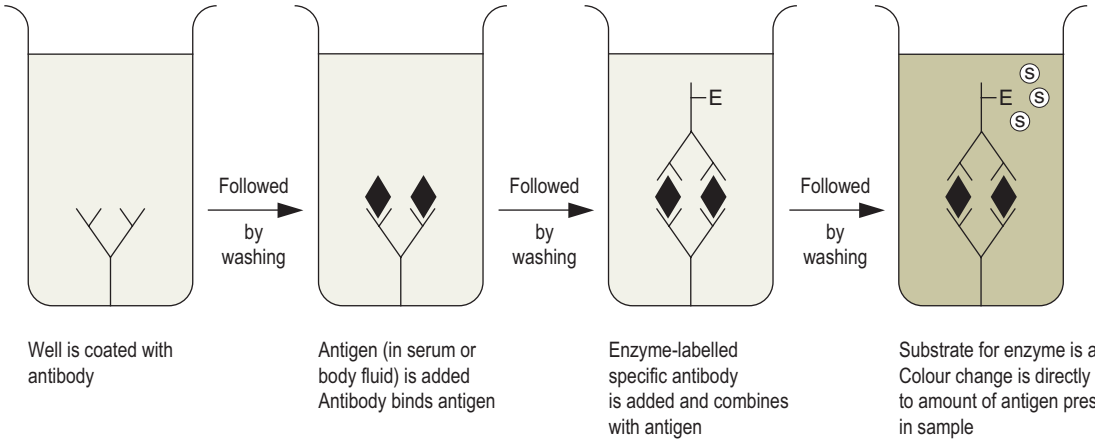
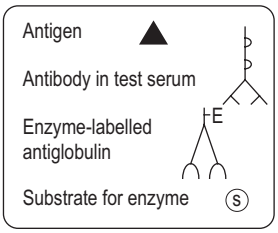


Figure 3.14 ELISA technique for detection or measurement of antigen in a test sample (antigen capture, sandwich or direct method).



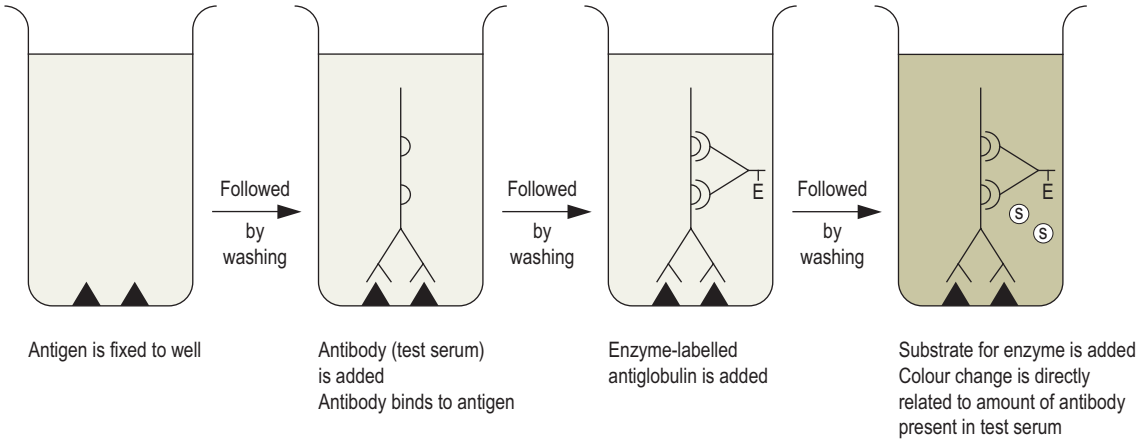


Figure 3.15 ELISA technique for measuring the amount of antibody in a test serum (indirect method).

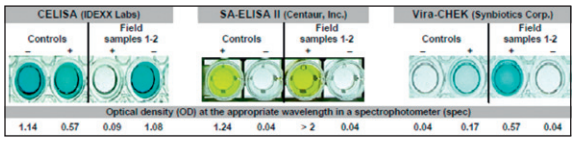


Figure 3.16 Three ELISAs for the detection of antibodies against equine infectious anaemia: In the SA-ELISA and the Vira-CHEK ELISA the colour reaction is directly related to the amount of antibody in the sample but the CELISA is a competition ELISA thus, the positive samples have less colour than the negative samples.

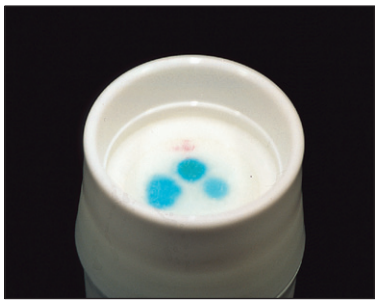


Figure 3.17 ELISA (CITE Combo IDEXX): feline immunodeficiency virus antibody positive (left), positive control (centre top), and feline leukaemia virus positive (left). Red dot is for orientation.

Competition ELISAs involve the detection of antibody by its ability to interfere in a pretitrated system resulting in a decrease in colour. The wells with the positive samples have little or no colour. This is because the antibody in the test serum has bound to the enzyme-linked antigen and prevented it binding to the antibodies fixed to the microtitre plate. This type of ELISA is widely used for the detection of agents such as blue tongue virus, classical swine fever virus, ovine herpesvirus 2 (malignant catarrhal fever), vesicular stomatitis virus and *Babesia* species.

Capture ELISAs have been developed for the detection of IgM, for example in the case of West Nile fever in horses. Anti-equine IgM is adsorbed to wells in polystyrene plates to capture the IgM in the test serum. Following incubation with the test serum the wells are washed and virus antigen is added. The antigen that binds to the specific IgM is then detected by addition of enzyme-conjugated anti-flavivirus monoclonal antibody.

One commercial system combines reagents for direct and indirect ELISA into one detection system (Fig. 3.17) thereby detecting antigens for one infectious agent (feline leukaemia) and antibodies for another (feline immunodeficiency virus).

ELISAs lend themselves to automation thus allowing the screening of large numbers of samples. Some ELISAs have the ability to distinguish between antibodies produced in

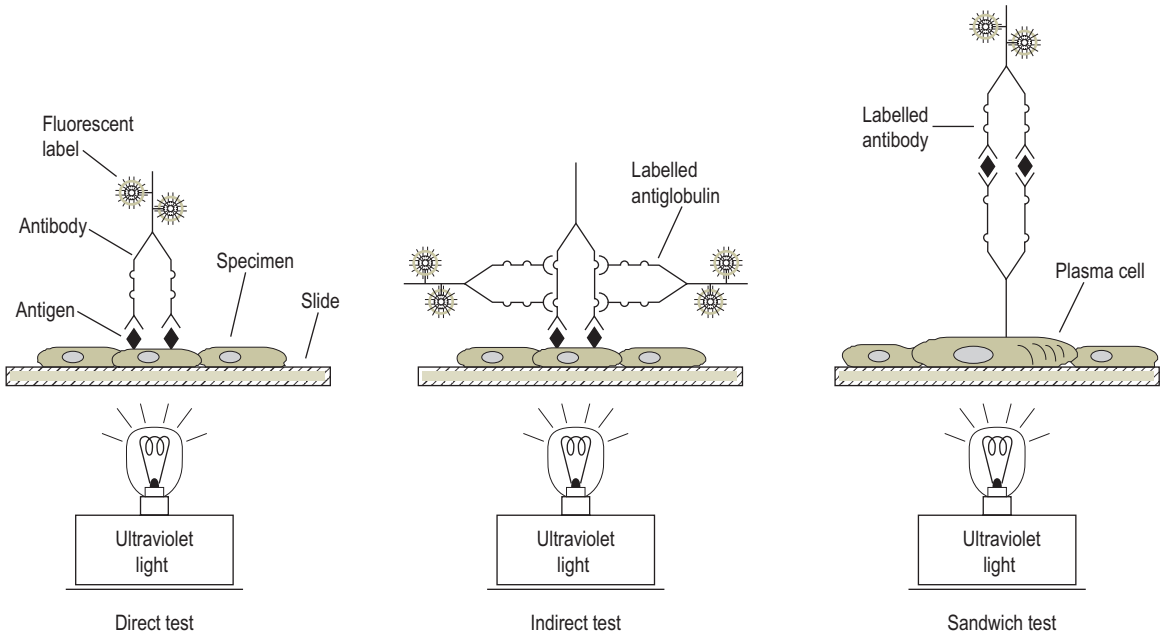


Figure 3.18 Fluorescent antibody techniques (immunofluorescence). In the direct technique, specific antibody is labelled with fluorescent dye (fluorescein isothiocyanate). Labelled antiglobulin is used in the indirect test. In the 'sandwich' method, which is used to detect antibody in tissues rather than antigen, the first reagent added is specific antigen. Following washing, labelled antibody is added which reacts with the antigen bound by antibodies in the tissue section.

response to natural infection and antibodies produced in response to marker vaccines that lack specific glycoproteins. Such marker or gene-deleted vaccines are available for the control of Aujeszky's disease or pseudorabies and infectious bovine rhinotracheitis virus. They are the vaccines of choice where the intention is to eradicate the disease. The ELISAs test for antibodies against the glycoprotein encoded by the gene that has been deleted from the vaccinal virus. These antibodies are present in animals that have been naturally infected but not in vaccinated animals.

Immunofluorescence

Immunofluorescence, like ELISA, uses a labelled immunoglobulin for detecting antigen or antibody (Fig. 3.18). The label employed is a fluorochrome, usually fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate, covalently attached to antibody molecules. When illuminated with light of a specific wavelength or wavelengths, fluorescein emits a characteristic green colour and rhodamine a red colour. A special fluorescence microscope with a mercury-vapour light source is required for immunofluorescence. A disadvantage of immunofluorescence is that it depends on the subjective interpretation of results by the microscopist. Indirect immunofluorescence is frequently used for the detection of antibodies in serum. Antigen smears prepared on slides are screened with dilutions of test serum and positives are identified using fluorescein-conjugated antiglobulin raised in another species. This technique is used for the detection of antibodies against *Toxoplasma gondii* (Fig. 3.19) and viruses such as capripoxvirus and bluetongue virus.

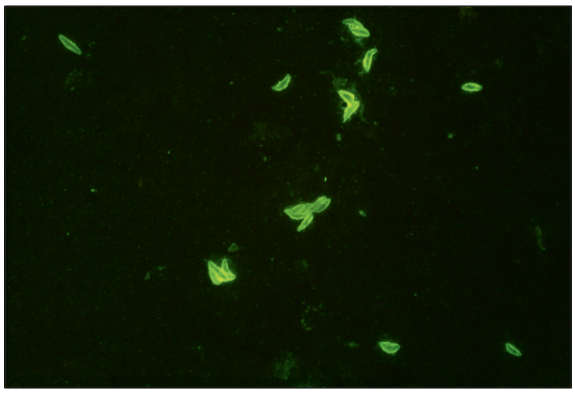


Figure 3.19 Tachyzoites of *Toxoplasma gondii* stained by indirect immunofluorescence. (×400)

Radioimmunoassay

Radioimmunoassay (RIA) uses isotopically labelled molecules and because of its sensitivity, extremely small

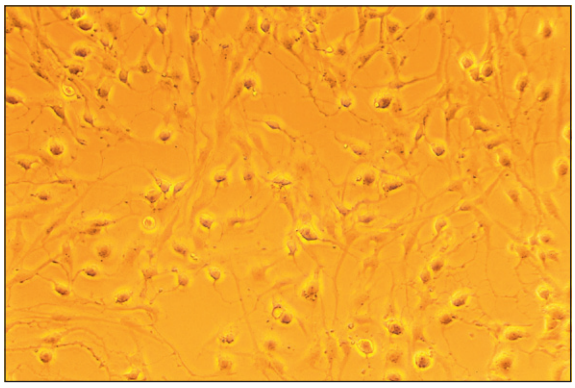


Figure 3.20 Virus neutralization test in tissue culture (control): foetal calf lung (fibroblasts) cells showing evidence of cytopathic effects (CPE) due to a bovine adenovirus.

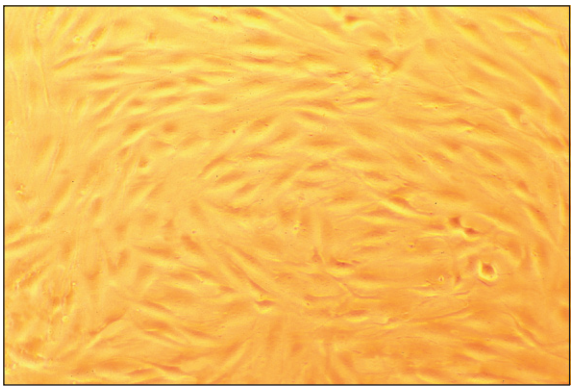


Figure 3.21 Virus neutralization test in tissue culture (positive test): virus (bovine adenovirus) neutralized by specific antiserum and hence no CPE.

amounts of antigen, antibody or immune complexes can be detected. The principle of this assay is similar in many respects to ELISA and immunofluorescence. RIA for detection of antibody is frequently performed as a competitive binding assay. A fixed concentration of radioactive antibody competes with non-radioactive test serum in the test system for attachment to solid-phase bound antigen. The radioactive label iodine-125 is often used for labelling antibody. The short half-life of this isotope necessitates frequent standardization of reagents. Disadvantages include potential radiation hazard and the relatively short half-life of gamma-emitting radioisotopes. Many RIA assays have been replaced by ELISA.

Neutralization Tests

Neutralization tests refer to the ability of antibody to neutralize the biological activity of toxins or viruses in vitro. Antibody, by combining with viruses, can neutralize their infectivity, thereby protecting the cells in a monolayer against virus destruction. Most changes induced by viral infections in monolayers or fertile eggs can be neutralized by specific antibody. This is the basis of virus neutralization tests used either for identification of unknown viruses or for measurement of specific antiviral antibody. Serum–virus mixtures are inoculated into appropriate cell cultures which are incubated until the virus control, that is, without antibody, develops cytopathic effects (Figs 3.20 and 3.21). There is a variety of tissue culture-based methods to detect antibody including plaque reduction, plaque inhibition and microtitre neutralization. If a virus is non-cytopathic then non-neutralized virus can be detected using fluorescence or immuoperoxidase as an indicator system. Virus neutralization tests are highly specific and extremely sensitive. However, they are time-consuming and require a significant level of technical expertise to deal with cell culture and infectious virus. Virus neutralization tests for antibodies against equine arteritis virus (EAV) and Aujeszky's disease are prescribed tests for the international trade of horses and pigs respectively. The fluorescent antibody virus neutralization test (FAVN) and the neutralizing peroxidase-linked assay (NPLA) are prescribed tests for classical swine fever as the causal virus is non-cytopathic. Similarly FAVN and a rapid fluorescent focus inhibition test (RFFIT) are used for the detection of antibodies to rabies virus.

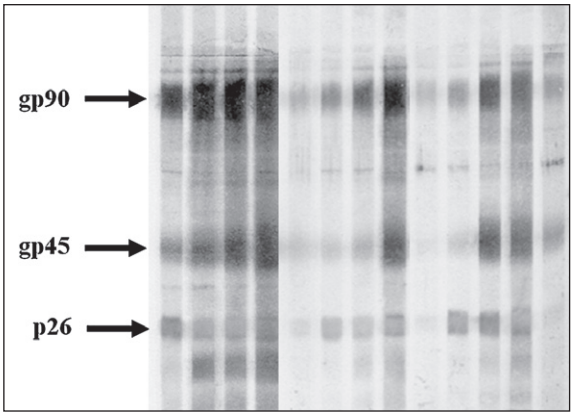


Figure 3.22 An immunoblot for equine infectious anaemia detects antibodies against p26, gp90 and gp45. It is not a routine diagnostic test or an official test but can be used to reach consensus when other diagnostic tests have yielded contradictory results.

Immunoblotting

Immunoblotting or Western blotting is a technique for detecting proteins that have been electrophoretically separated on a gel and transferred to a membrane. A positive immunoblot result appears as bands on the membrane (Fig. 3.22). Antibodies in the test serum detect

proteins on the membrane and are visualized using secondary antibodies conjugated to enzymes such as horseradish peroxidase or alkaline phosphatase. Three types of substrates are used. Chromogenic detection uses an enzyme to catalyse a reaction that results in insoluble precipitates requiring no special equipment for analysis. Chemiluminescent detection uses an enzyme to catalyse a reaction that results in the production of light which is detected by exposing the blot to X-ray film or by the use of a chemiluminescence-compatible digital imaging system. Chemiluminescent detection is more sensitive than chromogenic detection. Fluorescent detection employs either a fluorophore-conjugated antibody or fluorogenic substrates that are detected with fluorescent imaging equipment.

Immunoblot assays have been developed for the diagnosis of a number of infectious conditions including African horse sickness, equine infectious anaemia, contagious bovine pleuropneumonia and lumpy skin disease.

Chapter 4

Molecular techniques in diagnostic microbiology

The development of different nucleic acid amplification technologies has revolutionized the detection and characterization of microbial pathogens. The speed of these techniques has huge advantages over culture-based diagnostic techniques. Other advantages include the ready detection of fastidious, unculturable and/or dangerous pathogens and the ease of strain typing. Organisms that have been inactivated during the processing of vaccines and other biological products can be detected by molecular diagnostics. The polymerase chain reaction (PCR) is the most widely used method of nucleic acid amplification and has the potential to amplify a specific region of DNA millionsfold within a few hours. PCR can be used to identify the microorganism in a sample, from culture or in fixed tissue. PCR requires only small amounts of sample material for the detection of infection. PCR techniques can be adapted to detect actively replicating virus rather than transcriptionally dormant virus. A PCR assay protocol has been developed for virtually all major veterinary pathogens. Probe-based assays such as microarrays that enable the simultaneous detection of a large number of pathogens are being combined with amplification techniques to improve their diagnostic sensitivity.

Polymerase Chain Reaction (PCR)

A diagrammatic representation of the basic PCR procedure is shown in Figure 4.1. PCR is a cyclical process of copying DNA, which involves heating and cooling. Each cycle has three steps, the first of which involves the denaturation of double stranded DNA. The DNA double helix comprises two phosphate sugar (deoxyribose) polymers that are connected by hydrogen bonding between the bases adenine (A), guanine (G), thymine (T) and cytosine (C). Guanine pairs only with cytosine and adenine with thymine. The PCR method separates the two strands of the DNA helix by heating at a temperature of 93 to 94°C. The hydrogen bonds between the paired bases are disrupted giving rise to single stranded DNA.

The second step involves cooling to allow synthetic oligonucleotide primers to anneal to the strands of DNA. The specificity of the reaction is based on these primers which are generally 20 to 25 bases long. They are selected such that they flank the region of DNA to be amplified with one derived from each of the complementary strands. Computer programs are available to assist with the design of primers and avoid primers that self-anneal, that is, form dimers. The annealing temperature varies depending on the sequence of the primers but is usually between 50 and 60°C. When the primers come in contact with a complementary sequence they bind and create a binding site for the enzyme DNA polymerase.

The third step involves heating to allow primer extension. Primer extension is the synthesis of a complimentary strand of DNA using a single stranded template but starting from a double stranded region. In the PCR the primers are arranged so that each primer extension reaction directs the synthesis of DNA towards the other. The DNA polymerase catalyzes the extension of the annealed primers, that is,. it initiates DNA synthesis. It is necessary to use a heat-stable DNA polymerase such as Taq polymerase to withstand the repeated heating during multiple cycles. Extension usually occurs at 72°C as Taq polymerase, which was originally isolated from a bacterium *Thermus aquaticus* that inhabits hot springs, is most active at this temperature. Extension requires the four deoxynucleotides (dATP, dGTP, dCTP and dTTP) and a specific buffer containing magnesium which is essential for Taq polymerase activity.

The PCR cycle of denaturation, annealing and extension is repeated and the product resulting from the extension

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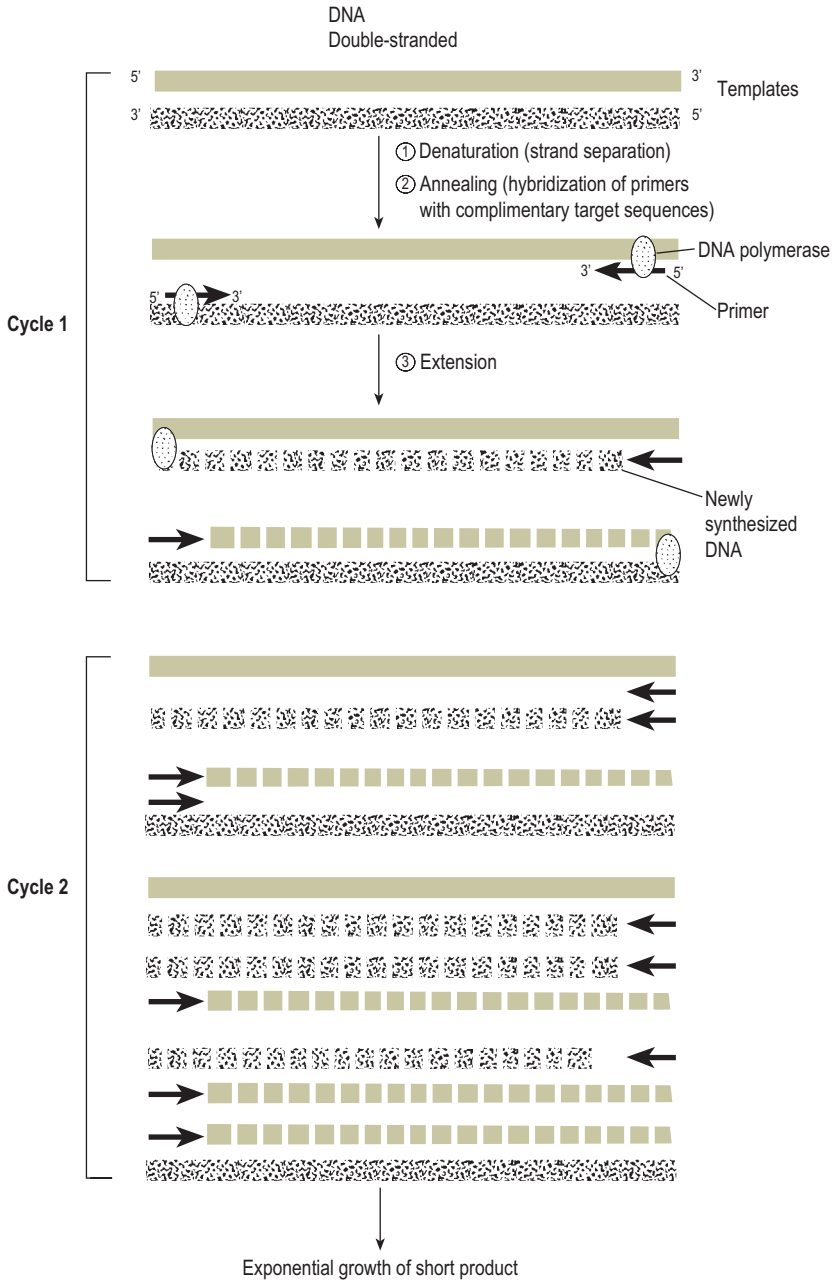

Figure 4.1 Diagram of PCR reaction. The products generated at cycle 1 are not of a defined length. The first PCR products of defined length are produced at cycle 2 due to priming on the products generated during cycle 1. A third cycle results in the first double-strand PCR products of defined length and subsequent cycles lead to exponential amplification of PCR product.

of one primer serves as the template for the second primer and vice versa. Initially synthesis goes beyond the sequence complimentary to the other primer but with each cycle of denaturation, annealing and primer extension the target, that is, the region of DNA flanked by the primers, increases almost exponentially. The PCR cycle is repeated 30 to 40 times to produce detectable amounts of PCR product or amplicon. The simplest way to detect the PCR product is to load a fraction of the reaction onto an agarose gel (Fig. 4.2). The product should be visible as a sharp band

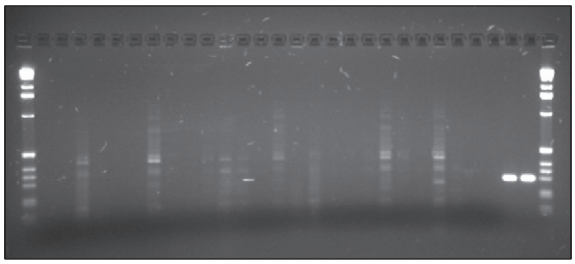

Figure 4.2 PCR products, following amplification of signature sequence of 16S region of chlamydiae, electrophoresed in agarose gel and stained with ethidium bromide. Two lanes (far right) contain amplicon of correct product of size 298 base pairs. First and last lanes contain a molecular size marker.

of expected size. Fluorimeter-based real-time detection systems (see below) are replacing conventional PCR and gel-based detection systems in many veterinary laboratories. Zinc-finger proteins, DNA-binding proteins that directly and specifically detect PCR products, have recently been used to detect microorganisms including *Salmonella* spp. and influenza A viruses.

PCR instrumentation

The PCR procedure is carried out in a programmable thermal cycler. Thermal cyclers differ in the method of thermoregulation. Most use a heating element in contact with a metal block which holds reaction tubes or microtitre plates. Some have gradient blocks that can operate at a range of temperatures allowing independent PCR reactions that require different annealing temperatures to be carried out simultaneously. Air cyclers do not have a block but heat and cool the air streams circulating around capillaries or tubes containing the PCR reaction mix. Initially air cyclers had faster cycling capacity than block thermocyclers but the latter have become more sophisticated and new fast-blocks have reduced their run times. Portable PCR machines for use in the field have been developed. A new PCR chemistry linear-after-the-exponential (LATE) PCR, has been adapted successfully to these portable machines. Other developments include loop-mediated isothermal amplification (LAMP) technology which does not require a thermocycler but uses a simple thermoblock. The results can be visualized by the naked eye. This type of effective but simpler technology has great potential for use in laboratories in less developed areas.

Reverse transcription PCR

As PCR is based on the amplification of DNA the detection of RNA viruses requires the conversion of their genomic RNA into DNA prior to PCR amplification. This is referred to as reverse transcription PCR (RT-PCR) as it is based on the ability of the RNA-dependent DNA polymerase reverse

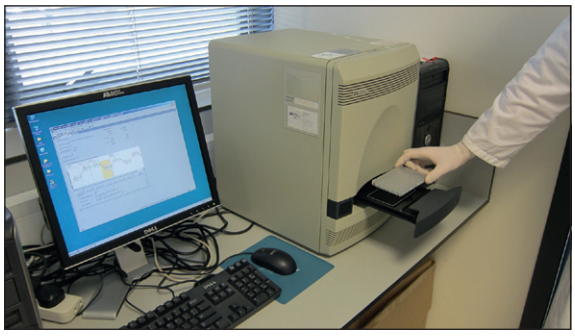

Figure 4.3 Real time PCR machine and associated computer.

transcriptase, to generate a complementary strand of DNA (cDNA) using the RNA as a template. The RT-PCR reaction and the subsequent PCR reaction can be carried out in the same tube, that is, a one-step reaction. This type of system is less time-consuming than a two-step reaction where the reverse transcriptase reaction is performed in one tube and the PCR is performed in a second tube. However, a one-step reaction may be less sensitive as neither reaction can be fully optimized. Also, all the cDNA generated in a one-step reaction is used in the PCR while only an aliquot need be used in a two-step reaction and the remainder can be stored for further tests if required.

Real-time PCR

Systems based on fluorescent detection of double-stranded DNA or specific target sequences by measurement of fluorescence resonance energy transfer (FRET) allow for the monitoring of the accumulation of product in real time after each cycle (Fig. 4.3). This capacity for kinetic measurement of product accumulation eliminates the need for detection of amplicon by gel electrophoresis when the PCR is completed. Real-time PCR involves the continuous collection of fluorescent signal with each cycle. The increase in fluorescence is directly proportional to the increase in PCR product. The number of cycles required for the fluorescence to be significantly above background is known as the cycle threshold or Ct (Fig. 4.4). The more template present in the sample the lower the Ct value, i.e. less cycles are required for detection. Quantitative real-time PCR (qPCR) is the conversion of the fluorescent signals into a numerical value. This method determines the amount of DNA or RNA in a sample which is then extrapolated to organism equivalents often referred to as bacterial or viral load. Quantitative PCR is used extensively in human medicine to quantify the virus load in clinical specimens. It is, for example, an invaluable tool for monitoring disease progression and response to antiviral treatment in individuals infected with human immunodeficiency virus (HIV). Commercial qPCR kits are available for several important human pathogens including HIV,

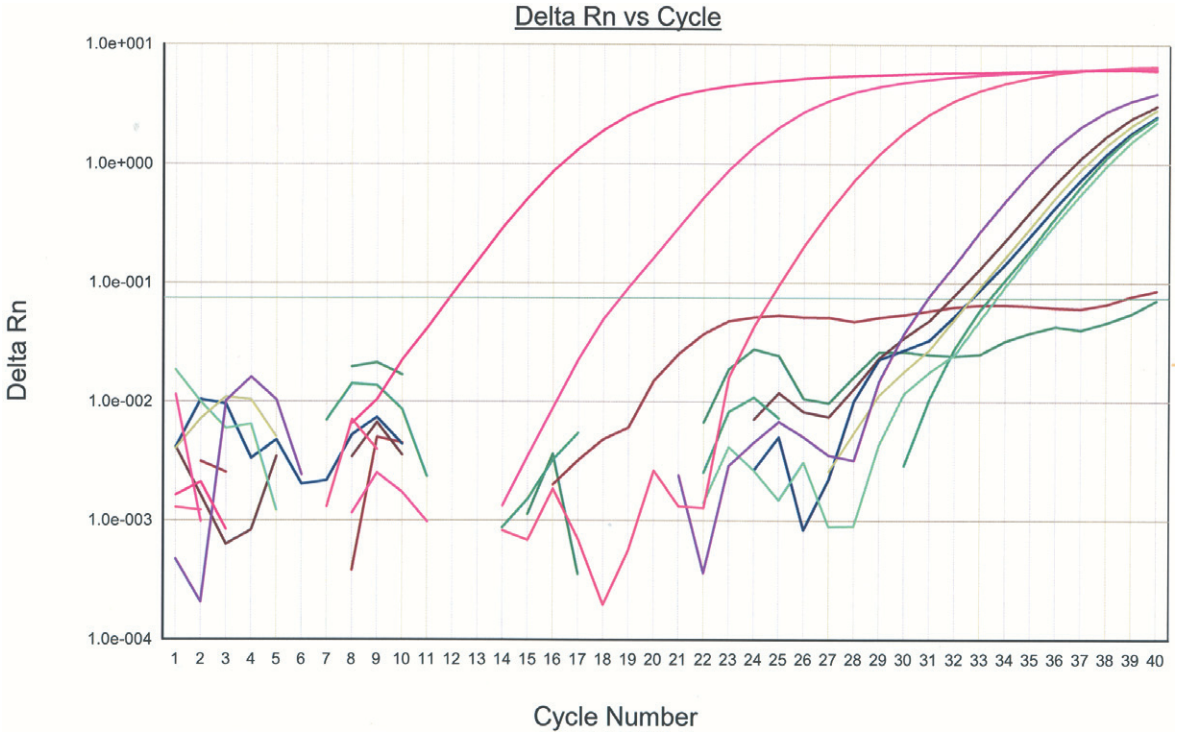

Figure 4.4 Amplification plot generated following real-time PCR reaction. The Delta Rn is the magnitude of the fluorescent signal generated by the PCR. The horizontal turquoise line is the threshold i.e. the level of Delta Rn whose intersection with the amplification plot determines the Ct. The Ct is the cycle number at which the fluorescence passes the threshold.

hepatitis viruses and cytomegalovirus. At present there is very limited availability of antiviral products for use in animals and qPCR is used primarily as a research tool in veterinary laboratories.

There are three major methodologies for the detection of product by real-time PCR all of which use fluorescent dyes. The product can be detected using a free dye in the reaction such as SYBR Green which causes a dramatic increase in fluorescence when it binds to double-stranded DNA. SYBR Green-based PCR assays are easy to develop as only a pair of primers are required. As a detection system SYBR Green is relatively inexpensive and quite sensitive. However, it lacks specificity as the dye binds to any double-stranded DNA including primer dimers. Other similar double-stranded DNA-binding flurophores include EvaGreen, BOXTO and LCGreen.

More specific detection systems are probe based and rely on FRET for product detection. The probe is a fluorescently labelled oligonucleotide that hybridizes to a complementary target sequence in an internal region of the PCR product, that is, between the primers. Primer dimers and extra-assay DNA are not detected by this system. FRET requires a fluorescent molecule known as a donor to interact with a second molecule known as the acceptor. During FRET the donor fluorescent dye is excited by an external light source and emits light. When the donor is in close proximity to the acceptor the light is absorbed by the acceptor, thus quenching the donor signal. The fluorescent signal from the donor commonly known as the reporter, is monitored in real-time PCR. TaqMan or dual labelled probes have a reporter at one end and a quencher at the other (Fig. 4.5). When free in the reaction mix the reporter signal is quenched by the acceptor. During PCR the probe anneals to the target sequence prior to the primers. When the primer binds and Taq DNA polymerase extends the new strand the probe is displaced. The probe is degraded and the dyes separate. FRET ceases and the reporter signal is detected by the PCR instrument. The fluorescence increases with each cycle as the probe degrades. The length of the probe, i.e. the distance between the reporter and the quencher, is inversely correlated to the quenching efficiency. Thus, TaqMan probes are usually less than 30 bases in length. The original donor and quencher dyes were 6-carboxy fluorescein (6-FAM) and 6-carboxy-tetramethylrhodamine (TAMRA). However, there are many different fluorescent dyes and quencher molecules on the market and most real-time PCR instruments have the capability to measure the light emitted from different reporters in a single reaction. Thus it is possible to multiplex and test for several pathogens in a single assay.

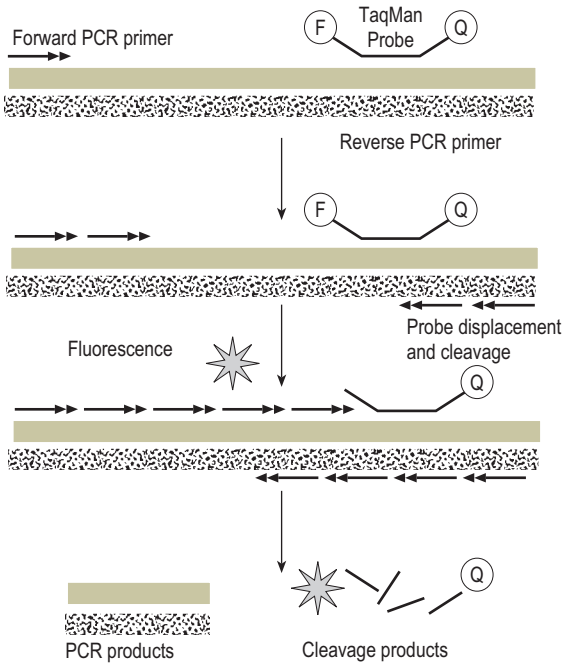

Figure 4.5 Diagram outlining TaqMan and fluorescence resonance energy transfer reaction.

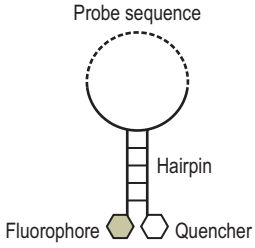

Figure 4.6 Diagram of a molecular beacon.

Molecular beacons are similar to TaqMan probes but they do not require probe cleavage as they are designed to fold into a hairpin structure that brings the reporter and quencher close together (Fig. 4.6). On annealing the structure unfolds to separate the dyes, FRET ceases and the reporter signal is detected.

The third methodology for the detection of real-time PCR product does not require a probe but uses fluorescently labelled primers such as LUX, Plexor and Scorpion. LUX has been applied to the development of a duplex PCR for the detection of avian influenza and Newcastle disease but in general these systems are not as commonly used in veterinary laboratories as the more popular SYBR Green and TaqMan assays.

Optimization of PCR

Notwithstanding the steady increase in the availability of commercial PCR assays for veterinary pathogens, many assays continue to be developed and validated in the diagnostic laboratory. Careful optimization of each PCR assay is essential to obtain consistent results. The primer and probe target sequences need to be specific for the target organism and conserved across different types or strains. For example, by choosing primers from a conserved region within the NS1 segment, a TaqMan assay has been developed for the detection of all nine African horse sickness virus (AHSV) serotypes. The potential of PCR to attain far greater sensitivity than traditional methods of isolation and detection can be seriously compromised by limitations due to sequence variation. Not every PCR test is successful. False-negative results may result from sequence variation in the PCR primer target sites. Degenerate PCR, that is, the use of several primers that vary at one or more nucleotide position may be used to detect viruses of high genetic diversity. Primer/probe design is particularly important in multiplex PCR where it is important to minimize cross-reactions which could result in a decrease in sensitivity and specificity. False-negative results may also be due to failure to optimize the PCR conditions. Once suitable primers and probes have been selected the most important parameters that affect the efficiency of PCR are the annealing temperature, the cycling regimen and the composition of the buffer. The operator can control the cycling parameters and the number of cycles, while some thermocyclers allow the rate of heating and cooling (ramping) to be modified. This flexibility is useful when optimizing PCR reactions. The concentration of magnesium in the buffer may also require optimization as it can affect the specificity and efficiency of the reaction. If there is too much magnesium there may be a loss of specificity leading to the amplification of nonspecific products and if there is too little the yield of PCR product may be reduced. PCR optimization kits are available and allow a range of buffers to be evaluated. Several types of recombinant Taq polymerase that work optimally under different conditions and are suited to different purposes are available. Hot-start enzymes limit the polymerase activity below a certain threshold and minimize the false priming that can sometimes occur within the first cycle when the reaction is heating. Once the assay is optimized individual sample quality, PCR inhibitors and poor nucleic extraction may have a negative impact on the accuracy of a result. Bile salts, polysaccharides in faeces and anticoagulants in blood samples may inhibit PCR.

There are many methods and a large number of kits available for DNA and RNA isolation from different samples. The choice of kit affects the sensitivity of the assay. Automated extraction systems have accelerated the diagnostic procedure and led to the establishment of high-throughput assays. However, these extraction systems are sometimes less sensitive than manual extraction.

Strain Typing and Characterization

PCR-based techniques can be used to type microorganisms and to identify specific genes or mutations that are associated with virulence factors or antimicrobial resistance. Nucleotide sequencing is a definitive method of genotyping. The PCR product can be sequenced directly using one of the PCR primers as the primer for the sequencing reaction and the sequence compared with the data in a sequence database, for example, Genbank. Variable genes such as the 65-kDa heat shock protein gene present in mycobacteria or the haemagglutinin gene of influenza viruses are used to identify related species and to establish phylogenetic relationships. The diversity of the intergenic spacer regions has been used for the identification of staphylococcal and streptococcal species. The sequencing of short polymorphic repeat regions in the protein A gene (spa) and the coagulase gene has proved very useful for the typing of *Staphylococcus aureus* including meticillin-resistant strains.

Mutations and different strains may be detected by melting-curve analysis when DNA-binding dyes or hybridization probes that are still intact after PCR are used. The melting point (Tm) is the temperature at which 50% of the double-stranded DNA dissociates and is single stranded (Fig. 4.7). As the Tm depends on the sequence a mutation may be detected by melt curve analysis. This type of approach has been successfully applied to the typing of cryptosporidia, herpesviruses, pox viruses and other organisms. The identification of a single nucleotide change such as the putative neurovirulence marker in the polymerase gene of equine herpesvirus 1 does not require the sequencing of the PCR amplicon but is rapidly and conveniently detected by differential probe hybridization. Similarly, a multiplex real-time PCR assay using a chemistry known as primer probe energy transfer (PriProET) targets the 3D gene of foot-and-mouth disease virus (FMDV) and detects and differentiates all seven serotypes. PriProET has also been used for the differential diagnosis of three vesicular diseases, FMDV, swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) and two swine haemorrhagic diseases: classical swine fever (CSF) and African swine fever (ASF). This chemistry tolerates mutations in the probe target better than many other probe-based real-time PCR systems. Different strains are detected by melting curve analysis.

Microarrays

Microarray probe-based diagnostics have recently expanded into the field of viral and bacterial diseases. Solid-phase microarrays consist of a panel or array of nucleic acid probes on slides, tubes or strips that bind

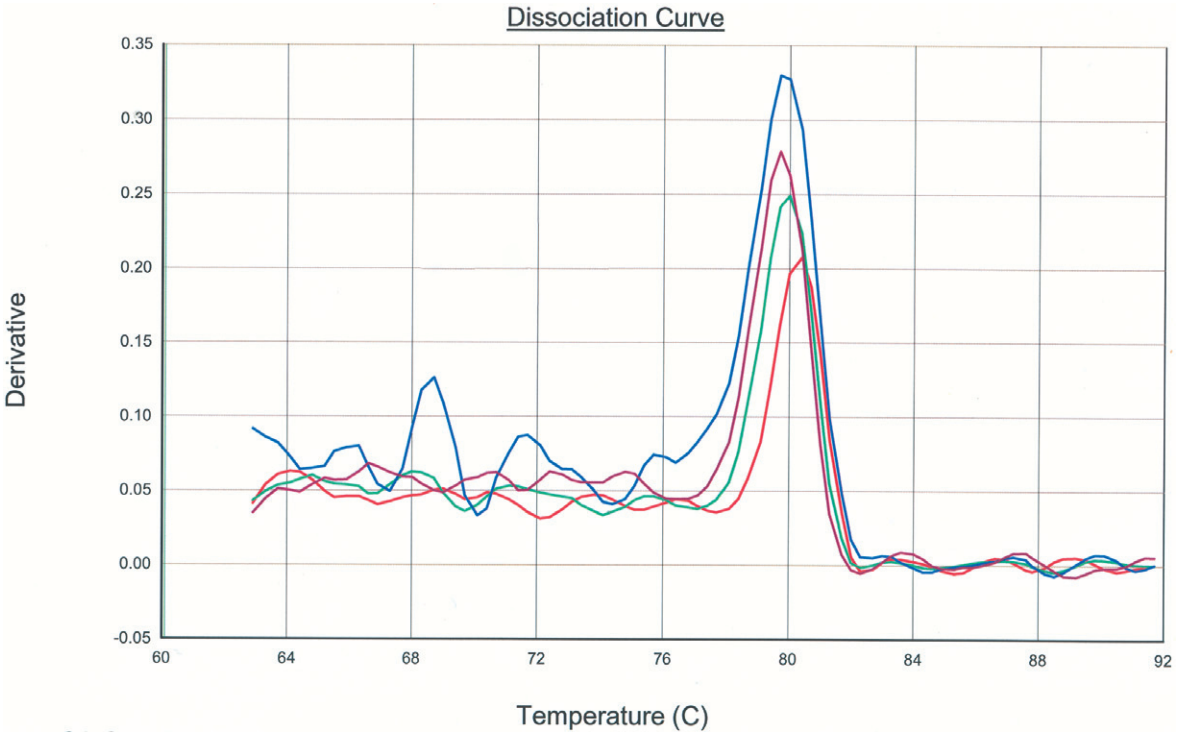

Figure 4.7 Example of melting curve analysis. The peaks indicate that these PCR products have the same Tm.

complementary pieces of nucleic acid. Nucleic acid probes linked to microspheres offer an alternative to the solid surface in the conventional microarray and should be easy to automate for high-throughput diagnostics. Microarray technology has been used to detect avian influenza, herpesviruses, pestiviruses and adenoviruses. Commercial microarrays include kits for the analysis of antimicrobial resistance and virulence in different bacteria and for the detection of enteric pathogens of swine. The sensitivity of diagnostic microarrays can be improved by the use of padlock probes. The target/probes are amplified with a universal primer set. This is more costly and less sensitive than conventional real-time PCR assays but allows simultaneous analysis of thousands of samples. The combination of padlock probes and microarrays has been used to develop assays that detect and type avian influenza and VSV. Microarrays can be adapted not just to detect infectious agents but to monitor cytokine expression and other components of the immune response as well as genetic and nutritional factors.

Conclusion

Molecular techniques provide rapid, sensitive, front-line diagnostic tests for infectious diseases. This type of technology is constantly improving and the development of less expensive more robust tests facilitates their transfer between laboratories. The worldwide application of these techniques will contribute to more effective disease control, sustainable livestock production and an improvement of animal welfare.

FURTHER READING

Belák, S., Thorén, P., LeBlanc, N., et al., 2009. Advances in viral disease diagnostic and molecular epidemiological technologies. Expert Review of Molecular Diagnostics 9, 367–381.

Dorak, M., 2006. Real-time PCR. Taylor and Francis, Oxford.

McPherson, M., Møller, S., 2006. PCR. Taylor and Francis, Oxford.

Pestana, E., Belak, S., Diallo, A., et al, 2010. Early, rapid and sensitive veterinary molecular diagnostics – real time PCR applications. Springer, Dordrecht.

Tang, Y.-W., Stratton, C.W., 2006. Advanced Techniques in Diagnostic Microbiology. Springer, Berlin.

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Chapter 5

The isolation of viruses and the detection of virus and viral antigens

VIRUS ISOLATION

Virus isolation is traditionally the definitive criterion against which other diagnostic methods are assessed. Viruses are obligatory intracellular parasites and can only be isolated in living systems. Isolation of a virus may be carried out in cell monolayers, fertile eggs or laboratory animals (Fig. 5.1). Thankfully, the use of laboratory animals has largely been supplanted. The vast majority of virus isolation is carried out in cells. Cell culture involves the growth of cells in vitro as monolayers attached to the surface of a glass or plastic culture vessel or, less frequently, as suspensions. Cell monolayers may be primary or continuous. A primary cell line is one derived from cells or tissue taken directly from the animal. Foetal tissues are frequently used as they grow well, disaggregate easily and the cells tend to be more permissive than those originating from adult tissue. Primary cell lines are labour-intensive to prepare and die or exhibit a decline in permissiveness after a limited number of passages. They have a finite life span. Thus, whenever possible, most laboratories opt to use continuous or established cell lines. These cell lines are usually available commercially and are capable of extended or indefinite propagation. They originate from malignant neoplasms or transformed cell lines. Many have a narrower range of virus susceptibilities than do primary cell lines. There is no single cell line that is susceptible to all viruses. Thus, the laboratory has to choose cell lines for routine use that are permissive for the viruses most commonly encountered.

Virus replication in cells is detected by observing the cytopathic effect (CPE) or the formation of plaques, that is, areas of cell lysis which do not take up stain such as neutral red. Inoculated cell monolayers are observed daily by microscopy for many days. Many viruses produce a characteristic type of CPE. When no CPE is observed blind passaging of the supernatant onto a fresh monolayer may result in amplification of virus, facilitating detection. Samples that are contaminated may require filtering to remove bacteria or fungi prior to inoculation. Some viruses are non-cytopathic but can be detected by a characteristic property. For example, some viruses of the orthomyxovirus and paramyxovirus groups cause the adherence of erythrocytes to cells (haemadsorption) and some viruses agglutinate erythrocytes (haemagglutination). Non-cytopathic viruses can also be detected in cell culture supernatant by the detection of viral antigen by immunofluorescence or immunoenzyme techniques or by detection of nucleic acid by the polymerase chain reaction (PCR) (see Chapter 4).

Some viruses, for example certain strains of influenza and bluetongue virus, grow more readily in embryonated eggs than in tissue culture. Fertile eggs for virus isolation should, if possible, be obtained from a specific pathogen-free flock to minimize problems with egg-transmitted viruses and maternal antibody. Before inoculation the eggs are candled to ensure the embryo is alive and to mark the position of the air sac and the major blood vessels. The eggs are inoculated between days five and 14 of incubation, depending on the route used (allantoic or amniotic cavity, yolk sac, chorioallantoic membrane or intravenous). Virus introduced into the allantoic cavity replicates in the endodermal cells of the allantois and is released into the allantoic fluid. Virus introduced into the amniotic cavity enters the respiratory and digestive tracts of the embryo and may therefore replicate in a variety of cell types depending on the tropism of the virus. These two

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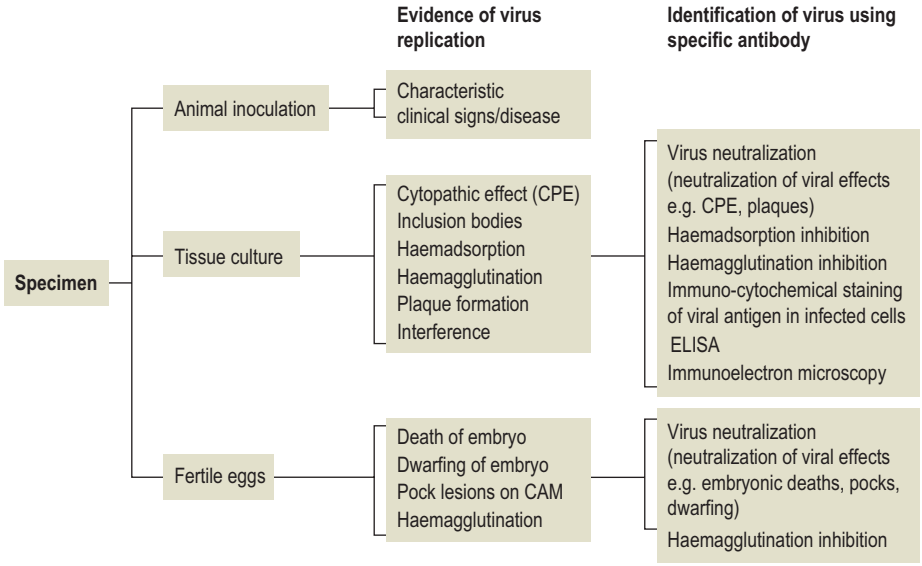

Figure 5.1 Virus isolation and identification. CAM = chorioallantoic membrane; ELISA = enzyme-linked immunosorbent assay.

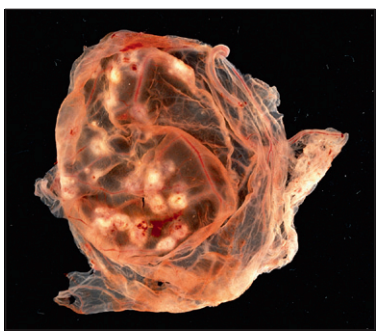

Figure 5.2 Chorioallantoic membrane with pock lesions caused by vaccinia virus.

routes are frequently used for the isolation of influenza viruses. The chorioallantoic route is used more frequently for viruses which produce plaques or pocks on the chorioallantois (Fig. 5.2), for example, fowlpox. The yolk-sac route is used for the isolation of rickettsiae and chlamydiae. Many viruses that replicate in eggs may be detected by examining the fluids or tissues harvested two to six days post inoculation. Influenza virus may be detected by the haemagglutinating activity of the harvested allantoic or amniotic fluid, and its inhibition with specific antiserum.

If optimal isolation conditions for the virus in question are provided, then isolation can be a very sensitive procedure for the detection of viruses, while permitting further study of the virus. However, virus isolation is labour-intensive, expensive and slow to yield results. A number of passes may be required to adapt the virus to growth in the laboratory and special care is required in the handling and transport of specimens (see Chapter 1) to ensure the viability of viruses in the sample upon arrival at the laboratory. A number of cultivation factors influence the reliability of virus isolation (Fig. 5.3). For example, rotaviruses and coronaviruses replicate best in the presence of trypsin. Rolling the inoculated cell culture tubes may enhance virus replication and CPE. Co-cultivation techniques may be required. Certain viruses, such as alcelaphine herpesvirus-1 cannot be isolated from dead cells but may be recovered from peripheral blood leukocytes if cell viability is preserved during processing. Other viruses such as papillomaviruses have not been grown in cell culture.

Following isolation, additional tests are usually required to specifically identify the virus (Tables 5.1 to 5.6). Most of the techniques discussed in the chapter on serological diagnosis (Chapter 3) may be used for this purpose as may PCR (Chapter 4). Methods that require specific antisera include immunocytochemical staining, haemagglutination inhibition, complement fixation and neutralization. PCR does not require specific antisera and can be used to rapidly and specifically identify virus isolates.

Finally, a negative virus isolation result does not eliminate the possibility that the disease is of viral aetiology. Conversely, a positive result does not automatically establish a causal relationship between the clinical signs and the virus isolate.

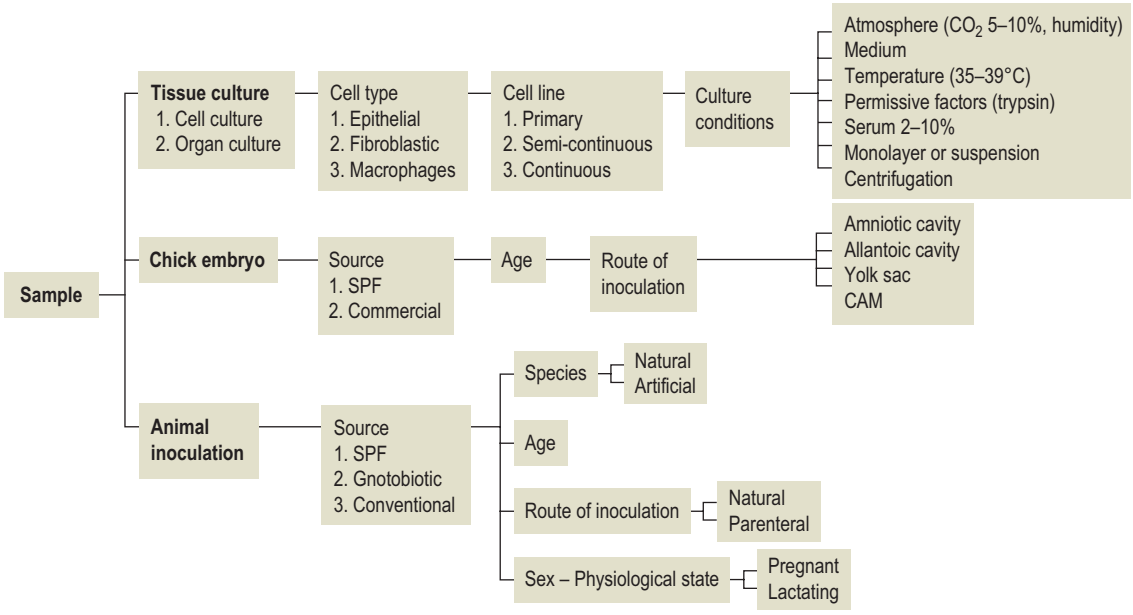

Figure 5.3 Factors for consideration when undertaking viral cultivation. SPF = specific pathogen-free; CAM = chorioallantoic membrane.

Table 5.1 Isolation and identification of viruses of veterinary importance: ruminants

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Bovine herpesvirus 1 (infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis)	Nasal and ocular swabs. Nasopharyngeal aspirate. Tracheal scraping. Foetal liver and kidney. Vaginal swab	Cell culture (bovine origin, e.g. calf kidney, calf testes, foetal calf lung, MDBK cell line, etc.)	CPE Intranuclear inclusions	VN IF Restriction endonuclease analysis PCR
Bovine herpesvirus 2 (mammilitis)	Scabs. Swabs from teat and udder lesions	Cell culture (bovine origin, e.g. MDBK cell line)	CPE	VN PCR
Foot-and-mouth disease virus	Vesicular fluid or lesion material Blood and oesophageal/ pharyngeal fluid	Cell culture (primary bovine thyroid cells, and primary calf, pig or lamb kidney cells)	CPE	ELISA CFT RT-PCR
Vesicular stomatitis virus	Vesicular fluid or lesion material	Cell culture (Vero, IB-RS-2 and BHK-21 cells)	CPE	IF ELISA PCR
Bluetongue virus	Buffy coat	Embryonated chicken eggs	Death Haemorrhage	ELISA VN RT-PCR
Rift Valley fever virus	Blood and tissues (liver, spleen and brain)	Cell culture (Vero and BHK-21 cells)	CPE	IF RT-PCR

Continued

Table 5.1 Isolation and identification of viruses of veterinary importance: ruminants—cont'd

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Bovine viral diarrhoea virus (bovine viral diarrhoea/mucosal disease)	Buffy coat (freshly collected heparinized blood). Spleen, abomasum, intestine, semen. Foetal tissues	Cell culture (bovine origin)	CPE IF (non-cytopathic isolates)	VN IF RT-PCR
Bovine coronavirus	Faeces	Cell culture (bovine kidney cells; trypsin enhances growth)	CPE	VN IF RT-PCR
Bovine parainfluenza virus 3	Nasal swabs. Nasopharyngeal aspirate. Lung	Cell culture (bovine origin)	CPE (variable) HA (guinea-pig RBCs)	VN IF ELISA HAI RT-PCR
Bovine respiratory syncytial virus	Nasal swabs. Nasopharyngeal aspirate. Lung	Cell culture (bovine origin; preferably derived from respiratory tract)	CPE (slow to develop)	IP IF RT-PCR
Border disease virus	Leukocytes Tissues (spleen, thyroid, thymus, kidney, brain, lymph nodes)	Cell culture (ovine origin) Co-cultivation of leukocytes	CPE or Non-cytopathic	IP IF RT-PCR
Louping ill virus	Brain	Cell culture (primary ovine embryo cell lines or pig kidney IB/RS-2 cell line) Suckling mice (intracerebral inoculation)	CPE (variable) Posterior paralysis. Death	VN RT-PCR HAI (pigeon or rooster RBCs)

CPE = cytopathic effect, CF = complement fixation, ELISA = enzyme linked immunosorbent assay, HA = haemagglutination, HAI = haemagglutination inhibition, IF = immunofluorescence, IP = immunoperoxidase, PCR = polymerase chain reaction, RT-PCR = reverse transcription polymerase chain reaction, VN = virus neutralization

Table 5.2 Isolation and identification of viruses of veterinary importance: pigs

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Porcine reproductive and respiratory syndrome virus	Serum, ascetic fluid and tissues (lungs, tonsils, lymph nodes and spleen)	Cell culture (porcine alveolar macrophages, and MARC-145 (MA-104 clone) cells)	CPE	IF RT-PCR
Porcine herpesvirus 1 (Aujeszky's disease)	Nasal and tonsil swabs, brain, tonsil,spleen and lung tissue	Cell culture (porcine origin, e.g. PK-15 or SK6 cell line or primary or secondary kidney cells)	CPE	VN IF IP PCR

Table 5.2 Isolation and identification of viruses of veterinary importance: pigs—cont'd

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Swine influenza virus	Nasal swabs and lung tissue	Embryonated chicken eggs Cell culture (porcine cells and MDCK cells)	HA CPE	HAI NI RT-PCR
Porcine parvovirus	Foetal tissues	Cell culture (porcine origin; freshly seeded)	CPE	VN IF PCR
Transmissible gastroenteritis virus	Faeces, intestine	Cell culture (primary pig kidney; trypsin enhances growth)	CPE Plaque formation IF	VN IF RT-PCR
Porcine teschovirus	Suspensions of brain and spinal cord	Cell culture (porcine kidney cells)	CPE	VN IF RT-PCR
Swine fever virus (hog cholera)	Blood (heparin or EDTA treated), spleen, tonsils, ileum, lymph nodes	Cell culture (PK-15 cell line)	IF IP	IF IP RT-PCR
African swine fever virus	Blood (EDTA treated), spleen, lymph nodes, tonsil and kidney	Primary pig leukocyte or bone marrow cultures	HAD	IF PCR
Swine vesicular disease virus	Vesicular fluid or lesion material Faeces (subclinical infection)	Cell culture (porcine cells IB-RS-2)	CPE	ELISA RT-PCR
Nipah virus	Urine, throat or nasal swabs Tissues (brain, lung, spleen and kidney)	Cell culture Vero and RK-13 cells	CPE	IP VN PCR
Vesicular stomatitis virus (see Table 5.1)				

CPE = cytopathic effect, ELISA = enzyme linked immunosorbent assay, HA = haemagglutination, HAD = haemadsorption, HAI = haemagglutination inhibition, IF = immunofluorescence, IP = immunoperoxidase, NI = neuraminidase inhibition, PCR = polymerase chain reaction, RT-PCR = reverse-transcription polymerase chain reaction, VN = virus neutralization

Table 5.3 Isolation and identification of viruses of veterinary importance: horses

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Equine herpesviruses 1 or 4 (abortion, rhinopneumonitis, neurological disease)	Nasal swabs Foetal lung, liver, kidney, spleen, adrenal and thymus Brain and spinal cord	Cell culture (RK13 for EHV-1 and equine cell lines for EHV-4)	CPE	IF PCR

Continued

Table 5.3 Isolation and identification of viruses of veterinary importance: horses—cont'd

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Equine influenza virus	Nasal swabs	Nine- to 12-day-old embryonated hen eggs (allantoic cavity) Cell culture (MDCK)	HA	HAI RT-PCR
Equine viral arteritis virus	Nasal swabs Conjunctival swabs Blood Semen (carrier stallion) Lung, liver, spleen and lymph glands	Cell culture (RK13)	CPE	VN IF IP RT-PCR
West Nile fever virus	Brain and spinal cord	Cell culture Vero and RK-13 cells	CPE	IF PRN RT-PCR
Eastern equine encephalomyelitis virus	Brain (tissue of choice) Liver and spleen	Cell culture – primary chicken or duck embryo fibroblasts; Vero, BHK-21 and RK-13 cells One- to four-day-old-mice (intracerebral inoculation)	CPE Death	CF HAI PRN IF RT-PCR
Western equine encephalomyelitis virus	Brain but WEE is rarely isolated from infected horses	Cell culture (BHK-21 and RK-13 cells) One- to four-day-old mice or hamsters (intracerebral inoculation)	CPE Death	CF HAI PRN IF RT-PCR
Venezuelan equine encephalomyelitis virus	Blood or sera of febrile horses Less frequently blood or brain of encephalitic horses	Cell culture (BHK-21 and RK-13 cells) 1–4-day-old mice or hamsters (intracerebral inoculation)	CPE Neurological signs	CF HAI PRN IF RT-PCR
Japanese encephalitis virus	Brain and spinal cord Blood	Cell culture (porcine or hamster kidney cells, Vero or MDBK cells) Two- to four-day-old mice (intracerebral inoculation)	CPE Neurological signs followed by death	IF RT-PCR
Hendra virus	Urine, tissues (brain, lung, spleen and kidney)	Cell culture Vero and RK-13 cells	CPE	IP VN PCR
Vesicular stomatitis virus (see Table 5.1)				

CPE = cytopathic effect, HA = haemagglutination, HAI = haemagglutination inhibition, IF = immunofluorescence, IP = immunoperoxidase, PCR = polymerase chain reaction, PRN = plaque reduction neutralization, RT-PCR = reverse-transcription polymerase chain reaction, VN = virus neutralization

Table 5.4 Isolation and identification of viruses of veterinary importance: dogs

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Canine distemper virus	Lymphocytes	Cell culture Canine macrophages or lymphocytes (cocultivation) Inoculation of ferret	CPE IF Clinical signs Death	IF RT-PCR ELISA
Canine parvovirus	Faeces	Cell culture (canine or feline origin, e.g. MDCK or CRFK cell lines; freshly seeded)	IF HA (pig RBCs at 4°C)	IF VN HAI PCR
Canine adenovirus 1 (infectious canine hepatitis)	Nasal swabs, urine, blood, lung, kidney and lymph nodes	Cell culture (canine origin)	CPE	VN PCR
Canine adenovirus 2	Nasal or throat swabs Lung	Cell culture (canine origin)	CPE	VN PCR
Canine herpesvirus	Kidney, liver, lung and spleen	Cell culture (canine origin)	CPE	VN PCR
Rabies virus	Brain	Cell culture (neuroblastoma cells) Weanling mice (intracerebral inoculation)	IF CNS signs, death	IF PCR

CPE = cytopathic effect, ELISA = enzyme linked immunosorbent assay, HA = haemagglutination, HAI = haemagglutination inhibition, IF = immunofluorescence PCR = polymerase chain reaction, RT-PCR = reverse-transcription polymerase chain reaction, VN = virus neutralization

Table 5.5 Isolation and identification of viruses of veterinary importance: cats

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Feline parvovirus (panleukopenia)	Faeces. Intestine	Cell culture (feline origin; freshly seeded)	CPE or Non-cytopathogenic IF Intranuclear inclusions	IF PCR
Feline herpesvirus 1 (rhinotracheitis)	Nasal, oropharyngeal and conjunctival swabs	Cell culture (feline origin)	CPE	VN PCR IF
Feline calicivirus	Nasal, oropharyngeal and conjunctival swabs Lung	Cell culture (feline origin)	CPE	VN IF RT-PCR

CPE = cytopathic effect, IF = immunofluorescence, PCR = polymerase chain reaction, RT-PCR = reverse-transcription polymerase chain reaction, VN = virus neutralization

Table 5.6 Isolation and identification of viruses of veterinary importance: poultry

Virus	Specimen(s)	Host system	Evidence of viral replication	Identification
Avian infectious bronchitis virus	Trachea, lung, large intestine and faeces	Nine- to 11-day-old embryonated hens' eggs (allantoic cavity)	Teratological changes in embryo, stunting of embryo and curling of down	VN IF PCR
		Tracheal organ culture (TOC)	Ciliostasis	
Avian infectious laryngotracheitis	Tracheal swabs and scrapings	CAM of 10- to 12-day-old embryonated hens' eggs Cell culture (chick embryo liver or kidney)	Pocks CPE	VN EM IF PCR
Avian paramyxovirus 1 (Newcastle disease)	Oro-nasal, tracheal and cloacal swabs and faeces. Tissues including trachea, lungs, intestine, spleen, kidney, brain and liver	Nine- to 11-day-old embryonated hens' eggs (allantoic cavity)	HA	HAI RT-PCR
Avian encephalomyelitis virus (epidemic tremor)	Brain, faeces	Chick embryo (six to seven days; yolk sac) Day-old chicks (intracerebral inoculation)	Clinical signs in hatched chicks Tremors, death	VN RT-PCR
Avian influenza virus (fowl plague)	Oro-nasal, tracheal and cloacal swabs and faeces. Tissues including trachea, lungs, intestine, spleen, kidney, brain and liver	Nine- to 11-day-old embryonated hens' eggs (allantoic cavity)	HA	AGID ELISA RT-PCR HAI
Fowlpox virus	Scabs, scrapings from lesions	Nine- to 12-day-old embryonated hens' eggs (allantoic cavity)	Focal white pock lesions. Thickening of the CAMs with intracytoplasmic inclusion bodies	VN PCR
Marek's disease virus	Buffy coat (freshly collected heparinized blood). Suspensions of lymphoma cells or spleen cells. Feather tips	Cell culture (chicken kidney cells or duck embryo fibroblasts)	CPE, plaque formation	IF PCR

AGID = agar gel immunodiffusion, CAM = chorioallantoic membrane, CF = complement fixation, CPE = cytopathic effect, ELISA = enzyme linked immunoassay, EM = electron microscopy, HA = haemagglutination, HAI = haemagglutination inhibition, IF = immunofluorescence, PCR = polymerase chain reaction, RT-PCR = reverse transcription polymerase chain reaction, PRN = plaque reduction neutralization, VN = virus neutralization

DIRECT DEMONSTRATION OF VIRUS AND VIRAL ANTIGENS

Electron Microscopy

Electron microscopy is a rapid though relatively insensitive means of detecting virus. It is usually a supplementary rather than a primary viral diagnostic technique. A negative result does not necessarily indicate the absence of virus. A virus concentration in excess of 10^6 per mL is required if viral particles are to be visualized. In certain clinical conditions such as enteric or skin infections, for example rotavirus diarrhoea or vesicular stomatitis (VS), the numbers of viral particles frequently exceeds this figure. Alternatively, it may be necessary to isolate and successfully passage the virus in order to attain titres of this magnitude. It is possible to detect non-viable virus provided that structural morphology has been retained. Combined viral infections can be readily diagnosed. The most serious drawback is the large expense incurred in purchasing and maintaining an electron microscope. Specialist expertise is also required for the processing of samples and the interpretation of results.

Clarification and subsequent concentration of the viral particles present in the sample by ultracentrifugation is usually required for visualization of the virus(es), particularly in the case of faecal specimens. Negative staining, using electron-dense heavy metals, such as phosphotungstic acid, ammonium molybate or uranyl acetate, is used to increase contrast. The viral particles are visualized as bright objects against a dark background (Figs 5.4 and 5.5). In general, members of a viral family have identical morphology and cannot be distinguished by electron microscopy. Exceptions include the *Poxviridae* and *Reoviridae* where there are significant ultrastructural differences between the genera in each family. Agar diffusion or the pseudoreplica technique can be used to concentrate virus particles and remove salts. Immunoelectron microscopy is a modification of the technique capable of enhancing its sensitivity. The antiserum used may be mixed with the sample and the immune complexes pelleted by centrifugation following incubation, or it may be coated onto the copper grid and the sample applied in the usual way. The antiserum may contain polyclonal or monoclonal antibodies and be species- or even serotype-specific. Immunogold labelling is a useful aid in the detection and identification of virus serotypes.

Electron microscopy can be used to detect viruses in thin sections of biopsy or postmortem tissue. The disadvantage of poor sensitivity is offset by the lack of a requirement for specific antiserum.

Immunofluorescence

Immunofluorescence uses a labelled immunoglobulin for detecting antigen or antibody. The label employed is a fluorochrome, usually fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate, covalently attached to antibody molecules. When examined by light of a specific wavelength or wavelengths, fluorescein emits a characteristic green colour and rhodamine a red colour. The two fluorochromes can be used together when testing for the presence of two separate antigens. A special fluorescence microscope with a mercury vapour light source is required for immunofluorescence.

There are many different methods of using fluorescent-labelled antibodies in microbiology and these include the direct, indirect and sandwich methods (see Figure 3.18). In direct immunofluorescence, conjugated antibody is added directly to a smear, tissue section, or monolayer fixed on a slide and after incubation, unbound antibody

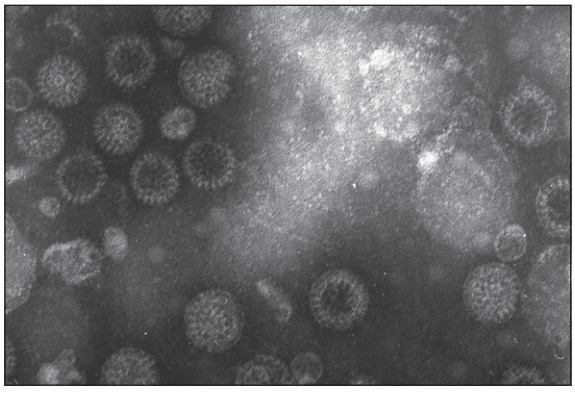

Figure 5.4 Electron micrograph of rotavirus in a faecal preparation showing the double capsid and typical 'wheel' appearance. Negatively stained with phosphotungstic acid (PTA). (×45,000)

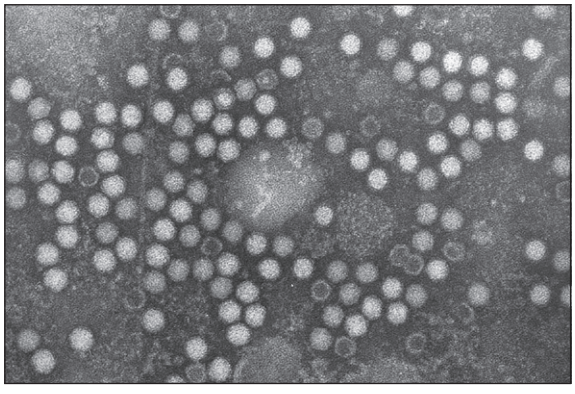

Figure 5.5 Electron micrograph of an enterovirus (*Picornaviridae*) in faeces illustrating both intact and 'empty' (stain penetrated) virions. (PTA, ×100,000)

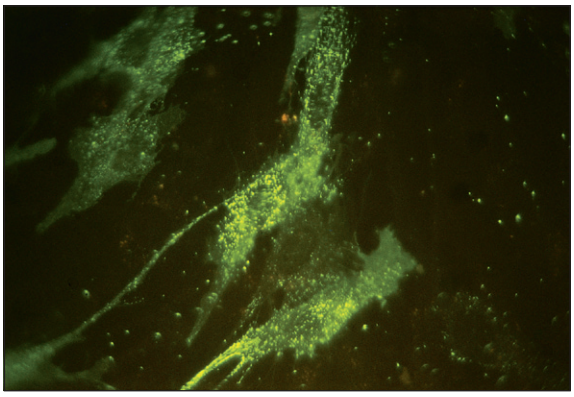

Figure 5.6 Direct immunofluorescence. Demonstration of parainfluenza 3 (PI3) virus in foetal calf lung cells. (×400)

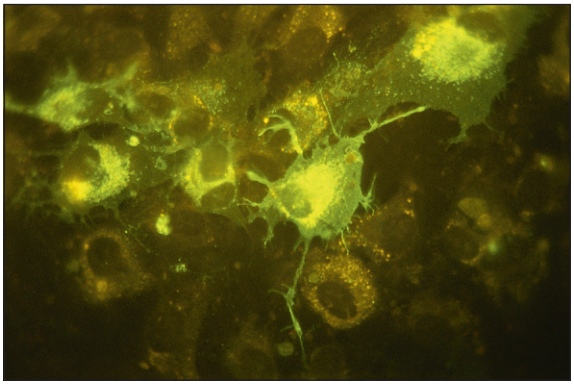

Figure 5.7 Bovine coronavirus in foetal calf lung cells. (Direct FA technique, ×400)

is removed by washing. The slide is examined in a fluorescence microscope and where labelled antibody binds to antigen bright fluorescence is evident (Fig. 5.6). Immunofluorescence can be used to detect viral antigen in smears such as nasopharyngeal aspirates, epithelial cells from the base of vesicular lesions and in cryostat sections of tissues. It may also be used to confirm the presence of virus in tissue culture (Fig. 5.7), in particular, non-cytopathic viruses (Fig. 5.8). Rabies virus may be detected by immunofluorescence of an acetone-fixed brain tissue smear. Fluorescent antibody (FA) methods are rapid and sensitive (a single infected cell may be detected), but require careful interpretation to avoid errors. Both monoclonal and polyclonal antibodies may be used in fluorescent antibody tests. Monoclonal antibodies are more specific and can be used to distinguish closely related viruses that cross-react with polyclonal antibodies, for example, equine herpesviruses 1 and 4. However, monoclonal antibodies may vary in sensitivity when a virus population is heterogeneous. In such instances a pool of different monoclonals should

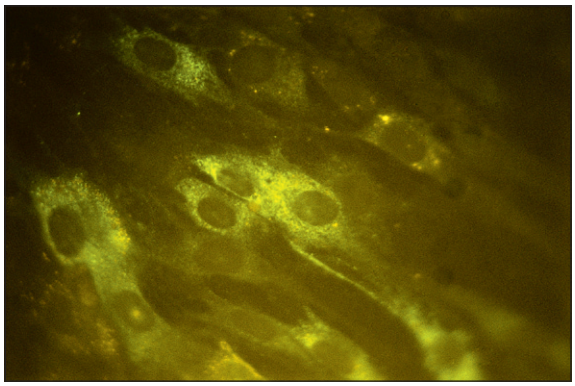

Figure 5.8 Bovine viral diarrhoea virus in foetal calf lung cells showing characteristic intracytoplasmic fluorescence. (Direct FA technique, ×400)

be employed to reduce the risk of obtaining false-negative results.

In indirect immunofluorescence the antiviral antibody is not conjugated and is detected by a conjugated anti-immunoglobulin. The indirect test is usually more sensitive than the direct test because more labelled antibody attaches per antigenic site. However, the extra incubation and washing requires more time than the direct test which can often be completed within an hour. The high affinity of avidin (an egg white protein) for biotin (a vitamin) can be exploited in immunofluorescence. Biotin is easily coupled to antigen or antibody, while avidin can be coupled to fluorescein. Avidin can bind up to four biotin molecules and their multivalent interaction provides amplification.

Immunofluorescence has proven to be particularly useful for the detection of intracellular viruses such as herpesviruses. A disadvantage of immunofluorescence is that it depends on the subjective interpretation of results by the microscopist. Autofluorescence may occur with some tissues and conjugates may bind non-specifically. Conjugates and other reagents need to be titrated to determine the optimal working dilution.

Histopathology and Immunochemical Staining

Some viruses induce the formation of inclusion bodies in infected cells. These accumulations of viral protein or characteristic degenerative changes may be identified by their staining properties and position within the host cell. Depending on the virus, inclusions may be intranuclear or intracytoplasmic, acidophilic or basophilic (Figs 5.9 and 5.10).

Tissue localization of viral antigens may be accomplished using enzyme-labelled reagents such as peroxidase-labelled antibody. Identification of bound antibody is possible through conversion of a colourless

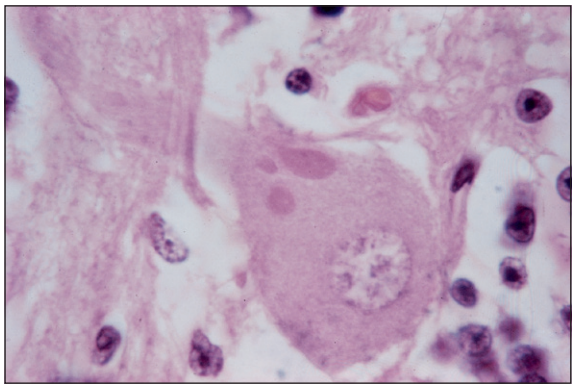

Figure 5.9 Three intracytoplasmic Negri bodies in a Purkinje cell from a dog with rabies showing the characteristic eosinophilic staining. (Haematoxylin and eosin (H&E) stain, ×1000)

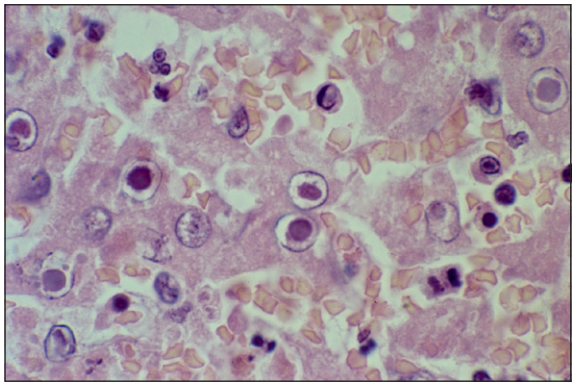

Figure 5.10 Numerous basophilic intranuclear inclusion bodies in hepatocytes from a dog with infectious canine hepatitis. (H&E stain, ×400)

soluble substrate to a coloured insoluble product by the enzyme (Fig. 5.11). Horseradish peroxidase and alkaline phosphatase are the enzymes most often used for this test and the technique has significant advantages over immunofluorescence in that paraffin-embedded or resin-embedded sections can be used. In addition, tissues can be examined by conventional light microscopy and stained preparations can be kept for long periods without fading. In reading immunoperoxidase reactions, care must be taken to distinguish specific from non-specific staining. Endogenous peroxidase is present in the cells of many tissues.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs can be designed to detect antigen or antibody (see also Chapter 3). Direct, indirect and competitive assays

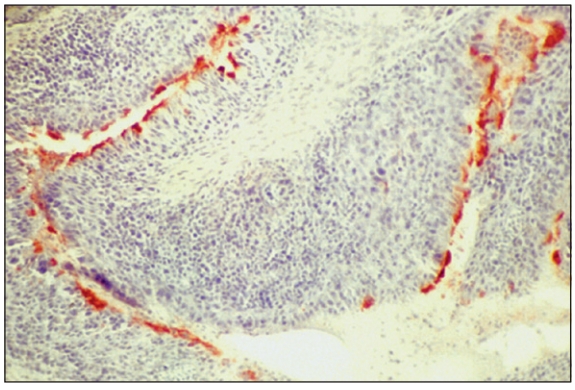

Figure 5.11 Immunoperoxidase staining with haematoxylin counter-stain. Turkey rhinotracheitis virus antigen (red) in bronchiolar epithelium in a lung section. (×400)

can be used to detect viral antigen in samples such as milk, serum and nasal secretions. The indirect sandwich ELISA is currently the diagnostic method of choice for identification of VS and swine vesicular disease. Automated systems allow the rapid processing of thousands of samples. Some ELISAs have been adapted for simple testing of individual animals, primarily companion animals, at the veterinary clinic.

Agglutination

This type of assay can be used for the detection of antigen in which case latex particles are coated with antiviral antibody. If virus is present in the sample the antibody-coated latex particles bind to the antigen and agglutinate. A latex agglutination test is commonly used for the detection of rotavirus in faeces. The result is available within minutes and can be read by eye.

Immunochromatography

Immunochromatography assay kits have been developed for some viruses and are most useful where laboratory support is limited. No instrumentation is required. These kits are based on the same principle as pregnancy testing kits. They include a membrane with immobilized capture antibody in a plastic cassette, enzyme-conjugated antiviral antibodies and substrate solutions. The results are seen as coloured spots or bands. Packet systems are available for rotavirus and influenza.

Agar Gel Immunodiffusion

The AGID test (see also Chapter 3) may be used to identify some viruses such as the morbilliviruses that cause rinderpest and peste des petits ruminants (PPR). It is a cheap and simple test that can be performed in the field if necessary.

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Chapter 6

Antimicrobial agents

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The purpose of antimicrobial susceptibility testing is to guide the clinician in the selection of an antimicrobial agent to which the clinical condition being treated will respond. Although standards and interpretive criteria have been available in human medicine for many years, the first standard for the performance of antimicrobial susceptibility tests (ASTs) in veterinary medicine was published in 1999 by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Veterinary Antimicrobial Susceptibility Testing. This standard is regularly revised with new editions being published at intervals.

There are three principal methods of antimicrobial susceptibility testing in common use: disc diffusion, broth dilution and agar dilution. The choice of method is determined by several factors including ease of use, flexibility, automation or semiautomation for larger-scale operations, cost, reliability and accuracy. The agar disc diffusion technique is probably the most commonly used method in veterinary diagnostic laboratories, particularly in smaller laboratories. The broth microdilution method is increasingly used because of demand for testing methods which give data on minimum inhibitory concentrations (MIC) rather than a simple qualitative result. Methods giving MIC data are usually automated and are more suitable for use in laboratories processing large numbers of samples.

Disc Diffusion Method

Disc diffusion is the simplest method to perform. It entails the placing of discs impregnated with antimicrobial agents onto an agar plate seeded with the bacterium to be tested. The antimicrobial agents diffuse into the agar creating a zone saturated with the agent, in which an organism susceptible to that agent will not grow. The edges of the zone are the point of minimum inhibitory concentration (MIC), however, due to inconsistent rates of dispersion an accurate MIC cannot be determined by this method. The method is simple to perform, reproducible and low cost to run. The method should only be used for the rapidly growing pathogens. In order for the results to be clinically reliable the technique must be carried out in a standardized manner. Standards for the performance of this test method have been described, with many laboratories following the methods described by the NCCLS, now renamed the Clinical and Laboratory Standards Institute (CLSI). The standard developed for testing of bacteria isolated from animals is described in document M31-A3 (CLSI, 2008).

Factors affecting the size of the zone of inhibition

One of the reasons for the strict standardization of the test procedures is that many factors can influence the size of the zone of inhibition including:

- The bacterial concentration of the inoculum: this is of great importance and is usually addressed by ensuring the turbidity of the inoculum is adjusted to a 0.5 McFarland opacity standard. The aim is to have a dense lawn of bacterial growth with the individual colonies just touching each other (Figs 6.1 and 6.2).
- The test medium: Mueller–Hinton or modifications of this medium (Iso-sensitest agar, Oxoid) is usually chosen for routine susceptibility tests. It gives good batch-to-batch reproducibility; is low in

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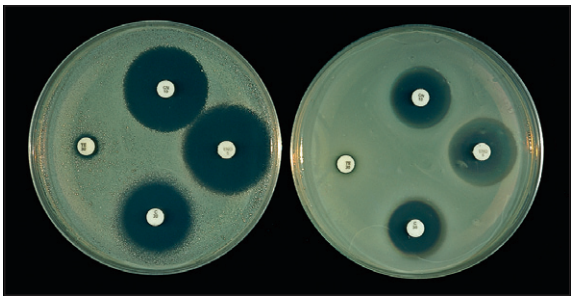

Figure 6.1 *Staphylococcus aureus* on Iso-Sensitest agar indicating the effect of a correct inoculum (left) and a heavy inoculum (right) on the size of the zone of inhibition.

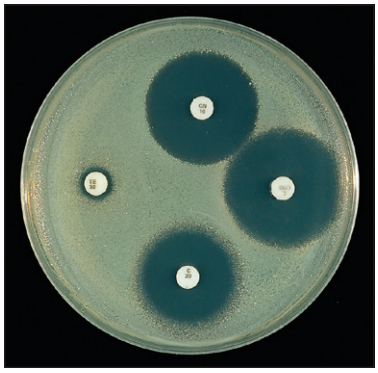

Figure 6.2 *Staphylococcus aureus* on Iso-Sensitest agar tested against gentamicin (CN), enrofloxacin (ENO), chloramphenicol (C) and tetracycline (TE) and illustrating an inoculum size that gives a lawn of the correct density.

sulphonamide, trimethoprim and tetracycline inhibitors; and most pathogens grow satisfactorily on the medium. However, quality control checks should be carried out on each new batch of medium.

- Excessive amounts of thymidine or thymine in a test medium can inhibit sulphonamides and trimethoprim, yielding smaller zones or no zones at all. Variation in divalent cations, mainly magnesium and calcium, will affect the zone size of tetracycline, polymyxin and aminoglycoside tests against *Pseudomonas aeruginosa*.
- For bacteria, such as streptococci, that are unable to grow on Mueller–Hinton agar, blood agar with 5–10% defibrinated sheep blood can be used. However, the zone size, particularly for nafcillin, novobiocin and meticillin, will be 2–3 mm smaller than the normal control limits (Figs 6.3 and 6.4).
- The depth of the agar and the pH of the medium must be standardized as these may also have an effect on the zone size (see Quality Control Methods).

Figure 6.3 *Staphylococcus aureus* on Isosensitest agar tested against novobiocin (NO), trimethoprim-sulfamethoxazole (SXT) and sulfafurazole (SF). Compare with Figure 6.4 when blood agar is used.

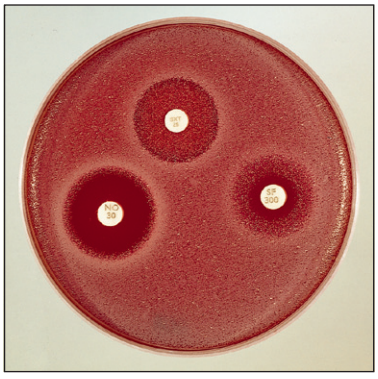

Figure 6.4 The same isolate of *S. aureus* as in Figure 6.3 on blood agar tested against novobiocin (NO), trimethoprim-sulfamethoxazole (SXT) and sulfafurazole (SF). Note the smaller zone sizes compared with Figure 6.3.

- The antimicrobial agent and its concentration in the disc: the ability of the agent to diffuse through the agar varies and the zone of inhibition for some drugs, such as streptomycin, is always comparatively small. The concentrations of the antimicrobial agents in the discs have been chosen to give zone sizes that correlate with achievable serum levels in the patient. The zone sizes and their interpretation are given in Table 6.1. At present there are insufficient data for the correlation of in vitro tests and the clinical use of topical agents for skin, eye and ear conditions. The potency of the agent in the discs must be maintained and storage and handling methods are discussed in Quality Control Procedures.
- Incubation conditions: these have been standardized for routine susceptibility tests to aerobic incubation at 35°C for 16–18 hours and 24 hours for the staphylococci. The plates must not be incubated under an increased concentration of carbon dioxide

Table 6.1 Zone size interpretation chart (Modified from CLSI (2008) M31-A3)

Antimicrobial agent	Disc content in μg unless otherwise stated	Diameter of zone of inhibition to nearest mm		
		Susceptible	Intermediate	Resistant
Amikacin	30	≥17	15–16	≤14
Amoxicillin-clavulanic acid				
Staphylococci	20/10	≥20	–	≤19
Other organisms	20/10	≥18	14–17	≤13
Ampicillin				
Enterobacteriaceae	10	≥17	14–16	≤13
Staphylococci	10	≥29	–	≤28
Streptococci (Not *S. pneumoniae*)	10	≥26	19–25	≤18
Enterococci	10	≥17		≤16
Cefazolin	30	≥18	15–17	≤14
Cefpodoxime Dogs (pathogens isolated from wounds or abscesses)	10	≥21	18–20	≤17
Ceftiofur	30	≥21	18–20	≤17
Streptococcus equi subsp. *zooepidemicus*	30	≥22	–	–
Cephalothin	30	≥18	15–17	≤14
Clindamycin (dogs) *Staphylococcus* spp.	2	≥21	15–20	≤14
Danofloxacin (*M. haemolytica* and *P. multocida* in cattle respiratory disease)	5	≥22	–	–
Difloxacin (skin and urinary tract infections in dogs)	10	≥21	18–20	≤17
Enrofloxacin	5	≥23	17–22	≤16
Bovine respiratory pathogens	5	≥21	17–20	≤16
Erythromycin				
Staphylococcus and *Enterococcus* spp.	15	≥23	14–22	≤13
Streptococci	15	≥21	16–20	≤15
Florfenicol				
Bovine respiratory pathogens	30	≥19	15–18	≤14
Porcine respiratory pathogens	30	≥22	19–21	≤18
Gentamicin	10	≥15	13–14	≤12
Imipenem	10	≥16	14–15	≤13
Kanamycin	30	≥18	14–17	≤13
Marbofloxacin (infections in dogs and cats)	5	≥20	15–19	≤14
Orbifloxacin (infections in dogs and cats)	10	≥23	18–22	≤17
Oxacillin				
Staphylococcus aureus	1	≥13	11–12	≤10
Staphylococcus spp.	1	≥18	–	≤17

Continued

Table 6.1 Zone size interpretation chart (Modified from CLSI (2008) M31-A3)—cont'd

Antimicrobial agent	Disc content in μg unless otherwise stated	Diameter of zone of inhibition to nearest mm		
		Susceptible	Intermediate	Resistant
Penicillin				
Staphylococci	10 units	≥29	–	≤28
Streptococci, β-haemolytic group (not *S. pneumoniae*)	10 units	≥24	–	–
Enterococci	10 units	≥15	–	≤14
Penicillin-novobiocin (pathogens causing bovine mastitis)	10 units/30	≥18	15–17	≤14
Pirlimycin (staphylococci and streptococci causing bovine mastitis)	2	≥13	–	≤12
Rifampin (*Enterococcus* spp.)	5	≥20	17–19	≤16
Spectinomycin (bovine respiratory pathogens)	100	≥14	14–17	≤10
Sulfisoxazole	300	≥17	13–16	≤12
Tetracyclines				
Organisms other than streptococci	30	≥19	15–18	≤14
Streptococcus species other than *S. pneumoniae*	30	≥23	19–22	≤18
Tiamulin (*Actinobacillus pleuropneumoniae*)	30	≥9	–	≤8
Ticarcillin				
Pseudomonas aeruginosa	75	≥15	–	≤14
Enterobacteriaceae	75	≥20	15–19	≤14
Ticarcillin-clavulanic acid				
Pseudomonas aeruginosa	75/10	≥15	–	≤14
Enterobacteriaceae	75/10	≥20	15–19	≤14
Tilmicosin				
Mannheimia haemolytica (bovine respiratory disease)	15	≥14	11–13	≤10
Pasteurella multocida and *Actinobacillus pleuropneumoniae* (porcine respiratory disease)	15	≥11	–	≤10
Trimethoprim-sulfamethoxazole (not *S. pneumoniae*)	1.25/23.75	≥16	11–15	≤10
Tulathromycin (bovine respiratory pathogens)	30	≥18	15–17	≤14
Vancomycin				
Enterococci	30	≥17	15–16	≤14
Streptococci	30	≥17	–	–
Staphylococcus spp.	30	≥15	–	–

as this can significantly alter the zones of inhibition with some antimicrobial agents. A modified method is used for *Haemophilus* species and other fastidious bacteria that require carbon dioxide for growth (CLSI 2012, M02-A11). Anaerobes should not be tested by the disc diffusion method and the CLSI (2012) has published a standard method for testing the susceptibility of anaerobes (document M11-A8).

- The test bacterium: the degree of resistance or susceptibility of a bacterium to selected antimicrobial agents will vary with the species and strain of the pathogen. There are specific difficulties in detecting meticillin-resistant staphylococci (see below).

Routine test procedure for the disc diffusion method

Standard method

- At least four to five well-isolated colonies of the same morphological type are selected from a non-selective agar plate. Just the tops of the colonies are touched and the growth transferred to a tube

containing 4–5 mL of soybean-casein digest broth or an equivalent (tryptone soya broth, Oxoid).
- The inoculated broth is incubated at 35–37°C until a slight visible turbidity appears, this is usually within two to eight hours.

Alternative method

- The colonies are selected as before and a suspension is made in saline or broth without the preincubation in broth. This is suggested as the best method for testing staphylococci, especially with suspected meticillin-resistant strains.
- The turbidity of both the pre-incubated broth and the suspension of bacteria (alternative method) is adjusted by comparison with a 0.5 McFarland turbidity standard. The standard and the test suspension are placed in similar 4–6 mL thin-glass tubes or vials. The turbidity of the test suspension is adjusted with broth or saline and compared with the turbidity standard, against a white background with contrasting black lines until the turbidity of the test suspension equates to that of the turbidity standard. Alternatively, turbidity can be measured using an instrument such as a Densimat (bioMérieux).

McFarland 0.5 turbidity standard

Solution A (0.048 M $BaC1_2$)
1.175 g $BaC1_2.2H_20$
Make up to 100 mL with distilled water.

Solution B (0.36 N H_2SO_4)
1.0 mL H_2SO_4 (Analar grade, sp.gr. 1.84)
Make up to 100 mL with distilled water.

Stock Solution
0.5 mL Solution A (0.048 M $BaC1_2$)
99.5 mL Solution B (0.36 N H_2SO_4)

Shake vigorously and dispense into 4–6 mL sealed tubes or screw-capped vials. Store in the dark at room temperature and replace three months after preparation. Always agitate the turbidity standard before use.

Test procedure:

- A sterile, non-toxic swab on an applicator stick is dipped into the standardized suspension of bacteria and excess fluid is expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level.
- The swab is streaked in three directions over the entire surface of the agar with the objective of obtaining a uniform inoculation. A final sweep with the swab can be made against the agar around the rim of the Petri dish.
- The test agar must be Mueller–Hinton agar or a satisfactory equivalent such as Iso-sensitest agar (Oxoid). The exception is the use of 5–10% sheep blood agar for streptococci or *Trueperella* (*Arcanobacterium*) *pyogenes* that are unable to grow on Mueller–Hinton agar. The surface of the agar should be moist but no droplets of moisture should be visible on the surface of the agar.
- The inoculated plates are allowed to stand for three to five minutes, but no longer than 15 minutes, for any excess moisture from the inoculum to be absorbed by the agar before applying the antimicrobial discs.
- The discs are placed onto the agar surface using sterile forceps or an antibiotic disc dispenser (Fig. 6.5). Each disc is gently pressed with the point of sterile forceps to ensure complete contact with the agar surface. The discs should be placed no closer together than 24 mm (centre-to-centre). This is equivalent to six discs per standard 90 mm Petri dish.
- The plates are inverted and placed in a 35°C incubator, within 15 minutes of applying the discs and incubated aerobically for 16–18 hours or 24 hours for staphylococci.
- After incubation the diameters of the zones of inhibition are measured to the nearest mm using a ruler or calipers. The diameters are read from the back of the plate when the test is on the comparatively clear Mueller–Hinton medium but over the surface of the agar with streptococci grown on blood agar. The diameter of the zones should be read across the centre of the discs. Automated devices for reading and interpretation of zone sizes are available (e.g,. Mastascan, Mast Group Ltd.). An interpretation of the size of the zones of inhibition is made with reference to available interpretative tables such as those produced by CLSI. Table 6.1 is modified from CLSI (2008) document M31-A3. The bacterium is reported as susceptible, intermediately

Figure 6.5 The materials required for susceptibility testing showing an antibiotic disc dispenser for the correct spacing of discs on the lawn of the test bacterium.

susceptible or resistant to each antimicrobial agent used in the test:

- **Susceptible:** the infection may respond to the treatment at the normal dosage.
- **Intermediate:** this category may be used when the pathogen may be inhibited by attainable concentrations of the antimicrobial agent provided a higher dosage is used or the pathogen is in a certain body site, such as urine, where the drug is physiologically concentrated. Classification of a result as intermediate can be used for technical reasons also.
- **Resistant:** the bacterium is not inhibited by the usually achievable systemic concentrations of the antimicrobial agent and efficacy has not been reliable in clinical studies.

Some observations on the interpretation of zones of inhibition

The zone of inhibition is that area showing no obvious growth that can be detected by the unaided eye. The diameter of the zone is measured to each edge of this clear area. However, there are occasions when the exact extent of the zone is difficult to determine:

1. Large colonies growing within an otherwise clear zone of inhibition should be subcultured, re-identified and retested. Faint growth of tiny colonies at the edge of the zone can be ignored.
2. *Proteus* species may swarm into areas of inhibited growth around certain antimicrobial discs. If the zones of inhibition are clearly outlined then the veil of swarming can be disregarded.
3. With trimethoprim and the sulphonamides, slight growth in the zone can be ignored and the zone measured to the margin of heavy growth.
4. For bacteria that have to be grown on blood agar plates, the zone size for nafcillin, novobiocin, oxacillin and meticillin will be 2–3 mm smaller than the normal control limits.
5. Transmitted light should be used to examine for light growth within the zone in the case of meticillin-resistant strains of staphylococci.

Meticillin-resistant staphylococci

There can be some difficulty in detecting these strains. Currently, resistance to cefoxitin (30 µg) is used as a marker for meticillin resistance in many laboratories. If cefoxitin discs are not used, the recommendations are as follows:

- An oxacillin disc is used because it is more stable in storage and is more likely to detect cross-resistance than a meticillin disc itself.
- The alternative method of standardizing the inoculum, without pre-incubation in broth, is preferred for testing staphylococci.
- If the incubator cannot be controlled at 35°C, then a separate test should be carried out with oxacillin and incubated at 30°C. Resistance to meticillin or oxacillin may not be seen at 37°C.
- Meticillin-resistant strains often have multiple resistance that includes the beta-lactams, aminoglycosides, marcrolides, clindamycin and tetracyclines. This may act as a clue to resistance to penicillinase-resistant penicillins.
- A film of growth within a zone of inhibition around a meticillin, oxacillin, nafcillin or cephalothin disc also indicates heteroresistance.
- Confirmation of intrinsic oxacillin-resistance in staphylococci can be made by inoculating the suspect strain onto a quadrant of Mueller–Hinton agar that has been supplemented with 4% NaC1 and 6 µg oxacillin per ml. The inoculation is made with a suspension adjusted to the 0.5 McFarland standard. The plate is incubated at 35°C for 24 hours. Any growth indicates intrinsic oxacillin-resistance.

Selection of antimicrobial discs

The selection of the types of antimicrobial agents for use in the disc diffusion test will, to a large extent, depend on clinical considerations including the drugs that are available and in general use by the veterinarian. However, to make routine susceptibility testing relevant and practical the following guidelines may be helpful:

- A tetracycline disc will predict the result against all the other tetracyclines
- Sulfisoxazole is a suitable representative for all the sulphonamides
- Erythromycin is the only macrolide that requires testing
- A clindamycin disc will predict the result for lincomycin
- Any aminoglycosides or quinolones required clinically should be tested separately as often the drugs are not related closely enough to assume cross-resistance
- Chloramphenicol, vancomycin, nitrofurantoin and trimethoprim/sulfamethoxazole are tested separately as required.
- Within the penicillins:
  - Staphylococci should be tested against penicillin G and oxacillin (for resistance to meticillin, cephalosporins and other betalactams)
  - *Enterococcus faecalis* tested against penicillin G will predict the result against ampicillin, ampicillin-analogues, amoxicillin and the acylamino-penicillins
  - Streptococci should be tested against either penicillin G *or* ampicillin, testing against both is unnecessary.

- Within the cephalosporins:
  - Staphylococci are usually susceptible except for the meticillin-resistant strains. These should be reported as resistant even if the result of the in vitro test suggests otherwise.
  - There is a lack of clinical correlation with *Enterococcus faecalis* against the cephalosporins and the results of the in vitro tests cannot be interpreted with accuracy.
  - A cephalothin disc will represent the result to cefaclor, cephapirin, cefazolin, cephradine, cephalexin and cefadroxil. Cefatozime represents ceftazidime, ceftizoxime and ceftriaxone.

Interpretive criteria and reporting of results

The disc diffusion method is extremely useful in veterinary diagnostic laboratories as almost all veterinary-specific antimicrobial agents are available in antimicrobial-impregnated discs. Care should be taken when reporting results of ASTs for veterinary pathogens as interpretative criteria have not been established for all clinical conditions or for all animal species. The establishment of such criteria requires microbiological data on the population distribution of MICs of the relevant microorganisms, information on the pharmacokinetic and pharmacodynamic properties of the antimicrobial agents and outcome data from clinical efficacy trials. Data on all these areas may not be available in all cases and if interpretation of susceptibility has been determined using criteria established for other animal species or for humans, this should be indicated to the veterinary practitioner concerned.

Quality control procedures

Quality control procedures are needed to monitor the precision and accuracy of the test procedure including a check on the potency of the antimicrobial discs and the satisfactory performance of the test medium. They are of great importance also when harmonization of methods between laboratories is desired and results generated in different laboratories are to be compared.

Control strains of bacteria

Stock cultures of control bacteria from the American Type Culture Collection (ATCC) or other national culture collections should be maintained. A table of selected quality control strains is given in Watts and Lindeman (2006). These reference strains when tested against the antimicrobial discs should yield zones of inhibition within the control limits indicated in Table 6.2. The suggested control bacteria are:

- *Escherichia coli* (*E. coli*) ATCC 25922
- *Staphylococcus aureus* ATCC 25923
- *Pseudomonas aeruginosa* ATCC 27853
- *Klebsiella pneumoniae* ATCC 700603 for testing extended-spectrum β-lactamase production
- *Enterococcus faecalis* ATCC 29211 or ATCC 33186 to establish that the test medium is relatively free of thymine.

The working control cultures are stored on soybean-casein digest agar or an equivalent (tryptone soya agar, Oxoid) at 4 °C and subcultured weekly. These working cultures are replaced at least monthly from stock cultures maintained lyophilized; frozen below –20 °C; or in liquid nitrogen. For testing, the control cultures are streaked out on an agar plate to obtain isolated colonies. The control tests are then conducted as for the routine test procedure.

Antimicrobial discs

1. Storage conditions of the discs must ensure that their potency is maintained. The discs should be kept under appropriate anhydrous conditions and stored at 4 °C for routine use. The discs should be allowed to equilibrate with room temperature before being used in the test.

Table 6.2 Zone diameter (mm) limits for quality control of antimicrobial disc diffusion-susceptibility tests on unsupplemented Mueller–Hinton medium (modified from CLSI (2008) M31-A3)

Antimicrobial agent	Disc content in μg	*E. coli* (ATCC 25922)	*S. aureus* (ATCC 25923)	*P. aeruginosa* (ATCC 27853)
Amikacin	30	19–26	20–26	18–26
Amoxicillin-clavulanic acid	20/10	18–24	28–36	–
Ampicillin	10	16–22	27–35	–
Apramycin	15	15–20	17–24	13–18
Cefazolin	30	21–27	29–35	–
Cefovecin	30	25–30	25–32	–

Continued

Table 6.2 Zone diameter (mm) limits for quality control of antimicrobial disc diffusion-susceptibility tests on unsupplemented Mueller–Hinton medium (modified from CLSI (2008) M31-A3)—cont'd

Antimicrobial agent	Disc content in μg	*E. coli* (ATCC 25922)	*S. aureus* (ATCC 25923)	*P. aeruginosa* (ATCC 27853)
Cefoxitin	30	23–29	23–29	–
Cefpodoxime	10	23–28	19–25	–
Cefquinome	30	28–36	25–33	–
Ceftiofur	30	26–31	27–31	–
Cephalothin	30	15–21	29–37	–
Clindamycin (dogs)	2	–	24–30	–
Danofloxacin	5	29–36	24–31	18–25
Difloxacin	10	28–35	27–33	16–22
Enrofloxacin	5	32–40	27–31	15–19
Erythromycin	15	–	22–30	–
Florfenicol	30	22–28	22–29	–
Gentamicin	10	19–26	19–27	16–21
Imipenem	10	26–32	–	20–28
Kanamycin	30	17–25	19–26	–
Marbofloxacin	5	29–37	24–30	20–25
Neomycin	30	17–23	18–26	–
Orbifloxacin	10	29–37	24–30	16–22
Oxacillin	1	–	18–24	–
Penicillin	10 units		26–37	–
Penicillin-novobiocin	10 units/30	–	30–36	–
Pirlimycin	2	–	20–25	–
Pradofloxacin	5	31–39	29–38	21–28
Rifampin	5	8–10	26–34	–
Spectinomycin	100	21–25	13–17	10–14
Sulfisoxazole	300	15–23	24–34	–
Tetracycline	30	18–25	24–30	–
Tiamulin	30	–	25–32	–
Ticarcillin	75	24–30	–	21–27
Ticarcillin-clavulanic acid	75/10	24–30	29–37	20–28
Tilmicosin	15	–	17–21	–
Trimethoprim- sulfamethoxazole	1.25/23.75	23–29	24–32	–
Tulathromycin	30	–	18–24	–
Tylosin	60	–	19–25	–
Vancomycin	30	–	17–21	–

2. Discs that have passed the manufacturer's expiry date must be discarded.
3. Quality control of discs containing combinations of beta-lactams and beta-lactamase inhibitors are monitored using *E. coli* and *K. pneumoniae* control strains.
4. The potency of a new batch of antimicrobial discs should be checked against the control bacteria and the zones of inhibition compared with the control limits shown in Table 6.2.

The test medium

1. The depth of agar in a Mueller–Hinton agar plate should be approximately 4 mm. This is equivalent to 60–70 mL of medium in a 140 mm Petri dish, or 25–30 mL of medium in a 90 mm Petri dish.
2. The pH of a new batch of medium should be checked and be between 7.2 and 7.4.
3. Each batch of poured medium should be tested for sterility by incubating a few randomly selected plates at 30 to 35 °C for 24 hours or longer. These plates are then discarded.
4. The medium is stored at 4 °C and should be used within seven days of preparation unless kept sealed inside a plastic sleeve.
5. To establish that a new batch of medium is free of thymidine and thymine, a control strain of *Enterococcus faecalis* (ATCC 29212) is tested with a trimethoprim/sulfamethoxazole disc. Satisfactory medium will show a clear and distinct zone of inhibition of 20 mm or more. Unsatisfactory media will produce no distinct zone or a hazy growth within the zone.
6. The control strain *Pseudomonas aeruginosa* (ATCC 27853) is tested against tetracycline and aminoglycosides to monitor satisfactory levels of calcium and magnesium cations in the medium. The zone sizes must conform to the control limits given in Table 6.2.

Zone size limits

Zone size limits for the control bacteria against antimicrobial discs on Mueller–Hinton medium without supplements are shown in Table 6.2. Daily testing to establish the accuracy of the test should yield no more than one zone size outside the control limits in 20 consecutive tests. The zone size should be no more than four standard deviations above or below the midpoint between stated limits (midpoint + (maximum – minimum zone limits)). Once satisfactory performance of the test has been established, weekly testing as well as testing each time a new batch of medium or antimicrobial discs is introduced may be sufficient. CLSI (2012) M02-A11 should be consulted for further details.

Checklist of common sources of error

One or more of the following causes may be the reason for a zone size being outside the control limits given in Table 6.2.

- Clerical or reader error.
- Contamination or genetic change in the control bacterium.
- Inoculum too heavy or too light. This may result from the turbidity standard being incorrectly prepared; used later than three months after preparation; or not agitated sufficiently before use. If a turbidity-measuring device is used, this may be faulty.
- A change in the composition of the Mueller–Hinton medium.
- Loss of potency of the antimicrobial agent in the disc.

Quantitative Methods of Antibiotic Susceptibility Testing

Quantitative methods which allow the calculation of the minimal inhibitory concentration of an antimicrobial agent are the preferred methods for surveillance studies and are increasingly being used in larger clinical diagnostic laboratories as well. The minimal inhibitory concentration (MIC) is the highest dilution of an antibiotic required to inhibit the growth of a bacterium. Conventional tests for determining the MIC include the broth dilution and agar dilution tests. Quality control procedures must be included with these methods, similar to those described for the disc diffusion technique.

Broth dilution method

This test is performed by preparing twofold dilutions of an antibiotic in a series of tubes containing a nutrient broth. Each tube is inoculated with a suspension of the test bacterium that contains between 10^4 and 10^5 bacteria/mL. The inoculated tubes of broth are incubated at 35–37 °C for 24 hours. The highest dilution of the antibiotic to inhibit growth (no turbidity in the tube) is the MIC. The test can be conducted in tubes containing at least 2 mL of suspension (macrodilution) or, more commonly now, in microtitration plates (microdilution). Microtitration plates with prediluted series of antimicrobial agents are available commercially as either dry or frozen panels.

The minimum bactericidal concentration (MBC) can also be determined by the broth dilution method. After the MIC has been read, a standard volume of broth is taken from the tubes showing no visible growth after 24 hours' incubation and subcultured onto agar media. The MBC is arbitrarily defined as the lowest antibiotic concentration that kills 99.9% of the original inoculum, or where a 1000-fold reduction in bacterial numbers has occurred.

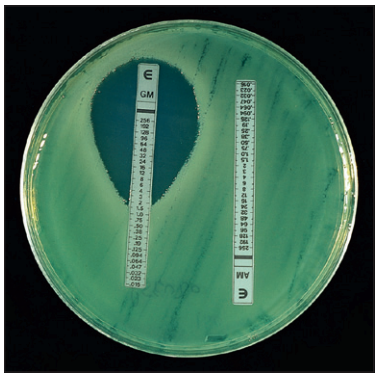

Figure 6.6 A commercial strip for the determination of MIC values. *Pseudomonas aeruginosa* tested against gentamicin (left) giving a MIC of 2 µg/mL and ampicillin (right) to which *P. aeruginosa* is resistant.

Agar dilution tests

These tests are similar to the broth dilution method except that the antibiotic dilutions are incorporated into an agar medium in a series of Petri dishes. These are spot-inoculated with a number of test bacteria, between 20–36 bacterial isolates can be accommodated on a 90 mm Petri dish. A series of control bacteria, of known sensitivity, should be included on each plate.

E test

Several commercial systems are available for determining MIC values, not all of which involve preparation of a dilution series of the antimicrobial agent. The E test (AB Biodisk) is illustrated (Fig. 6.6). This test consists of a thin, inert plastic test carrier with a predefined exponential gradient of antibiotic. After incubation, the MIC value is read from the scale at the point of intersection between the zone edge and the test carrier.

Permission to excerpt Tables 6.1 and 6.2 from M31-A3 (2008; Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals 3rd edn; approved standard) has been granted by the Clinical Laboratory Standards Institute. CLSI is not responsible for errors or inaccuracies. The interpretive data are valid only if the methodology in M31-A3 is followed. It is assumed that users of this table have M31-A3 available. The current M31 edition may be obtained from Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

Molecular Methods for the Detection of Antimicrobial Resistance

Although phenotypic methods are routinely used in diagnostic laboratories, molecular methods for the detection of antimicrobial resistance are increasingly employed in research laboratories. Molecular methods involve the detection of resistance genes and can have advantages over conventional methods, particularly when the organism is slow-growing or difficult to isolate. Direct detection of resistance genes in clinical specimens can be performed, which may allow the institution of effective antimicrobial therapy sooner than when phenotypic methods are used. The major disadvantage of methods involving detection of resistance genes is that the presence of a gene does not necessarily imply the expression of that gene and thus a strain possessing a particular resistance gene may not be invariably resistant phenotypically. In addition, phenotypic methods are necessary for determination of MICs. Most of the genetic methods in current use for the detection of resistance are based on PCR, either conventional or real-time PCR (see Chapter 4). Microarray analysis is likely to be increasingly used in the future as the number of targets to be investigated becomes larger. As with conventional methods, quality control is an essential part of antimicrobial resistance determination using molecular methods and standards for the performance of such tests are available (CLSI 2006, 2010).

ANTIBACTERIAL AND ANTIFUNGAL CHEMOTHERAPY

Successful use of antimicrobial drugs for the treatment of clinical disease depends on many factors, some relating to the sick animal and the reliability of the diagnosis, others relating to the specimen collected and its processing by the laboratory. The ultimate determining factor is the selection and administration of an appropriate drug at a frequency suitable to maintain blood levels at the optimal concentration for the duration of the treatment. Increasingly, successful antimicrobial therapy is defined as that which minimizes the development of resistance as well as resulting in a therapeutically successful outcome. Thus the most important decision is the initial one in which the clinician decides whether the administration of antimicrobial therapy is required. Inappropriate prescription of antibiotics is a major problem and has been documented in up to 50% of cases in human medicine and up to 80% of cases in veterinary medicine (Sarkar and Gould, 2006).

The clinical use of antimicrobial drugs begins with the clinician and the detailed examination that allows a specific clinical diagnosis to be made. With experience, it may be possible to relate clinical signs to a particular causative agent and initiate treatment with an antimicrobial drug, selected on the basis of clinical impression alone. However, it is preferable to obtain a representative specimen for diagnostic microbiology as a safeguard against error in diagnosis **before** giving antimicrobial drugs. In many

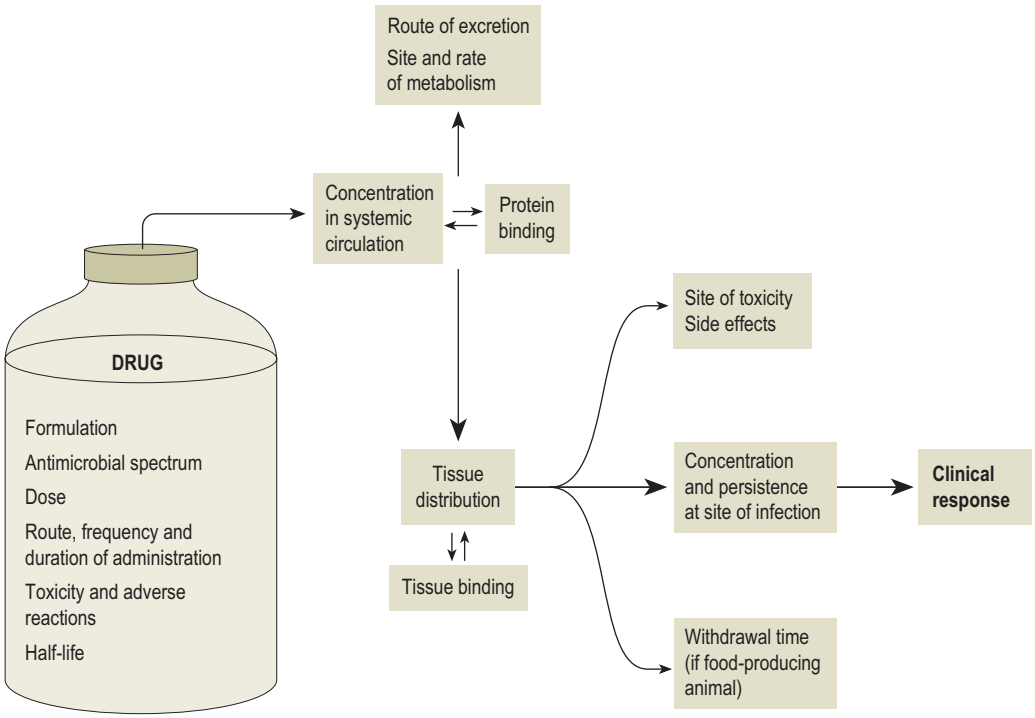

Figure 6.7 Considerations involved in the selection and administration of antimicrobial drugs. Inherent toxicity or side effects arising from chemotherapy require careful consideration in some species and also in young animals. Legal regulations in some countries may prohibit the use of certain antibiotics, such as chloramphenicol, in food-producing animals.

instances, the relationship between causative agent and clinical picture is not constant. Chemotherapy can be initiated as soon as specimens are collected and empirical chemotherapy, can, if necessary, be modified when laboratory data are at hand.

Effective antimicrobial therapy depends on the susceptibility of the pathogen, pharmacokinetic characteristics of the drug, the amount of drug given at one time, the route, frequency of administration and the duration of treatment (Fig. 6.7). Other variables relating to chemotherapy include the toxicity of the drug for the host, its half-life, concentration and persistence at the site of infection and its effect on the normal flora of the host. Because of the many species encountered in clinical practice and their individual sensitivities to drug toxicity, care should be exercised in the selection and administration of antimicrobial drugs. Differences between ruminant and monogastric species may be particularly marked. Apart from species variation, unexpected responses in young animals, especially in the neonatal period, may also occur. Figure 6.8 shows the factors relevant to the drug, infectious agent and the host which influence the outcome of antimicrobial chemotherapy.

Drug Distribution

When treating bacterial or fungal infections, it is essential that an effective concentration of the drug is rapidly attained at the site of infection and that it is maintained for sufficient time to achieve the desired effect on the target organisms. The location of the infection can have a major influence on the drug concentration attained in a particular tissue and some sites such as the central nervous system are protected by barriers which limit or prevent the entry of many drugs except highly lipid-soluble compounds. The amount of drug, therefore, that enters each organ or tissue is determined by physiochemical properties of the drug as well as physiological factors. Protein binding is a major determinant of drug distribution and it is worth noting that published interpretative criteria for resistance do not yet take into account the extent to which antimicrobial agents are bound by plasma proteins. For agents that are bound by plasma proteins to a significant extent, the MIC breakpoint should be lowered by the fraction of the drug which is bound (Boothe, 2006). The effect of the inflammatory response on the structural integrity of physiological barriers may have a direct influence on drug

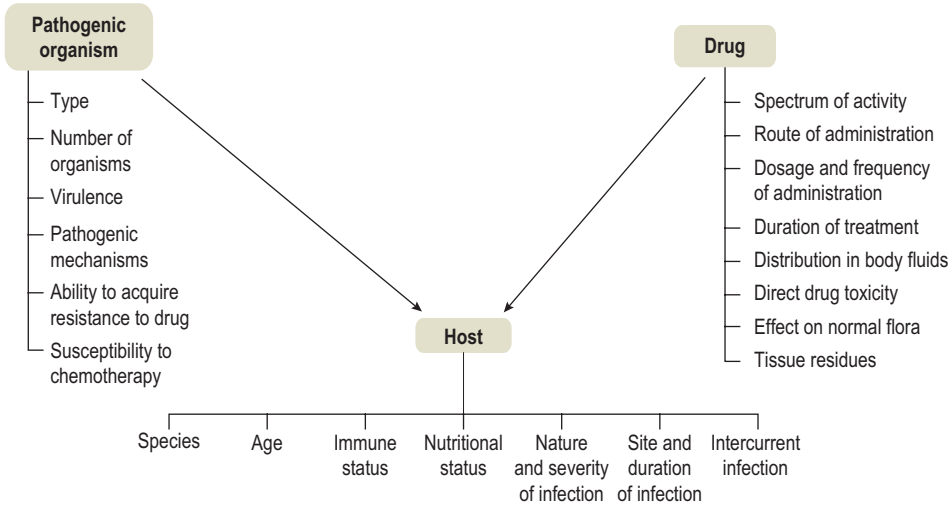

Figure 6.8 Factors relevant to the infectious agent, host and drug which influence the outcome of antimicrobial chemotherapy.

permeation of tissues and the intracellular or extracellular replication of individual pathogens may determine the final concentration of the drug to which they are exposed. Necrotic tissue or accumulated pus may further hinder drug penetration into lesions caused by pyogenic bacteria, hence the need for thorough debridement and drainage if the site of infection is accessible to direct surgical intervention.

Selection of Antimicrobial Drugs

If chemotherapy has to be administered before culture and susceptibility patterns are available, the body system affected, the nature and location of the infectious agent(s) and the most suitable drug likely to be effective against the pathogen should be considered. Route and frequency of administration of the drug, cost of treatment, adverse effects on the host and public health aspects of treatment are matters relevant to the veterinarian and the owner. Suggested antibacterial and antifungal drugs, for specific bacterial and fungal pathogens are presented in Tables 6.3 and 6.4. For pathogenic bacteria that commonly exhibit drug resistance such as staphylococci, members of the *Enterobacteriaceae* and pseudomonads, routine culture and susceptibility testing should be carried out. Specimens should be collected during the clinical examination procedure and before any antimicrobial drugs are administered. Sources of failure or adverse reactions following antimicrobial chemotherapy are shown in Figure 6.9.

Antimicrobial Drug Interactions

Antimicrobial substances, like many other classes of drugs, may interact with other medication given to animals, with possible adverse results. In addition, when antimicrobial drugs are combined, their effect may be quite different from that achieved by the individual drugs used separately. When two antimicrobial drugs act simultaneously on a homogeneous microbial population the effect may be one of the following:

- Indifference, where the combined action is no greater than that of the more effective drug used alone
- Additive, where the combined action is equivalent to the sum of the actions of each drug when used alone
- Synergism, where the combined action is significantly greater than the sum of both effects
- Antagonism, where the combined action is less than that of the more effective agent when used alone.

Antimicrobial synergy, implying the beneficial interaction between two drugs exceeding their additive effects, may take several forms.

1. Two drugs may sequentially block a microbial metabolic pathway. One of the best examples is the combination of a sulphonamide with trimethoprim. Sulphonamides compete with para-aminobenzoic acid, which is required by some bacteria for the synthesis of dihydrofolate. Folate antagonists such as trimethoprim inhibit the enzyme (dihydrofolic acid reductase) that reduces dihydrofolate to tetrahydrofolate. The presence of a sulphonamide and trimethoprim results in the simultaneous blocking of sequential steps leading to the synthesis of purines and nucleic acid and can achieve a greater inhibition of growth than either drug alone.
2. One drug may prevent the inactivation of a second drug by microbial enzymes. Inhibition of

Table 6.3 Suggested antimicrobial treatment for pathogenic bacteria and related organisms

Pathogenic bacterium	Disease	Suggested drugs	Alternative drugs	Comments
Actinobacillus lignieresii	'Wooden or timber tongue' in cattle, superficial lesions in sheep	Potassium iodide *per os*, sodium iodide I/V, streptomycin (+ penicillin)	Tetracycline	Drug penetration into lesion may be limited in chronic cases
A. equuli	Septicaemic and multifocal infections in foals	Gentamicin, ampicillin	Erythromycin Cephalosporins*	Supportive treatment is necessary
A. pleuro-pneumoniae	Pleuropneumonia in pigs	Trimethoprim-sulfamethoxazole Penicillins	Tilmicosin Cephalosporins*	Elimination of infected breeding animals and mass medication of in-contact pigs may eradicate infection
Actinobaculum suis	Cystitis and pyelonephritis in sows	Trimethoprim-sulfameth-oxazole, penicillin G	Ampicillin	Antimicrobial therapy is likely to be effective only in the early stages of infection
Actinomyces bovis	'Lumpy jaw' in cattle	Potassium iodide *per os*, sodium iodide I/V, penicillin + streptomycin	Tetracycline	In advanced cases, treatment will not restore normal bone structure
A. viscosus	Granulomatous lesions in skin or thoracic cavity in dogs	Penicillins	Tetracycline	Drainage of suppurative lesions is beneficial
Trueperella (Arcano-bacterium) pyogenes	Purulent infections of traumatic or opportunistic origin in cattle and sometimes in sheep, goats and pigs. Implicated in 'summer mastitis' (cattle)	Penicillins, tetracycline	Trimethoprim-sulfameth-oxazole. Florfenicol (respiratory infections), Cefquinome* (arthritis/ osteomyelitis)	Response to treatment is disappointing in 'summer mastitis' presumably due to active involvement of other bacteria and nature of lesions
Bacillus anthracis	Anthrax in cattle, sheep, pigs and other animals. Important zoonosis	Penicillin G	Tetracycline	Acute disease, hence prompt treatment required; chemopro-phylaxis of in-contact animals indicated where disease is endemic
Bordetella bronchiseptica	Kennel cough in dogs Atrophic rhinitis in pigs	Treatment not required		Kennel cough responds poorly to therapy and vaccination is prefer-able. Atrophic rhinitis is not treatable; preventative measures include vaccination, prophylactic treatment or elimination of carrier sows
	Pneumonia (small animals)	Trimethoprim-sulfamethoxazole	Amoxycillin-clavulanate	
	Pneumonia (horses)	Gentamicin		

Continued

Table 6.3 Suggested antimicrobial treatment for pathogenic bacteria and related organisms—cont'd

Pathogenic bacterium	Disease	Suggested drugs	Alternative drugs	Comments
Borrelia burgdorferi	Lyme disease in humans and arthritic manifestations in other animals	Tetracycline	Amoxicillin	Prompt treatment is desirable
Brachyspira hyodysenteriae	Swine dysentery	Tiamulin	Valnemulin, lincomycin	Antimicrobial drugs do not eliminate the organisms
Brucella canis	Canine brucellosis	Trimethoprim-sulphamethoxazole, Tetracycline		Antimicrobial treatment is usually unsuccessful. Control is based on testing and elimination of infected dogs
Campylobacter species	Diseases of the reproductive tract in cattle and sheep; enteritis in other animals	Penicillin G + streptomycin	Erythromycin, tetracycline	Disease is best controlled by prevention, not by treatment
Chlamydophila species	Wide spectrum of diseases ranging from infections in birds (psittacosis/ornithosis) to chlamydial abortion in sheep	Tetracycline	Erythromycin	Vaccines are beneficial for the prevention of enzootic ovine abortion
Clostridium species	Gas gangrene, tetanus and enteric disease	Penicillins, Tylosin	Cephalosporins*, tylosin Metronidazole (*C. difficile*)	Clostridial diseases are best controlled by vaccination
Corynebacterium pseudo-tuberculosis	Caseous lymphadenitis in sheep; abscess formation in cattle and horses	Antimicrobial treatment ineffective in sheep. Prolonged penicillin or trimethoprim/sulfamethoxazole therapy used in horses		Segregation and culling of affected sheep followed by thorough disinfection is the preferred control procedure
C. renale	Bovine pyelonephritis; ovine posthitis	Penicillin G	Trimethoprim/sulfamethoxazole	Antimicrobial therapy only useful in early stage of infection
Dermatophilus congolensis	Streptothricosis in cattle, 'mycotic dermatitis' and 'strawberry foot rot' in sheep, 'rain scald' in horses	Penicillin G + streptomycin at high dose rates	Oxytetracycline (long-acting), trimethoprim/sulfamethoxazole, topical disinfectants	Treatment in tropical Africa can be disappointing. Minimizing trauma to skin and control of ectoparasites is beneficial

Table 6.3 Suggested antimicrobial treatment for pathogenic bacteria and related organisms—cont'd

Pathogenic bacterium	Disease	Suggested drugs	Alternative drugs	Comments
Dichelobacter nodosus	Footrot in sheep and goats; implicated in infections of interdigital skin in cattle	Penicillin G + streptomycin, topical oxytetracycline, tilmicosin	Footbaths containing agents such as zinc sulphate	Foot trimming and topical application of disinfectants or antibiotics are essential; foot baths beneficial in control programmes
Enterococcus spp.	Urinary tract infections in dogs and other species. Opportunistic infections in many animals	Penicillins	Trimethoprim-sulfameth-oxazole, fluoroquinolones*	
Escherichia coli	Septicaemia or diarrhoea in calves, young pigs, lambs; oedema disease in weaned pigs; mastitis in dairy cows; urinary tract infections in many animals	Gentamicin, trimethoprim-sulfamethoxazole	Ampicillin, cephalosporins*, fluoroquinolones*	Essential to establish an antimicrobial susceptibility pattern at an early stage. Fluid-replacement therapy required in enteric infections of young animals
Fusobacterium necrophorum (often associated with other bacteria)	Pyonecrotic lesions in calves (calf diphtheria); bull-nose in pigs; liver abscesses and interdigital necrobacillosis in cattle and sheep	Penicillin G, sulphonamides, metronidazole, ampicillin	Florfenicol, ceftiofur*	Surgical drainage of foot conditions may be necessary. Footbaths can be beneficial in control programmes
Histophilus somni	Infectious thromboembolic meningo-encephalitis, septicaemia, pneumonia in cattle	Penicillin G, ampicillin, trimethoprim/ sulfamethoxazole	Tetracycline, florfenicol, tilmicosin, third- and fourth-generation cephalosporins*	
Klebsiella pneumoniae	Coliform mastitis in dairy cows, reproductive diseases in mares and urinary tract infections in bitches	Gentamicin, Cephalosporins*	Trimethoprim-sulfamethoxazole	Sawdust or wood shavings used as bedding may increase the incidence of coliform mastitis in dairy cattle
Leptospira interrogans serovars	Abortion in cattle and pigs; septicaemia, hepatic and renal involvement in dogs; ocular lesions in horses	Penicillin G, ampicillin, dihydrostreptomycin	Tetracycline	Vaccination is used in dogs, cattle and pigs. The carrier state may persist after chemotherapy

Continued

Table 6.3 Suggested antimicrobial treatment for pathogenic bacteria and related organisms—cont'd

Pathogenic bacterium	Disease	Suggested drugs	Alternative drugs	Comments
Listeria monocytogenes	Septicaemia in young animals of many species, meningo-encephalitis and abortion in cattle and sheep	Penicillin G + gentamicin, tetracycline	Trimethoprim-sulfamethoxazole	Often associated with silage feeding in cattle and sheep; bacterium widely distributed in the farm environment
Mannheimia haemolytica	Broncho-pneumonia in cattle; septicaemia, broncho-pneumonia and mastitis in sheep	Tetracycline, florfenicol	Macrolides, fluoroquinolones, third- and fourth-generation cephalosporins*	Primary viral damage may facilitate proliferation of pasteurellae with subsequent pneumonia
Moraxella bovis	Infectious bovine keratoconjunctivitis	Tetracycline, penicillin G + streptomycin	Chloramphenicol	Subconjunctival administration of suitable antibiotics preferable to topical applications
Mycoplasma spp.	Associated with pneumonia in many species; arthritis; occasionally mastitis and conjunctivitis	Tetracycline, tiamulin,	Macrolides, fluoroquinolones*	*Mycoplasma* species vary widely in their susceptibility to antimicrobial therapy
Nocardia species	Pyogranulomatous infections in dogs and other species; mastitis in dairy cattle	Trimethoprim-sulfamethoxazole,	Amikacin	Abscesses and empyema require drainage. Chemotherapy often disappointing
Pasteurella multocida	Haemorrhagic septicaemia in cattle and buffaloes; pneumonic lesions in cattle; fowl cholera; atrophic rhinitis in pigs; respiratory disease in rabbits	Ampicillin, florfenicol fluoroquinolones*	Cephalosporins*	Vaccination is effective in some animal species where disease seems to be independent of viral involvement
Proteus mirabilis or *P. vulgaris*	Often associated with otitis externa and urinary tract infections in dogs and occasionally other animals	Ampicillin, amoxicillin – clavulanate	Cephalosporins*	Routine susceptibility testing is required with these organisms
Pseudomonas aeruginosa	Otitis externa in dogs; mastitis in dairy cows; pneumonia in mink; opportunistic skin infections in many species	Gentamicin, tobramycin, fluoroquinolones*	Cephalosporins*	Multiple resistance commonly encountered

Table 6.3 Suggested antimicrobial treatment for pathogenic bacteria and related organisms—cont'd

Pathogenic bacterium	Disease	Suggested drugs	Alternative drugs	Comments
Rhodococcus equi	Suppurative bronchopneumonia in foals. Abscesses in older horses	Erythromycin or azithromycin + rifampicin	Trimethoprim sulfamethoxazole may be effective if given early post infection	Treatment should be instituted immediately the disease is recognized
Rickettsial organisms	Febrile diseases in many species of animals. Often transmitted by ticks and occasionally by files	Tetracycline	Chloramphenicol, Imidocarb	Vector control should be a priority in endemic areas
Salmonella species	Septicaemia and enteritis in many species, particularly ruminants where abortion may occur. Horses, pigs and poultry also affected. The infection has zoonotic implications	Trimethoprim-sulfamethoxazole, colistin, spectinomycin	Ampicillin, Fluoroquinolones*, Cephalosporins*	Control measures should be aimed at prevention. Vaccination is of value for some serotypes. Chemotherapy may prolong the excretion of salmonellae
Staphylococcus species	Pyogenic infections in many species of animals. *S. aureus* is a major cause of mastitis in dairy cattle	Penicillin G sensitive: Penicillin G Penicillin G resistant: A penicillinase-resistant penicillin Meticillin resistant: as indicated by susceptibility testing	Cephalosporins * Cephalosporins*, lincosamides, erythromycin, fluoroquinolones* Variable, depending on resistance pattern of isolate	The susceptibility pattern of each isolate should be established before treatment is instituted
Streptococcus spp. *S. agalactiae* *S. dysgalactiae* *S. uberis*	Mastitis in dairy cows	Penicillin G, cloxacillin	Ampicillin, cephalosporins*	Intramammary preparations used. Control programmes should include teat dipping and attention to milking-machine hygiene and performance
S. equi subsp. *equi*	Strangles in horses	Penicillin G	Ceftiofur*	Immediate isolation of affected or suspect horses is essential. Treatment with antibiotics is controversial
S. suis	Meningitis in pigs	Penicillin G	Trimethoprim-sulfamethoazole, cephalosporins*	
Yersinia enterocolitica	Latent infections with sporadic enteritis or generalized infection in many animals	Trimethoprim-sulfamethoxazole	Ampicillin, Tetracycline	Resistance to antimicrobial drugs is commonly encountered

* Fluroquinolones and third- and fourth-generation cephalosporins should be reserved for treatment of clinical conditions which respond poorly to other antimicrobial agents and where antimicrobial susceptibility testing has been carried out.

Table 6.4 Suggested antifungal treatment for superficial and systemic mycoses

Fungus	Diseases	Suggested drugs		Comments
		Topical administration	Systemic administration	
Aspergillus fumigatus	Infections of mucous membranes in dogs; lungs and air sacs in poultry; guttural pouch in horses and mastitis and abortion in cattle	Antifungal agents such as iodine or nystatin may be applied to superficial or accessible lesions	Amphotericin B, terbinafine, voriconazole, itraconazole (orally)	Aspergillosis in poultry is preventable by efficient management. Immunosuppression may lead to tissue invasion by aspergilli and other opportunistic fungi. Successful treatment for nasal granulomas in dogs using topical perfusion has been reported
Blastomyces dermatitidis	Disseminated disease in many species particularly dogs. Lungs, lymph nodes, skin and other tissues may be involved		Ketoconazole (orally), itraconazole (orally), amphotericin B, voriconazole	Combined ketoconazole-amphotericin B treatment may be necessary if disease is well established and treatment may be long term
Candida albicans	Mucocutaneous candidiasis occurs in many species of animals, often as a consequence of prolonged antibiotic treatment or because of immunosuppression	Clotrimazole, nystatin	Ketoconazole (orally), amphotericin B ± flucytosine, miconazole, voriconazole	The factors which precipitated the disease should be identified and dealt with as candidiasis is usually a secondary problem
Coccidioides immitis	Usually occurs in geographically defined areas of North and South America, affecting dogs and other species. In dogs, pulmonary involvement with dissemination to other organs occurs		Amphotericin B, ketoconazole, itraconazole	Long-term treatment is required. If the disease is at an advanced stage, euthanasia should be considered
Cryptococcus neoformans	Infections occur in many species, particularly cats and often involves soft tissues with a tendency to localize in the CNS. Pigeon droppings frequently contain this fungus		Amphotericin B + flucytosine (orally), fluconazole	In advanced cases, the response to treatment is variable
Histoplasma capsulatum	Histoplasmosis affects many animals, particularly dogs. Primary lesions are in the lungs with dissemination to intestines, spleen, liver and lymph nodes		Ketoconazole or itraconazole (orally), amphotericin B	Early in disease treatment may be successful but with disseminated disease the prognosis is poor

Table 6.4 Suggested antifungal treatment for superficial and systemic mycoses—cont'd

Fungus	Diseases	Suggested drugs		Comments
		Topical administration	Systemic administration	
Malassezia pachydermatis	Can be present, in small numbers, in ears of normal dogs. Increased numbers in otitis externa associated with other pathogenic organisms	Nystatin, natamycin miconazole		*Malassezia* infections frequently recur. Repeated use of antibiotics may allow yeast proliferation. Attention to predisposing factors is helpful. Aeration and drainage hasten recovery
Microsporum species	Invasion of the keratinized layers of the skin, hair, feathers, claws and nails. 'Ringworm' of many species including poultry	Clotrimazole, tolnaftate, natamycin, ketoconazole	Terbinafine, griseofulvin* (orally)	Griseofulvin is teratogenic early in gestation for dogs and cats. Thorough disinfection of premises and fittings with iodine-based or chlorine-based disinfectants essential
Sporothrix schenckii	Chronic ulcerative lesions of the skin and subcutaneous tissue, often accompanied by lymphangitis. Dissemination to viscera, bones and CNS may occur in rare cases		Potassium iodide (orally), sodium iodide (I/V), itraconazole, amphotericin B	Surgery is usually contraindicated
Trichophyton species	Invasion of keratinized layers of skin and hair. 'Ringworm' in many animal species	Clotrimazole, natamycin, tolnaftate	Terbinafine, griseofulvin* (orally)	Thorough disinfection of premises and fittings with iodine-based or chlorine-based disinfectants is essential
Zygomycetes (*Lichtheimia, Mortierella, Mucor, Rhizopus, Rhizomucor,* and others)	Opportunistic infections. Because of the multiple aetiology, many tissues may be invaded including respiratory, intestinal and genital tracts. Mucocutaneous granulomas occur in many animal species		Amphotericin B, ketoconazole	Excision of lesion, where possible, followed by systemic treatment

* Griseofulvin has been withdrawn from the market in many countries

beta-lactamases by clavulanic acid can protect penicillin G or other susceptible antibiotics from inactivation by bacteria that produce beta-lactamase.

3. One drug may promote the uptake of a second drug thereby increasing the overall antimicrobial effect. This appears to be a widely applicable mechanism of synergism with considerable clinical importance. Penicillins enhance the uptake of aminoglycosides by enterococci and in human medicine this combination has proved highly beneficial for the treatment of enterococcal endocarditis. Cell wall inhibitors such as penicillins and cephalosporins may enhance the entry of aminoglycosides into some Gram-negative bacteria and produce synergistic effects.
4. One drug may affect the cell membrane and facilitate the entry of a second drug. Amphotericin B may be

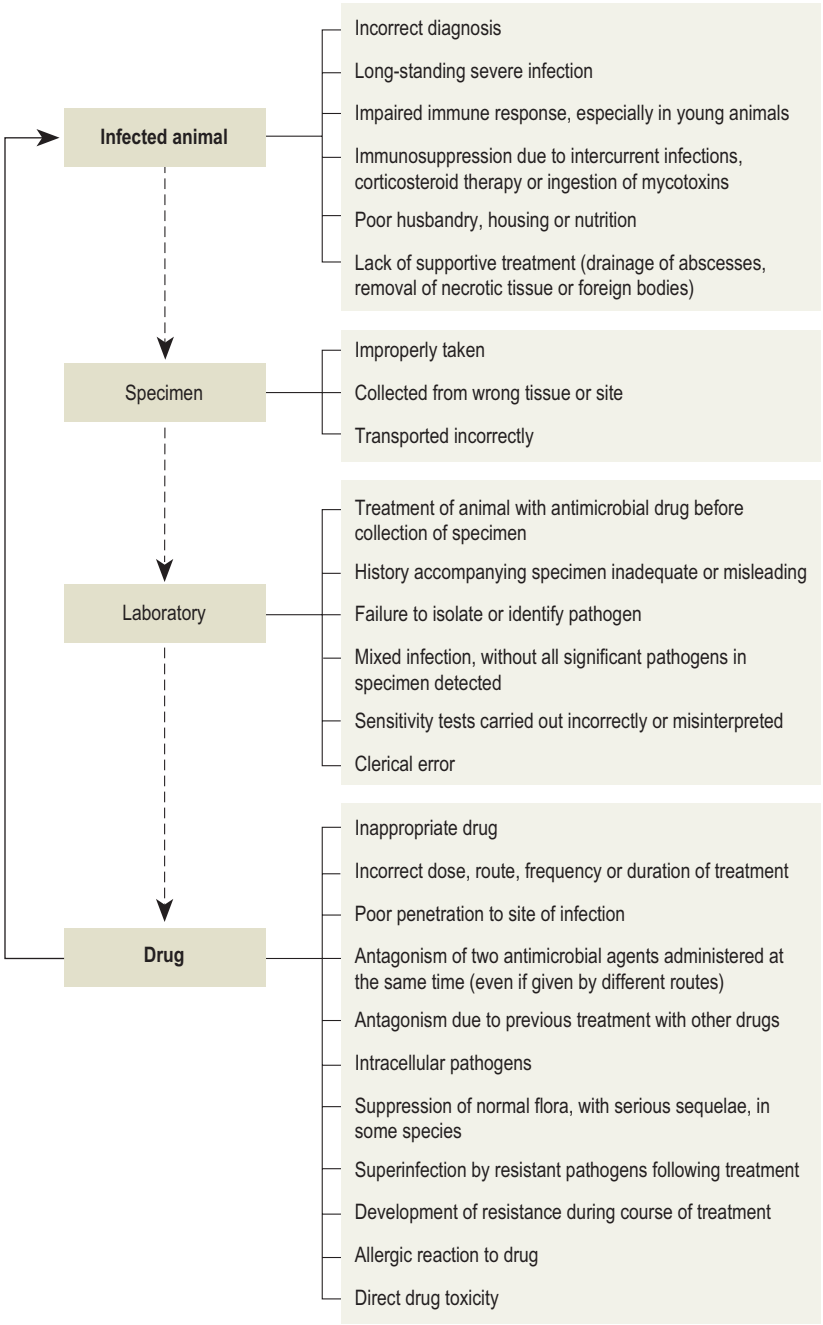

Figure 6.9 Sources of failure or adverse response following prescribed antimicrobial therapy.

synergistic with flucytosine against *Cryptococcus neoformans*.

5. A drug combination may also prevent the emergence of resistant populations. Treatment of *Rhodococcus equi* infection in foals with erythromycin-rifampicin is used synergistically for this purpose.

Just as two antibacterial compounds can be combined in a mutually beneficial way, they can also interfere with each other's activity. A form of antagonism which has received much attention is that occurring between predominantly bacteriostatic drugs such as chloramphamicol or a tetracycline and bactericidal drugs such as penicillin

or an aminoglycoside. Clearly, if bacterial growth is halted by a bacteriostatic compound, the bactericidal activity of a second drug that is effective against actively dividing cells may be abolished. Antagonism is more likely to occur if the bacteriostatic drug reaches the site of infection before the bactericidal drug. A recognized mechamism of drug antagonism is in the combined use of beta-lactam-stable cephalosporins and extended-spectrum penicillins. The enzyme-stable cephalosporin induces the production of beta-lactamase in certain bacteria and this enzyme may then inactivate the unstable penicillin.

Administration of two antimicrobial drugs (provided that they are not antagonists) may be justified when (1) treating mixed bacterial infections, where each drug has activity against one of the pathogens; (2) treating severe infections of uncertain aetiology; (3) using synergistic combinations with documented efficacy against specific infections. There are also inherent disadvantages in combined antimicrobial treatment regimens. These include an additive toxic effect from the drugs used, an increased risk of superinfection with overgrowth of fungi or resistant bacteria and a danger of enhanced spread of R plasmids. There is the additional risk that if antimicrobial compounds are not carefully selected, antagonism may occur. Using combinations of antimicrobial drugs can lead to a more casual approach in clinical diagnosis of disease with less interest in establishing the infectious agents involved in the disease process.

Resistance to Antimicrobial Agents

When antibacterial or antifungal drugs are used to treat infections, the outcome is influenced by many factors. Therapeutic success depends not only on the intrinsic activity of the antimicrobial drug against the invading pathogen, but relies on the drug reaching the site of infection in sufficient concentration. The response of the animal to the infectious agent often determines the course of the infection subsequently. The resistance of bacteria and fungi to antimicrobial drugs is an increasing problem in human and in veterinary medicine. Microorganisms may be resistant to certain antimicrobial drugs because the cellular mechanisms required for antimicrobial susceptibility are absent from the cell. This is sometimes referred to as constitutive or intrinsic resistance. Acquired, genetically based resistance can arise because of chromosomal mutation or through the acquisition of transferrable genetic material. Acquired resistance is not present in the entire species but only within certain strains of the organism (Harbottle et al. 2006). Chromosomal mutations occur at a low rate and thus it was considered that this method of acquiring resistance was slower than resistance developing through the horizontal transfer of resistance elements. However, there is recent evidence that some bacteria increase their rate of mutation in the presence of antimicrobial agents, which may increase the rate of resistance development through mutation (Cirz et al. 2005). Thus, it is likely that some individual organisms in a bacterial population causing infection will develop resistance and will have a higher MIC than the mean of the population detected by laboratory testing. This gives rise to the concept of mutant prevention concentration (MPC), which can be determined in the laboratory using multiple testing steps. Helfand et al. (2003) reported that the ratio of MPC to MIC for veterinary fluoroquinolones varied from 6–10 for *E. coli*.

Horizontal transmission of resistance arises through the incorporation of resistance genes from donor organisms to acceptor bacteria by conjugation, transduction, transformation or via mobile genetic elements, such as transposons. Conjugation, the process whereby a donor bacterium transfers plasmid DNA to a recipient organism, usually via a sex pilus, is considered a major route of transmission of resistant genes between different species and genera. Transfer of resistance by transduction occurs when bacterial resistance genes are transferred between organisms by means of bacteriophages. Occasionally resistance may be transmitted between organisms by means of transformation, which is the uptake of free DNA by a competent bacterium. The latter is not thought to play an important role in resistance transfer. Horizontal transfer of resistance can take place via transposons and integrons also. These mobile genetic elements may contain antimicrobial resistance genes which are transferred with the element. Transposons are short, mobile sequences of DNA which carry the gene for a transposase enzyme and may contain other genes encoding antimicrobial resistance. The transposase enzyme is required to incorporate the transposon into a new location, either on a chromosome or plasmid. Integrons are mobile genetic elements that can capture and carry genes, located on gene cassettes, which sometimes encode antimicrobial resistance. Integrons contain an integrase enzyme which is required for integration of its DNA into a chromosome or plasmid of the recipient organism and a promoter for expression of the genes carried on the gene cassette. Integrons can move only by site-specific recombination.

Mechanisms of resistance usually involve either modification of the antimicrobial agent itself, reduced intracellular accumulation of the agent or modification of the target site of the agent. A summary of mechanisms of resistance is presented in Table 6.5.

Frequently, development of resistance to one drug confers cross resistance to other agents. This form of resistance is encountered with the sulphonamides, tetracyclines, aminoglycosides and macrolides and may explain findings such as those reported by DANMAP (2004), in which persistently high levels of resistance to tetracycline are detected in the absence of significant use of this agent. Organisms which are resistant to multiple antimicrobial agents and which cause severe disease, particularly in hospitalized humans or animals, are sometimes termed

Table 6.5 Mechanisms of resistance to antimicrobial agents and antimicrobial classes in which the mechanism may be present in selected organisms against agents within the class

Antimicrobial class	Resistance mechanism
	Alteration of target site
Aminoglycosides	Altered ribosomal protein
Beta-lactam antibiotics	Altered or new penicillin-binding proteins
Erythromycin	Ribosomal RNA methylation
Quinolones	Altered DNA gyrase
Sulphonamides	New drug-insensitive dihydropteroate synthase
Tetracyclines	Ribosomal protection
Trimethoprim	New drug-insensitive dihydrofolate reductase
Vancomycin	Altered cell-wall stem peptide
	Drug-destroying mechanisms
Aminoglycosides	Acetyltransferase Nucleotidyltransferase Phosphotransferase
Beta-lactamase antibiotics (penicillins, cephalosporins)	Beta-lactamase
Chloramphenicol	Acetyltransferase
	Decreased uptake (decreased permeability)
Beta-lactam antibiotics, chloramphenicol, quinolones, tetracyclines, trimethoprim	Alteration in the permeability of the bacterial cell envelope
	Increased expulsion
Aminoglycosides, fluoroquinolones, glycylcyclines, macrolides, beta-lactam antibiotics	Efflux pumps

'superbugs'. Frequently, these organisms fall into two classes, the first class being well-known pathogens which have acquired resistance to multiple antimicrobial agents and the second class being opportunistic, environmental pathogens which are intrinsically resistant to many antibiotics (Wright 2007). Examples of the first class include meticillin-resistant *Staphylococcus aureus* and multi-drug-resistant *E. coli*, whereas environmental organisms such as *Pseudomonas aeruginosa* and *Acinetobacter baumanii* are included in the second class. The emergence of such organisms as major nosocomial pathogens in human and veterinary medicine, in addition to the increasing resistance of pathogens of intensively farmed animals and companion animals has led to major problems for the efficacy of antimicrobial therapy worldwide.

Guidelines for the prudent use of antimicrobial agents have been developed by many agencies worldwide and include recommendations such as improving infection control, increasing appropriate prescribing practice and better use of disease prevention practices. Appropriate prescribing should be designed to ensure efficacy of the agent and prevention of resistance development. Account must be taken of the pharmacokinetics and pharmacodynamics of the drug, in addition to use of MIC data. Thus, quantitative susceptibility testing methods will be the ideal in all diagnostic laboratories in the future.

Adverse Reactions to Antimicrobial Drugs

Adverse effects of antimicrobial drugs include dose-related toxic reactions, usually involving a specific organ or system, hypersensitivity reactions, alteration in host microflora, and abnormal tissue residues. Other undesirable consequences include the risk of superinfection with overgrowth of fungi or resistant bacteria. Considerable species variation occurs and drugs which are relatively non-toxic in one species may be profoundly toxic in another species (Table 6.6). Drug administration in the presence of liver or kidney disease requires careful consideration and adjustment of dosage is required. In the presence of renal failure, aminoglycosides should be given at more widely spaced intervals and neomycin is contraindicated. Sulphonamides should be used with care and alternative drugs should be considered in severe renal failure. Severe hepatic disease usually decreases the clearance of drugs that are metabolized by the liver. Tetracyclines, chloramphenicol, lincomycin, erythromycin and oxacillin are among the antibiotics which should be avoided in the presence of severe hepatic insufficiency.

Antimicrobial drugs do not distinguish between pathogenic microorganisms and those that constitute the normal flora of the host. Some broad-spectrum antibiotics may suppress the normal flora, especially if treatment is prolonged, leading to the proliferation of drug-resistant organisms which in turn may give rise to superinfection. Even narrow-spectrum antibiotics like penicillin have a profound effect on the intestinal flora of some species, such as the guinea pig, often with fatal consequences.

Table 6.6 Adverse effects of antimicrobial drugs in different species of animals

Drug	Animal species	Toxic effect	Comments
Aminoglycosides	Many species	Ototoxic, nephrotoxic and cause neuromuscular blockade	Prolonged therapy with aminoglycoside antibiotics should be avoided even in large animals
Streptomycin	Cats, dogs, mice, hamsters, guinea pigs and ferrets. Especially young animals	Vomition, salivation and ataxia in cats; vestibular damage in dogs and cats; directly toxic for mice and very young animals; toxic reactions reported in hamsters, guinea pigs and ferrets	Avoid completely in susceptible animals species
Neomycin	Cats, cattle, pigs	Nephrotoxicity, deafness and neuromuscular blockade	
Chloramphenicol	Many animal species, humans	Bone marrow depression and fatal aplastic anaemia	There is an absolute ban on the use of chloramphenicol in food-producing animals in many countries
Erythromycin and other macrolides	Rabbits, gerbils, guinea pigs, hamsters	Contraindicated, causes enterocolitis	
	Ruminants	Severe diarrhoea if used orally	
	Adult horses	Severe diarrhoea	
Fluoroquinolones	Dogs, especially greyhounds, horses	Contraindicated in young animals due to cartilage erosions in weight-bearing joints	These drugs should be administered with caution to all young animals
Griseofulvin	Cats (dogs)	Teratogenic effects in cats (dogs) early in gestation	Topical treatment with anti-fungal drugs may be preferable in pregnant animals
Lincomycin	Horses, cattle	This drug is contraindicated in the horse in which it may cause fatal haemorrhagic colitis and intractable diarrhoea. Oral administration in dairy cattle produces severe depression, a drop in milk production and diarrhoea, sometimes with high mortality	Even extremely low doses may produce serious disease in horses and cattle
	Rabbits, guinea pigs, hamsters	This antibiotic is highly toxic for these animals, apparently allowing overgrowth of *Clostridium* species in the large intestine	
Penicillins	Some animals	Hypersensitivity reactions	Most likely to develop from repeated systemic administration
	Guinea-pigs, hamsters	Destroys the normal flora with resultant overgrowth of organisms such as *Clostridium* species, causing toxaemia and death	
Procaine penicillin	Mice, guinea pigs and rabbits	Procaine hydrochloride is toxic	

Continued

Table 6.6 Adverse effects of antimicrobial drugs in different species of animals—cont'd

Drug	Animal species	Toxic effect	Comments
Sulphonamides	Many animals	Respiratory distress and collapse if given rapidly I/V in large animals. Crystalluria, renal tubular damage and blood dyscrasias may occur in some animal species	All animals receiving sulphonamide therapy should have ad libitum access to water. These drugs are contraindicated in severe renal disease
Tetracycline	Many animals	Deposited in bones and teeth of young animals with discolouration evident. Irritant if injected locally into tissues	Toxic reactions also reported in rats, hamsters and guinea pigs
	Horses	These drugs may induce intractable diarrhoea in horses	

REFERENCES

Boothe, D.M., 2006. Principles of antimicrobial therapy. Veterinary Clinics of North America, Small Animal Practice 36, 1003–1047.

Cirz, R.T., Chin, J.K., Andes, D.R., et al., 2005. Inhibition of mutation and combating the evolution of antibiotic resistance. PLOS Biology 3 (6), e176.

Clinical and Laboratory Standards Institute (CLSI), 2006. Molecular diagnostic methods for infectious diseases; approved guideline, second ed, CLSI Document MM03-A2. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard, eighth ed (M11-A8). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, third ed (M31-A3). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance standards for antimicrobial disk susceptibility tests; approved standard, eleventh ed (M02-A11). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2010. Quantitative molecular methods for infectious diseases; approved guideline, second ed. CLSI document MM06-A2. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

DANMAP, 2004. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. ISSN 1600–2032, Available at www.dfvf.dk (accessed 14 January 2013).

Harbottle, H., Thakur, S., Zhao, S., et al., 2006. Genetics of antimicrobial resistance. Animal Biotechnology 17, 111–124.

Helfand, M.S., Bonomo, R.A., 2003. β-Lactamases: a survey of protein diversity. Current Drug Targets – Infectious Disorders 3, 9–23.

Wright, G.D., 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. Nature Reviews Microbiology 175–186.

Sarkar, P., Gould, I.M., 2006. Antimicrobial agents are societal drugs: how should this influence prescribing? Drugs 66, 893–901.

Watts, J.L., Lindeman, C.J., 2006. Antimicrobial susceptibility testing. In: Aarestrup, F.M. (Ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington, DC, pp. 29–35.

FURTHER READING

Brooks, G.F., Butel, J.S., Morse, S.A., 2007. Medical Microbiology, twenty-fourth ed. Appleton and Lange, Norwalk, Connecticut.

Brunton, L., Lazo, J., Parker, K., 2006. Goodman and Gilman's The Pharmacological Basis of Therapeutics, eleventh ed. McGraw-Hill Medical, New York.

Giguere, S., Prescott, J.F., Baggot, J.D., et al. (Eds.), 2007. Antimicrobial Therapy in Veterinary Medicine, fourth ed. Wiley-Blackwell.

Guardabassi, L., Jensen, L.B., Kruse, H. (Eds.), 2008. Guide to Antimicrobial Use in Animals, Wiley-Blackwell.

Section 2

Bacteriology

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Chapter 7

Staphylococcus species

Genus Characteristics

The staphylococci are Gram-positive cocci with an average diameter of 0.8 to 1 μm, that tend to be arranged in pairs, tetrads, or more often, grouped in irregular clusters or 'bunches of grapes' (Fig. 7.1). Colonies are usually white with regular edges. They are non-motile, non-sporulating and most species are facultative anaerobes, with a fermentative metabolism. They are sensitive to lysostaphin (MIC of 12.5 μg/ml) and furazolidone (100 μg/disc) and resistant to lysozyme (MIC of 1000 μg/ml), bacitracin (0.04 unit disc) and to O/129 (0.5 mg). They are usually catalase-positive and oxidase-negative. Growth occurs on nutrient and blood agars but not on MacConkey agar. They are usually not capsulated or have a limited amount of capsule. There are about 30 species of staphylococci and most are found in animals but few are pathogenic. They are considered opportunistic pathogens. Infections with staphylococci are often acute and pyogenic. The two major pathogenic staphylococci, *Staphylococcus aureus* and *S. pseudintermedius* are coagulase-positive. The coagulase test usually correlates well with pathogenicity. However, the cause of exudative epidermitis in young pigs, *Staphylococcus hyicus*, can be coagulase-negative as only 24 to 56% of isolates are coagulase-producing isolates. Coagulase-negative staphylococci occur as commensals and in the environment. They are considered a major component of the normal microflora of animals and humans and occasionally cause opportunistic infections. In this chapter, we will focus primarily on the identification of *S. aureus*, *S. pseudintermedius*, *S. hyicus*, *S. chromogenes*, *S. aureus* subsp. *anaerobius*, *S. delphini*, *S. schleiferi* subsp. *coagulans* and *S. felis*, the species most commonly associated with animal infections.

Staphylococci Compared with Other Gram-Positive Cocci

Micrococci are non-pathogenic, Gram-positive cocci that could be confused with coagulase-negative staphylococci. However, micrococci are variably positive to conventional oxidase tests, oxidase-positive in a modified oxidase test (Faller & Schleifer 1981), are oxidative in the O-F test and have a different susceptibility pattern to bacitracin and furazolidone. The colonies of the micrococci can be white but are often pigmented, the pigmentation ranging from a garish-yellow through cream, to buff or pink (*M. roseus*) (Fig. 7.2). Streptococci and enterococci are distinguished from staphylococci by the catalase test. Macrococci cells are approximately 4 to 5 times bigger than staphylococcal cells with a diameter of about 2 μm. The pertinent reactions for the commonly isolated Gram-positive cocci are summarized in Table 7.1.

Natural Habitat

Staphylococci are widespread in nature and occur worldwide in mammals and birds. They colonize the nasal cavity, naso-pharynx, skin, and mucous membranes. They can be transient in the intestinal tract. Many infections are endogenous but prolonged survival of staphylococci in the environment (some resistance to heat, NaCl and certain disinfectants) permits indirect transmission.

Pathogenesis and Pathogenicity

The staphylococci are pyogenic bacteria associated with abscess formation and suppuration. Pathogenic *Staphylococcus* species can infiltrate the tissues following a

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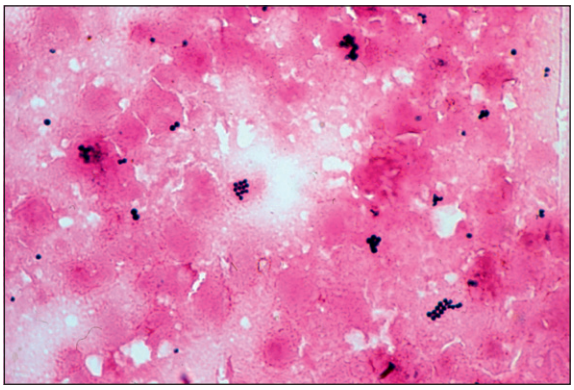

Figure 7.1 *Staphylococcus aureus* in a Gram-stained smear of a bovine mastitic milk showing the characteristic 'bunches of grapes' arrangement of cells. (×1000)

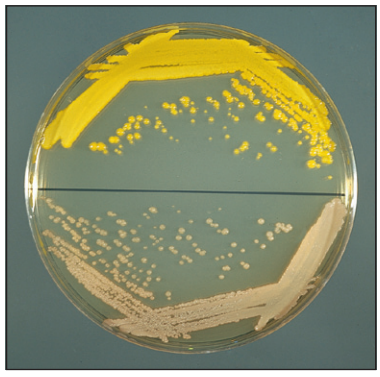

Figure 7.2 Two pigmented *Micrococcus* species on nutrient agar. Colonies of *Micrococcus* may be white or pigmented. They are aerobic (oxidative) and variably oxidase-positive, distinguishing them from the staphylococci.

Table 7.1. The main differentiating characteristics of the Gram-positive cocci

Genus	Coagulase	Catalase	Oxidase	O-F glucose	Haemolysis	Bacitracin 0.04 unit disc[1]	Furazolidone 100 μg disc[2]
Staphylococcus							
Pathogenic	+	+	–	F	+ (–)	R	S
Non-pathogenic	–	+	–[3]	F	– (+)	R	S
Macrococcus	–	+	+	F	– (+)	R	S
Micrococcus	–	+	+[4]	O	– (+)	S	R
Streptococcus	–	–	–	F	(+)	R	S
Enterococcus	–	–	–	F	(+)	R	S

+ = 90% or more strains positive, – = 90% or more strains negative, (+) = some strains positive, (–) = some strains negative, F = Fermentative, O = oxidative, R = resistant, S = susceptible

[1]Susceptible to bacitracin = zone 10–25 mm

[2]Susceptible to furazolidone = zone 15–35 mm

[3]Except *Staphylococcus sciuri* which is oxidase positive

[4]Modified oxidase test (Faller & Schleifer 1981)

cutaneous lesion and produce suppurative lesions which usually remain localized at the infection site. Pus is composed of the debris of dead leukocytes and both living and dead bacteria. This is surrounded by intact phagocytic cells and fibrin strands. A fibrous capsule will eventually be formed around an abscess. In chronic staphylococcal wound infections ('botryomycosis') the lesion is granulomatous with pockets of pus throughout the tissue.

The pathogenic staphylococci produce a 'battery' of toxins and enzymes, but the significance of many of them in the pathogenesis of disease is not fully understood. Enterotoxins (A–E) are involved in human food poisoning (mainly enterotoxin A). They act by reflex stimulation of the emetic centre. Exfoliatin produces staphylococcal scalded skin syndrome (SSSS) in human infants and dogs. Human toxic shock syndrome (TSS) is caused by TSS toxin-1. Epidermolytic toxins are implicated in porcine exudative epidermitis and in staphylococcal skin conditions in humans and dogs. The alpha toxin (a haemolysin) is associated with gangrenous mastitis in cattle. This toxin is a pore-forming toxin and causes lysosomal disruption in leukocytes and also affects smooth muscle, leading to constriction, paralysis and finally necrosis of the smooth muscle cells of the walls of blood vessels. A leukocidin kills neutrophils and macrophages of cattle, rabbits and humans. Protein A, a surface component of most strains

of virulent *S. aureus*, binds to the fragment crystallizable (Fc) region of IgG and may play a part in the pathogenesis of staphylococcal diseases.

Enzymes include staphylokinase which is a plasminogen activator; coagulase which causes plasma coagulation in vitro; hyaluronidase ('spreading factor'); lipase; collagenase; proteases; nucleases and urease, all of which may have a role in the pathogenesis of staphylococcal infections. Table 7.2 lists the main diseases caused by the pathogenic staphylococci while their main virulence factors are presented in Table 7.3.

Species Characteristics

The species characteristics discussed in the paragraphs below are those usually used to identify these organisms in a diagnostic veterinary bacteriology laboratory. Key tests for rapid identification of the most clinically significant *Staphylococcus* species are found in Table 7.4 while a schematic representation is shown in Figure 7.3.

Staphylococcus aureus subsp. *anaerobius*

Staphylococcus aureus subsp. *anaerobius* is catalase-negative and grows more fully in anaerobic conditions. After 48 hours, on blood culture, the colonies are between 0.5 and 2 mm, circular, convex, smooth, non-pigmented with regular edges and haemolytic. Haemolysis can be readily seen after incubating the plates at 4°C. This organism will only grow anaerobically upon first isolation. It is coagulase- and hyaluronidase-positive. It causes abscessation in small ruminants, principally in sheep. The disease resembles caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis.*

Staphylococcus chromogenes

Colonies of *S. chromogenes* are non-haemolytic and it is reported that 82% of the isolates have a yellowish pigment. The catalase test is positive and the coagulase and clumping factor tests are both negative. They are sensitive to novobiocin and oxidase-negative. *Staphylococcus chromogenes* does not produce hyaluronidase and is sensitive to bacitracin (MIC of 2 U/mL). These two characteristics can be used to differentiate the non-haemolytic isolates from *S. hyicus*. *Staphylococcus chromogenes* causes subclinical mastitis in bovine, ovine and caprine species. It has been isolated rarely from dermatitis cases in both horses and cats.

Staphylococcus delphini

Colonies of *S. delphini* are haemolytic and non-pigmented. The catalase and coagulase tests are positive but the clumping factor test is negative. They are sensitive to novobiocin and oxidase-negative. Few reports are available on *Staphylococcus delphini* but it has been associated with purulent cutaneous lesions in dolphins.

Staphylococcus felis

Colonies of *S. felis* are weakly haemolytic and non-pigmented. The catalase test is positive and the coagulase test is negative. They are sensitive to novobiocin and oxidase-negative. *Staphylococcus felis* isolates closely resemble *Staphylococcus simulans*. Fermentation of mannose, alkaline phosphatase and sensitivity to bacitracin may help in their differentiation. This organism has been reported as a cause of otitis, abscessation, dermatitis, cystitis and conjunctivitis in cats.

Staphylococcus hyicus

Colonies of *S. hyicus* are non-pigmented and non-haemolytic. The catalase test is positive and the coagulase test is variable. For those isolates that are coagulase-positive, coagulation is observed only after 18 to 24 hours' incubation at 35°C. The identification of *S. hyicus* is

Table 7.2 Main diseases caused by the pathogenic staphylococci in veterinary medicine

Species	Host(s)	Diseases
Staphylococcus aureus	Many animal species	Abscesses and suppurative conditions. Infection can be systemic. Important cause of infections following surgery
	Cattle	Mastitis: subclinical, chronic, acute, peracute or gangrenous Udder impetigo: small pustules, often at base of teats
	Sheep	Mastitis: acute, peracute or gangrenous Tick pyaemia of lambs (two to five weeks old): associated with heavy tick (*Ixodes ricinus*) infestation Periorbital eczema (dermatitis): infections of abrasions, associated with communal trough feeding Dermatitis: predisposed to by scratches from vegetation such as thistles

Continued

Table 7.2 Main diseases caused by the pathogenic staphylococci in veterinary medicine—cont'd

Species	Host(s)	Diseases
	Goats	Mastitis: subacute or peracute Dermatitis
	Pigs	Mastitis: acute, subacute and chronic (botryomycosis) Necrotizing endometritis Udder impetigo: after abrasions from teeth of piglets
	Horses	Mastitis: acute Botryomycosis (spermatic cord) after castration
	Rabbits	Exudative dermatitis in neonates Abscesses, conjunctivitis and pyaemic conditions
	Poultry	'Bumble-foot': pyogranulomatous lesion of subcutaneous tissue of foot that can involve the joints Arthritis and septicaemia in turkeys Omphalitis (more commonly caused by *Escherichia coli*)
	Dogs, cats	Suppurative conditions similar to those listed for *S. pseudintermedius*
Staphylococcus aureus subsp. *anaerobius*	Sheep	Lesions similar to those of caseous lymphadenitis (*Corynebacterium pseudotuberculosis*)
Staphylococcus pseudintermedius	Dogs, cats	Canine (feline) pyoderma (juvenile and adult). Chronic and recurrent pyoderma is a complex syndrome possibly involving cell-mediated hypersensitivity, endocrine disorders and a genetic predisposition. Responds poorly to antibiotic therapy alone Pustular dermatitis occurs in neonates or in adults under conditions of poor hygiene. Responds readily to antibiotic therapy Pyometra Otitis externa (usually in concert with other pathogens) Infections involving respiratory tract, bones, joints, wounds, eyelids and conjunctiva
	Horses, cattle	Rare infections in these species
Staphylococcus hyicus	Pigs	Exudative epidermitis (greasy pig disease), usually in pigs under seven weeks old, there is systemic involvement and the condition can be fatal Septic polyarthritis, metritis, vaginitis
	Cattle	Rare cases of mastitis and cutaneous infections
	Horses	Cutaneous infections
Staphylococcus chromogenes	Ruminants Pigs Horses, cats	Subclinical mastitis Exudative epidermitis Dermatitis (rare)
Staphylococcus delphini	Dolphins	Purulent cutaneous lesions
Staphylococcus felis	Cats	Otitis, abscesses, dermatitis, cystitis, conjunctivitis
Staphylococcus schleiferi subsp. *coagulans*	Dogs	Otitis externa

Table 7.3 Principal virulence factors of pathogenic staphylococci in veterinary medicine

Species	Virulence factors	Functions
Staphylococcus aureus	Coagulase (extracellular protein)	Adherence to prothrombin, transforms fibrinogen into fibrin
	Protein A	Adherence to Fc fragment of immunoglobulin G, inhibits phagocytosis
	Proteases	Hydrolysis of proteins
	Enterotoxins (A, B, C, D, E, and F)	Neurotoxins with superantigen activity on T lymphocytes: vomiting, diarrhoea, possible shock
	Haemolysins (alpha, beta, delta, gamma)	Lysis of erythrocytes. Alpha toxin is a pore-forming toxin and may also have a role in escape from the phagosome of intracellular *S. aureus*
	Exfoliatins A and B	Pustular dermatitis
	Lipases and phospholipases	Enzymatic activity on lipids and phospholipids
	Deoxyribonuclease	Exotoxin, hydrolysis of DNA
	Staphylokinase	Adherence to plasminogen and transformation into plasmin which has fibrinolytic and proteolytic activity. Aids dissemination and nutrition
	Toxic-Shock-Syndrome Toxin 1 (TSST-1)	Superantigen activity
	Clumping factors	Adherence to fibrinogen
	Leucocidin	Lysis of leucocytes
	Surface proteins	Adherence to fibronectin, collagen, vitronectin
	Capsule	Inhibits phagocytosis
	Beta-lactamases	Hydrolysis of the beta-lactam ring of penicillins
	Staphyloferrin B	Siderophore
	Bacteriocins (Staphylococcin)	Inhibit Gram-negative bacteria
Staphylococcus aureus subsp. *anaerobius*	Coagulase	Adherence to prothrombin, transforms fibrinogen into fibrin
	Hyaluronidase	Spreading factor, hydrolysis of extracellular matrix
	Protein A	Adherence to Fc fragment of immunoglobulin G, inhibits phagocytosis
	TSST-1	Superantigen activity
Staphylococcus pseudintermedius	Surface proteins	Adherence to fibronectin, collagen, lactoferrin
	Clumping factor	Adherence to fibrinogen
	Coagulase (extracellular protein)	Adherence to prothrombin, transforms fibrinogen into fibrin
	Glycocaylyx (exopolysaccharides)	Inhibits phagocytosis
	Protein-A like	Adherence to Fc fragment of immunoglobulin G, inhibits phagocytosis

Continued

Table 7.3 Principal virulence factors of pathogenic staphylococci in veterinary medicine—cont'd

Species	Virulence factors	Functions
	Exfoliatin toxin	Pustular dermatitis
	Leucocidin	Lysis of leucocytes
	Haemolysin beta (beta-toxin)	Sphingomyelinase C, lysis of erythrocytes
	Enterotoxins	Superantigen activity on T lymphocytes: vomiting, diarrhoea, shock
Staphylococcus hyicus	Coagulase (extracellular protein)	Adherence to prothrombin, transforms fibrinogen into fibrin
	Protein A-like (porcine isolates)	Adherence to Fc fragment of immunoglobulin G, inhibits phagocytosis
	Staphylokinase	Adherence to plasminogen and transformation into plasmin which has fibrinolytic and proteolytic activity. Aids dissemination and nutrition
	Lipase	Enzymatic activity on lipids and phospholipids
	Metalloproteases	Possible cytotoxicity
	Exfoliatins (A, B, and C)	Pustular dermatitis

Table 7.4 Key tests for rapid identification of most clinically significant *Staphylococcus* species

Species	Tests					Acid from (aerobically):	
	Coagulase	Polymyxin B (300 unit disc)	VP	ONPG (B-galactosidase)	Haemolysis	Mannitol	Maltose
S. aureus	+	R	+	–	+	+	+
S. hyicus	d	R	–	–	–	–	–
S.pseud-intermedius	+	S	–	+	+	(d)	+/–
S. aureus subsp. *anaerobius*	+	nk	–	–	+	nk	+
S. chromogenes	–	R	–	–	–	d	d
S. delphini	+	nk	–	nk	+	+	+
S. felis	–	nk	–	+	+/–	v	–
S. schleiferi subsp. *coagulans*	+	nk	+	d	+	d	_

+ = 90% or more of strains positive, – = 90% or more of strains negative, d = 11 to 89% of strains positive, () parentheses indicate a delayed reaction, +/–, = 90% or more of strains weakly positive, nk, = not known, R = resistant, S = susceptible, v = variable

Resistance to polymyxin B = zone of less than 10 mm

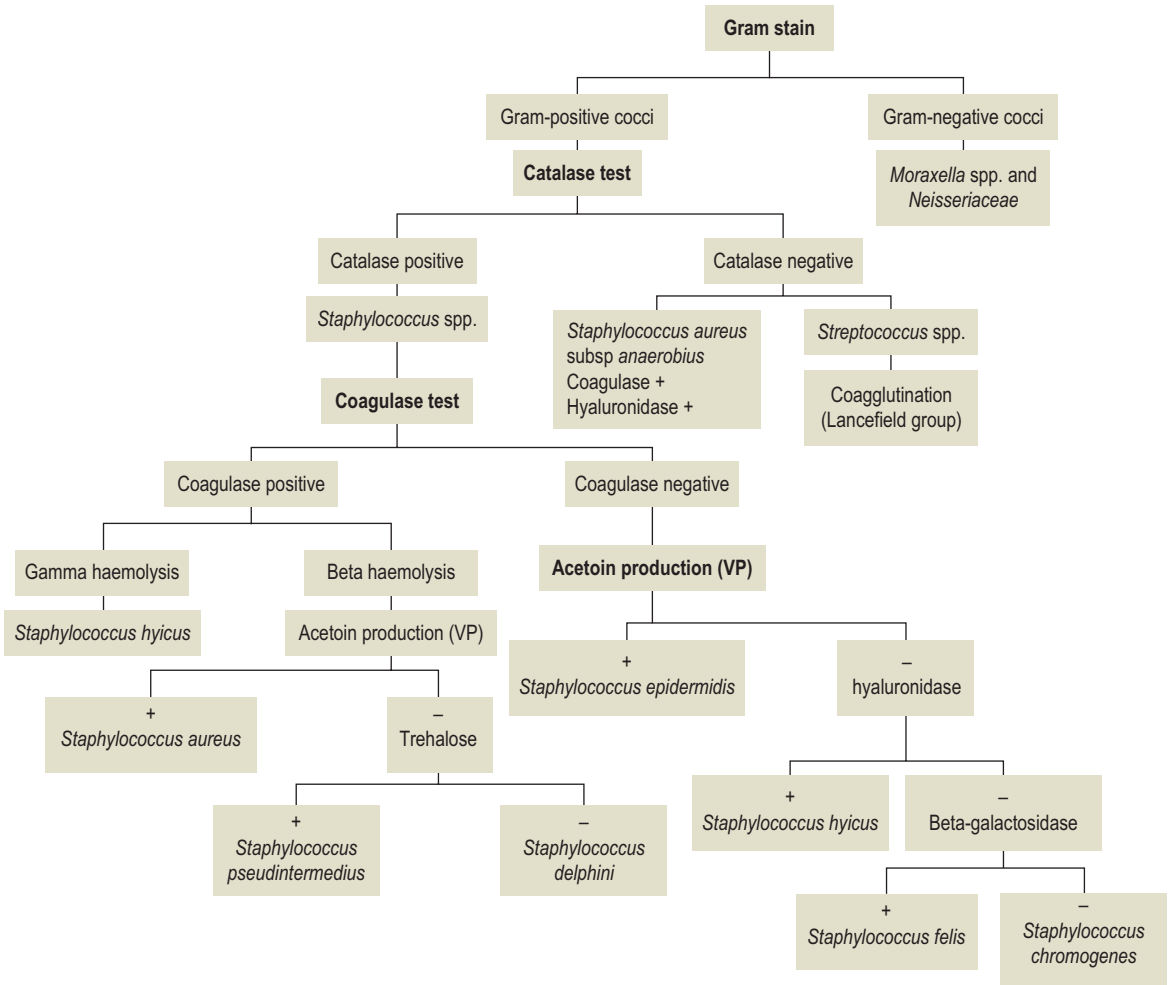

Figure 7.3 Flow chart outlining rapid identification of most clinically significant *Staphylococcus* species.

usually based on the following tests: absence of haemolysis, beta-galactosidase-negative, acetoin-negative, mannitol-negative and hyaluronidase-positive. The hyaluronidase and bacitracin tests can be used to differentiate *S. hyicus* from *S. chromogenes*. This organism is responsible for various infections in animals including cutaneous infections in horses, cattle and pigs (exudative epidermitis or greasy pig disease), as well as metritis and vaginitis in pigs.

Staphylococcus aureus

Colonies of *S. aureus* often have a typical double zone of haemolysis. Animal isolates are usually non-pigmented. The catalase and coagulase tests are positive. For those isolates that are coagulase-positive, coagulation can usually be observed after four hours' incubation at 35°C. The beta-galactosidase test and the fermentation of mannitol and maltose can be used to differentiate this species from *S. pseudintermedius*. *Staphylococcus aureus* is the cause of many diseases; the main ones are summarized in Table 7.2.

Staphylococcus pseudintermedius

Many isolates formerly identified phenotypically as *S. intermedius* have been shown to be distinct and have been reclassified as *S. pseudintermedius* (Devriese et al. 2009). Colonies of *S. pseudintermedius* are usually haemolytic. The catalase and coagulase tests are positive, coagulation can usually be observed after four hours' incubation at 35°C. The beta-galactosidase test, the fermentation of trehalose and the absence of fermentation of xylose can be used to differentiate this species from other staphylococci. However, definitive identification is most reliable using molecular methods. *Staphylococcus pseudintermedius* is the most common cause of canine skin infections and

staphylococcal scalded skin syndrome has been reported in this species.

Staphylococcus schleiferi subsp. *coagulans*

Colonies of *Staphylococcus schleiferi* subsp. *coagulans* are similar to those of *S. pseudintermedius,* being 1–2 mm in diameter, smooth, slightly convex, opaque and non-pigmented. Beta-haemolysin is produced. The catalase and tube-coagulase tests are positive. Clumping factor test is negative. Isolates are resistant to bacitracin, do not ferment maltose or trehalose and produce acetoin from glucose (positive Voges–Proskauer test). *Staphylococcus schleiferi* subsp. *coagulans* causes otitis externa in dogs.

Other *Staphylococcus* species isolated from animals with uncertain clinical significance

- *S. caprae* has been isolated from goat's milk
- *S. gallinarum* and *S. arlettae* have been isolated from the skin of chickens
- *S. lentus* has been isolated from the skin of sheep and goats
- *S equorum* has been isolated from the skin of horses
- *S. simulans* has been isolated from clinical specimens in cats.

Laboratory Diagnosis

Specimens

Clinical material from sites of infection is suitable and may include exudates, pus from abscesses, mastitic milk, skin scrapings, urine and affected tissues. No special precautions are required to preserve the viability of the bacterium since staphylococci are relatively resistant to drying and temperature changes.

Direct microscopy

Preparation and examination of Gram-stained smears of pus or exudates is often rewarding and may show the Gram-positive cocci of staphylococci in the typical 'bunches of grapes' formation.

Isolation

The usual medium for inoculation of specimens is blood agar (preferably sheep blood). For most staphylococcal species, the inoculated plates are incubated aerobically at 35–37°C for 24 to 48 hours. On blood plates, abundant growth of staphylococci occurs usually within 18 to 24 hours. A MacConkey agar plate is inoculated in parallel to detect any Gram-negative bacteria that may also be present in the specimens.

Specimens from contaminated sources could also be streaked onto a selective medium, which will inhibit the growth of Gram-negative microorganisms but allow staphylococci and some other Gram-positive cocci to grow, such as mannitol-salt agar (MSA, Fig. 7.4), Columbia colistin-nalidixic acid (CNA) agar, phenylethyl alcohol (PEA) agar and Baird-Parker medium (Fig. 7.5). On selective media, incubation may have to be extended. A medium selective for Gram-positive bacteria is useful particularly if *Proteus* species (indicative of faecal contamination) is also present in the specimens (Figs. 7.6 and 7.7).

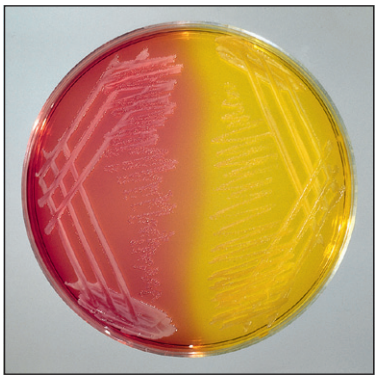

Figure 7.4 *S. aureus* (left) and *S. epidermidis* (right) on mannitol salt agar. Most pathogenic bacteria, other than the staphylococci, are inhibited by the 7.5% NaCl. Coagulase-positive strains usually ferment the mannitol producing a pH change from pink to yellow. (Phenol red indicator).

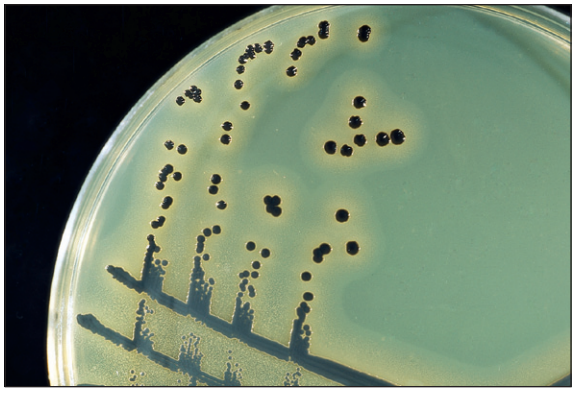

Figure 7.5 Close-up of *S. aureus* colonies on selective Baird-Parker medium. *S. aureus* reduces tellurite to form black shiny colonies. Opaque halos surrounding them are probably due to the action of a lipase. Some strains also produce a smaller, clear zone around the colonies due to proteolytic activity.

Identification

Colonial characteristics

Colonies usually appear in 24 hours. After 48 hours' incubation, well-isolated colonies can reach 4 mm in

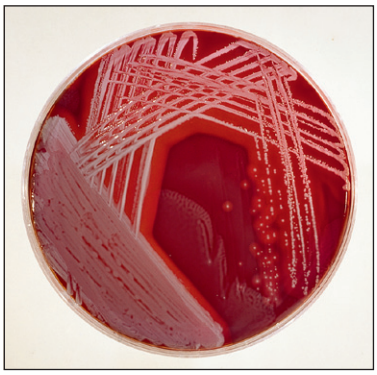

Figure 7.6 *Staphylococcus pseudintermedius* on sheep blood agar overgrown by a swarming *Proteus* sp.

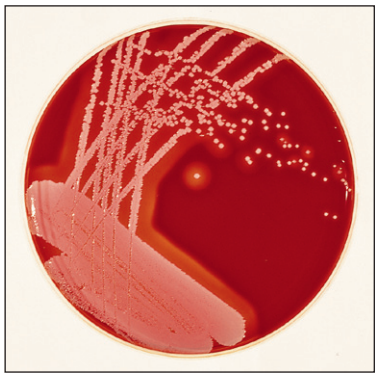

Figure 7.7 *S. pseudintermedius* from the same sample as Figure 7.6, giving an almost pure culture, but plated on selective sheep blood agar.

Table 7.5. The staphylococcal haemolysins

Haemolysin	Type of haemolysis	Erythrocytes		Characteristics
		Affected	Not affected	
Alpha	Complete	Rabbit Sheep Cattle	Horse Human Chicken	Cytotoxic for a large range of tissue culture cells; lethal and necrotizing in rabbits. Acts on membrane lipids and causes spasm of smooth muscle
Beta	Incomplete Becomes complete after further storage at 4–15°C (hot–cold lysis)	Sheep and cattle at 37°C	Rabbit Chicken Horse	It is a phospholipase C, unique to animal strains of staphylococci. May mask the effect of the alpha haemolysin. Potentiation of the partial haemolysis of this haemolysin is the basis of the CAMP test (see Chapter 8)
Delta	Complete	Wide spectrum including human, rabbit, sheep, horse, rat, guinea pig and some fish erythrocytes	–	This haemolysin migrates through agar more slowly than the alpha-haemolysin so the effect takes longer to express. Lethal and necrotizing effects in rabbits. It is a polypeptide and is inhibited by normal serum and phospholipids
Gamma	None	Rabbit Sheep Human Guinea pig	–	Cytopathic for some tisue culture cells and causes oedema in rabbits (intradermal injection). Inhibited by agar, heparin, phospholipids and cholesterol

diameter. They are round, smooth, and glistening and on blood agar tend to appear substantial and opaque (white) compared to the smaller, translucent (grey) colonies of beta-haemolytic streptococci.

1. **Pigmentation:** *Staphylococcus aureus* strains from domestic animals are almost always non-pigmented (white) while human isolates are usually pigmented, ranging from cream yellow to orange. The colonies of *S. pseudintermedius* and *S. hyicus* are also non-pigmented. Some of the coagulase-negative staphylococci produce pigment, especially strains of *S. chromogenes* whose colonies are orange-yellow. Pigment enhancement in the staphylococci is said to be induced by the addition of milk, fat or glycerol monoacetate to the medium.
2. **Haemolysis:** the staphylococcal haemolysins (alpha, beta, delta and gamma) can be produced singly, in combination or not at all. The haemolysins differ antigenically, biochemically and in their effect on the red cells of various animal species. Table 7.5

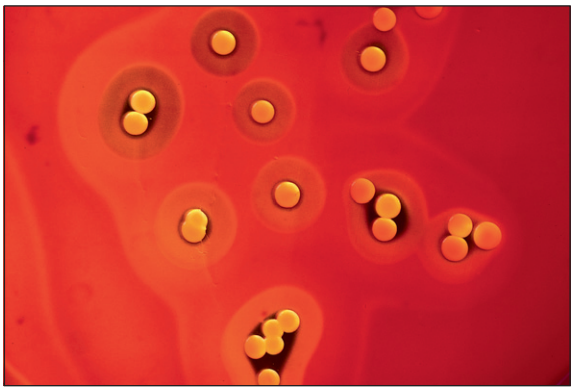

Figure 7.8 A close-up of the characteristic 'target-haemolysis' of a bovine isolate of *S. aureus* on sheep blood agar.

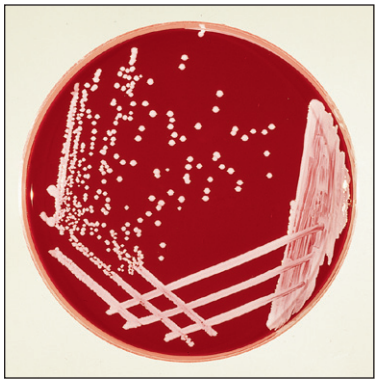

Figure 7.9 *S. hyicus* subsp. hyicus (non-haemolytic) on sheep blood agar.

summarizes the main properties of the haemolysins. Blood agar prepared with either ovine or bovine erythrocytes is preferable in veterinary diagnostic work as the red cells from both these animal species are susceptible to the alpha-haemolysins and beta-haemolysins that are produced commonly by staphylococcal isolates from animals. Both *S. aureus* and *S. pseudintermedius* are usually haemolytic and often produce both the alpha-lysins and beta-lysins and so exhibit 'double-haemolysis' (Fig. 7.8). The alpha-lysin is responsible for the narrow zone of clear haemolysis immediately around the colony and the beta-lysin for the broader zone of incomplete (partial) haemolysis outside the zone caused by the alpha-lysin. *S. hyicus* is non-haemolytic (Fig. 7.9). Haemolytic activity among the coagulase-negative staphylococci is variable and often slow in appearing.

Microscopic appearance

A Gram-stained smear from colonies will reveal Gram-positive cocci randomly distributed over the field (Fig. 7.10).

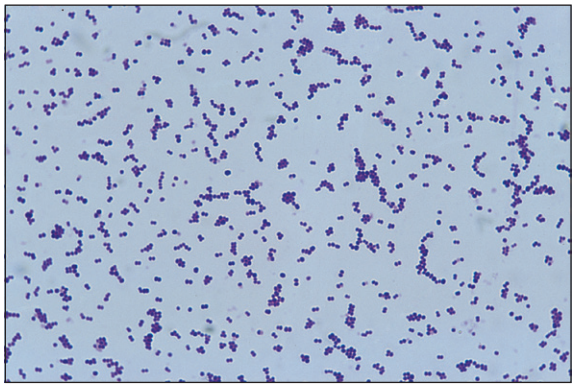

Figure 7.10 *S. pseudintermedius* in a Gram-stained smear from a colony. (×1000)

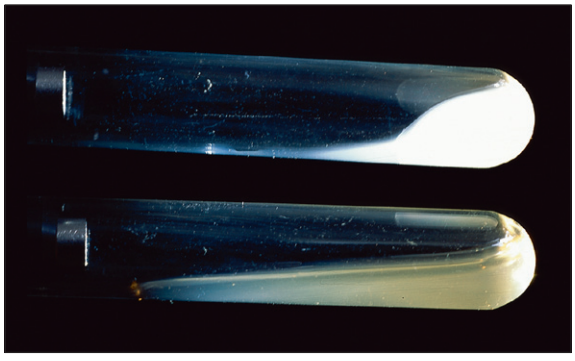

Figure 7.11 Tube coagulase test: positive (top) and negative (bottom).

Coagulase production

The coagulase test usually correlates well with pathogenicity. As some pathogenic staphylococci can be negative to the slide coagulase test but positive to the tube test (e.g. *S. pseudintermedius*), some laboratories perform only the tube coagulase test (Fig. 7.11). Fresh or reconstituted commercial freeze-dried rabbit plasma is used as the reagent. Rabbit plasma contains fibrinogen that is converted to fibrin by the staphylococcal coagulase enzymes. 'Bound' coagulase (clumping factor) is detected by the slide test and 'free' coagulase by the tube test. While the tube test is definitive, the slide test can be used as a rapid screening test for *S. aureus*.

- **Slide coagulase test:** a heavy loopful of the staphylococcal culture is emulsified in a drop of water on a microscope slide. A loopful of rabbit plasma is added and mixed well with the bacterial suspension. The slide is gently rocked and a positive reaction is indicated by clumping within 10 seconds. Commercial latex agglutination tests for clumping factor are available.

Table 7.6 Biochemical reactions and other characteristics of staphylococci isolated from animals

Species	Test									Acid from: (aerobically)				
	Coagulase	DNase	Haemolysis	Alkaline phosphatase	Urease	Beta-galactosidase	Acetoin production	Novobiocin 5 µg disc	Polymyxin B 300 unit disc	Mannitol	Maltose	Mannose	Trehalose	Xylose
S. aureus	+	+	+	+	d	–	+	S	R	+	+	+	+	–
S. pseudintermedius	+	+	+	+	+	+	–	S	S	d	+/–	+	+	–
S. hyicus	d	+	–	+	d	-	–	S	R	–	–	+	+	–
S. epidermidis	–	d	d	d	+	–	+	S	R	–	+	+	–	–
S. saprophyticus	–	–	–	–	+	+	+	R	S	d	+	–	+	–
S. aureus subsp. *anaerobius*	+	+	+	+	nk	–	–	S	nk	nk	+	–	–	–
S. caprae	–	–	d	+	+	–	+	S	S	d	d	+	+	–
S. gallinarum	–	–	d	+	+	d	–	R	S	+	+	+	+	+
S. arlettae	–	–	–	+	–	d	–	R	nk	+	+	+	+	+
S. lentus	–	–	–	+/–	–	–	–	R	S	+	d	+	+	(±)
S. equorum	–	–	d	+	+	d	–	R	nk	+	+	d	+	+
S. simulans	–	–	d	d	+	+	d	S	S	+	+/–	d	d	–
S. delphini	+	–	+	+	+	nk	–	S	nk	+	+	+	–	–
S. chromogenes	–	–	–	+	+	–	–	S	R	d	d	+	+	–
S. felis	–	–	+/–	+	+	+	–			+/–	–	+	+	–
S. schleiferi subsp. coagulans	+	+	+	+	+	d	+	S	nk	d	-	+	_	_

+ = 90% or more of strains positive, – 90% or more of strains negative, d = 11 to 89% of strains positive, () parentheses indicate a delayed reaction, +/– = 90% or more of strains weakly positive, nk not known, R = resistant, S = susceptible

Resistance to novobiocin = zone of 16 mm or less

Resistance to polymyxin B = zone of less than 10 mm

- **Tube coagulase test:** 0.5 ml of rabbit plasma is placed in a small (7 mm) test tube. A suspension (0.1 ml) of an overnight broth culture (Brain Heart Infusion) is added to the rabbit plasma test tube. The tube is rotated gently to mix the contents and then incubated at 35–37°C, preferably in a water bath. Alternatively, one to three large well-isolated colonies can be tranferred into 0.5 mL of rabbit plasma in a tube and incubated as described above. A positive test, with any degree of clotting of the plasma, can occur in two to four hours. However, many weak coagulase-positive strains will coagulate the plasma only after overnight incubation.

Biochemical tests

These are given in Table 7.6. The table also summarizes the reactions of staphylococci to the coagulase and DNase tests (Fig. 7.12) and indicates which species produce haemolysins or pigment. Commercial systems are available for the identification of staphylococci.

Purple agar base (Difco) with the addition of 1% maltose (Fig. 7.13) is a useful medium to distinguish

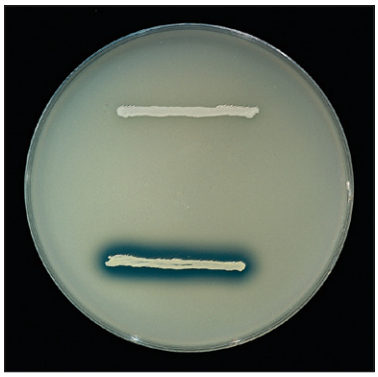

Figure 7.12 DNase agar: *S. epidermidis* (top) negative and *S. pseudintermedius* (bottom) showing DNase activity.

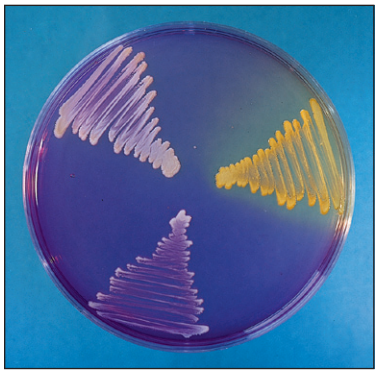

Figure 7.13 Purple agar base containing 1% maltose: *S. pseudintermedius* (left), *S. aureus* (right) and *S. hyicus* (bottom).

between the pathogenic staphylococci, particularly with coagulase-positive isolates from dogs that might be either *S. aureus* or *S. pseudintermedius*. This presumptive identification is based on the fact that *S. aureus* strains rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. pseudintermedius* gives a weak or delayed reaction while *S. hyicus* does not ferment maltose but attacks the peptone in the medium causing the bromocresol purple to indicate an alkaline reaction (a deeper purple) around the colonies. The reactions of the pathogenic staphylococci on this medium are summarized in Table 7.7.

Phage typing

Phage typing is carried out for epidemiological purposes, particularly for *S. aureus* strains from human cases of food poisoning (Figs. 7.14 and 7.15) and occasionally, in reference laboratories, for *S. aureus* isolates from bovine mastitis. Animal staphylococcal phage types are usually different from those of human origin.

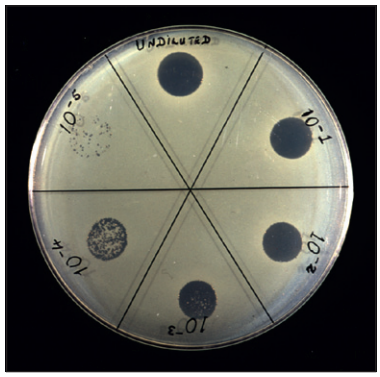

Figure 7.14 Titration of a *S. aureus* bacteriophage to determine the RTD (routine test dilution) for phage typing. The RTD in this case is 10^{-3} (confluent lysis). Dilutions of the phage (20 μL amounts) were added to a lawn of the host bacterium on a nutrient agar plate.

Table 7.7. Reactions of coagulase-positive staphylococci on purple agar with 1% maltose

Species	Maltose fermentation	Reactions on purple agar with 1% maltose
Staphylococcus aureus	+++	Diffuse yellow colour around colonies. Rapid reaction within 24 hours' incubation
Staphylococcus pseudintermedius	±	Little change in the medium. Slight yellowish zone under colonies and occasionally isolated colonies may have a yellowish tinge
Staphylococus hyicus	–	Diffuse deep purple (alkaline) zone around the colonies

+++ = rapid fermentation of maltose, ± = weak and slow fermentation, – = negative

Antibiotic Susceptibility Testing and Antimicrobial Resistance

Staphylococci acquire resistance easily. Vaccination is not used routinely although a vaccine against *S. aureus* mastitis

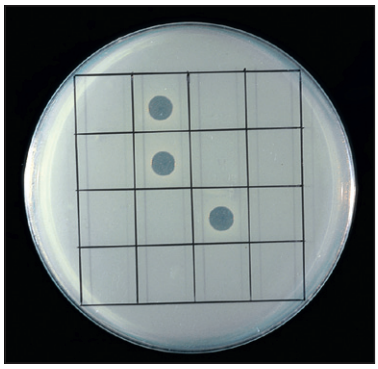

Figure 7.15 Phage typing of a *S. aureus* strain using 16 different staphylococcal phages. *S. aureus*, grown as a lawn on a nutrient agar plate, has been lysed by the second phage across in rows 1 and 2 and by the third phage across in row 3 of the grid.

in cows is now available commercially. It does not prevent infection but will reduce the severity of clinical and subclinical infections.

Susceptibility testing should be conducted on all coagulase-positive isolates and also when a coagulase-negative isolate appears to be significant. Resistance to the beta-lactam antibiotics is frequently due to a plasmid-encoded penicillinase (beta-lactamase). Tolerance is a less common form of penicillin resistance and is thought to be due to the failure of the autolytic cell wall enzymes. Resistance to other antimicrobial agents is also common among the staphylococci.

Meticillin-resistant *Staphylococcus aureus* is an important nosocomial pathogen in humans, and is increasingly recognized in community-acquired infections as well as in veterinary medicine. There are several reports of MRSA infection in horses, dogs and cattle (Leonard & Markey 2008). In addition, MRSA ST 398 is an important zoonotic organism isolated mainly from pigs which was first reported in the Netherlands (Voss et al. 2005). Although this strain of MRSA seldom causes clinical disease in pigs, a high percentage of pigs carry the organism and humans in contact with live pigs frequently become colonized with it. Serious infections in humans have been reported although transmission between humans does not occur as readily as with human nosocomial MRSA strains. A variety of commercially available selective/indicator media are available for the presumptive identification of MRSA. Alternatively, resistance to cefoxitin (30 µg discs) can be used as indicative of MRSA (CLSI 2012).

Staphylococcus aureus resistance to oxacillin/meticillin occurs when an isolate carries an altered penicillin-binding protein, PBP2a, which is encoded by the *mecA* gene. The alteration of the penicillin-binding protein does not allow the drug to bind well to the bacterial cell, resulting in resistance to beta-lactam antimicrobial agents. MRSA can be confirmed by PCR for the *nuc* (specific for *S. aureus*) and *mecA* genes (Poulsen et al. 2003) or by commercially available agglutination kits which detect PBP2a.

Resistance to meticillin in *S. pseudintermedius* (MRSP) has emerged worldwide since approximately 2000 and can be screened for by testing for oxacillin resistance. Bemis et al. (2009) reported that MIC values for oxacillin of ≥0.5 mg/L or a zone diameter of ≤17 mm around a 1 µg oxacillin disc were highly correlated with the detection of *mecA*. Unlike MRSA, cefoxitin resistance testing is not useful in the identification in MRSP. Confirmation of resistance is by detection of the *mecA* gene as for MRSA. MRSP strains are usually resistant to several other classes of antimicrobial agents in addition to the beta-lactam antibiotics, including most agents licensed for use in veterinary medicine (Kadlec & Schwarz 2012). Thus, treatment of MRSP infections poses a major challenge for the veterinary practitioner.

Molecular diagnosis

Staphylococcus species are easily identified using conventional phenotypic approaches, therefore molecular methods for identification of these pathogens are seldom used in veterinary medicine. However, the sensitivity of culture may not be sufficient to detect the microorganism and this could be a disadvantage. For example, in bovine *S. aureus* mastitis detection, intermittent *S. aureus* shedders, intracellular *S. aureus*, or *S. aureus* inhibited by residual therapeutic antimicrobials or leukocytes could remain undetected by culture. Therefore, simplex, multiplex and real-time PCR protocols have been developed for identification of the various mastitis pathogens including *S. aureus* (Riffon et al. 2001; Phuektes et al. 2001, 2003, Gillespie & Oliver 2005). A species-specific multiplex PCR based on detection of the 16S to 23S rRNA spacer region was developed and compared to traditional culture by Phuektes et al. (2001) for four mastitis pathogens, including *S. aureus*. Riffon et al. (2001) have developed molecular probes reacting in PCR with bacterial DNA from bovine milk, providing direct and rapid detection of various mastitis pathogens. The primers for *S. aureus* were designed based on the 23S rRNA sequence.

Definitive identification of *S. pseudintermedius* is most easily performed using molecular methods. Bannoehr et al. (2009) developed a test for differentiation of the *S. intermedius* group (*S. intermedius, S. pseudintermedius* and *S. delphini*) based on PCR amplification of a fragment of the *pta* gene followed by the use of MboI in a RFLP procedure. A multiplex PCR test for differentiation of the coagulase-positive staphylococci was reported by Sasaki et al. (2010). The taxonomy, ecology, diagnostics, epidemiology and pathogenicity of *S. pseudintermedius* are reviewed by Bannoehr and Guardabassi (2012).

Strain typing

A number of different methods are commonly used for the molecular typing of staphylococci. Typing is most frequently carried out for MRSA, reflecting its importance as a major pathogen of humans worldwide. A MLST scheme for international comparison of strains is available (http://saureus.mlst.net/ accessed 2 December 2012). PFGE following digestion with the SmaI enzyme is commonly employed for epidemiological investigations and typing based on the sequence of the gene encoding staphylococcal protein A (*spa* typing) is frequently carried out. For MRSA, PCR-based typing of the staphylococcal chromosomal cassette *mec* (SCC*mec*) region is also used and MRSA clones are assigned to epidemic lineages based on their MLST profile, SCC*mec* type and antimicrobial resistance profile. These typing methods can be employed for MRSP also, although considerably less data are available on these strains compared to MRSA (Bannoehr & Guardabassi 2012).

REFERENCES

Bannoehr, J., Franco, A., Iurescia, M., Battisti, A., et al., 2009. Molecular diagnostic identification of *Staphylococcus pseudintermedius*. Journal of Clinical Microbiology 47, 469–471.

Bannoehr, J., Guardabassi, L., 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Veterinary Dermatology 19 April 2012. doi: 10.1111/j.1365-3164.2012.01046.x. (EPUB ahead of print).

Bemis, D.A., Jones, R.D., Frank, L.A., et al., 2009. Evaluation of susceptibility test breakpoints used to predict mecA-mediated resistance in *Staphylococcus pseudintermedius* isolated from dogs. Journal of Veterinary Diagnostic Investigation 21 (1), 53–58.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance standards for antimicrobial susceptibility testing, 22nd Informational Supplement. CLSI document M100-MS22. Clinical and Laboratory Standards Institute, Wayne, PA.

Devriese, L.A., Hermans, K., Baele, M., et al., 2009. *Staphylococcus pseudintermedius* versus *Staphylococcus intermedius*. Veterinary Microbiology 133 (1–2), 206–207.

Faller, A., Schleifer, K.H., 1981. Modified oxidase and benzidine test for separation of staphylococci from micrococci. Journal of Clinical Microbiology 13, 1031–1035.

Gillespie, B.E., Oliver, S.P., 2005. Simultaneous detection of mastitis pathogens, *Staphylococcus aureus, Streptococcus uberis* and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. Journal of Dairy Science 88, 3510–3518.

Kadlec, K., Schwarz, S., 2012. Antimicrobial resistance of *Staphylococcus pseudintermedius*. Veterinary Dermatology 11 June 2012, doi: 10.1111/j.1365-3164.2012.01056.x. (EPUB ahead of print).

Leonard, F.C., Markey, B.K., 2008. Methicillin-resistant *Staphylococcus aureus* in animals: a review. Veterinary Journal 175 (1), 27–36.

Phuektes, P., Browning, G.F., Anderson, G., et al., 2003. Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* in bulk milk samples. Journal of Dairy Science 70, 149–155.

Phuektes, P., Mansell, P.D., Browning, G.F., 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. Journal of Dairy Science 84, 1140–1148.

Poulsen, A.B., Skov, R., Pallesen, L.V., 2003. Detection of methicillin resistance in coagulase-negative staphylococci and in staphylococci directly from simulated blood cultures using the EVIGENE MRSA Detection Kit. Journal of Antimicrobial Chemotherapy 51, 419–421.

Riffon, R., Sayasith, K., Khalil, H., et al., 2001. Development of rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. Journal of Clinical Microbiology 39, 2584–2589.

Sasaki, T., Tsubakishita, S., Tanaka, Y., et al., 2010. Multiplex-PCR method for species identification of coagulase-positive staphylococci. Journal of Clinical Microbiology 48, 765–769.

Voss, A., Loeffen, F., Bakker, J., 2005. Methicillin-resistant *staphylococcus aureus* in pig farming. Emerging Infectious Diseases 11, 1965–1966.

FURTHER READING

De la Fuente, R., Suarez, G., Schleifer, K.H., 1985. *Staphylococcus aureus* subsp. *anaerobius* subsp. nov., the causal agent of abscess disease of sheep. International Journal of Systematic Bacteriology 35, 99–102.

Hàjek, V., 1976. *Staphylococcus intermedius*, a new species isolated from animals. International Journal of Systematic Bacteriology 26, 401–408.

Hajek, V., Devriese, L.A., Mordarski, M., et al., 1986. Elevation of *Staphylococcus hyicus* subsp. *chromogenes* (Devriese et al. 1978) to species statues: Staphylococcus chromogenes (Devriese et al. 1978) comb. nov. Systematic and Applied. Microbiology 8, 169–173.

Higgins, R., Gottschalk, M., 1991. Isolation of *Staphylococcus felis* from case of external otitis in cats. Canadian Veterinary Journal 32, 312.

Igimi, S., Kawamura, S., Takahashi, E., et al., 1989. *Staphylococcus felis:* a new species from clinical specimens from cats. International Journal of Systematic Bacteriology 39, 373–377.

Kloos, W.E., T.L. Bannerman, 1999. *Staphylococcus* and *Micrococcus*. In: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H. (Eds.), Manual of Clinical Microbiology, seventh ed. ASM Press, Washington, DC, pp. 264–282.

Kloos, W.E., Ballard, D.N., George, C.G., et al., 1998. Delimiting the genus *Staphylcoccus* through description of *Macrococcus caseolyticus* gen. nov., comb. nov. and *Macrococcus equipercicus* sp. nov. and *Macrococcus bovicus* sp. nov. and *Macrococcus carouselicus* sp. nov. International Journal of Systematic Bacteriology 48, 859–877.

Terauchi, R., Sato, H., Hasegawa, T., et al., 2003. Isolation of exfoliative toxin from *Staphylococcus intermedius* and its local toxicity in dogs. Veterinary Microbiology 94, 19–29.

Varaldo, P.E., Kilpper-Bålz, R., Biavasco, F., et al., 1988. *Staphylococcus delphini* sp. nov.: a coagulase-positive species isolated from dolphins. International Journal of Systematic Bacteriology 38, 436–439.

Waage, S., Mork, T., Roros, A., et al., 1999. Bacteria associated with clinical mastitis in dairy heifers. Journal of Dairy Science 82, 712–719.

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Chapter 8

The streptococci and related cocci

Genus Characteristics

The streptococci and enterococci are Gram-positive cocci that occur singly, in pairs, or in chains of varying lengths. Each coccus is less than 2 μm in diameter and can appear as spherical or ovoid. They are facultative anaerobes, catalase-negative, oxidase-negative and non-motile with the exception of some of the enterococci. The streptococci are fastidious and require the addition of blood or serum to media for growth. The enterococci tolerate the bile salts in MacConkey agar and appear as small pin-point colonies on this medium. Strictly anaerobic streptococci of veterinary importance, previously classified in the genus *Peptostreptococcus*, have been reclassified in the genus *Peptoniphilus*.

Natural Habitat

Streptococci are worldwide in distribution. Most of the streptococci of veterinary interest live as commensals on the mucosa of the upper respiratory and lower urogenital tracts. They are susceptible to desiccation and do not usually survive for long away from the animal host. The enterococci are opportunists and can be found in the intestinal tract of many animals.

General Differentiation of the Streptococci

- Haemolysis: the type of haemolysis produced by a streptococcal species can be variable. The main types of haemolysis are:
  - (β) Beta-haemolysis: a clear zone of haemolysis around the colony (Fig. 8.1)
  - (α) Alpha-haemolysis: a zone of greening or of partial haemolysis (Fig. 8.2).
  - (γ) Gamma-haemolysis: no haemolysis.

Generally the beta-haemolytic streptococci tend to be the most pathogenic for animals. Lancefield Group C streptococci from horses usually produce large zones of clear, beta-haemolysis (Fig. 8.3).

- *Lancefield Groups*: The serological Lancefield grouping scheme is based on group-specific carbohydrate cell wall antigens, with groups from A to H and K to V. Some isolates are not groupable. The methods for Lancefield grouping include:
  1. Conventional method: the C-substance (antigen) is extracted either by autoclaving or by acid extraction (hydrochloric acid). A ring precipitation test is conducted by layering the extracted antigen over known antisera that can be obtained commercially for some Lancefield Groups.
  2. Latex agglutination test: This method is the most commonly used and kits are available commercially for identifying Lancefield Groups A, B, C, D, F and G (Fig. 8.4).

Pathogenesis and Pathogenicity

Streptococci are pyogenic bacteria that are commonly associated with suppuration and abscess formation while enterococci are opportunistic pathogens. The polysaccharide capsules of *S. pyogenes*, *S. pneumoniae* (Fig. 8.5) and some strains of *S. equi* subsp. *equi* and *S. agalactiae* are anti-phagocytic. The principal diseases and associated virulence factors of the streptococci and enterococci of veterinary importance are given in Tables 8.1 and 8.2.

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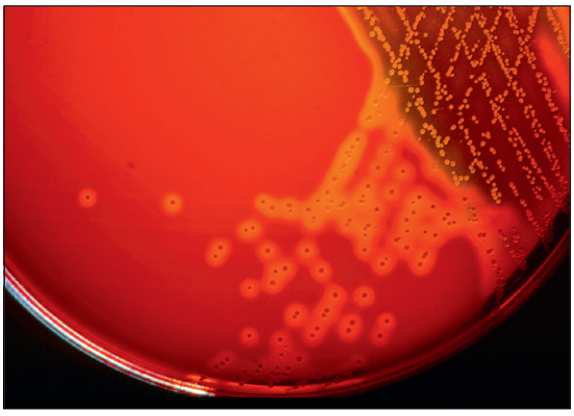

Figure 8.1 Beta-haemolytic *Streptococcus* on sheep blood agar.

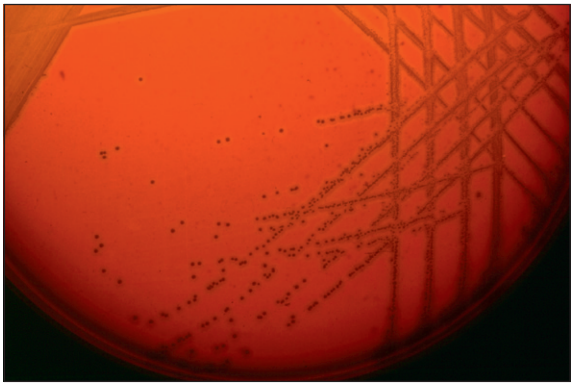

Figure 8.2 Alpha-haemolytic *Streptococcus* on sheep blood agar.

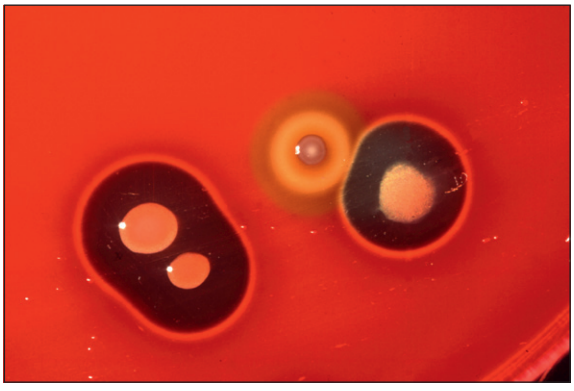

Figure 8.3 Close-up of colonies of *Streptococcus equi* subsp. *equi* (beta-haemolytic) after four days' incubation compared with the greening-type haemolysis of an alpha-haemolytic *Streptococcus* (contaminant).

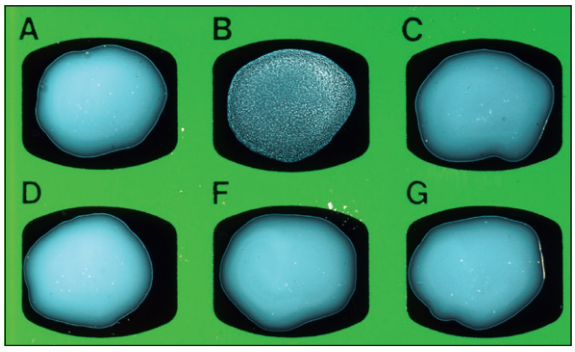

Figure 8.4 Latex agglutination test for Lancefield grouping of streptococci. In this instance the *Streptococcus* under test belonged to Lancefield Group B.

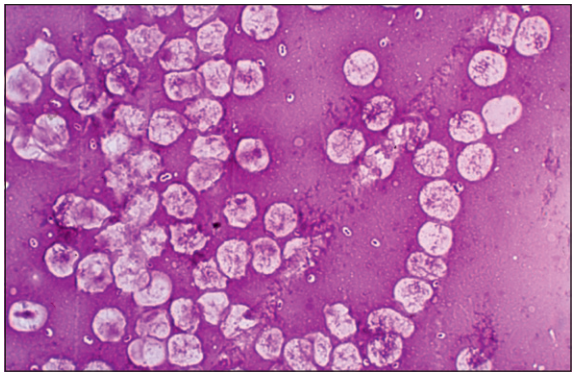

Figure 8.5 *Streptococcus pneumoniae* in a blood smear showing the distinctive capsule. (Nigrosin stain, ×1000)

Streptococcus pyogenes is a rare agent of mastitis in cattle and lymphangitis in foals but is an important human pathogen causing serious respiratory and soft tissue infections. These include necrotizing fasciitis and toxic shock-like syndrome. *Streptococcus agalactiae* is an obligate parasite of the mammary gland of cattle and a well-known agent of chronic contagious mastitis. It enters through the teat orifice and adheres to the mammary epithelium, following which colonization of the lactiferous ducts occurs. Inflammation and fibrosis of the gland leads to fibrin plug formation in ducts, involution of glandular tissue and agalactia. Infection may become chronic if not treated with antimicrobial agents. This microorganism may also cause occasional cases of neonatal septicaemia, kidney and uterine infections in dogs and cats. *Streptococcus dysgalactiae* subsp. *dysgalactiae* is an opportunistic pathogen and causes sporadic cases of acute or subclinical mastitis. Insect bites or other injury to the epithelium of the teat or the gland are reported to facilitate infection. This streptococcal organism has also been associated with cases of suppurative polyarthritis in lambs, bacteraemia in dogs and disease in fish. *Streptococcus uberis* is a commensal

Table 8.1 Streptococci of veterinary importance, the diseases they produce and their principal virulence factors

Species	Lancefield group	Haemolysis	Host	Diseases	Natural habitat	Virulence factors
S. pyogenes	A	β	Human	Scarlet fever, septic sore throat, puerperal fever, erysipelas, abscesses and rheumatic fever	Human upper respiratory tract	M protein, pyrogenic exotoxins (superantigens), hyaluronic acid capsule, haemolysins, and other factors
			Cattle	Mastitis (rare)		
			Foal	Lymphangitis		
S. agalactiae	B	β (α, γ)	Cattle, goat and sheep	Chronic mastitis	Milk ducts	Hyaluronidase, CAMP factor, proteases (EspA), lipoteichoic acid, beta-haemolysin, collagenase, capsular polysaccharide, C5a peptidase, proteins (C, R and X), lactoferrin binding-protein, group B streptococcal protective surface antigen (BPS) and other factors
			Human and dog	Neonatal septicaemia	Maternal vagina	
			Cat	Kidney and uterine infections		
S. dysgalactiae subsp. *dysgalactiae*	C	α (β, γ)	Cattle	Acute mastitis	Buccal cavity and genitalia	Hyaluronidase, streptodornase, streptokinase, fibronectin-binding protein (fnbA, fnbB), plasminogen receptor, protein (G, M-like), L2 macroglobulin receptor, streptolysin S and O (for subspecies *equisimilis*) and other factors
			Lamb	Polyarthritis		
S. dysgalactiae subsp. *equisimilis*	C (A, G or L)	β	Horse	Abscesses, endometritis, abortion and mastitis	Skin and vagina	
			Pig, cattle, dog and bird	Various suppurative conditions	Tonsils	
S. equi subsp. *equi*	C	β	Horse	Strangles, genital and suppurative conditions, mastitis and purpura haemorrhagica	Equine tonsils	Capsular polysaccharide (hyaluronic acid) , streptokinase, streptolysin S, peptidoglycan, fibronectin-binding protein, proteases, adhesins, SzPSe protein, antiphagocytic SeM, equibactin (*S. equi),* exotoxins (SePE-H, SePE-I) and other factors

Continued

Table 8.1 Streptococci of veterinary importance, the diseases they produce and their principal virulence factors—cont'd

Species	Lancefield group	Haemolysis	Host	Diseases	Natural habitat	Virulence factors
S. equi subsp. *zooepidemicus*	C	β	Horse	Joint infection, mastitis, abortion, secondary pneumonia and navel infections	Skin and vagina	Capsular polysaccharide (hyaluronic acid), hyaluronidase, streptokinase, streptolysin S, peptidoglycan, fibronectin-binding protein, IgG binding proteins, proteases, SzP protein and other factors
			Cattle	Metritis and mastitis		
			Pig	Septicaemia and arthritis in 1–3-week-old piglets	Skin and mucous membranes of sows	
			Poultry	Septicaemia and vegetative endocarditis		
			Lamb	Pericarditis and pneumonia		
Enterococcus faecalis, E. faecium, E. durans	D	α (β, γ)	Many species	Opportunistic infections such as septicaemia in chickens, bovine mastitis, endocarditis in cattle and lambs, and urinary tract infections in dogs	Intestinal tract of many animals	Gelatinase, haemolysin, enterococcal surface protein (Esp), aggregation substance (AS), MSCRAMM Ace, serine protease, capsule, cell wall polysaccharide, superoxide and other factors
Streptococcus gallolyticus	D	α	Pigeons Goslings Chickens Human	Septicaemia, endocarditis, arthritis, osteomyelitis and opportunistic infections	Intestinal tract	Capsule, fimbriae, biofilm formation and other factors
S. porcinus	E (P, U or V)	β	Pig	Jowl abscesses and lymphadenitis	Mucous membranes	Streptokinase, protein (M) and other factors
S. canis	G	β	Dogs and cats	Neonatal septicaemia, genital, skin and wound infections, otitis, mastitis, prostatis, abortion, conjunctivitis, lymphadenitis	Genital tract and anal mucosa	Streptolysin O, protein (M) and other factors
			Cattle	Occasional mastitis		

Table 8.1 Streptococci of veterinary importance, the diseases they produce and their principal virulence factors—cont'd

Species	Lancefield group	Haemolysis	Host	Diseases	Natural habitat	Virulence factors
S. suis	D	α	Pig	Meningitis, arthritis, pneumonia, endocarditis and septicaemia	Tonsils, nasal cavity, genital and digestive tracts of pigs	Polysaccharide capsule, serum opacity-like factor, muramidase-released protein (MRP), extracellular factor (EF), suilysin (SLY), modification of peptidoglycan by N-deacetylation, D-alanylation of lipoteichoic acid, fibronectin/fibrinogen-binding protein, IgA1 protease, and other factors Pathogenicity islands 89K (human epidemic clone in China)
			Human	Meningitis, permanent hearing loss, septic shock (toxic shock syndrome) and even death		
S. uberis	Not assigned	α (γ)	Cattle	Mastitis	Skin, vagina and tonsils	Hyaluronic acid capsule, plasminogen activator proteins (PauA, PauB, SK), lactoferrin binding proteins, SUAM, CAMP factor, a surface dehydrogenase protein GapC, Opp proteins, and other factors
S. pneumoniae	Not assigned	α	Human and primate	Pneumonia, septicaemia, and meningitis	Upper respiratory tract	Capsule, teichoic acid, peptidoglycan, neuraminidases, IgA protease, adhesins, fibronectin binding proteins and other factors
			Horses	Pneumonia		
			Guinea pigs and rat colonies	Pneumonia (outbreaks can occur)		

organism, found in the tonsils, gastrointestinal and genital tracts of the cow and can cause mastitis via an ascending infection of the teat canal. It is also found in the environment and is associated with subclinical and clinical intramammary infections of both housed animals and those at pasture.

Streptococcus equi subsp. *equi* is the agent of equine strangles, a contagious upper respiratory tract disease with abscessation of the local lymph nodes. Clinical signs include fever, nasal discharge and swelling of the mandibular and retropharyngeal lymph nodes and intermandibular region. Dissemination of the organism may occur with abscesses forming in the lungs or other locations. The source of the infection is usually pus from abscesses or nasal discharges from infected horses. However, feed and/or water can also be contaminated.

Table 8.2 General functions of the main virulence determinants of streptococci and enterococci

Virulence determinant	Functions
Beta-haemolysin	Cytotoxic for bacterial and eukaryotic cells by pore formation in the target cell membrane
CAMP factor (ceramide-binding protein) of *S. agalactiae*	Enhances the haemolysis of staphylococcal sphingomyelinase beta-toxin, cytotoxic and lethal for cell cultures, possibly cytotoxic for mammary tissues
Capsular polysaccharide (polysaccharide type-specific antigen, hyaluronic acid)	Antiphagocytic, type-specific antibodies can be protective
Collagenase	Extracellular enzyme which disrupts collagen and likely contributes to endothelial cell damage, tissue destruction, and haemodynamic derangement
Streptococcal C5a peptidase (SCPA)	Highly specific endopeptidase which acts primarily to eliminate C5a chemotactic signal from inflammatory foci
Exotoxins (such as SePE-H, SePE-I, SePE-M, SePE-G)	Pyrogenic exotoxins, mitogens (superantigens): non-specific T cell stimulation and cytokine release
Hyaluronidase	Promotes tissue dissemination, increased activity in *S. equi* subsp. *zooepidemicus* compared to *S. equi* subsp. *equi* may explain the more frequent dissemination of the former throughout the body
Equibactin of *S. equi* subsp. *equi*	Siderophore, iron acquisition
IgA protease	Cleaves immunoglobulin A in its hinge region, evades host responses
IgG binding protein (Protein G)	Evades host responses, likely to reduce phagocytosis
Leukocidal toxin of *S. equi* subsp. *equi*	Damages polymorphonuclear leukocytes, imparing phagocytosis and killing
L2 macroglobulin receptor	Binds L2 macroglobulin
Fibronectin-binding protein (such as fnbA and fnbB)	Evades host responses, likely to reduce phagocytosis
Muramidase-released protein (MRP) of the cell wall of *S. suis*	Unknown functions but contributes to protection
Extracellular factor (EF) of *S. suis*	Unknown functions but associated with virulence
Peptidoglycan	Potent activator of alternative complement pathway
Plasminogen receptor	Binds plasminogen, may reduce phagocytosis
Proteins C and R of *S. agalactiae*	C: Unknown functions but promotes protective and opsonophagocytic antibodies R: Unknown functions but likely to enhance epithelial colonization
Proteins M-like (SzPSe protein) of *S. equi* subsp. *equi*	Binds equine fibrinogen, may reduce phagocytosis
Proteins (M)	Antiphagocytic
SeM protein of *S. equi* subsp. *equi*	Binds equine fibrinogen, antiphagocytic and protective antigen
Serine protease (EspA) of *S. agalactiae*	Cleaves fibrinogen, and blocks phagocytosis
Streptokinase	Plasminogen activation (active plasmin hydrolyses fibrin which may promote dispersion)
Streptodornase (DNase)	Escape killing in neutrophils
Streptolysin S and O	Beta-haemolysis, a bacteriocin-like cytotoxin

Table 8.2 General functions of the main virulence determinants of streptococci and enterococci—cont'd

Virulence determinant	Functions
Suilysin of *S. suis*	Thiol-activated haemolysin that is either secreted or lightly cell-attached which produces transmembrane pores in target cells
Opp proteins	Involved in the active transport of solutes, essential for growth in milk
SUAM	Mediates adherence to and invasion of epithelial cells
MSCRAMM Ace of enterococci	Microbial surface component recognizing adhesive matrix molecules, adhesion of collagen
Gelatinase	Protease capable of hydrolysing collagen, casein, haemoglobin and other peptides
Haemolysin	Cytolytic protein capable of lysing human, horse and rabbit erythrocytes
Esp	Cell wall associated protein involved in biofilm formation

A small proportion of animals fail to clear the infection from the guttural pouches and these healthy carriers are likely to be responsible for spread of infection between epidemics. Depending on the immunity status, the disease can take an acute or mild course with most horses recovering relatively quickly. *Streptococcus equi* subsp. *zooepidemicus* is a commensal organism with a large host range, which opportunistically causes disease following viral infections or tissue injury. It is a frequent cause of pneumonia and joint infections in horses. There have been several reports of haemorrhagic pneumonia in dogs associated with this organism in recent years (Priestnall & Erles 2011). It is also a zoonotic agent with infection following close contact with horses or contaminated milk products. *Streptococcus dysgalactiae* subsp. *equisimilis* may be a more significant pathogen of horses than previously thought as a recent study showed that it was frequently isolated from cases of infection of the equine genital tract (Erol et al. 2012).

Streptococcus suis is associated with septicaemia, meningitis, polyarthritis and pneumonia in pigs. It is also a zoonotic pathogen capable of causing severe invasive disease in humans exposed to pigs or pork products. Most pigs are healthy carriers with *S. suis* located in their tonsils, nasal cavities, genital or digestive tracts. Disease development is incompletely understood but is believed to be related to the strain and immune status of the host. Some virulence factors appear to be associated with more severe, invasive disease (Beineke et al. 2008, Wei et al. 2009). Stress and intensive management practices are also likely to predispose to disease occurrence. Most cases of disease in both pigs and humans are caused by *S. suis* serotype 2. In both species the main clinical manifestations of *S. suis* are meningitis and septicaemia although some serotypes are commonly associated with pneumonia. *Streptococcus suis* outbreaks in pigs are common while most reports in humans describe sporadic cases (Gottschalk et al. 2007). However, *S. suis* has recently emerged as an important zoonotic agent in some Asian countries, and has been reported as the primary cause of adult meningitis in Vietnam (Mai et al. 2008). *Streptococcus porcinus* causes a variety of pathological conditions in pigs including contagious cervical lymphadenitis, 'porcine strangles', endocarditis, and abortion. It is believed that the organism enters its host through the mucosa of the pharyngeal or tonsillar surfaces. Organisms are then carried to the lymph nodes, primarily of the head and neck region, where abscesses are formed. *Streptococcus porcinus* is also an opportunistic agent in many other hosts. It has rarely been implicated as a human pathogen with only a few cases reported. *Streptococcus dysgalactiae* subsp. *equisimilis* is a commensal of the porcine tonsil and a cause of suppurative athritis in piglets. Acquisition of this microorganism is usually via the tonsils, umbilicus or a skin abrasion.

Streptococcus canis is an opportunistic pathogen found as part of the normal flora of the oropharyngeal, anal and genital mucosa in both dogs and cats. A variety of infections have been reported associated with this organism. Contagious lymphadenitis is possibly the most frequent manifestation in cats. In dogs, sporadic necrotizing fasciitis and toxic shock syndrome have been associated with *S. canis*. This syndrome may be related to fluoroquinolone usage, which was found to induce a lytic bacteriophage encoding a homologue of pokeweed mitogen (Ingrey et al. 2003).

Streptococcus pneumoniae is an important human pathogen which causes invasive disease in adults and children but is rarely isolated from clinical disease in animals. It has been reported to infect and cause pneumonia in horses. Equine isolates of *S. pneumoniae* are reported to

represent a tight clonal group and to be genetically distinct from human isolates (Whatmore et al. 1999).

The agent of streptococcosis in pigeons is *Streptococcus gallolyticus* subsp. *gallolyticus,* which was formerly classified as *S. bovis* biotype I (Devriese et al. 1998). Streptococcosis is an important septicaemic disease in pigeons. Clinical signs include lameness, emaciation, inability to fly, polyuria, production of green and slimy droppings and sudden death. Extensive and well-circumscribed areas of necrosis in the pectoral muscles and arthritis of the knee, hock and shoulder joints are observed at necropsy. *Streptococcus gallolyticus* is an opportunistic pathogen of humans and *S. gallolyticus* subsp. *gallolyticus* is the subspecies most often linked with human endocarditis-associated colonic cancer.

Enterococci are opportunistic pathogens found in the intestinal tracts of animals and humans. There are many species of enterococci but *E. faecalis* and *E. faecium* are the species most frequently isolated from infections in animals. Types of infection include wound infections in all species, mastitis in cattle and infections of the ears and urinary tract in dogs. Enterococci are frequently intrinsically resistant to many classes of antimicrobial agents and vancomycin-resistant enterococci are important nosocomial pathogens in human medicine. Vancomycin resistance in animal isolates of enterococci was associated with avoparcin use but resistance levels have decreased following a ban on the use of this antibiotic in animals since 1997.

Finally, the anaerobic *Streptococcus*, *Peptoniphilus indolicus* has been described in association with *Trueperella pyogenes* in 'summer mastitis' of cattle in Europe. *Anaerococcus tetradius* and *Peptostreptococcus anaerobius* have been associated with abscesses in dogs and cats respectively.

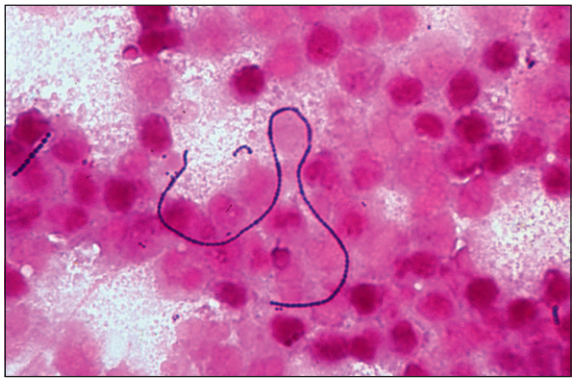

Figure 8.6 *Streptococcus equi* subsp. *equi* in a smear of pus from a case of strangles. The long chain of Gram-positive cocci is characteristic both of this bacterium and of the disease. (Gram stain, ×1000)

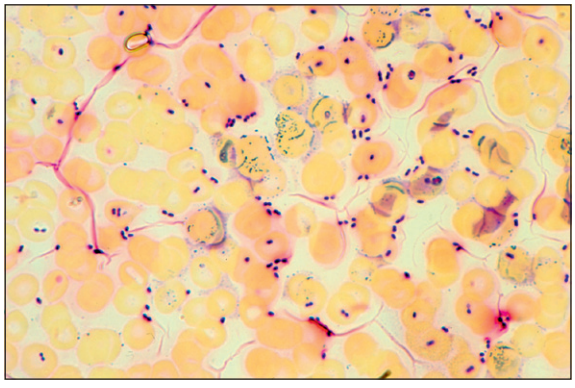

Figure 8.7 *Streptococcus pneumoniae* in a blood smear demonstrating the pairs of cocci characteristic for this bacterium. (Leishman stain, ×1000)

Laboratory Diagnosis

Specimens

Depending on the pathological condition these may include exudates, pus, mastitic milk, skin scrapings, cerebrospinal fluid, urine and affected tissues. Swabs should be submitted in transport medium as streptococci are very susceptible to desiccation.

Direct microscopy

Smears from pus, exudates or centrifuged deposits of milk or urine can be fixed and stained by the Gram method (Fig. 8.6). *Streptococcus pneumoniae* (the *Pneumococcus*) occurs as pairs of cocci (Fig. 8.7). Fluorescent antibody tests have been employed to identify streptococci, such as *S. suis* type 2, in tissues (Robertson & Blackmore 1987).

Isolation

The streptococci are fastidious and require the addition of blood or serum to media for growth. The growth of many streptococci is stimulated by a CO_2-enriched atmosphere. Glucose and other sugars are fermented but gas is not produced. Routine media include sheep or ox blood agar, selective blood agar (as described for the staphylococci) and MacConkey agar.

Mastitic milk samples can be inoculated on blood agar, Edwards medium (Oxoid) and MacConkey agar. Herd mastitic milk samples can be quarter-plated on blood agar containing 0.1 or 0.05% aesculin to indicate aesculin hydrolysis.

Inoculated plates are incubated aerobically at 37°C for 24 to 48 hours.

Identification

Colonial appearance

Most streptococci produce small colonies (about 1 mm after 48 hours' incubation) and in the case of the

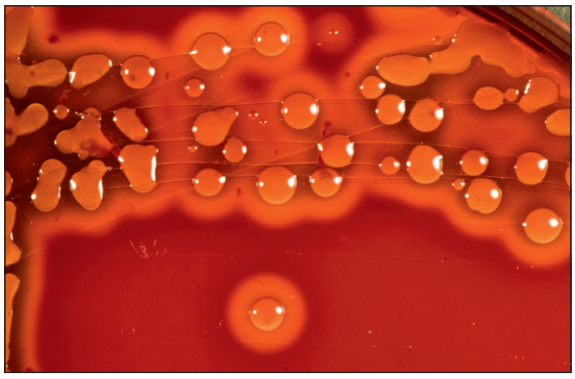

Figure 8.8 *Streptococcus equi* subsp. *equi*: primary isolation from bastard strangles in a foal. Note the comparatively large, mucoid colonies and wide zones of haemolysis.

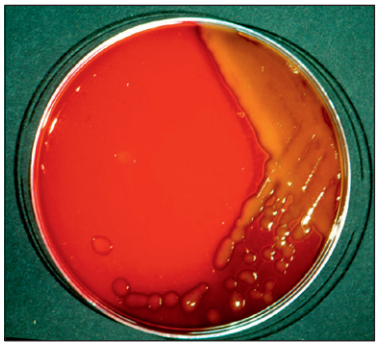

Figure 8.9 Mucoid-type colonies of *S. pneumoniae* on sheep blood agar showing the colonial form and alpha-haemolysis.

beta-haemolytic streptococci the colonies appear translucent. Colonial variation can occur and mucoid strains of *S. equi* subsp. *equi* (Fig. 8.8), *Streptococcus equi* subsp. *zooepidemicus* and *S. pneumoniae* (Fig. 8.9) are not uncommon. Although variation in the type of haemolysis within a species occurs, haemolytic activity is a useful diagnostic characteristic. The clear zones of the Group C streptococci from horses are often large. *S. pneumoniae* is alpha-haemolytic and produces either mucoid colonies or flat colonies with smooth borders and a central concavity ('draughtsman' colonies) after 48 to 72 hours on blood agar (Figs 8.10 and 8.11).

- **Gram-stained smears:** when made from the colonies Gram-positive cocci are seen. The characteristic chains only occur in broth cultures or in animal tissues.
- **Catalase test**: the streptococci are catalase-negative which helps to distinguish them from the catalase-positive staphylococci.
- **Lancefield grouping:** this can be carried out by the methods previously described.

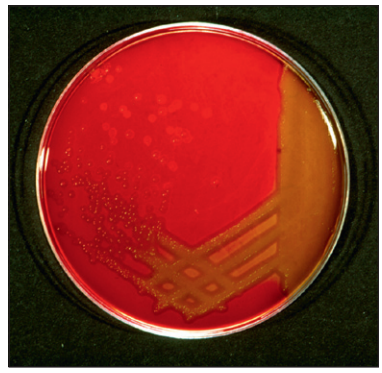

Figure 8.10 Non-mucoid colonies of *S. pneumoniae* on sheep blood agar. They are alpha-haemolytic.

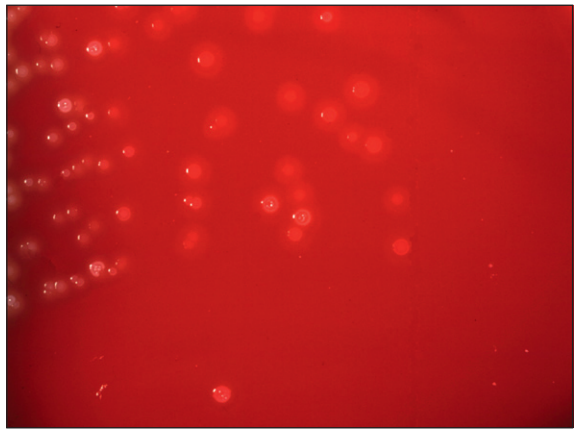

Figure 8.11 A close-up of the 'draughtsman' colonies characteristic of *S. pneumoniae*.

Biochemical tests

The methods for conducting biochemical tests include:

1. Identification using commercial kits such as the rapid ID 32 STREP system (bioMérieux).
2. Conventional peptone water sugars with 45 drops of serum added to 2 mL of medium can be used for fastidious streptococci. An alternative is cystine tripticase agar (CTA) medium with the appropriate carbohydrate discs. Biochemical reactions for the streptococci of veterinary significance are shown in Table 8.3. A short range of sugar fermentation tests may be carried out if a Group C *Streptococcus* is isolated from a horse (Fig. 8.12) although commercial test kits are now more usually employed. The biochemical differentiation of Group C streptococci from horses is given in Table 8.4.

If the bacterium appears to be *S. equi* subsp *equi*, capable of causing the highly contagious disease strangles, the result should be confirmed by a more extensive range of

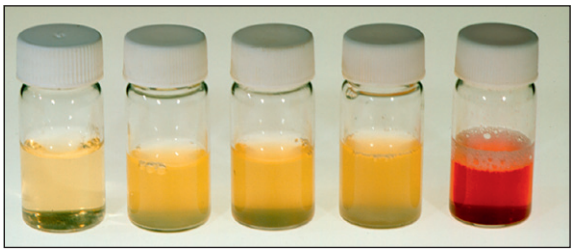

Figure 8.12 *Streptococcus equi* subsp. *equi* in a short range of peptone water sugars (with the addition of a few drops of sterile serum in each). Phenol red pH indicator. From left, uninoculated, trehalose (–), sorbitol (–), lactose (–) and maltose (+).

biochemical tests or by PCR-based tests. The enterococci and Group D streptococci are distinguished by growth at 45°C and toleration of 40% bile. They all hydrolyse aesculin. A short range of tests can differentiate members of this group (Table 8.5).

Presumptive identification tests for streptococci are summarized in Table 8.6:

1. **CAMP test:** a culture of a *Staphylococcus aureus*, with a wide zone of partial haemolysis (beta-haemolysin), is streaked across the centre of a sheep or ox blood agar plate. A streak of the suspect Group B *Streptococcus* is made at right angles to, and taken to within 1 to 1.5 mm of the staphylococcal streak. The plate is incubated at 37°C for l8 to 24 hours. A positive CAMP test is indicated by an arrowhead of

Table 8.3 Biochemical reactions of important streptococci from animals

		Acid from:									
	Lancefield group	Inulin	Lactose	Mannitol	Raffinose	Salicin	Sorbitol	Trehalose	Aesculin hydrolysis	Sodium hippurate	Growth in 6.5% NaCl
S. pyogenes	A	–	+	v	–	+	–	+	–	–	–
S. agalactiae	B	–	+	–	–	(+)	–	+	–	+	–
S. dysgalactiae subsp. *dysgalactiae*	C	–	+	–	–	–	–	+	–	–	–
S. dysgalactiae subsp. *equisimilis*	C (A, G or L)	–	v	–	–	(+)	–	+	–	–	–
S. equi subsp. *equi*	C	–	–	–	–	+	–	–	–	–	–
S. equi subsp. *zooepidemicus*	C	–	+	–	–	+	+	–	–	–	–
Enterococcus faecalis	D	–	+	+	–	+	+	+	+	v	+
Streptococcus equinus	D	d	d	–	d	NA	NA	d	+	–	–
Streptococcus gallolyticus subsp. gallolyticus	D	+	+	+	+	NA	–	+	+	–	–
S. porcinus	E (P,U or V)	–	(+)	+	–	+	+	+	+	–	+
S. canis	G	–	(+)	–	–	NA	–	(+)	v	–	–
Enterococcus avium	Q	–	+	+	–	+	+	+	+	v	(+)
Streptococcus suis	D	(+)	+	–	v	+	–	+	v	–	–
S. uberis	Not assigned	+	+	+	–	+	+	+	+	+	(+)
S. pneumoniae	Not assigned	+	+	–	+	v	–	+	(+)	–	–

(+) = majority of strains positive, v = variable reactions, d = strains previously designated as *S. bovis* positive, those designated as *S. equinus* negative, NA = information not available.

Table 8.4 Differentiation of equine Group C streptococci

	Trehalose	Sorbitol	Lactose	Maltose
S. dysgalactiae subsp. *equisimilis*	+	–	v	+
S. equi subsp. *equi*	–	–	–	+
S. equi subsp. *zooepidemicus*	–	+	+	+(–)

v = variable reactions, (–) = a few strains are negative

Table 8.5 Differentiation of the enterococci and group D streptococci

	Lancefield group	Lactose	Arabinose	Sorbitol	Mannitol	Growth in 6.5% NaCl
E. faecalis	D	+	–	+	+	+
E. faecium	D	+	+	–	+	+
E. durans	D	+	–	–	–	+
E. avium	Q	+	+	+	+	+
S. equinus	D	v	v	–	v	–

v = variable reactions

Table 8.6 Presumptive identification tests for the streptococci

	Bacitracin (0.04 units) susceptibility	CAMP test	Sodium hippurate hydrolysis	Optochin susceptibility	Bile susceptibility
Group A streptococci	+	–	–	–	–
Group B streptococci	–	+	+	–	–
S. pneumoniae	–	–	–	+	+

complete haemolysis (Fig. 8.13). The Group B streptococci produce a diffusible metabolite that completes the lysis of the red cells, only partially haemolysed by the beta-haemolysin of the *Staphylococcus*.

2. **Hydrolysis of sodium hippurate:** this is another test that distinguishes the Group B streptococci from the other streptococci (Fig. 2.52).
3. **Production of a carotenoid pigment by Group B streptococci under anaerobic incubation:** media such as GBS agar (Oxoid) have been designed to exploit the ability of about 97% of Group B streptococci to produce an orange to red pigment when incubated anaerobically (Fig. 8.14).
4. **Susceptibility to a 0.04 unit disc of bacitracin:** distinguishes the Group A streptococci from the other beta-haemolytic streptococci. The discs are available commercially and the test is carried out by the disc diffusion method (Fig. 8.15).

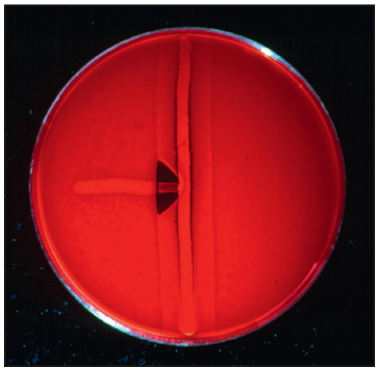

Figure 8.13 CAMP test for Group B streptococci. *Streptococcus agalactiae* is causing the characteristic 'arrowhead' clearing of the partial haemolysis (beta-haemolysin) of *Staphylococcus aureus* (vertical streak).

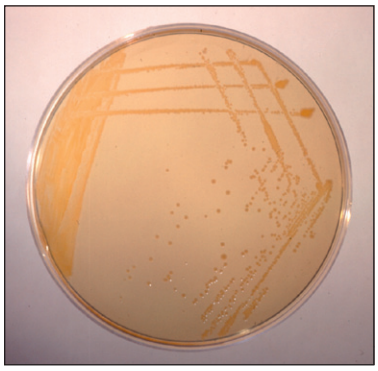

Figure 8.14 Group B streptococci showing the carotenoid pigment on GBS agar under anaerobic conditions.

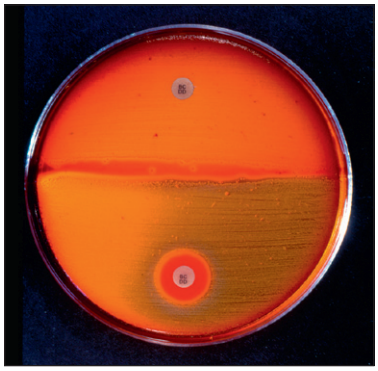

Figure 8.15 Bacitracin (0.04 units) susceptibility test to distinguish Group A streptococci from other beta-haemolytic streptococci. Group B (above) resistant and Group A (below) susceptible.

5. **Susceptibility to optochin:** *Streptococcus pneumoniae* can be distinguished from the other alpha-haemolytic streptococci by its susceptibility to low levels of optochin (ethylhydrocuprein HCl). The test is performed as a disc diffusion test using commercially available discs. The zone of inhibition when using a 6 mm disc should be equal to or greater than 14 mm (Fig. 8.16).
6. **Bile solubility:** the test is conducted by adding a suspension of the suspect *S. pneumoniae* in physiological saline or in a broth culture to an equal amount of 10% solution of sodium taurocholate. Within 10 to 15 minutes a *Pneumococcus* will autolyse and the suspension will become clear.

Identification of the streptococci causing bovine mastitis

The identification of these streptococci is summarized in Table 8.7. Edwards medium is highly selective for streptococci. As it contains both red blood cells and aesculin, haemolysis and aesculin hydrolysis can be observed (Fig. 8.17).

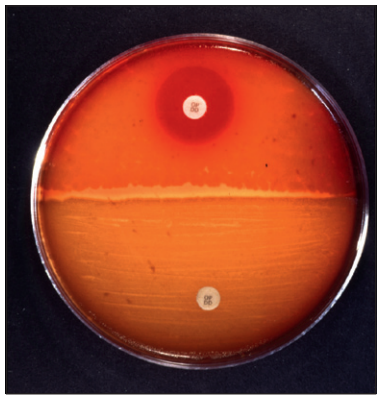

Figure 8.16 Optochin susceptibility test to distinguish *S. pneumoniae* from other alpha-haemolytic streptococci. *Streptococcus pneumoniae* (above) susceptible and *Enterococcus faecalis* (below) resistant.

Table 8.7 Differentiation of streptococci causing bovine mastitis

	CAMP test	Aesculin hydrolysis on Edwards medium	Growth on MacConkey agar
S. agalactiae	+	–	–
S. dysgalactiae	–	–	–
S. uberis	–	+	–
E. faecalis	–	+	+

Antigen preparation for Lancefield grouping by the ring precipitation test

Hot HCl extraction

- A pure culture of the *Streptococcus* is grown in 25 mL of Todd–Hewitt broth at 37°C for 24 to 48 hours.
- Centrifuge the broth to concentrate the cells. Discard the supernatant.
- Add 1 mL of the stock HC1-saline mixture (1 ml conc. HCl + 99 mL of N saline) and resuspend the cells.
- Place in a boiling water bath for 15 minutes and allow to cool.
- Add one drop of phenol red indicator and neutralize with N/10 NaOH until the suspension is a pale pink colour.

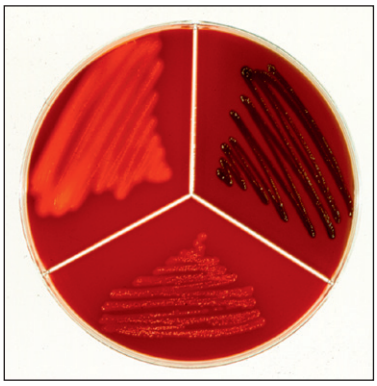

Figure 8.17 Edwards medium is highly selective for the streptococci and also indicates aesculin hydrolysis and the type of haemolysis. *Streptococcus agalactiae* (left) and *S. dysgalactiae* (bottom) are non-aesculin splitters whereas *E. faecalis* (right) shows aesculin hydrolysis with darkening of the colonies and medium.

- Centrifuge and use the supernatant as the antigen for the test.

Autoclave extraction

- A pure culture of the *Streptococcus* is grown in 25 mL of Todd–Hewitt broth at 37°C for 24 to 48 hours.
- Centrifuge the broth to concentrate the cells. Discard the supernatant.
- Add 0.5 mL of 0.85% NaCl solution to the cells and shake to resuspend.
- Autoclave the suspension for 15 minutes at 121°C.
- Cool and centrifuge. Decant the supernatant into a clean tube for use as an antigen in the test.

Ring precipitation test for Lancefield grouping

To economize on commercial antisera use a capillary tube of outside diameter 1.2 to 1.5 mm. Autoclave the capillary tubes and store in a sterile container. The antisera chosen to test against the antigen extract will depend on the animal species and lesion from which the *Streptococcus* was isolated. For example, the majority of the streptococci isolated from horses are Lancefield Group C.

- Dip a sterile capillary tube into the antiserum until a column of about 1 cm long has been drawn into the tube. Plunge the lower end of the capillary tube into plasticine stuck on a microscope slide so that the tube is held upright.
- With a fine pipette carefully layer the antigen solution on top of the antiserum, taking care that there are no air bubbles and that no mixing of the antiserum and antigen occurs.
- Examine the tube in bright light against a dark background. A white ring of precipitate should appear, in a positive reaction, in five to 30 minutes. Precipitate formation after 30 minutes should be disregarded.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Antimicrobial susceptibility testing of streptococci can be carried out using agar disk diffusion, broth microdilution or commercial Sensititre (Thermo Scientific) methods. Resistance patterns vary depending on species and geographical region. Streptococcal mastitis pathogens in Sweden were reported as broadly susceptible to all agents tested other than penicillin G (Persson et al. 2011). Resistance to sulphonamides, lincomycin and ampicillin was recorded in 10–20% of *S. uberis* and *S. dysgalactiae* isolates in New Zealand with even greater levels of resistance, almost 90%, shown by *S. dysgalactiae* against tetracyclines (Petrovski et al. 2011). Equine beta-haemolytic streptococci were susceptible to most agents commonly used in equine practice in a study published by Erol et al. (2012). However, resistance to gentamicin (up to 14% of isolates), sulphonamides (up to 95%) and tetracyclines (up to 30%) was recorded. The penicillins retain high activity against *S. canis* in most reports but resistance to tetracycline (50%), chloramphenicol (44.4%), gentamicin (33.3%) and orbifloxacin (16.7%) in isolates from Korea was found in a recent study by Gebru et al. (2012). Similarly, *S. suis* remains susceptible to beta-lactam antibiotics in most countries, but resistance to these agents is beginning to emerge in China (Zhang et al. 2008).

Strain Typing

A variety of different molecular techniques have been developed to type streptococci of veterinary importance for diagnostic and epidemiological purposes. Multilocus sequence typing schemes are available for *S. equi* subsp. *zooepidemicus* (Webb et al. 2008), *S. uberis* (Coffey et al. 2006) and *S. suis* (King et al. 2002). MLST sequence types of *S. suis* appear to correlate with virulence; with ST1 containing all of the virulence marker genes encoding suilysin, extracellular factor and muramidase-released protein (Fittipaldi et al. 2011). Sequencing of the variable region of the *SeM* gene encoding the M protein of *S. equi* subsp. *equi* (Ivens et al. 2011) is a useful technique for investigating the molecular epidemiology of this pathogen at both regional and national levels. PFGE techniques have also been employed for molecular typing of many animal streptococcal species.

Molecular Diagnosis

Many PCR-based methods have been developed for the detection and identification of streptococci of veterinary

importance including both conventional and 'real-time' techniques. The 16S rRNA gene is frequently employed as a target gene but the use of many different genes has been described. These may include, for example, the *see1* or *SeM* genes for *S. equi* subsp. *equi*, possession of *sodA* and lack of *see1* for *S. equi* subsp. *zooepidemicus* and strepokinase genes for *S. dysgalactiae* subsp. *equisimilis* (Casagrande Proietti *et al.*, 2011). Recently a kit-based assay for detection of *S. equi* subsp. *equi* in equine specimens was developed (Artiushin et al. 2011). This assay is similar to PCR-based techniques but is based on thermophilic helicase-dependent DNA amplification which does not require a thermocycler. Thus the test result may be visualized in a disposable cassette and the kit can be used outside a laboratory setting. Multiplex PCR methods for detection of bovine mastitis pathogens, including the streptococci, have been described in recent years.

REFERENCES

Artiushin, S., Tong, Y., Timoney, J., et al., 2011. Thermophilic helicase-dependent DNA amplification using the IsoAmp™ SE experimental kit for rapid detection of *Streptococcus equi* subspecies *equi* in clinical samples. Journal of Veterinary Diagnostic Investigation 23 (5), 909–914.

Beineke, A., Bennecke, K., Neis, C., et al., 2008. Comparative evaluation of virulence and pathology of *Streptococcus suis* serotypes 2 and 9 in experimentally infected growers. Veterinary Microbiology 128 (3–4), 423–430.

Casagrande Proietti, P., Bietta, A., Coppola, G., et al., 2011. Isolation and characterization of β-haemolytic streptococci from endometritis in mares. Veterinary Microbiology 152 (1–2), 126–130.

Coffey, T.J., Pullinger, G.D., Urwin, R., et al., 2006. First insights into the evolution of *Streptococcus uberis*: a multilocus sequence typing scheme that enables investigation of its population biology. Applied Environmental Microbiology 72 (2), 1420–1428.

Devriese, L.A., Vandamme, P., Pot, B., et al., 1998. Differentiation between *Streptococcus gallolyticus* strains of human clinical and veterinary origins and *Streptococcus bovis* strains from the intestinal tracts of ruminants. Journal of Clinical Microbiology 36 (12), 3520–3523.

Erol, E., Locke, S.J., Donahoe, J.K., et al., 2012. Beta-hemolytic *Streptococcus* spp. from horses: a retrospective study (2000–2010). Journal of Veterinary Diagnostic Investigation 24 (1), 142–147.

Fittipaldi, N., Xu, J., Lacouture, S., et al., 2011. Lineage and virulence of *Streptococcus suis* serotype 2 isolates from North America. Emerging Infectious Diseases 17 (12), 2239–2244.

Gebru, E., Damte, D., Lee, S.J., et al., 2012. The in vitro activity of 15 antimicrobial agents against bacterial isolates from dogs. Journal of Veterinary Medical Science 20 April 2012 (EPUB ahead of print).

Gottschalk, M., Segura, M., Xu, J., 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Animal Health Research Reviews 8 (1), 29–45.

Ingrey, K.T., Ren, J., Prescott, J.F., 2003. A fluoroquinolone induces a novel mitogen-encoding bacteriophage in *Streptococcus canis*. Infection and Immunity 71 (6), 3028–3033.

Ivens, P.A., Matthews, D., Webb, K., et al., 2011. Molecular characterisation of 'strangles' outbreaks in the UK: the use of M-protein typing of *Streptococcus equi* ssp. *equi*. Equine Veterinary Journal 43 (3), 359–364.

King, S.J., Leigh, J.A., Heath, P.J., et al., 2002. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis:* identification of virulent clones and potential capsular serotype exchange. Journal of Clinical Microbioly 40 (10), 3671–3680.

Mai, N.T., Hoa, N.T., Nga, T.V., et al., 2008. *Streptococcus suis* meningitis in adults in Vietnam. Clinical Infectious Diseases 46 (5), 659–667.

Persson, Y., Nyman, A.K., Grönlund-Andersson, U., 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Veterinaria Scandinavica 53, 36.

Priestnall, S., Erles, K., 2011. *Streptococcus zooepidemicus:* an emerging canine pathogen. Veterinary Journal 188 (2), 142–148.

Petrovski, K.R., Laven, R.A., Lopez-Villalobos, N., 2011. A descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to commercial diagnostic laboratories in New Zealand (2003–2006). New Zealand Veterinary Journal 59 (2), 59–66.

Robertson, I.D., Blackmore, D.K., 1987. The detection of pigs carrying *Streptococcus suis* type 2. New Zealand Veterinary Journal 35, 1–4.

Webb, K., Jolley, K.A., Mitchell, Z., et al., 2008. Development of an unambiguous and discriminatory multilocus sequence typing scheme for the *Streptococcus zooepidemicus* group. Microbiology 154, 3016–3024.

Wei, Z., Li, R., Zhang, A., et al., 2009. Characterization of *Streptococcus suis* isolates from the diseased pigs in China between 2003 and 2007. Veterinary Microbiology 137 (1–2), 196–201.

Whatmore, A.M., King, S.J., Doherty, N.C., et al., 1999. Molecular characterization of equine isolates of *Streptococcus pneumoniae:* natural disruption of genes encoding the virulence factors pneumolysin and autolysin. Infection and Immunity 67 (6), 2776–2782.

Zhang, C., Ning, Y., Zhang, Z., et al., 2008. In vitro antimicrobial susceptibility of *Streptococcus suis* strains isolated from clinically healthy sows in China. Veterinary Microbiology 131, 386–392.

Chapter 9

Corynebacterium species and *Rhodococcus equi*

Genus Characteristics

The *Corynebacterium* species belong to the *Corynebacteriaceae* family of the *Actinomycetales* order. They are small, short, pleomorphic Gram-positive rods (about 0.5 µm in width) that occur in rod, coccoid and club-shaped forms. The corynebacteria are non-spore-forming, non-acid fast, catalase-positive, oxidase-negative and usually facultatively anaerobic. The animal pathogens are non-motile. Species of this genus infect humans and animals (Table 9.1). Stained smears from animal tissues often reveal groups of cells in parallel ('palisades') or cells at sharp angles to each other ('Chinese letters') (Fig. 9.1). Many have metachromatic granules (high-energy phosphate stores) and these are seen best in *Corynebacterium diphtheriae* (Fig. 9.2). Their nomenclature has undergone many changes over time:

Previous name	Present name
Corynebacterium equi	*Rhodococcus equi*
Corynebacterium murium	*C. kutscheri*
Corynebacterium ovis	*C. pseudotuberculosis*
Actinomyces (Corynebacterium) pyogenes	*Trueperella (Arcanobacterium) pyogenes* (Chapter 10)
Eubacterium (Corynebacterium) suis	*Actinobaculum suis* (Chapter 10)
C. renale type I	*C. renale*
C. renale type II	*C. pilosum*
C. renale type III	*C. cystitidis*

Rhodococcus equi also belongs to the *Corynebacteriaceae* family of the *Actinomycetales* order. *Rhodococcus* species are aerobic and can appear as a Gram-positive coccus or as a rod. The bacterial cell is capsulated and sometimes weakly acid-fast. *R. equi* is catalase-positive and possesses an oxidative metabolism. This species is the only significant veterinary pathogen in the genus (Table 9.1) and is considered as an emerging human opportunistic pathogen in immunocompromised humans.

Natural Habitat

Corynebacteria are found on the skin, mucous membranes (nasopharynx), and in the intestinal tracts of animals and humans. *Corynebacterium pseudotuberculosis* survived for one to eight days on inanimate surfaces and for seven to 55 days on particulate fomites such as wood shavings, hay, straw, and faeces (Augustine & Renshaw 1986). Lower temperatures generally extended the survival potential of this microorganism.

The natural habitat of *R. equi* is soil, particularly soil contaminated with manure from horses and farm animals. Faecal contamination increases the rate of multiplication in soil because the volatile fatty acids in faecal material promote growth of the bacterium. The prevalence of *R. equi* pneumonia has been shown to be associated with the airborne burden of virulent *R. equi* (Muscatello et al. 2006). Lower soil moisture concentrations and lower pasture heights give rise to elevated airborne concentrations of virulent *R. equi*. Acidic soils may contribute to an increased proportion of virulent strains within an *R. equi* population (Muscatello et al. 2006).

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Table 9.1 Main diseases caused by the major pathogenic *Corynebacterium species* and *Rhodococcus equi* in veterinary medicine

Species	Host(s)	Typical diseases
Corynebacterium bovis	Cattle	Commensal and subclinical mastitis
	Rabbit	Abscesses
Corynebacterium kutscheri	Laboratory rodents: mice, rats, guinea pigs	Abscesses in liver, kidneys, lungs, and lymph nodes, pseudotuberculosis (rodents), pneumonia
Corynebacterium pseudotuberculosis	Goats, sheep, camels	Caseous lymphadenitis
	Horses, cattle, pigs	Ulcerative lymphangitis, ventral abscessation, contagious acne (Canadian horse pox)
	Cattle	Mastitis
Corynebacterium renale	Cattle	Pyelonephritis and cystitis
	Pigs	Kidney abscesses
	Male sheep	Balanoposthitis (pizzle rot)
	Goats	Osteomyelitis
Corynebacterium cystitidis	Cattle	Cystitis, pyelonephritis
Corynebacterium pilosum	Cattle	Cystitis , pyelonephritis
Corynebacterium ulcerans	Cattle	Mastitis
	Cats	Respiratory disease (rare)
Rhodococcus equi	Foals (4–12 weeks old)	Suppurative bronchopneumonia (subacute or chronic) possibly with: ulcerative typhlocolitis, mesenteric lymphadenitis, osteomyelitis, purulent arthritis, and ulcerative lymphangitis
	Older foals	Abscesses
	Pigs, cattle	Cervical lymphadenitis
	Goats (young)	Granulomatous lesions in the liver

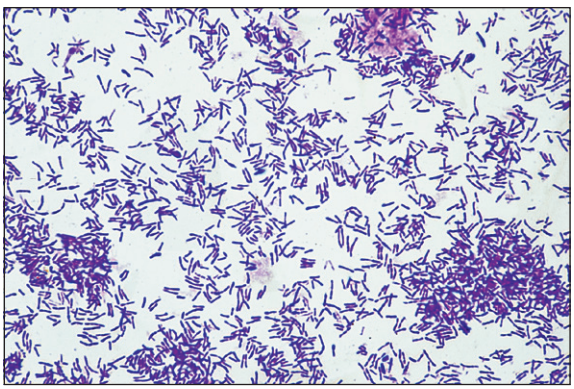

Figure 9.1 *C. diphtheriae* demonstrating club-shaped cells and 'Chinese-letter' patterns characteristic of the genus. (Gram stain, ×1000)

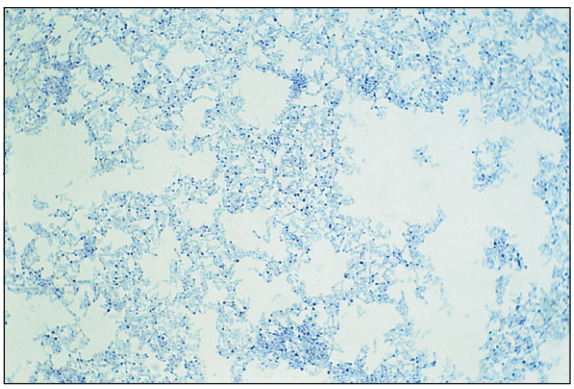

Figure 9.2 *C. diphtheriae* with dark-staining (reddish-purple) metachromatic granules of polyphosphate. (Methylene blue stain, ×1000)

Table 9.2 Main virulence factors of pathogenic *Corynebacterium* spp. and *Rhodococcus equi* in veterinary medicine

Species	Virulence determinants (gene)	Functions
Corynebacterium pseudotuberculosis	Phospholipase D	Increases vascular permeability and facilitates dissemination, inhibition of chemotaxis, degranulation and death of neutrophils, inactivation of complement
	Cell-surface lipids	May facilitate intracellular survival
Corynebacterium renale	Pili	Adherence
	Urease	Production of ammonia and mucosal inflammation
	Renalin: extracellular protein	May facilitate lysis of host cell membrane
Corynebacterium cystitidis	Pili	Adherence
	Urease	Production of ammonia and mucosal inflammation
Corynebacterium pilosum	Pili	Adherence
	Urease	Production of ammonia and mucosal inflammation
Corynebacterium ulcerans	Phospholipase D	Increases vascular permeability and facilitates dissemination, inhibition of chemotaxis, degranulation and death of neutrophils, inactivation of complement
	Diphtheria toxin when lysogenized by a phage with the gene *tox*	
Rhodococcus equi	Plasmid encoded VapA protein (*vapA*)	Surface protein, interferes with phagolysosome formation/function
	Plasmid encoded Vap proteins (*vap* A, C, D, E, F, G, and H)	*Vaps* C, D and E are secreted proteins, exact functions remain unknown
	Plasmid encoded *VapB* protein (*vapB*)	Surface protein, exact functions remain unknown but found in pig-virulent *R. equi*
	Cell-wall mycolic acid	May facilitate intracellular survival
	Lipoarabinomannam	Exact functions remain unknown but may modulate immune responses
	Capsular polysaccharides	Likely prevent phagocytosis and resistance to complement
	Exoenzymes	May promote cellular membrane destruction

Pathogenesis and Pathogenicity

The main diseases caused by the major pathogenic *Corynebacterium* species and *Rhodococcus equi* in veterinary medicine are summarized in Table 9.1 and their virulence determinants are shown in Table 9.2.

Corynebacteria are pyogenic bacteria causing a variety of suppurative conditions. *Corynebacterium pseudotuberculosis* is the aetiological agent of caseous lymphadenitis, a chronic contagious disease that affects goats and sheep, and can cause severe economic losses. It has also been recovered from other animal species (Table 9.1). It is a facultative intracellular bacterium which forms abscesses in the skin, lymph nodes (external or internal) and organs such as the spleen, lungs, liver, and kidneys. Transmission is usually via shearing, castration or any other skin wounds. Inhalation and ingestion are also possible ports of entry of the microorganism. Infected phagocytes disseminate the bacteria via the lymph or the blood to secondary sites where they cause lesions. The virulence of *C. pseudotuberculosis* is attributed to an exotoxin, phospholipase D (PLD) and to the surface lipid layer adjacent to the cell wall. The expression of *fagB C D* genes, which encode a putative iron uptake system, appears to contribute to virulence (Billington et al. 2002). *Corynebacterium pseudotuberculosis* can cause lymphadenitis and abscesses with granulomatous necrotizing lesions in humans and should be regarded as an occupational disease (Peel et al. 1997).

Corynebacterium ulcerans is a rare cause of mild to severe (loss of the affected quarter) bovine mastitis. Virulence is attributed to the production of a PLD that genetically resembles the one produced by *C. pseudotuberculosis*. *Corynebacterium ulcerans* can also carry a phage that encodes for the diphtheria toxin. As a result, human diphtheria has been reported after consumption of raw milk (Barrett 1986). This pathogen is also occasionally associated with disease in cats and dogs and transmission from pets to humans can occur (Hogg et al. 2009).

Corynebacterium bovis rarely causes clinical mastitis. In fact, it is considered to be a commensal of the bovine mammary gland and may protect the gland from infection with more virulent pathogens (Pociecha 1989).

Corynebacterium kutscheri is a commensal of the upper respiratory and intestinal tracts of laboratory rodents. Infection is usually subclinical in mice and rats. However, when immunosuppressed, a pseudotuberculosis with high morbidity and mortality can appear in these animals. *Corynebacterium renale, Corynebacterium cystitidis* and *Corynebacterium pilosum* are commensals of the bovine reproductive tract. They are opportunistic pathogens causing simple or complicated ascending urinary tract infections (Table 9.1). *C. renale* is the most common cause of cystitis, ureteritis and pyelonephritis among the three species. *C. pilosum* is a milder pathogen often resulting in an uncomplicated cystitis. The virulence of these species is attributed to pili, which mediate adhesion to epithelial cells of the bladder, urease, which is responsible for hydrolysing urea and releasing nitrogen, and renalin (mainly in *C. renale*) that may play a role in lysis of host cells.

R. equi is the cause of suppurative bronchopneumonia in foals. This disease increases in prevalence where high stocking rates occur. It is usually seen in foals of four to 12 weeks of age, possibly due to a decline in maternal antibody at about six weeks of age. The main route of infection is by inhalation. Heavily infected sputum may be swallowed by the affected foal leading to ulcerative colitis and mesenteric lymphadenitis. Infection has been seen in other species (Table 9.1). The bacterium can also multiply in soil enriched with equine faeces and may be a commensal in the intestine of horses. *Rhodococcus equi* is a facultative intracellular bacterium able to survive intracellularly through suppression of phagolysosomal fusion. The organism multiplies in and eventually destroys alveolar macrophages while neutrophils remain bactericidal. *Rhodococcus equi* has many virulence factors (Table 9.2) which probably play a part in the pathogenesis; however, the mechanisms involved are not all fully understood. In foals, *R. equi* virulence is associated with 80–90 kb plasmids, which include a pathogenicity island containing the virulence-associated protein (*vap*) gene family. In addition, nine chromosomal genes involved in fatty acid and lipid metabolism (*choD, fadD13, fbpB*), heme biosynthesis (*hemE*), iron utilization (*mbtF*), heat shock resistance and genes encoding chaperones (*clpB, groEL*), a sigma factor (*sigK*), and a transcriptional regulator (*moxR*) were significantly induced in *R. equi* cells growing inside macrophages (Rahman et al. 2005).

In immunocompromized humans, *R. equi* necrotizing pneumonia is the commonest form of infection. However, extrapulmonary infections, such as wound infections and subcutaneous abscess, have also been described (Puthucheary et al. 2006).

Laboratory Diagnosis

Specimens

Pus or exudates should be collected from suppurative conditions. Mid-stream urine is suitable for isolation of the members of the *C. renale* group. A tracheal wash technique, with infusion of saline, can be used for the recovery of *R. equi* from affected foals.

Direct microscopy

The corynebacteria are Gram-positive rods with varying degrees of pleomorphism (Fig. 9.3). *R. equi* is usually coccal but can be rod-shaped particularly in animal tissue (Fig. 9.4) and can be MZN-positive (weakly acid-fast).

Isolation

For routine isolation sheep or ox blood agar is used with MacConkey agar to detect any Gram-negative contaminants that may be present. The plates are incubated at 35°C for 24 to 48 hours. A modified NANAT *R. equi*-selective agar medium has been used to recover *R. equi* from faecal samples (Grimm et al. 2007).

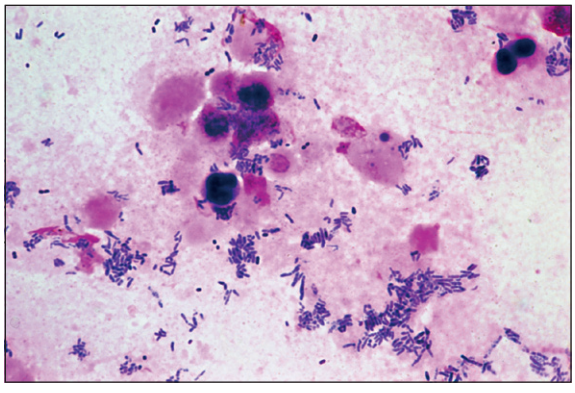

Figure 9.3 *C. renale* in a Gram-stained smear of bovine urine from a case of pyelonephritis. It shows extreme pleomorphism from club-shaped rods to coccal forms. (Gram stain, ×1000)

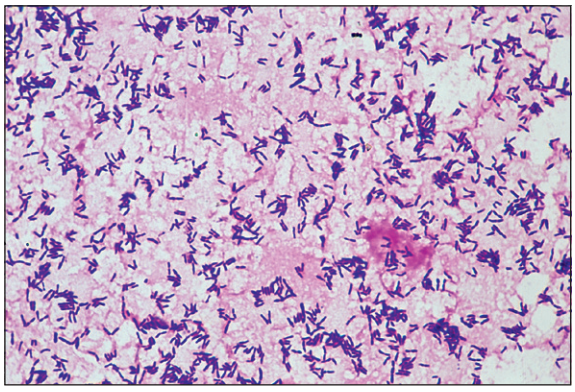

Figure 9.4 *R. equi* in a smear of pus from a lung abscess in a case of suppurative bronchopneumonia in a foal. In this smear rod-shaped forms predominate. (Gram stain, ×1000)

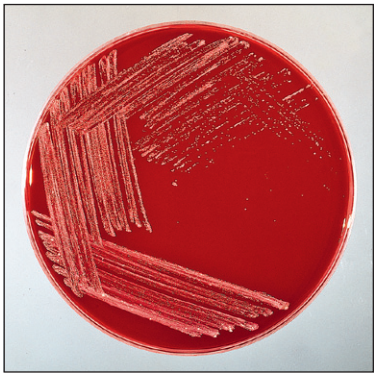

Figure 9.5 *C. pseudotuberculosis*: small, white and dry colonies on sheep blood agar. Non-haemolytic at 24 hours' incubation (see Fig. 9.6).

Identification

Colonial morphology

Corynebacterium pseudotuberculosis produces small, white, dry colonies (Fig. 9.5). They can be surrounded by a narrow zone of haemolysis but often not until after 48 to 72 hours' incubation (Fig. 9.6). After several days' incubation the colonies can reach 3 mm in diameter and appear dry, crumbly and cream in colour. *C. kutscheri* produces small, whitish colonies that bear a resemblance to those of *C. pseudotuberculosis* (Fig. 9.7). Occasional strains are haemolytic. The colonies of *C. bovis* are small, white, dry, and non-haemolytic with a tendency to appear in the wells of plates inoculated with a milk sample as it is a lipophilic bacterium (Fig. 9.8). *C. ulcerans* produces slightly haemolytic, small, whitish colonies.

The colonies of *C. renale* are non-haemolytic and very small after 24 hours' incubation, but become opaque and

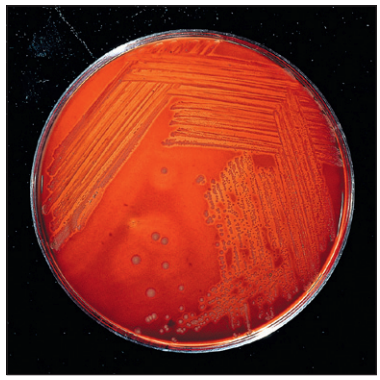

Figure 9.6 *C. pseudotuberculosis* on sheep blood agar demonstrating haemolysis after 72 hours' incubation.

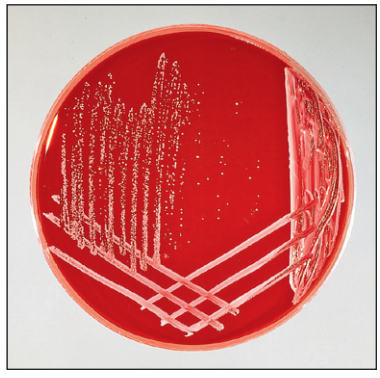

Figure 9.7 *C. kutscheri* on sheep blood agar: small, whitish colonies after 48 hours' incubation. This strain, which is non-haemolytic, was isolated from a mouse colony where there were recurring deaths. Liver abscesses are a common postmortem finding in this disease.

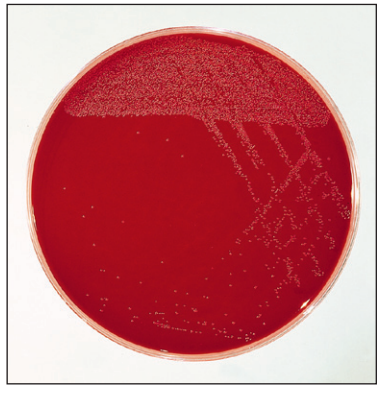

Figure 9.8 *C. bovis* on sheep blood agar: small, white, dry, non-haemolytic colonies after 48 hours' incubation.

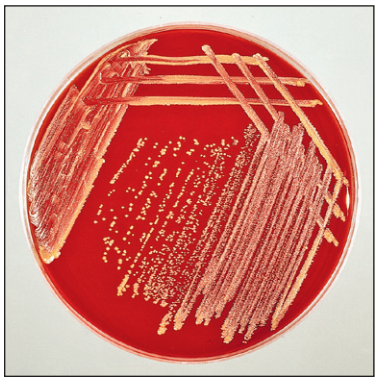

Figure 9.9 *C. pilosum* on sheep blood agar showing yellow pigmentation.

Figure 9.11 *R. equi* on nutrient agar (4-day culture) demonstrating the mucoid colonies and 'salmon-pink' pigmentation.

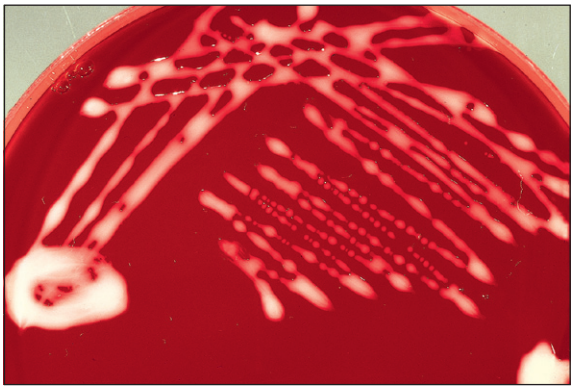

Figure 9.10 *R. equi* on sheep blood agar showing the typical mucoid colonies (4-day culture). The 'salmon-pink' pigmentation is not easily seen against a red background.

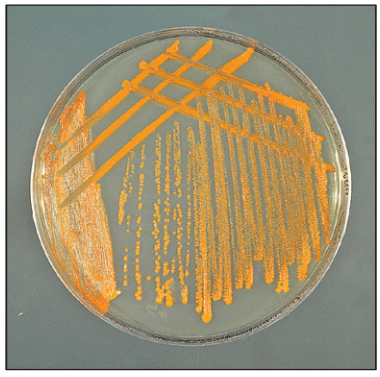

Figure 9.12 *R. equi* on pigment-enhancing medium after 48 hours' incubation, showing the typical (but enhanced) pigment and mucoid colonies.

a dull yellow colour as they age. *Corynebacterium pilosum* colonies resemble those of *C. renale* but become cream to yellow in colour with time (Fig. 9.9). The colonies of *C. cystitidis* are similar to the above two species but are transparent to white.

Rhodococcus equi produces small, smooth, shiny and non-haemolytic colonies after 24 hours' incubation which become larger, mucoid and salmon-pink in colour with age, usually after 48 hours' incubation (Figs 9.10 and 9.11). A medium useful for enhancing the pigment of *R. equi* contains yeast extract (10 g), glucose (10 g) and agar (15 g) in 1 litre of tap water (Fig. 9.12).

Microscopic appearance

Gram-stained smears from colonies will show pleomorphic Gram-positive rods for the *Corynebacterium* species, while *R. equi* can have either cocci (Fig. 9.13) or rods predominating.

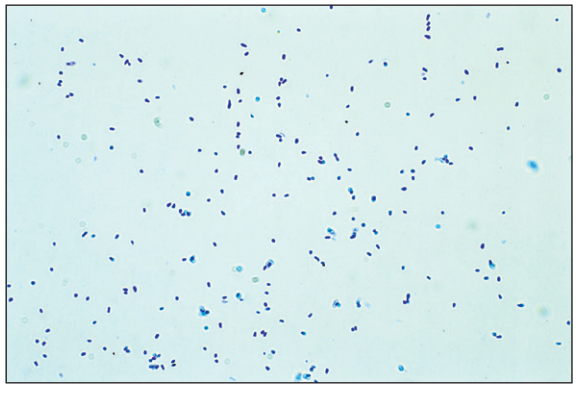

Figure 9.13 *R. equi* in a Gram-stained smear from a colony with coccal forms predominating. (×1000)

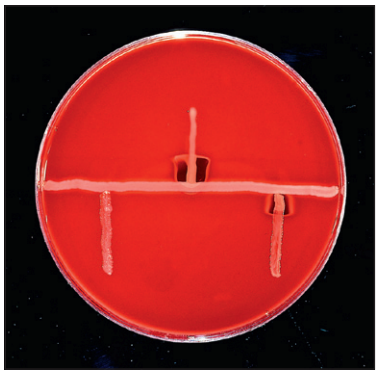

Figure 9.14 CAMP test with a *Staphylococcus aureus*. *C. pseudotuberculosis* (left), *R. equi* (centre) and *C. renale* (right). The latter two bacteria give an enhancement of the effect of the staphylococcal beta-haemolysin.

Figure 9.15 A CAMP test with *C. pseudotuberculosis* drawn across (left to right) *S. aureus*, demonstrating inhibition of the effect of the staphylococcal haemolysins.

Figure 9.16 CAMP test with *R. equi* against *S. aureus* (horizontal) showing the typical shovel-shaped enhancement of the effect of the staphylococcal beta-haemolysin that tends to extend to the opposite side of the *S. aureus* streak.

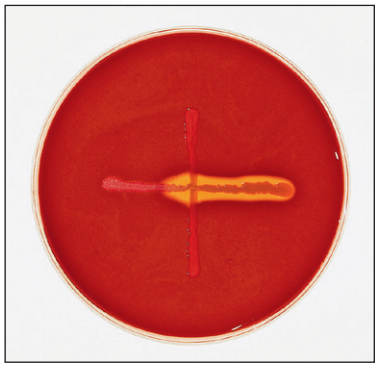

Figure 9.17 CAMP test with *C. pseudotuberculosis* (horizontal, left to right) drawn across *R. equi* (vertical) demonstrating synergistic haemolysis.

CAMP tests

CAMP tests can be used as quick presumptive tests for *C. pseudotuberculosis*, *R. equi* and *C. renale*, interacting with the beta-haemolysin of *Staphylococcus aureus* (Fig. 9.14). *Corynebacterium pseudotuberculosis* inhibits the action of the staphylococcal beta-haemolysin (reverse CAMP test) (Fig. 9.15), while *R. equi* (Fig. 9.16) and *C. renale* enhance the action of the staphylococcal beta-haemolysin. There is also a synergistic haemolytic effect seen between *R. equi* ('*R. equi* factors') and *C. pseudotuberculosis* (Fig. 9.17) on sheep blood agar.

Biochemical characteristics

Definitive identification of the corynebacteria and *R. equi* is based on differential biochemical tests (Table 9.3). Differentiation of members of the *C. renale* group is based on the colonial pigmentation (Fig. 9.18), presence or absence of growth at pH 5.4 and biochemical reactions (Table 9.4). The formula of the media for demonstrating casein digestion (Fig. 9.19) and hydrolysis of Tween 80 (Fig. 9.20) are given in Appendix 2.

Many commercial biochemical identification systems are available. However, clinically significant coryneforms that are difficult to identify should be confirmed with molecular genetic-based identification systems or be sent to a reference laboratory where chromatographic techniques can be used for definitive identification.

Serology

Several serodiagnostic tests for the detection of antibodies to *C. pseudotuberculosis* have been used, and these include haemolysis inhibition (Burrell 1980), indirect haemagglutination, anti-haemolysin inhibition, complement fixation tests (Shigidi 1979), immunodiffusion (Burrell 1980)

Table 9.3 Differentiation of corynebacteria and *Rhodococcus equi*

Test[a]	*C. bovis*	*C. kutscheri*	*C. pseudotuberculosis*	*C. ulcerans*	*C. renale group*	*R. equi*
Beta-hemolysis	–	v	+	+	–	–
Aesculin hydrolysis	–	+	–	–	–	–
Nitrate reduction	–	+	v	+	v	+
Urease	–	+	+ (>18h)	+	+ (<1h)	+(>18h)
Casein digestion	–	–	–	+	v	–
Fermentation of:						
Glucose	+	+	+	–	+	–
Maltose	–	+	+	+	–	–
Sucrose	–	+	–	–	–	–

[a]+ = greater than or equal to 90% positive; – = less than or equal to 10% positive; v = variable, 11 to 89% positive

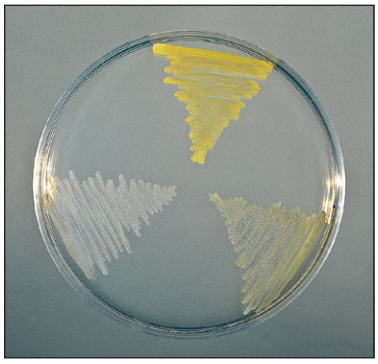

Figure 9.18 Pigments of the *C. renale* group: *C. pilosum* (top) distinctly yellow; *C. cystitidis* (left) white and *C. renale* (right) a dull yellow (48 hours' incubation).

Table 9.4 Differentiation of the *Corynebacterium renale* group

Test[a]	*C. renale*	*C. pilosum*	*C. cystitidis*
Colony colour (48 h)	Yellowish	Yellow	White
Growth at pH 5.4 (broth)	+	–	–
Nitrate reduction	–	+	–
Casein digestion	+	–	–
Hydrolysis of Tween 80	–	–	+
Acid from:			
Xylose	–	–	+
Starch	–	+	+

[a]+ = greater than or equal to 90% positive; – = less than or equal to 10 % positive; v = variable, 11 to 89% positive

and enzyme-linked immunosorbent assays (ELISA) (Sutherland et al. 1987a, 1987b, Binns et al. 2007). Serology can be used to detect infected goats and sheep which may have only internal abscesses, most often in the lungs or mediastinal lymph nodes, and which show no other clinical signs of infection. Many antigen preparations have been used in the ELISA tests such as cell wall antigens, whole cell extract, phospholipase D, cell supernatant and recombinant exotoxin. The most specific diagnostic test seems to be the ELISA based on recombinant phospholipase D expressed in *E. coli* (Menzies et al. 1994). Even if such tests perform well in goats, they may show reduced sensitivity in sheep. For such cases, there is a modified double antibody sandwich ELISA with both good sensitivity and specificity in sheep (Dercksen et al. 2000).

Serological diagnosis has also been developed for *R. equi* with the agar gel immunodiffusion test, the ELISA (Cuteri et al. 2003) and the synergistic haemolysis inhibition test. A peptide-based ELISA that detects VapA-specific IgGb antibodies has been shown to be a useful predictor of *R. equi* disease in foals aged between three weeks and six months (Phumoonna et al. 2006).

Antimicrobial Susceptibility Testing

There are at present no guidelines for the antimicrobial susceptibility testing of coryneform bacteria from the CLSI

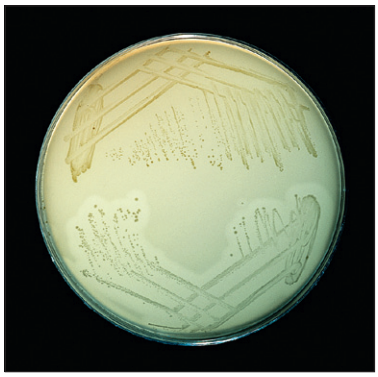

Figure 9.19 *C. renale* (top) showing casein digestion on milk agar. *C. pilosum* (bottom) and *C. cystitidis* do not give this reaction.

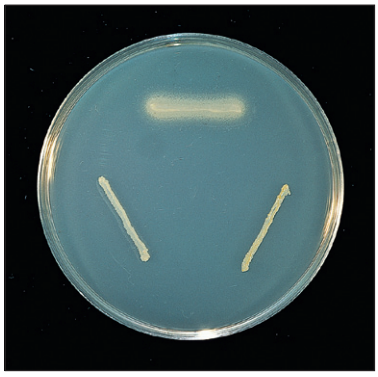

Figure 9.20 Hydrolysis of Tween 80 by *C. cystitidis* (top) on Tween 80 medium. Both *C. renale* (left) and *C. pilosum* (right) are negative.

(Clinical and Laboratory Standards Institute). However, when necessary, a disk diffusion test can be performed on Mueller–Hinton agar supplemented with 5% sheep blood. Incubation is carried out at 35°C for 24 hours (48 hours may be required). The recommended interpretation guidelines for streptococci should be used. The determination of minimum inhibitory concentrations (MICs) can be done on Mueller–Hinton agar with 5% sheep blood using the E-test (AB Biodisk, Solna, Sweden), the agar dilution method or the broth microdilution technique.

Rhodococcus equi antimicrobial susceptibility testing can be performed, when necessary, using the disk diffusion test on Mueller–Hinton agar (Approved Standard M31-A3; CLSI 2008). Minimum inhibitory concentrations (MICs) can be determined using the agar dilution method or the broth microdilution technique according to the guidelines of the CLSI (Approved standard M7-A4 or M31-A3). Alternatively, MIC can be determined using the E-test (AB Biodisk) on Mueller–Hinton agar in accordance with the manufacturer's recommended protocol (Brown 1991). *Rhodococcus equi* MICs have also been determined using JustOne microtitration strips (AccuMed International Ltd, Westlake, Ohio) (Jacks et al. 2003). This technique has previously been shown to correlate closely with the standard broth dilution method (Jones et al. 1980).

Antimicrobial resistance

For *C. pseudotuberculosis* infections, penicillin is usually the antibiotic of choice. However, because there is poor penetration into abscesses, antimicrobial therapy is not recommended for this type of infection.

Rhodococcus equi infections in foals are usually treated with a combination of erythromycin and rifampin. Azithromycin and clarithromycin have both been proposed as alternatives to eythromycin for the treatment of *R. equi* infections. Azithromycin has been shown to reduce the cumulative incidence of pneumonia due to *R. equi* among foals at breeding farms where endemic *R. equi* infections occur (Chaffin et al. 2008). Rifampin resistance in *R. equi* strains has been reported (Takai et al. 1997). Clarithromycin has been reported to be more active than azithromycin against *R. equi*, while rifampin, gentamicin, and imipenem were also highly active in vitro against at least 90% of *R. equi* isolates (Jacks et al. 2003). Antimicrobial susceptibility testing of *R. equi* cultures isolated from diseased horses and humans, revealed that all isolates were susceptible to erythromycin, gentamicin, imipenem, minocycline, neomycin, rifampicin, streptomycin and vancomycin; while 71% and 75% were resistant or moderately susceptible to tetracycline and penicillin G, respectively (Fuhrmann & Lämmler, 1997).

Strain Typing

Phenotypic typing methods based on metabolic and biological properties have shown limited discriminatory power between closely related isolates for both corynebacteria and *Rhodococcus* species. Seven capsular serotypes of *R. equi* have been described with most isolates belonging to serotype 1 or 2 (Prescott 1981). Molecular typing methods offer good discriminatory power, reproducibility and convenience.

Random amplification of polymorphic DNA (RAPD) and amplification of DNA fragments surrounding rare restriction site (ADSRRS)-fingerprinting have been shown to be suitable for epidemiological studies of *C. pseudotuberculosis*. Six pulsotypes were identified among 50 UK isolates of *C. pseudotuberculosis* following genotyping by pulsed-field gel electrophoresis (PFGE) using *Sfi*I restriction (Connor et al. 2000) and Morton et al. (1998) also used PFGE-based techniques successfully for investigation of *R. equi* isolate diversity. Restriction enzyme digestion patterns of virulence plasmids have been used to identify several different plasmid types among virulent *R. equi* isolates (Takai et al. 2001). A plasmid typing scheme termed

TRAVAP is based on three plasmid gene markers; *traA* (conserved conjugal transfer machinery), *vapA* (virulence associated VapA protein) and *vapB* (VapA-related surface protein of larger size and found mainly in non-equine isolates). This scheme classifies *R. equi* isolates into four categories (Ocampo-Sosa et al. 2007).

Molecular Diagnosis

Accurate diagnosis of caseous lymphadenitis generally relies on microbiological examination, followed by biochemical identification of isolates. To facilitate *C. pseudotuberculosis* detection, a multiplex PCR (mPCR) assay was developed targeting three genes of this bacterium: the 16S rRNA gene, *rpoB* and *pld* (Pacheco et al. 2007).

Molecular techniques are available for the detection of *R. equi* in clinical specimens but sensitivity and specificity are variable. A PCR assay based on primers for the *vapA* gene has been reported as having 100% sensitivity and 90.6% specificity on tracheal wash fluid from foals (Sellon et al. 2001). A dual-reaction real-time PCR assay based on detection of the species-specific cholesterol oxidase gene and the pathogenicity marker gene *vapA* is reported to be both sensitive and specific (Rodríguez-Lázaro et al. 2006).

REFERENCES

Augustine, J.L., Renshaw, H.W., 1986. Survival of *Corynebacterium pseudotuberculosis* in axenic purulent exudate on common barnyard fomites. American Journal of Veterinary Research 47, 713–715.

Barrett, N.J., 1986. Communicable disease associated with milk and dairy products in England and Wales: 1983–1984. Journal of Infection 12, 265–272.

Billington, S.J., Esmay, P.A., Songer, J.G., et al., 2002. Identification and role in virulence of putative iron acquisition genes from *Corynebacterium pseudotuberculosis*. FEMS Microbiology Letters 208, 41–45.

Binns, S.H., Green, L.E., Bailey, M., 2007. Development and validation of an ELISA to detect antibodies to *Corynebacterium pseudotuberculosis* in ovine sera. Veterinary Microbiology 123, 169–179.

Brown, D.F., Brown, L., 1991. Evaluation of the E test: a novel method of quantifying antimicrobial activity. Journal of Antimicrobial Chemotherapy 27 (2), 185–190.

Burrell, D.H., 1980. A simplified double immunodiffusion technique for detection of *Corynebacterium ovis* antigen. Research in Veterinary Science 28, 234–237.

Chaffin, M.K., Cohen, N.D., Martens, R.J., 2008. Chemopropylactic effects of azithromycin against *Rhodococcus equi*-induced pneumonia among foals at equine breeding farms with endemic infections. Journal of the American Veterinary Medical Association 232, 1035–1047.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard, 3rd edn (M31-MA3). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Connor, K., Quirie, M.M., Baird, G., et al., 2000. Characterization of United Kingdom isolates of *Corynebacterium pseudotuberculosis* using pulsed-field gel electrophoresis. Journal of Clinical Microbiology 38, 2633–2637.

Cuteri, V., Takai, S., Moscati, L., et al., 2003. A serological survey of *Rhodococcus equi* infection in foals in central Italy: comparison of two antigens using an ELISA test. Comparative Immunology, Microbiology and Infectious Diseases 26, 17–23.

Dercksen, D.P., Brinkhof, J.M.A., Dekker-Nooren, T., et al., 2000. A comparison of four serological tests for the diagnosis of caseous lymphadenitis in sheep and goats. Veterinary Microbiology 75, 167–175.

Fuhrmann, C., Lämmler, C., 1997. Characterization of *Rhodococcus equi* isolates from horse and man. Berliner Munchener Tierarztlicher Wochenschrift 110 (2), 54–59.

Grimm, M.B., Cohen, N.D., Slovis, N.M., et al., 2007. Evaluation of fecal samples from mares as a source of *Rhodococcus equi* for their foals by use of quantitative bacteriologic culture and colony immunoblot analyses. American Journal of Veterinary Research 68, 63–71.

Hogg, R.A., Wessels, J., Hart, J., et al., 2009. Possible zoonotic transmission of toxigenic *Corynebacterium ulcerans* from companion animals in a human case of fatal diphtheria. Veterinary Record 165 (23), 691–692.

Jacks, S.S., Giguere, S., Nguyen, A., 2003. In vitro susceptibilities of *Rhodococcus equi* and other common equine pathogens to azithromycin, clarithromycin and 20 other antimicrobials. Antimicrobial Agents and Chemotherapy 47, 1742–1745.

Jones, R.N., Gavan, T.L., Barry, A.L., 1980. Evaluation of sensititre microdilution antibiotic susceptibility system against recent clinical isolates: three laboratory collaborative study. Journal of Clinical Microbiology 11, 426–429.

Menzies, P.I., Muckle, C.A., Hwang, Y.T., et al., 1994. Evaluation of an enzyme-linked immunosorbent assay using an *Escherichia coli* recombinant phospholipase D antigen for the diagnosis of *Corynebacterium pseudotuberculosis* infection. Small Ruminant Research 13, 193–198.

Morton, A.C., Baseggio, N., Peters, M.A., et al., 1998. Diversity of isolates of *Rhodococcus equi* from Australian thoroughbred horse farms. Antonie Van Leeuwenhoek 74, 21–25.

Muscatello, G., Anderson, G.A., Gilkerson, J.R., et al., 2006.

Associations between the ecology of virulent Rhodococcus equi and the epidemiology of *R. equi* pneumonia on Australian thoroughbred farms. Applied and Environmental Microbiology 72, 6152–6160.

Ocampo-Sosa, A.A., Lewis, D.A., Navis, J., et al., 2007. Molecular epidemiology of *Rhodococcus equi* based on traA, vapA and vapB virulence plasmid markers. Journal of Infectious Diseases 196, 763–769.

Pacheco, L.G., Pena, R.R., Castro, T.L., Dorella, F.A., et al., 2007. Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. Journal of Medical Microbiology 56, 480–486.

Peel, M.M., Palmer, G.G., Stacpoole, A.M., et al., 1997. Human lymphadenitis due to *Corynebacterium pseudotuberculosis:* report of ten cases from Australia and review. Clinical Infectious Diseases 24, 185–191.

Phumoonna, T., Muscatello, G., Chicken, C., et al., 2006. Clinical evaluation of a peptide-ELISA based upon N-terminal B-cell epitope of the VapA protein for diagnosis of *Rhodococcus equi* pneumonia in foals. Journal of Veterinary Medicine B 53, 126–132.

Pociecha, J.Z., 1989. Influence of *Corynebacterium bovis* on constituents of milk and dynamics of mastitis. Veterinary Record 125, 628.

Prescott, J.F., 1981. Capsular serotypes of *Corynebacterium equi*. Canadian Journal of Comparative Medicine 45, 130–134.

Puthucheary, S.D., Sangkar, V., Hafeez, A., et al., 2006. *Rhodococcus equi* – an emerging human pathogen in immunocompromised hosts: a report of four cases from Malaysia. Southeast Asian Journal of Tropical Medicine and Public Health 37, 157–161.

Rahman, M.T., Parreira, V., Prescott, J.F., 2005. In vitro and intra-macrophage gene expression by *Rhodococcus equi* strain 103. Veterinary Microbiology 110, 131–140.

Rodríguez-Lázaro, D., Lewis, D.A., Ocampo-Sosa, A.A., et al., 2006. Internally controlled real-time PCR method for quantitative species-specific detection and vapA genotyping of *Rhodococcus equi*. Applied and Environmental Microbiology 72, 4256–4263.

Sellon, D.C., Besser, T.E., Vivrette, S.L., 2001. Comparison of nucleic acid amplification: serology, and microbiologic culture for diagnosis of *Rhodococcus equi* pneumonia in foals. Journal of Clinical Microbiology 39 (4), 1289–1293.

Shigidi, M.T.A., 1979. A comparison of five serological tests for the diagnosis of experimental *Corynebacterium ovis* infection in sheep. British Veterinary Journal 135, 172–177.

Sutherland, S.S., Ellis, T.M., Mercy, A.R., et al., 1987a. Evaluation of an enzyme-linked immunosorbent assay for the detection of *Corynebacterium pseudotuberculosis* infection in sheep. Australian Veterinary Journal 64, 263–266.

Sutherland, S.S., Paton, M.W., Mercy, A.R., et al., 1987b. A reliable method for establishing caseous lymphadenitis infection in sheep. Australian Veterinary Journal 64, 323–324.

Takai, S., Takeda, K., Nakano, Y., et al., 1997. Emergence of rifampin-resistant *Rhodococcus equi* in an infected foal. Journal of Clinical Microbiology 35, 1904–1908.

Takai, S., Chaffin, K., Cohen, N.D., et al., 2001. Prevalence of virulent *Rhodococcus equi* in soil from five *R. equi*-endemic horse-breeding farms and restriction fragment length polymorphisms of virulence plasmids in isolates from soil and infected foals in Texas. Journal of Veterinary Diagnostic Investigation 13, 489–494.

FURTHER READING

Meijer, W.G., Prescott, J.F., 2004. *Rhodococcus equi*. Veterinary Research 35, 383–396.

Muscatello, G., Leadon, D.P., Klay, M., et al., 2007. *Rhodococcus equi* infection in foals: the science of 'rattles'. Equine Veterinary Journal 39, 470–478.

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Chapter 10

The Actinobacteria

The class *Actinobacteria* comprises a heterologous group of procaryotes, containing some genera which have the ability to form Gram-positive, branching filaments of less than 1 µm in diameter. Thus, these bacteria were originally thought to be fungi. However, fungi are eucaryotes and their filaments (hyphae) are always greater than 1 µm in width. The main animal pathogens in the *Actinobacteria* are found in the genera *Actinomyces*, *Actinobaculum* and *Trueperella* in the family *Actinomycetaceae*, the genera *Mycobacterium*, *Corynebacterium*, *Rhodococcus* and *Nocardia* in the family *Corynebacteriaceae* and in the genus *Dermatophilus*. Members of the genus *Nocardia* are closely related to *Corynebacterium*, *Mycobacterium* and *Rhodococcus* species but *Actinomyces* differs from these in its DNA guanine/cytosine ratio and in the chemical composition of its cell wall. Information about the genera *Corynebacterium* and *Rhodococcus* is presented in Chapter 9 and the mycobacteria are discussed in Chapter 11. Other genera included in the *Actinobacteria* are *Streptomyces* and *Actinomadura*; some species of each produce mycetomas in humans but are rarely pathogenic for animals. *Streptomyces* species are prolific producers of antimicrobial substances (Fig. 10.1) and are common contaminants on laboratory agar media. Species in the genera *Saccharopolyspora* and *Thermoactinomyces* are not invasive but inhalation of their spores can cause allergic pulmonary disease in man and horses and possibly in other domestic animals fed or exposed to mouldy hay. The general characteristics of the genera *Actinomyces*, *Nocardia*, *Trueperella*, *Streptomyces* and *Dermatophilus* are presented in Table 10.1.

Changes in Nomenclature

Previous name	Present name
Arcanobacterium (Actinomyces) pyogenes	*Trueperella pyogenes*
Actinomyces suis	*Actinobaculum suis*
Nocardia caviae	*Nocardia otitidiscaviarum*

Natural Habitat

Actinomyces, *Arcanobacterium*, *Trueperella* and *Actinobaculum* species, with the exception of *Actinomyces hordeovulneris*, are present on mucous membranes of the host animal, often in the oral cavity or nasopharynx. *Nocardia* and *Streptomyces* species are soil microorganisms. *Dermatophilus congolensis*, the only species in the genus, is thought to maintain itself in small foci of infection on a carrier animal or within scab particles in dust. It can survive in scab material for periods of up to three years. The pathogenic *Actinobacteria* have a worldwide distribution but severe clinical disease caused by *D. congolensis* is most common in tropical and subtropical regions.

Pathogenicity

The pathogenic *Actinobacteria* and the diseases that they cause are summarized in Table 10.2.

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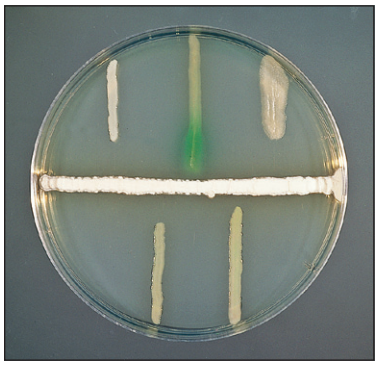

Figure 10.1 *Streptomyces* species (horizontal streak) on Isosensitest agar demonstrating antimicrobial activity against other bacteria that were streaked to within 2 mm of the *Streptomyces* species: *S. aureus* (top left), *Pseudomonas aeruginosa* (top centre), *Bacillus cereus* (top right), *Salmonella* species (bottom left) and *E. coli* (bottom right). *Pseudomonas aeruginosa* is the only bacterium not inhibited by the antimicrobial factor(s) from the *Streptomyces* species.

Actinomyces, *Trueperella* and *Actinobaculum* species

Infections by these organisms tend to be endogenous and most of the *Actinomyces* species cause pyogranulomatous reactions in animal tissues. Specific virulence factors of *Actinomyces* species of veterinary importance have not been identified.

Actinomyces bovis gains access to the alveolar region of the jaw in cattle from the oral cavity, probably through trauma to the mucosa. It initiates a rarefying osteomyelitis and soft tissue reaction, the condition being referred to as 'lumpy jaw'. Bacterial colonies form in the tissues with 'clubs' of mineralized calcium phosphate forming around them to create microscopic 'club colonies' or 'rosettes'. Figure 10.2 shows a diagram of a 'club colony'. The club formation is the result of phosphatase activity and is a host reaction to a chronic infection. Granulation, mononuclear infiltration and fibrosis occur in the lesions with sinus tracts leading to the outside. Exudate from the tracts contains pus with 'sulphur granules' that are about 1–2 mm

Table 10.1 General features of *Actinomyces*, *Nocardia*, *Trueperella*, *Streptomyces* (non-pathogenic) and *Dermatophilus* species

Characteristics	*Actinomyces*	*Nocardia*	*Trueperella*	*Streptomyces*	*Dermatophilus*
Atmospheric requirement	Anaerobic or capnophilic*	Strict aerobe	Capnophilic	Aerobe	Aerobe/capnophilic*
Catalase	–(+)	+	–	+	+
Partially acid-fast (MZN-positive)	–	+	–	–	–
Motility	–	–	–	–	+ (zoospores)
Growth on Sabouraud dextrose agar	–	+	–	+	–
Aerial filaments	–	+	–	+	–
Spores	–	+ (conidia)	–	+ (conidia)	+ (zoospores)
Fragmentation of filaments	–	+	– (coryneform morphology)	+	+
Odour of colonies	–	–	–	Pungent and earthy	–
Metabolism	Fermentative	Oxidative	Fermentative	Oxidative	Weakly fermentative
Reservoir	Oral muosa and nasopharynx	Soil	Mucous membranes	Soil and common laboratory contaminant	Foci on skin of carrier animal or within scabs in environment
Veterinary importance	Focal or systemic disease with characteristic granulomas in skin, subcutaneous tissue and internal organs		Pyogenic disease in many species	Non-pathogenic but similar to *Nocardia* in cultures. Some species produce antibiotics	Skin disease

*capnophilic (carboxyphilic) = carbon dioxide required for maximum growth, as opposed to microaerophilic, used to describe an organism that requires a reduced oxygen tension,. + = positive reaction, – = negative, –(+) = most species are negative (a few positive)

Table 10.2 Diseases caused by selected pathogenic actinobacteria of veterinary importance

Pathogen	Host(s)	Disease
Actinomyces bovis	Cattle	Bovine actinomycosis ('lumpy jaw')
A. viscosus	Dogs	Canine actinomycosis: Localized cutaneous granulomatous abscess Pyothorax and granulomas in the thoracic cavity
A. hordeovulneris	Dogs	Localized abscesses and systemic infections such as pleuritis, peritonitis, visceral abscesses and sepic arthritis. Often associated with tissue-migrating awns of the grass *Hordeum* species ('foxtails') which are common in Western USA
A. israelii	Humans	Human actinomycosis
	Pigs and cattle	Bovine and porcine actinomycosis (rare)
A. hyovaginalis	Pigs	Abortion, purulent vaginal discharge, lung disease, necrotic lesions in major organs
	Sheep	Abscesses
Actinomyces species (unclassified)	Pigs	Pyogranulomatous mastitis
	Horses	Poll evil and fistulous withers
Trueperella pyogenes	Cattle, sheep and pigs mainly	Chronic or acute suppurative mastitis, suppurative pneumonia, septic arthritis, vegetative endocarditis (cattle), endometritis, umbilical infections, wound infections and seminal vesiculitis (bulls and boars). Common in mixed infections with *Fusobacterium necrophorum* 'Summer mastitis' (cattle), a mixed infection with *Peptoniphilus indolicus* and *Streptococcus dysgalactiae*
Actinobaculum suis	Pigs	Cystitis, pyelonephritis,
Nocardia species	Dogs (cats)	Canine nocardiosis Localized cutaneous granulomatous abscess Pyothorax and granulomas in the thoracic cavity
	Cattle	Chronic granulomatous mastitis. Bovine farcy in tropical regions*
	Pigs, sheep, goats and others	Occasional infections: pneumonia, mastitis and lymphadenitis
	Whales, dolphins and birds	Uncommon infections: respiratory involvement with dissemination to other tissues
Dermatophilus congolensis	Cattle, horses, sheep and goats mainly but many animal species and man can be infected	The disease has many names: 'rain-scald', streptothricosis, dermatophilosis and in sheep: mycotic dermatitis (general infection), 'lumpy wool' (wool-covered skin) and 'strawberry footrot' (skin of lower leg and coronet) Skin infection, either focal or spreading over large areas of the body. Most common in tropical and subtropical regions
Saccharopolyspora rectivirgula	Horses	The spores, in hay, of this thermophilic actinobacterium have been associated with the allergic component of recurrent airway disease

*some mycobacteria have also been implicated in bovine farcy

in diameter, within which club colonies can be found if the granules are crushed and examined microscopically. Strains of *Actinomyces* similar to *A. bovis* have been isolated from sows with granulomatous lesions in the mammary glands. These porcine strains show minor biochemical and antigenic differences from the bovine strains. *Actinomyces hyovaginalis* is associated with abortions and purulent vaginal discharge in sows and with infections in various sites in pigs and in sheep (Collins et al. 1993, Storms et al. 2002, Foster et al. 2012). *A. viscosus* causes a clinical

syndrome in dogs indistinguishable from that initiated by *Nocardia* species. Two syndromes can occur, either separately or together. One is a localized granulomatous lesion involving skin and subcutis, the other is a pyothorax with granulomas in thoracic tissue and often a large accumulation of sanguineopurulent pleural fluid containing soft white granules about 1 mm in diameter.

Trueperella pyogenes produces a range of virulence factors, the most significant of which is a haemolytic exotoxin, pyolysin (Table 10.3). This toxin is cytolytic for neutrophils and macrophages. Adhesion to host tissues is facilitated by neuraminidases and extracellular matrix-binding proteins. The organism also possesses fimbriae, produces proteases and can form biofilms but the exact role of these factors in disease production has not been definitively established.

Nocardia species

Nocardiae are difficult to definitively identify using phenotypic methods and many species have been reclassified in recent years based on molecular techniques. These aerobic, and essentially saprophytic, bacteria cause suppurative and pyogranulomatous reactions in immunosuppressed hosts or animals that have been exposed to large doses of the bacterium. The pathogenic nocardiae survive within phagocytic vacuoles by preventing phagolysosome formation. This is probably due to the surface lipids; *Nocardia* species have a cell wall similar to the mycobacteria. Genes encoding putative virulence factors have been identified in some *Nocardia* species but the molecular pathogenesis of nocardial infections in animals is currently unclear. Superoxide dismutases and catalases appear to be important in resistance to phagocytosis and cell lipids may provoke the characteristic granulomatous reaction. Exudates are sanguineopurulent and can sometimes contain soft granules consisting of bacteria, neutrophils and debris. They lack the microstructure of the sulphur granules produced by some of the *Actinomyces* species. *Nocardia asteroides* was previously considered to account for the majority of infections in animals but following their reclassification, a number of species are now known to be associated with animal disease (Table 10.2). Nocardiform placentitis associated with a number of different members of the *Actinobacteria*, in particular *Amycolatopsis* species and *Crossiella equi*, have been documented in the USA and sporadically elsewhere (Erol et al. 2012).

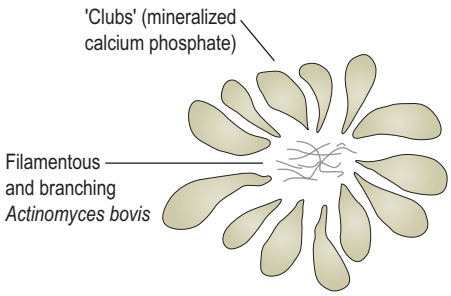

Figure 10.2 Diagrammatic representation of a club colony.

Dermatophilus congolensis

Dermatophilus congolensis causes skin infections that can affect many animal species and humans, but the condition is most commonly seen in cattle, sheep, goats and horses and polar bears in zoological collections. The infection is characterized by the formation of thick crusts which come away easily with a tuft of hair, leaving a moist, depressed area with bleeding points from capillaries. Virulence factors include an alkaline ceramidase and a number of proteases which facilitate invasion of the epidermis (García-Sánchez et al. 2004, Norris et al. 2008). Infections can be localized but have a tendency to spread over large areas of the body and the morbidity and mortality can be high, especially in tropical regions. The position of the lesions varies with the predisposing conditions. In periods of high rainfall the lesions tend to occur along the backs of animals. Where there is a heavy infestation with *Amblyomma* ticks the lesions are present in the predilection sites of the ticks: dewlap, axillae, udder, scrotum and escutcheon. In the dry season, in tropical regions, when feed is scarce the lesions are on the muzzle, head and lower limbs due to the animals foraging in thorn-covered scrub.

Table 10.3 Important virulence factors of *Trueperella pyogenes* and *Dermatophilus congolensis*

	Genes encoding virulence factor	Virulence factor	Role in virulence
Trueperella pyogenes	*plo*	Pyolysin	Cytolytic
	nanH, nanP	Neurominidases	Adhesion
	cbpA	Collagen binding protein A	Adhesion
	fimA, fimB, srt	Fimbriae	Adhesion
Dermatophilus congolensis	*agc*	Alkaline ceramidase	Facilitates spread in the epidermis
	nasp	Protease	Tissue/keratin breakdown

The pathogenic actinobacteria and the diseases that they cause are summarized in Table 10.2 and virulence factors of *T. pyogenes* and *D. congolensis* are given in Table 10.3.

Laboratory Diagnosis of *Actinomyces*, *Trueperella* and *Actinobaculum* Species

Specimens

Specimens include pus, exudates, aspirates, tissue and scrapings from the walls of abscesses if they have been incised. A volume of fluid or pus should be collected and submitted, if possible, rather than just a small amount on a swab. Thin sections of granulomas in 10% formalin are useful for histopathology.

Direct microscopy

Sulphur granules are the best specimens for direct examination in infections caused by *A. bovis* or *A. viscosus*. The pus or exudate is placed in a Petri dish and washed carefully with a little distilled water to expose the yellowish sulphur granules of *A. bovis* (Fig. 10.3) or the softer greyish-white granules of *A. viscosus*. A granule is placed on a microscope slide in a drop of 10% KOH and gently crushed by applying pressure on the coverslip. The characteristic clubs can be seen if the preparation is examined under the low-power objective of a microscope. A view of a club colony, in situ, can be seen in stained histological sections (Fig. 10.4). If smears are made from the granules and stained with the Gram stain, delicate, Gram-positive, branching filaments (Fig. 10.5) can be observed. Occasionally short filaments or pleomorphic diphtheroidal forms may predominate.

Gram-stained smears of pus or mastitic milk samples in *T. pyogenes* infections usually reveal large numbers of small, highly pleomorphic, Gram-positive forms (Fig. 10.6). They tend to be a mixture of cocci, rods and pear-shaped cells. Occasionally short branching forms may be seen.

Isolation

The *Actinomyces* species grow well on sheep or ox blood agar. *Actinomyces bovis* requires anaerobic conditions with 5–10% CO_2 added (H_2 + CO_2 commercial envelope). *Actinomyces viscosus* and *T. pyogenes* will grow aerobically but 5–10% CO_2 will enhance their growth. They are all incubated at 37 °C, *A. bovis* and *A. viscosus* usually require two to four days but the growth of *T. pyogenes* can usually be seen in 24 hours. The isolation of *A. hordeovulneris* is greatly enhanced by 10–20% foetal calf serum, although it will grow on blood agar at 37 °C under 10% CO_2 with either aerobic or anaerobic conditions. Colonies are visible in about three days.

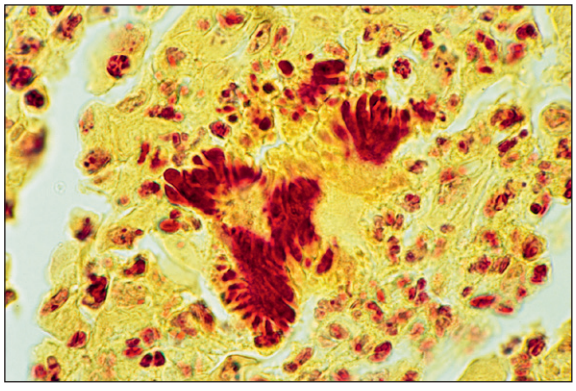

Figure 10.4 Club colonies in a tissue section from an *A. bovis* infection in a cow with lumpy jaw. (Plaut stain, ×400)

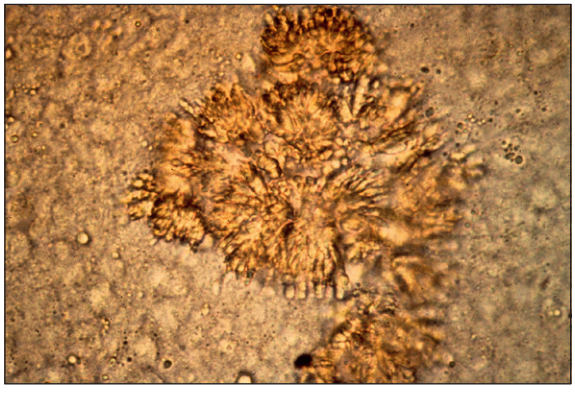

Figure 10.3 Sulphur granule in pus from an *Actinomyces bovis* infection. (Unstained, ×25)

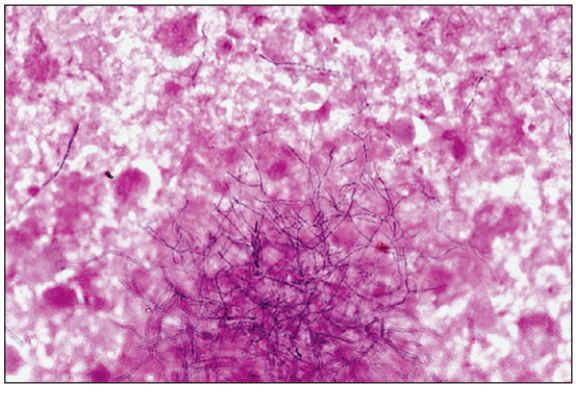

Figure 10.5 Gram-positive branching filaments of *A. bovis* in bovine tissue. (Gram stain, ×1000)

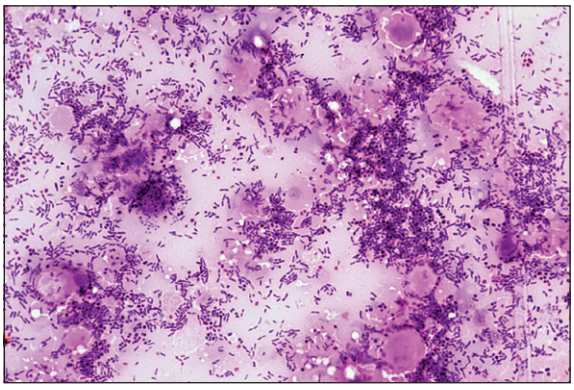

Figure 10.6 Gram-positive pleomorphic rods of *T. pyogenes* in a bovine mastitic milk sample. (Gram stain, ×1000)

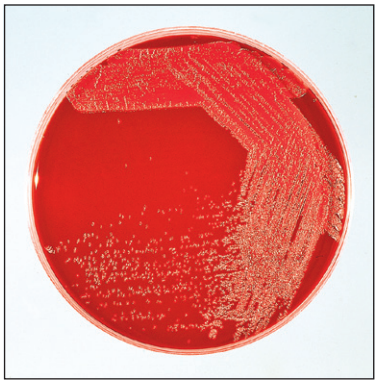

Figure 10.7 *Actinomyces bovis* on sheep blood agar after four days' incubation.

Identification

Colonial morphology and microscopic appearance

- *Actinomyces bovis* colonies are non-haemolytic, white, rough or smooth and adhere tenaciously to solid medium (Fig. 10.7). The colonies never attain a diameter of much more than 1 mm. Gram-stained smears show Gram-positive, slightly branched filaments or short forms. On subculture the bacterium may become diphtheroidal or coccobacillary (Fig. 10.8). *Actinomyces bovis* grows well in thioglycollate medium giving a diffuse growth in about 7–10 days (Fig. 10.9).
- *Actinomyces viscosus* commonly produces two colonial forms, one being smooth, entire, convex and glistening, while the other is smaller, rough, dry and irregular (Fig. 10.10). Neither form is haemolytic. The larger colonial type yields Gram-positive diphtheroidal forms (Fig. 10.11) and the smaller colony has short branching filaments (Fig. 10.12).

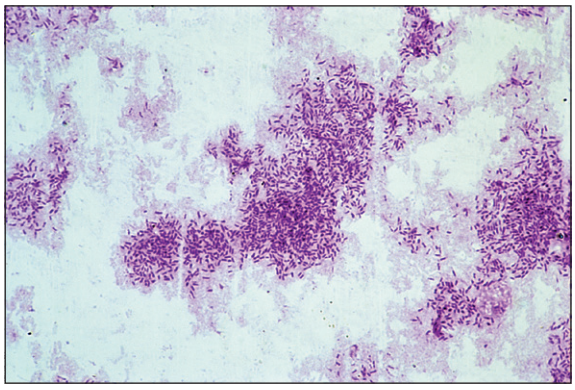

Figure 10.8 A Gram-stained smear from an *A. bovis* culture showing diphtheroidal forms typical of repeatedly subcultured isolates. (×1000)

Figure 10.9 *Actinomyces bovis* in thioglycollate medium after 10 days' incubation showing characteristic diffuse growth.

- *Trueperella pyogenes*: after 24 hours' incubation a hazy haemolysis may be noticed along the streak lines before the minute colonies can be seen. At 48 hours' incubation the tiny 1 mm colonies are visible surrounded by a narrow zone of complete haemolysis (Fig. 10.13). A Gram-stained smear

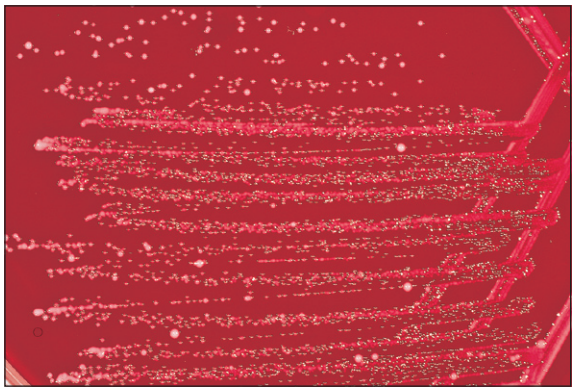

Figure 10.10 Close-up of *A. viscosus* on sheep blood agar showing the two colonial types. The smaller, dry, irregular type is predominating but about ten of the larger, smooth, glistening colonial variants are evident.

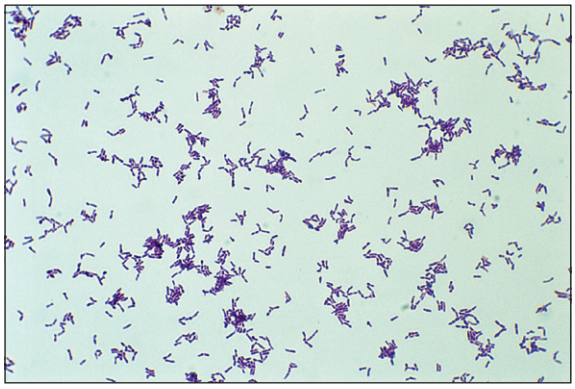

Figure 10.11 Gram-stained smear of the larger, smooth colonial variant of *A. viscosus* yielding Gram-positive diphtheroidal forms. (×1000)

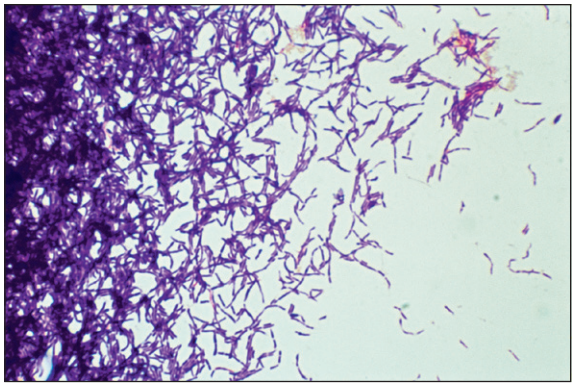

Figure 10.12 Gram-stained smear of the smaller, dry colonial variant of *A. viscosus* showing short branching filaments. (×1000)

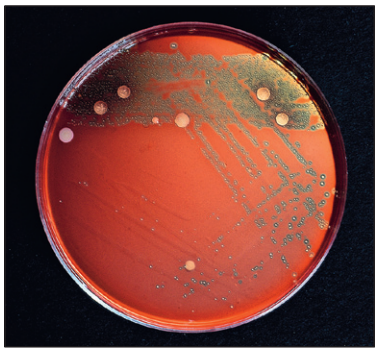

Figure 10.13 Small haemolytic colonies of *T. pyogenes* on a sheep blood agar diagnostic plate after 72 hours' incubation (large colonies are contaminants).

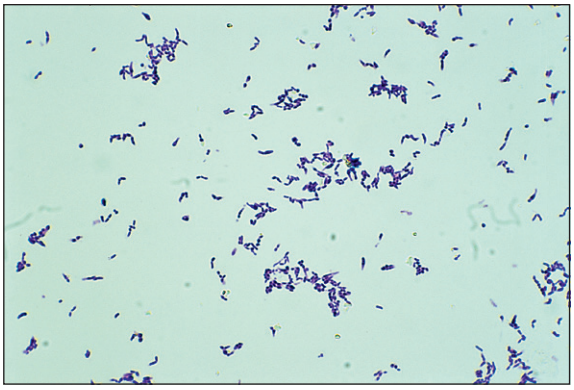

Figure 10.14 A Gram-stained smear taken from a *T. pyogenes* culture showing the typical pleomorphic appearance of the bacterium. (×1000)

reveals the typical pleomorphic, Gram-positive rods (Fig. 10.14).

- *Actinomyces hordeovulneris* colonies are about 2 mm in diameter after 72 hours' incubation. They are white, non-haemolytic, 'molar-toothed' (with ridges and valleys) but become conical and domed after further incubation. The colonies adhere firmly to the agar and a weak haemolysis may be produced after seven days' incubation.

Biochemical tests

Specialized methods are required for the identification of most of the *Actinomyces* species and are usually only performed in reference laboratories. These laboratories may use a fluorescent antibody technique to differentiate the species but molecular methods are more frequently employed currently. A rapid, presumptive test for *T. pyogenes* is to demonstrate its ability to pit a Loeffler serum slope in 24–48 hours (Fig. 10.15). A loopful of a pure culture of the bacterium is taken and a heavy inoculum is

made in a small area in the centre of the slope, taking care not to break the surface of the medium. The medium is incubated at 37°C for 24–48 hours. Table 10.4 lists the main features of the actinobacteria covered in this chapter.

Summary of the features allowing a presumptive identification of Actinomyces, Trueperella *and* Actinobaculum *species*

- Clinical and pathological findings and the animal species affected.
- The presence of granules in pus or exudates (*A. bovis* and *A. viscosus*).
- Demonstration of fine Gram-positive, branching filaments or pleomorphic diphtheroidal forms on direct microscopic examination. To distinguish *Actinomyces* species from *Nocardia* species:
  - The filaments of *Actinomyces* species do not fragment into bacillary forms.
  - *Nocardia* species are partially acid-fast (MZN-positive) but the *Actinomyces* species are MZN-negative.
- The presence of club-colonies in histopathological sections (*A. bovis*), although these can also occur in some other chronic infections such as bovine actinobacillosis and botryomycosis (*Staphylococcus aureus*).
- Isolation of the *Actinomyces* species on blood agar, under the appropriate atmospheric conditions, and with a consistent colonial appearance. The *Actinomyces* species cannot grow on Sabouraud dextrose agar while *Nocardia asteroides* tolerates this medium.
- The characteristic appearance in Gram-stained smears from the colonial growth that fits the *Actinomyces* species under investigation.
- Pitting of Loeffler serum slope and CAMP test for the presumptive identification of *T. pyogenes*.
- Urease is produced by *Actinobaculum suis*.

Figure 10.15 Loeffler serum slope: 'pitting' of slope by *T. pyogenes*, uninoculated or negative slope (right).

Laboratory Diagnosis of *Nocardia* Species

Specimens

Specimens should include exudates, aspirates, mastitic milk samples, tissue from granulomas and thin sections from granulomas in 10% formalin for histopathology.

Direct microscopy

The soft granules are not common in exudates from nocardial infections. Gram- and MZN-stained smears are made

Table 10.4 Characteristics of *Actinomyces* species, *Trueperella pyogenes* and *Actinobaculum suis*

Feature	*Actinomyces bovis*	*Actinomyces viscosus*	*Trueperella pyogenes*	*Actinobaculum suis*	*Actinomyces hordeovulneris*
Granules in pus	+ (sulphur granules)	+ (soft and whitish)	–	–	–(+)
Direct microscopic examination	F(D)	F/D	D	D	F/D
Atmospheric growth requirements	Anaerobic + CO_2	Aerobic (CO_2)	Aerobic (CO_2)	Anaerobic	Aerobic + CO_2 or anaerobic + CO_2
Catalase	–	+ (weak)	–	–	+(weak)
Pitting of Loeffler serum slope	–	–	+	–	–

+ = positive reaction, – = negative, –(+) = most species are negative (a few positive), F = filamentous and branching form, D = shorter diptheroidal form, CO_2 = growth enhanced by carbon dioxide

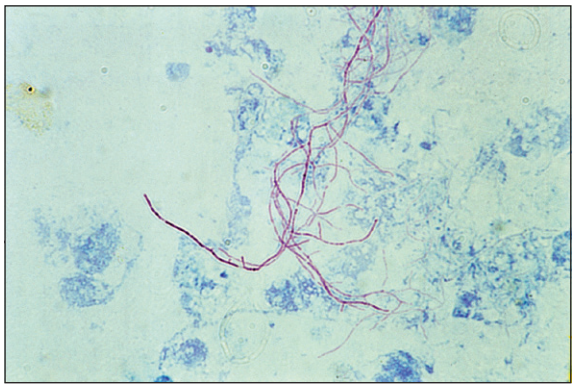

Figure 10.16 A modified Ziehl–Neelsen (MZN)-stained smear of *Nocardia asteroides* in a canine thoracic aspirate: red (MZN-positive) branching filaments. (×1000)

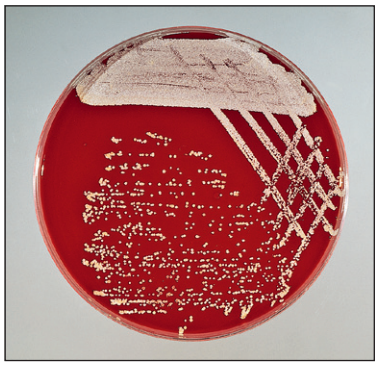

Figure 10.18 *Nocardia asteroides* on sheep blood agar after five days' incubation. The vivid white, powdery colonies are firmly adherent to the medium.

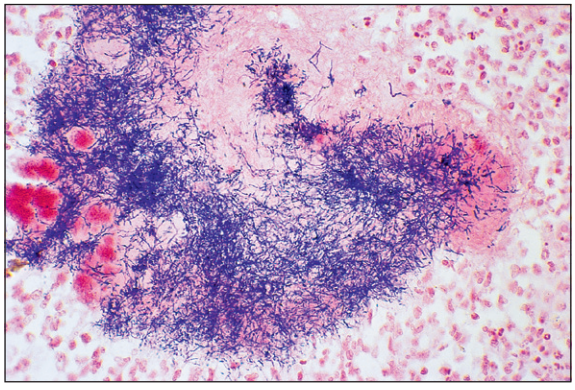

Figure 10.17 A Gram-stained section of a thoracic granuloma in a dog showing a microcolony of *N. asteroides*, present as branching Gram-positive filaments. (×400)

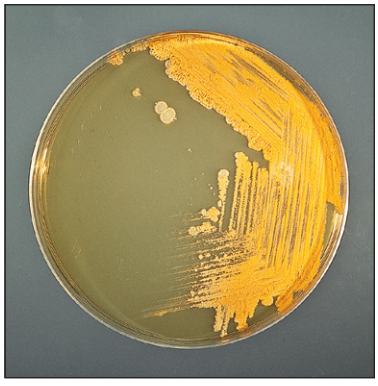

Figure 10.19 *Nocardia asteroides* on Sabouraud dextrose agar after seven days' incubation. The colonies are orange, dry and wrinkled.

from exudates, aspirates, granulomatous tissue and from centrifuged deposits of bovine mastitic milk. Gram-stained smears reveal Gram-positive branching filaments that often show some fragmentation into coccobacillary elements. The MZN-stained smears exhibit a similar morphology but most of the filaments retain the carbol fuchsin dye and stain red (Fig. 10.16). Microcolonies of nocardiae can be demonstrated in stained histopathological tissue sections (Fig. 10.17).

Isolation

Blood agar plates are inoculated with the specimens and incubated aerobically at 37°C for up to seven days, although growth should be evident in four to five days. Any suspect colonies could be used to heavily inoculate a Sabouraud dextrose agar plate. This is incubated at 37°C for up to 10 days.

Identification

Colonial appearance

The colonies on blood agar are often a vivid white and powdery in appearance if aerial filaments and spores are formed (Fig. 10.18). Use of a stereomicroscope facilitates the early identification of aerial filaments. Occasionally the colonies are smooth, heaped and variably pigmented. Both types of colonies are firmly adherent to the agar surface. The colonies on Sabouraud dextrose agar are dry, wrinkled and yellow, becoming deep orange with age (Fig. 10.19). The non-pathogenic *Streptomyces* species form white, powdery colonies, embedded in the agar, on blood agar and nutrient agar (Fig. 10.20). These are very similar in appearance to those of *Nocardia* species. *Streptomyces* species are also able to grow on Sabouraud dextrose agar. However, *Streptomyces* species have a characteristic and powerful earthy odour.

Figure 10.20 *Streptomyces* species on nutrient agar showing the white, powdery colonies, similiar to those of *N. asteroides*. *Streptomyces* species have a characteristic earthy odour.

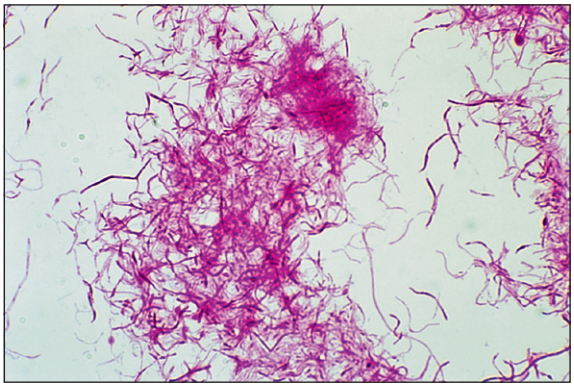

Figure 10.21 A modified Ziehl–Neelsen (MZN)-stained smear from a culture of *N. asteroides* showing red (MZN-positive) filaments. (×1000)

Microscopic appearance

Gram-stained smears from colonies show Gram-positive branching filaments that characteristically break up into rods or coccobacillary elements with age. A MZN-stained smear from young cultures reveals red-staining, branching filaments (Fig. 10.21). The filaments of *Streptomyces* and *Actinomyces* species are MZN-negative and stain blue with the counter-stain.

Biochemical reactions

Tests such as decomposition of casein, hypoxanthine, tyrosine, urea and xanthine are carried out in reference laboratories to differentiate between the *Nocardia* species. Reactions of many of the described nocardial species in these tests are given in Brown-Elliott et al. (2006). However, molecular methods are now preferred as these organisms are relatively unreactive biochemically.

Table 10.5 Differentiation of the causative agents of canine nocardiosis and canine actinomycosis

	Canine nocardiosis	Canine actinomycosis
Aetiology	*Nocardia* species	*Actinomyces* viscosus
Granules in exudates	Not common	Usually present
Filaments MZN-positive	+	–
Fragmentation of filaments	+	–
Growth on Sabouraud dextrose agar	+	–
Powdery white colonies (aerial hyphae)	+	
Susceptibility to penicillin	–	+

Differentiation of A. viscosus *and* Nocardia *species*

This is important in infections in dogs as the two bacteria cause indistinguishable clinical syndromes but *A. viscosus* infections respond well to penicillin and other commonly used antibiotics, whereas nocardial infections are often refractory to treatment and are only susceptible to a comparatively limited range of antimicrobial agents. Trimethoprim-sulfamethoxazole or erythromycin have been suggested as therapeutic agents in nocardial infections. Table 10.5 summarizes the differences in laboratory findings for canine nocardiosis and canine actinomycosis.

Laboratory Diagnosis of *Dermatophilus congolensis*

Specimens

A tuft of hair that is plucked from the lesion usually detaches with scab material adhering to it (Fig. 10.22).

Direct microscopy

Small pieces of material are shaved from the scab with a scalpel and the flakes of scab are softened in a few drops of distilled water on a microscope slide. A smear is made, taking care to leave a few flakes of scab material intact. The smear can be stained by either Giemsa or Gram stains. The

Figure 10.22 A tuft of hair being plucked from a horse with streptothricosis (*Dermatophilus congolensis*). The hairs came away with an adherent crust. The uneven appearance of the coat correlates with the distribution of lesions.

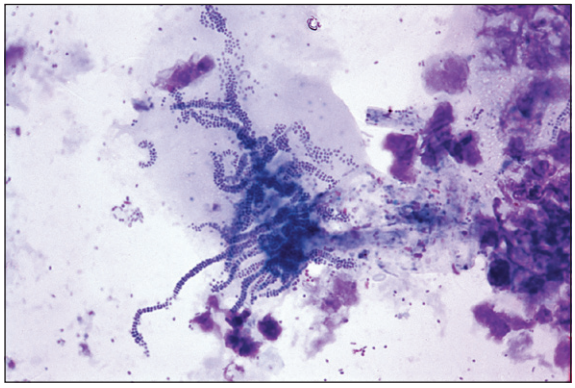

Figure 10.23 A Giemsa-stained smear of scab material from a case of bovine streptothricosis showing the branching filaments and zoospores of *D. congolensis*. (×1000)

Giemsa is the better stain to show the characteristic morphology of the bacterium (Fig. 10.23). If the conventional Gram stain is used, both the cells of *D. congolensis* and surrounding debris seem to absorb the crystal violet-iodine complex avidly and stain too darkly. A modification of the Gram stain is to leave the crystal violet on the smear for only two to three seconds, after which the morphology of the bacterium is easier to see (Fig. 10.24). The appearance of *D. congolensis* is so unique that a strong presumptive diagnosis of streptothricosis (dermatophilosis) can be made based on the direct examination of stained smears alone.

Dermatophilus congolensis is filamentous and branching. Mature filaments are composed of motile, coccal zoospores, in parallel lines, at least two abreast, resulting in a

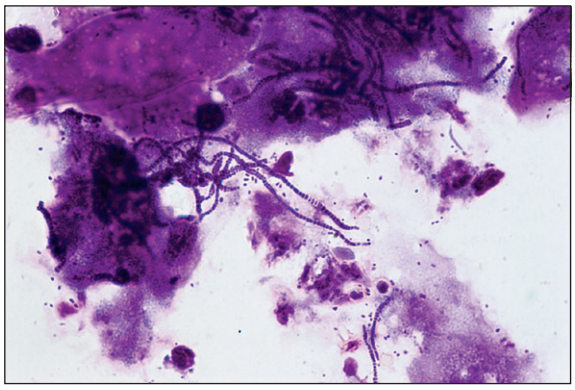

Figure 10.24 A Gram-stained smear of *D. congolensis* in bovine scab material. The zoospores take up the crystal violet dye avidly and are sometimes less easy to visualize than in a Giemsa-stained smear. (×1000)

'tram-track'-like appearance. The zoospores are about 1 µm in diameter. If the flakes of scab are treated too roughly, when the smears are made, the filaments will disintegrate and only Gram-positive cocci (zoospores) will be seen. Figure 10.25 shows, diagrammatically, the developmental cycle of *D. congolensis*. Transverse, horizontal and vertical septa form in the immature filaments dividing them into coccal zoospores. When mature, these zoospores are motile by polar flagella and are infective. They can initiate an infection in macerated or traumatized skin of the host animal.

Isolation

Although the isolation of *D. congolensis* may not be necessary for a diagnosis of streptothricosis, the bacterium is comparatively easy to culture and grows well on sheep or ox blood agar. An atmosphere of 5–10% CO_2 enhances the growth of the organism, especially on primary isolation. The inoculated plates are incubated at 37°C for up to five days, although colonies may be seen after 24–48 hours' incubation.

Scab material contains many contaminants and Haalstra's method (Box 10.1) was developed to overcome this problem.

Identification

Colonial appearance

Small (about 1 mm) greyish-yellow, distinctly haemolytic colonies can be seen after 24–48 hours' incubation. They are firmly adherent to the medium and appear to be embedded in the agar. After three to four days, isolated colonies can be 3 mm in diameter and are rough, wrinkled with a golden-yellow colour (Figs 10.26 and 10.27). Older colonies can become mucoid. No growth occurs on Sabouraud dextrose agar.

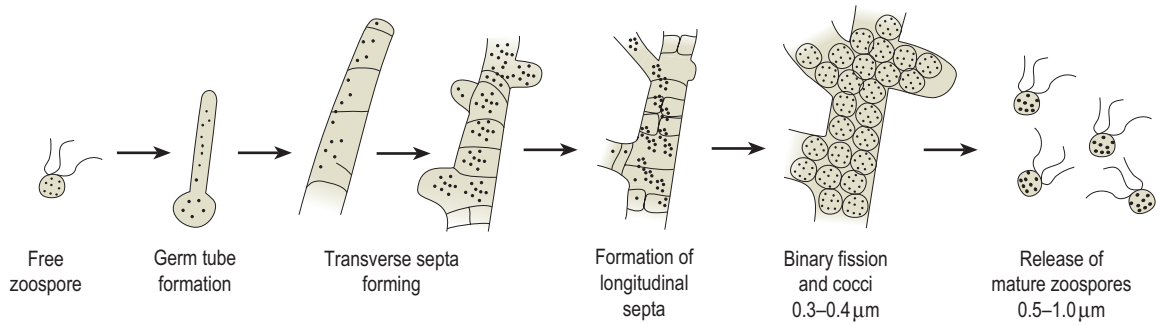

Figure 10.25 Developmental cycle of *Dermatophilus congolensis*.

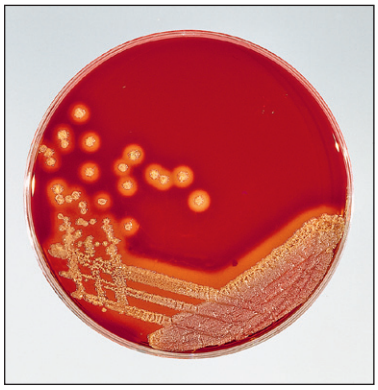

Figure 10.26 A culture of *D. congolensis* on sheep blood agar after three days' incubation in 10% CO_2.

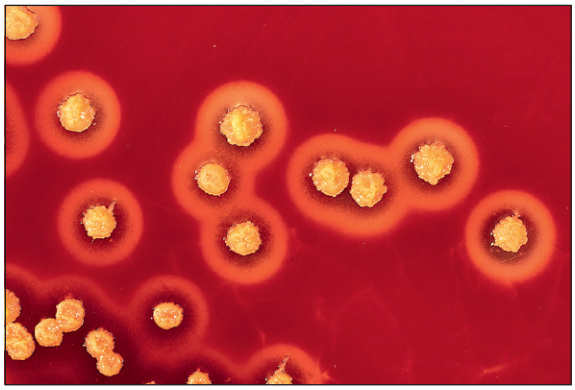

Figure 10.27 A close-up of the three-day-old haemolytic colonies of *D. congolensis* on sheep blood agar showing the rough, dry, golden-yellow appearance. They are firmly embedded in the medium.

Box 10.1 **Haalstra's method for the primary isolation of *Dermatophilus congolensis***

- Grind up a small amount of scab material and place a little in 2 ml distilled water in a bijou bottle for three hours at room temperature.
- Place the container, with lid removed, in a candle jar at room temperature for 15 minutes.
- The motile zoospores are chemotactically attracted to the carbon-dioxide-enhanced atmosphere in the candle jar and move to the surface of the distilled water. Remove a loopful of fluid from the surface and inoculate a blood agar plate. Incubate the inoculated plate at 37°C for 72 hours under 5–10% CO_2.

Microscopic appearance

Gram-stained smears from colonies do not show the characteristic 'tramtrack' appearance seen on direct microscopy. Usually the smears reveal uniformly staining, Gram-positive, branching filaments but sometimes coccal forms predominate.

Biochemical reactions

Biochemical tests are not usually carried out as a firm diagnosis will already have been made based on the unique microscopic appearance and the characteristic colonies. *Dermatophilus congolensis* is catalase-positive, urease-positive, gelatin-positive and produces acid from glucose, fructose and maltose. It is indole-negative, does not reduce nitrate and does not attack sucrose, salicin, xylose, lactose, sorbitol, mannitol or dulcitol.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

The preferred method of antimicrobial susceptibility testing for the organisms described in this chapter is broth microdilution (CLSI 2011). However, methods and interpretive standards are not available for all actinobacteria of veterinary importance. The pyogranulomatous nature of the lesions produced by many of the actinobacteria necessitates prolonged courses of antimicrobial agents for

treatment. Isolates of *T. pyogenes* are usually susceptible to many antimicrobial agents. However, macrolide resistance encoded by *ermB* and *ermX* has been recorded (Jost et al. 2003a, 2003b) and multiresistant *T. pyogenes* containing Class 1 integrons have been isolated from bovine endometritis in China (Liu et al. 2009). Resistance to sulphonamides and tetracycline has been recorded in up to 50% of isolates of *T. pyogenes* in Germany (Werckenthin et al. 2007). Ribeiro et al. (2008) reported that sulfamethoxazole-trimethoprim, amikacin, ceftiofur and gentamicin were the most effective agents in vitro against 28 veterinary clinical isolates of *Nocardia* species. Multiple drug resistance to three or more antimicrobials was observed in 36% of isolates. However, these authors used disk diffusion rather than the recommended broth microdilution method for testing.

Strain Typing

Although molecular typing methods have been widely employed in the investigation of human isolates of the actinobacteria, few studies have been performed in animals. Restriction fragment length polymorphism analysis of chromosomal DNA and ribotyping were used to characterize *N. farcinica* isolates from an outbreak of bovine mastitis. Results suggested that all isolates belonged to a single clone thought to have originated from contaminated intramammary tubes (Brown et al. 2007).

Random amplification of polymorphic DNA (RAPD) or PFGE has been used to differentiate epidemiologically unrelated isolates of *D. congolensis* from sheep, cattle, horse and deer. Both typing methods showed highly reproducible results. However, the discriminatory power of RAPD was greater than that of PFGE as the latter could not distinguish between epidemiologically related isolates. It was suggested that at least two independent primers should be used for RAPD typing in order to improve its discriminatory power, and that PFGE could be used for confirmation of RAPD results (Larrasa et al. 2004).

Molecular Diagnosis

PCR-mediated characterization of a number of gene targets including 16S rDNA, *sodA*, *plo* and the 16S-23S rDNA intergenic spacer region (ISR) can be used for the definitive identification of *T. pyogenes* (Hijazin et al. 2011). Amplification of the 16S rRNA gene or *hsp65* gene using PCR and subsequent digestion with specific endonucleases can be employed to identify *Nocardia* species (Brown-Elliott et al. 2006). In addition, PCR amplification followed by gene sequencing of the rRNA gene is frequently employed for nocardial identification. Sequencing of the rRNA gene has been used to confirm various *D. congolensis* infections (Sebastian et al. 2008; Byrne et al. 2010).

REFERENCES

Brown, J.M., Cowley, K.D., Manninen, K.I., et al., 2007. Phenotypic and molecular epidemiologic evaluation of a *Nocardia farcinica* mastitis epizootic. Veterinary Microbiology 125 (1/2), 66–72.

Brown-Elliott, B.A., Brown, J.M., Conville, P.S., et al., 2006. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. Clinical Microbiology Reviews 19 (2), 259–282.

Byrne, B.A., Rand, C.L., McElliott, V.R., et al., 2010. Atypical *Dermatophilus congolensis* infection in a three-year-old pony. Journal of Veterinary Diagnostic Investigation 22, 141–143.

Clinical and Laboratory Standards Institute (CLSI), 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes, approved standard, 2nd edn. CLSI document M24-MA2. Clinical and Laboratory Standards Institute, Wayne, PA.

Collins, M.D., Stubbs, S., Hommez, J., et al., 1993. Molecular taxonomic studies of *Actinomyces*-like bacteria isolated from purulent lesions in pigs and description of *Actinomyces hyovaginalis* sp. nov. International Journal of Systematic Bacteriology 43, 471–473.

Erol, E., Sells, S.F., Williams, N.M., et al., 2012. An investigation of a recent outbreak of nocardioform placentitis caused abortions in horses. Veterinary Microbiology 21 February 2012 (EPUB ahead of print).

Foster, G., Wragg, P., Koylass, M.S., et al., 2012. Isolation of *Actinomyces hyovaginalis* from sheep and comparison with isolates obtained from pigs. Veterinary Microbiology 157, 471–475.

García-Sánchez, A., Cerrato, R., Larrasa, J., et al., 2004. Identification of an alkaline ceramidase gene from *Dermatophilus congolensis*. Veterinary Microbiology 99 (1), 67–74.

Hijazin, M., Ulbegi-Mohyla, H., Alber, J., et al., 2011. Molecular identification and further characterization of *Arcanobacterium pyogenes* isolated from bovine mastitis and from various other origins. Journal of Dairy Science 94 (4), 1813–1819.

Jost, B.H., Field, A.C., Trinh, H.T., et al., 2003a. Tylosin resistance in *Arcanobacterium pyogenes* is encoded by an Erm X determinant. Antimicrobial Agents and Chemotherapy 47, 3519–3524.

Jost, B.H., Trinh, H.T., Songer, J.G., et al., 2003b. Identification of a second tylosin resistance determinant, Erm B, in *Arcanobacterium pyogenes*.

Antimicrobial Agents and Chemotherapy 48, 721–727.

Larrasa, J., Garcia-Sanchez, A., Ambrose, N.C., et al., 2004. Evaluation of randomly amplified polymorphic DNA and pulsed field gel electrophoresis techniques for molecular typing of *Dermatophilus congolensis*. FEMS Microbiology Letters 240, 87–97.

Liu, M.C., Wu, C.M., Liu, Y.C., et al., 2009. Identification, susceptibility, and detection of integron-gene cassettes of *Arcanobacterium pyogenes* in bovine endometritis. Journal of Dairy Science 92, 3659–3666.

Norris, B.J., Colditz, I.G., Dixon, T.J., 2008. Fleece rot and dermatophilosis in sheep. Veterinary Microbiology 128 (3/4), 217–230.

Ribeiro, M.G., Salerno, T., Mattos-Guaraldi, A.L., 2008. Nocardiosis: an overview and additional report of 28 cases in cattle and dogs. Revista Instituto Medicina Tropical de São Paulo 50 (3), 177–185.

Sebastian, M.M., Giles, R.C., Donahu, J.M., et al., 2008. *Dermatophilus congolensis*-associated placentitis, funisitis and abortion in a horse. Transboundary and Emerging Diseases 55, 183–185.

Storms, V., Hommez, J., Devriese, L.A., et al., 2002. Identification of a new biotype of *Actinomyces hyovaginalis* in tissues of pigs during diagnostic bacteriological examination. Veterinary Microbiology 84, 93–102.

Werckenthin, C., Alesík, E., Grobbel, M., et al., 2007. Antimicrobial susceptibility of *Pseudomonas aeruginosa* from dogs and cats as well as *Arcanobacterium pyogenes* from cattle and swine as determined in the BfT-GermVet monitoring program 2004–2006. Berliner und Munchener Tierarztliche Wochenschrif 120 (9–10), 412–422.

Chapter 11

Mycobacterium species

Genus Characteristics

The mycobacteria usually form thin rods of varying lengths (0.2–0.6 × 1.0–10.0 μm). Sometimes branching filamentous forms occur but these easily fragment into rods. They are non-motile, non-sporing, aerobic and oxidative. Although cytochemically Gram-positive, the mycobacteria do not take up the dyes of the Gram-stain because the cell walls are rich in lipids, particularly mycolic acid. They are characteristically acid-fast; once a dye has been taken up by the cells they are not easily decolourized, even by acid-alcohol. The rods tend to stain irregularly and often have a beaded appearance. The mycobacteria are closely related to the genera *Nocardia* and *Rhodococcus* and all three genera have a similar cell wall type. A comparatively slow growth rate is a characteristic of the mycobacteria, with generation times ranging from 2–20 hours. Some species (chromogens) produce carotenoid pigments. The genus includes animal and human pathogens as well as saprophytic members often referred to as 'atypical', 'anonymous' or 'non-tuberculous' mycobacteria. Some of these can occasionally cause disease in animals.

Runyon's Groups

Runyon (1959) grouped the atypical mycobacteria on the basis of pigmentation, colonial morphology and growth rate. The scotochromogens are those that produce yellowish-orange pigments whether incubated in the light or in the dark. The photochromogens will produce pigment only if exposed to light. For practical purposes, the slow-growing mycobacteria are defined as those that require over seven days' incubation, under optimal conditons, to produce easily seen colonies and the rapid growers as those requiring less than seven days. Although not all recently isolated species fit within the Runyon scheme, it is still a useful method of categorizing the atypical mycobacteria.

Natural Habitat

The source of the pathogenic mycobacteria is usually infected animals. *Mycobacterium bovis* is excreted in respiratory discharges, faeces, milk, urine and semen. *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* are shed in faeces while *M. tuberculosis* is mainly shed in respiratory discharges. Tuberculosis is typically a disease of captivity or domestication. However, wild animal reservoirs of *M. bovis* occur, such as badgers in Europe, brush-tailed opossums in New Zealand, Cape buffalo in East Africa, wild boar in Spain and deer in Europe and America. These hosts may be important in maintaining infection in cattle populations and interfere with the success of eradication programmes. So-called spillover hosts which can be infected by *M. bovis* but which do not maintain infection in the absence of contact with cattle may also be important sources of infection in some situations (Corner 2006).

The mycobacteria are resistant to physical influences and will retain their viability in soil and particles of dried faeces for many months. The atypical mycobacteria are widespread in soil, pastures, bogs and water.

Pathogenesis

Table 11.1 gives the mycobacterial diseases that occur in animals and Table 11.2 indicates the susceptibility of domestic animals and birds to the mycobacteria that cause tuberculosis.

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Table 11.1 Mycobacteria capable of causing disease in animals

Species	Host(s)	Significance
***M. tuberculosis* complex: slow growing**		
M. africanum	Humans	Human tuberculosis (mainly West Africa)
'M. canettii'	Humans	Human tuberculosis (mainly East Africa)
M. tuberculosis	Humans, captive primates, dogs, cattle, psittacine birds, canaries	Human tuberculosis (worldwide)
M. bovis	Cattle, deer, badgers, possums, humans, cats, other mammalian species	Bovine tuberculosis
M. microti	Voles, occasionally other mammalian species	Vole tuberculosis. Localized lesions seen in rabbits, calves and guinea pigs
M. caprae	Goats, cattle	Tuberculosis in goats
M. pinnipedii	Seals, sea-lions, occasionally other mammalian species including man	Tuberculosis in pinnipeds
Runyon's groups		
I. Photochromogens: slow-growing (over seven days' incubation) saprophytes but rare disease in man and animals		
M. kansasii	Deer, pigs and cattle	Tuberculosis-like disease. Isolated from lungs and lymph nodes
M. simiae	Humans (monkeys)	Isolated from lymph nodes of healthy monkeys. Pulmonary disease in man
M. marinum	Marine fish, aquatic mammals and amphibians	Fish tuberculosis: granulomatous and disseminated disease
M. vaccae	Saprophytic	Non-pathogenic
II. Scotochromogens: slow-growing, ubiquitous saprophytes found commonly in grasslands. Occasional disease in animals and humans		
M. scrofulaceum	Domestic and wild pigs, cattle and buffaloes	Tuberculosis-like lesions in cervical and intestinal lymph nodes
III. Non-chromogens: (slow growing)		
M. avium complex	Poultry and wild birds	Avian tuberculosis. Generalized form rare in mammals. Lesions in cervical lymph nodes
	Pigs	Intestinal lesions (rare)
	Horses, pigs and others	
M. intracellulare	Poultry and wild birds	Avian tuberculosis. Saprophyte in soil and water
	Pigs and cattle	Can be present in intestinal lymph nodes
	Non-human primates	Granulomatous enteritis (resembles paratuberculosis in cattle)
M. ulcerans	Cats	Nodulo-ulcerative skin lesions
M. xenopi	Cats	Nodulo-ulcerative skin lesions
	Pigs	Tuberculous lesion in lymph nodes of the alimentary tract

Table 11.1 Mycobacteria capable of causing disease in animals—cont'd

Species	Host(s)	Significance
IV. Rapidly growing mycobacteria: need less than seven days' incubation. Pigmentation variable. Saprophytes in soil, water and on plants. They are found regularly in intestines of pigs, ruminants and other animals. Occasionally pathogenic for animals.		
M. chelonae	Fish	Disseminated granulomatous lesions
	Turtles	Tuberculosis-like lesions in lungs
	Cattle	Granulomatous lesions in lymph nodes
	Manatees, cats and pigs	Abscesses and nodulo-ulcerative lesions in various tissues
	Monkeys	Abscesses in lymph nodes or disseminated disease
M. fortuitum	Cattle	Granulomatous lesions in lymph nodes and mammary glands
	Cats	Ulcerative, pyogranulomatous lesions of skin
	Dogs	Granulomatous lesions in skin and lungs
	Pigs	Granulomas in lymph nodes, joints and lungs
M. phlei	Cats	Nodulo-ulcerative lesions of skin (rare)
M. smegmatis	Cattle	Granulomatous mastitis
	Cats	Ulcerative skin lesions
Other mycobacteria		
M. avium subsp. *paratuberculosis*	Cattle, sheep, goats and other ruminants	Paratuberculosis (Johne's disease). Chronic, progressive, intestinal wasting disease
M. lepraemurium	Cats and rodents	Feline and murine leprosy respectively
M. leprae	Humans and 9-banded armadillo	Leprosy in humans. Replication in armadillos. Not isolated in vitro
Unidentified acid-fast bacterium	Cattle	Skin tuberculosis (lymphangitis)

Table 11.2 Susceptibility of animals to the mycobacteria that cause tuberculosis

	Mycobacterium tuberculosis	***Mycobacterium bovis***	***Mycobacterium avium* subsp. *avium***
Primates	+	+	–
Cattle	(+)	+	(+)
Sheep and goats	–	+	(+)
Pigs	(+)	+	(+)
Horses	_	+	+ (intestinal)
Dogs	+	+	–
Cats	+	+	–
Poultry	–	–	+
Canaries	+	–	+
Psittacine birds	+	–	–

+ = susceptible, (+) = slightly susceptible or may become sensitized, – = resistant to infection

Mycobacterium bovis

The pathogenesis of *M. bovis* infection in cattle involves the interplay of both host and bacterial factors. Genetic susceptibility of the host is thought to be important in determining the outcome of infection (Allen et al. 2010) as is magnitude of the infectious dose and route of infection. Infection is usually via the respiratory and intestinal tracts. However, the number of organisms required to produce infection in cattle is many times greater via the oral compared to the respiratory route. The organism has not been shown to produce toxic factors, rather virulence appears to reside largely in the lipids and waxes of the cell wall. Mycosides, phospholipids and sulpholipids are thought to protect the tubercle bacilli against phagocytosis. Glycolipids cause a granulomatous response and enhance the survival of phagocytosed mycobacteria. Waxes and various tuberculoproteins induce the delayed-type hypersensitivity detected in the tuberculin test. In previously unexposed animals, multiplication of the mycobacteria occurs within macrophages as phago-lysosome fusion is inhibited. Resistance to phagocytic killing allows continued intracellular and extracellular replication. Infected host cells and mycobacteria can reach local lymph nodes and from there may pass to the thoracic duct followed by general dissemination.

After the first week, cell-mediated immune reactions begin to modify the host response and activated macrophages are able to kill some mycobacteria. The cytokines TNF-α and IFN-γ are essential in the activation of macrophages and are produced by both the innate and adaptive immune responses. The aggregation of macrophages contributes to the formation of a tubercle or granuloma (Fig. 11.1) and a fibrous layer may encompass the lesion. Caseous necrosis occurs at the centre of the lesion and this may proceed to calcification (cattle) or liquefaction. Once cell-mediated immunity is established, lymphatic spread is retarded but T-lymphocyte-mediated reactions cause tissue damage and mycobacterial spread occurs by contiguous extension or via the erosion of bronchi, blood vessels or viscera to new areas. Haematogenous dissemination may produce miliary tuberculosis in animals such as deer, characterized by multifocal granuloma formation in major organs or on serosal surfaces.

Figure 11.1 A tuberculous lesion in a bovine lymph node (*Mycobacterium bovis* infection).

Mycobacterium avium

Transmission is usually by the faecal–oral route and in birds the lesions are characteristically found in the liver and can also be present in the intestines, spleen and bone marrow. Infection with *M. avium* may sensitize cattle to the tuberculin test.

Mycobacterium tuberculosis

Mycobacterium tuberculosis is transmitted by aerosols or fomites and lesions are principally found in the lungs and lymph nodes. Non-human primates, dogs, canaries and psittacine birds are susceptible to human tuberculosis.

Mycobacterium lepraemurium

Mycobacterium lepraemurium is thought to cause both feline and murine leprosy. The disease in cats is characterized by the formation of single and multiple nodules or granulomas in the skin, often with the development of non-healing ulcers. Less commonly, skin lesions in cats can be caused by some of the atypical mycobacteria.

Mycobacterium avium subspecies *paratuberculosis*

Cell-mediated immune phenomena are involved in the pathogenesis of disease caused by *M. avium* subsp. *paratuberculosis*. The organisms are found within macrophages, which are unable to kill them, in the submucosa of the ileocaecal area and adjacent lymph nodes. The ileum and colon are usually involved with extension to the rectum. The mucous membrane becomes thickened and permanently corrugated as a result of cellular infiltration. Lesions may be of either of two types, multibacillary (lepromatous) or paucibacillary (tuberculoid) and appear to be correlated with host immune response. Extensive pathology is correlated with high levels of IL-10 production whereas subclinical disease is associated with increased IFN-γ production (Sweeney et al. 1998, Khalifeh and Stabel 2004). Clinical signs include profuse diarrhoea and the disease is progressive, leading to emaciation and death. Diarrhoea may not necessarily be seen in sheep and goats. Mortality is ultimately caused by malabsorption of nutrients and loss of protein into the intestine.

Large numbers of mycobacteria are present in epithelioid and giant cells in the mucosa of clinically affected cattle and are shed in the faeces. Animals are thought to be infected in the neonatal period, primarily through faecally contaminated milk. Organisms are also shed in

colostrum and in utero infection may occur. Not all infected animals become clinical cases but remain subclinical excretors and these animals shed small numbers of mycobacteria intermittently. The incubation period is long, usually 18–24 months.

Atypical mycobacteria

Cases of disease involving these mycobacteria are comparatively rare. Predisposing factors may include a large infective dose and/or immunosuppression in the host. Some atypical mycobacteria can sensitize cattle to the tuberculin test.

Laboratory Diagnosis of Mycobacteria Causing Tuberculosis and Atypical Mycobacteria

Strict safety precautions must be enforced when working with specimens suspected of containing *M. bovis* or *M. tuberculosis*. These include preparing smears, processing specimens, inoculating media and adding reagents to biochemical tests. Both species are classified as Hazard Group 3 pathogens and all procedures should be carried out in a Category 3 laboratory although it may be acceptable to process some suspect diagnostic speciments using a Level 2 biological safety cabinet. As the mycobacteria are very resistant to disinfectants, care must be taken to use an effective one for discarding contaminated slides and instruments. All contaminated materials must be autoclaved before leaving the laboratory. Individuals who are immunocompromised in any way should not work with these organisms.

Specimens

Specimens from live animals might include aspirates from cavities, lymph nodes, biopsies, tracheobronchial lavages and the centrifuged deposit from about 50 mL milk in the case of suspected tuberculous mastitis. In the case of dead animals, fresh and fixed (in 10% formalin for histopathology) samples of lesions or a selection of lymph nodes from a tuberculin-reactor with no visible lesions are taken.

Direct microscopy

The Ziehl–Neelsen (ZN) stain is used to stain smears from lesions and other specimens. The mycobacteria appear as slender, often beaded, red-staining rods against a blue background (if methylene blue is the counter-stain). These can only be visualized if at least 5×10^4 mycobacteria/mL of material are present. The numbers of *M. bovis* are often low in bovine specimens but lesions of avian tuberculosis in poultry (Fig. 11.2) and *M. bovis* lesions in animals such as deer (Fig. 11.3) and badgers usually yield large numbers of mycobacteria.

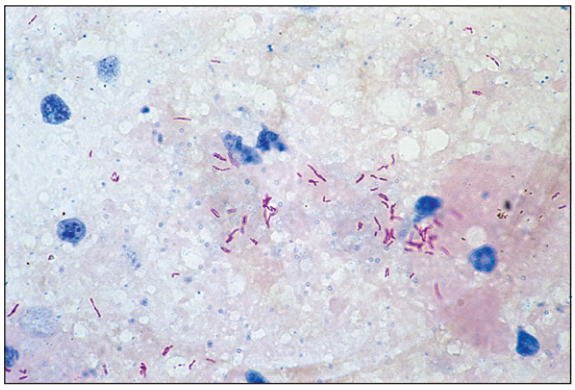


Figure 11.2 *Mycobacterium avium* in a ZN-stained smear of material from a tubercle in a pigeon demonstrating the numerous, slender, red-staining rods characteristic of avian tuberculosis. (×1000)

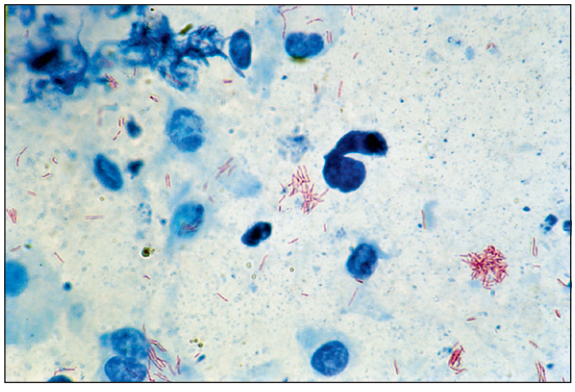


Figure 11.3 *Mycobacterium bovis* in a ZN-stained smear of material from a tubercle in a deer. The slender, beaded, red-staining (ZN-positive) rods tend to be more numerous in lesions from deer and badgers compared to the low numbers in bovine lesions. (×1000)

Smears stained by fluorescent dyes, such as auramine, acridine orange or fluorochrome, can be examined using a fluorescence microscope. These stains allow the mycobacteria to be seen more easily if relatively small numbers are present.

Isolation

Details of isolation procedures for *M. bovis* are given in Cousins (2009). Several preliminary procedures are necessary in order to recover the comparatively slow-growing mycobacteria:

- Selective decontamination to significantly reduce the number of fast-growing contaminant bacteria.
- Digestion or liquefaction of mucus. Mucin-trapped mycobacteria in specimens, such as tracheobronchial exudates, may not be available for growth in cultures.

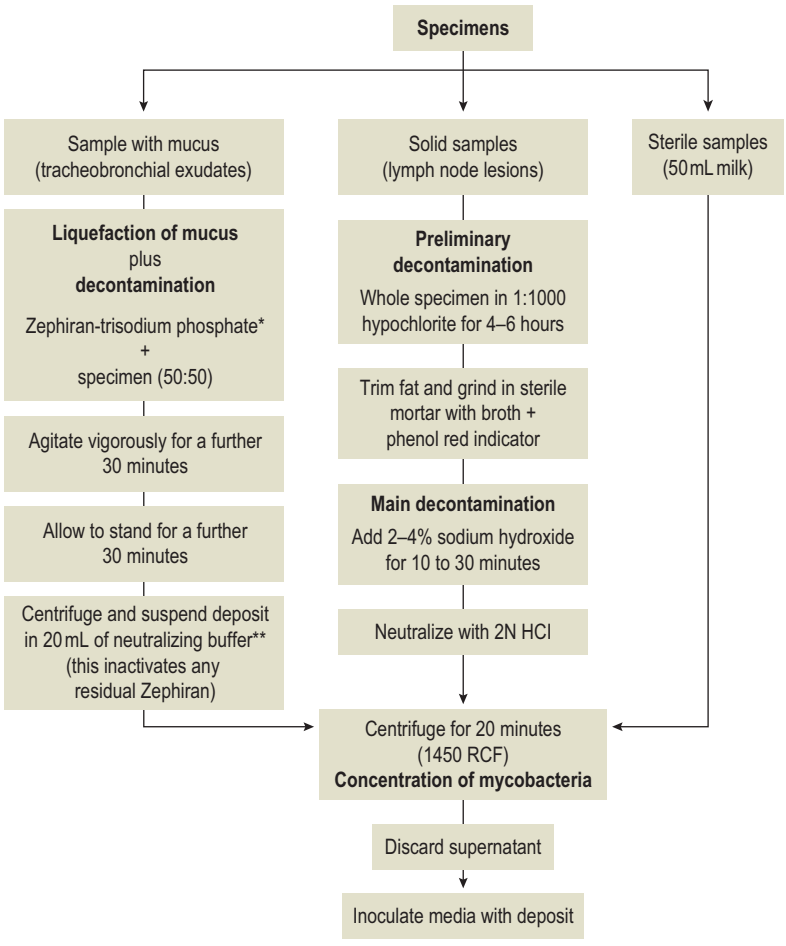


Figure 11.4 Procedure for recovery of mycobacteria from clinical specimens.

*Dissolve 1 kg trisodium phosphate (Na_3PO_4. 12 H_2O) in 4 litres of hot water
Add 7.5 mL of Zephiran concentrate (17% benzalkonium chloride, Winthrop Labs, NY)

**Add 37.5 mL of 0.067 M disodium phosphate to 62.5 mL of 0.067 M monopotassium phosphate. Final pH 6.6

- If mycobacteria are present in small numbers they must be concentrated to allow detection by stained smears and culture. This concentration can be done by centrifugation.

Mycobacteria are comparatively resistant to acids, alkalis and quaternary ammonium compounds. Decontaminating agents, such as 5% oxalic acid, 2–4% NaOH and 1% quaternary ammonium compound can be used on specimens. The mildest decontaminatng procedure that gives sufficient control over contaminants is desirable but even under optimal conditions 80–90% of the mycobacteria in the specimen may be killed by the decontaminating agent. Each laboratory will need to find the best compromise between destruction of contaminants and survival of sufficient mycobacteria for isolation. Examples of the procedures for recovering mycobacteria from specimens are given in Figure 11.4.

Media for the mycobacteria

The egg-based Lowenstein–Jensen and Stonebrinks media are most commonly used in veterinary bacteriology. Lowenstein–Jensen medium can be obtained commercially and the formula and method of preparation of Stonebrinks medium is given in Appendix 2. The media are prepared as solid slants in screw-capped bottles. Malachite green dye (0.025 g/100 mL) is commonly used as the selective agent. *Mycobacterium tuberculosis*, *M. avium* and many of the atypical mycobacteria require glycerol for growth. However, glycerol is inhibitory to *M. bovis* while sodium pyruvate (0.4%) enhances its growth. Thus media

with glycerol and without glycerol (but with Na pyruvate) should be inoculated. The media can be made more selective by the addition of cycloheximide (400 µg/mL), lincomycin (2 µg/mL) and nalidixic acid (35 µg/mL). Each new batch of culture medium should be inoculated with stock strains of mycobacteria to ensure that the medium supports satisfactory growth. The inoculated media may have to be incubated at 37°C for up to eight weeks for the mycobacteria in the tuberculosis group. *Mycobacterium tuberculosis* and *M. avium* prefer the caps on the culture media to be loose while *M. bovis* grows best in airtight containers. Liquid culture media may also be used for the isolation of mycobacteria and commercially available systems suitable for use in specialized laboratories processing large numbers of samples have been developed. Detection of growth of mycobacteria in these systems is based on radiometric or fluorometric methods. Examples include the BACTEC™ and MGIT™(Becton Dickinson) systems.

Most of the atypical mycobacteria will grow on media suitable for the tuberculosis-causing mycobacteria. *Mycobacterium leprae* and the mycobacterium causing bovine skin tuberculosis have not yet been cultured in vitro.

Identification

Colonial morphology

A summary of the colonial types of the mycobacteria of the tuberculosis group is given in Table 11.3. The luxuriant growth of *M. tuberculosis* on glycerol-containing media, giving the characteristic 'rough, tough and buff' colonies (Fig. 11.5), is known as eugonic. The growth of *M. avium* on media containing glycerol is also described as eugonic (Fig. 11.6). *Mycobacterium bovis* has sparse, thin growth on glycerol-containing media and this is referred to as dysgonic. However, *M. bovis* grows well on pyruvate-containing media without glycerol (Fig. 11.7). Cultures on Lowenstein–Jensen medium of some commonly isolated mycobacteria are illustrated (Figs. 11.8, 11.9, 11.10, and 11.11).

Pigment production and response to light

The mycobacteria that produce yellowish-orange carotenoid pigments are called chromogenic (Fig. 11.12). The

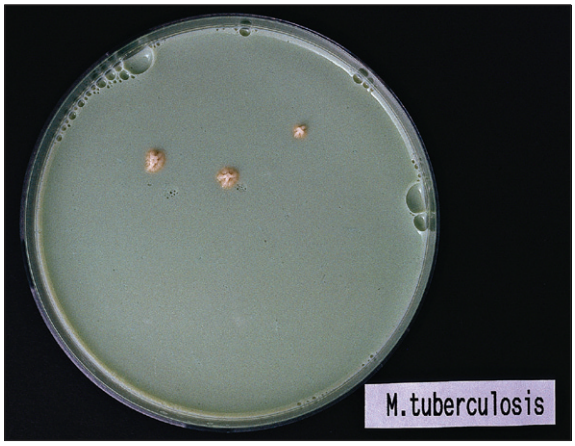


Figure 11.5 *Mycobacterium tuberculosis* on Lowenstein–Jensen medium with glycerol showing the typical colonial morphology ('rough, tough and buff').

Table 11.3 Differentiation of mycobacteria that cause tuberculosis

	Mycobacterium tuberculosis	***Mycobacterium bovis***	***Mycobacterium avium* subsp. *avium***
Growth-type and colony form on media with glycerol	Eugonic, 'rough, tough and buff'. Colonies hard to break up	Dysgonic. Small, moist-sheen colonies that break up easily	Eugonic. Whitish, sticky colonies that break up easily
Growth time	3–8 weeks	3–8 weeks	2–6 weeks
Glycerol required for growth	+	– (inhibited by glycerol)	+
Enhanced growth with 0.4% sodium pyruvate	–	+	–
Niacin production	+	–	–
Pyrazinamidase	+	–	+
Urease	+	+	–
Nitrate reduction	+	–	–
Inhibited by TCH (10 mg/mL)	–	+	–

+ = positive reaction, – = negative reaction, TCH = thiophen-2-carbonic acid hydrazide.

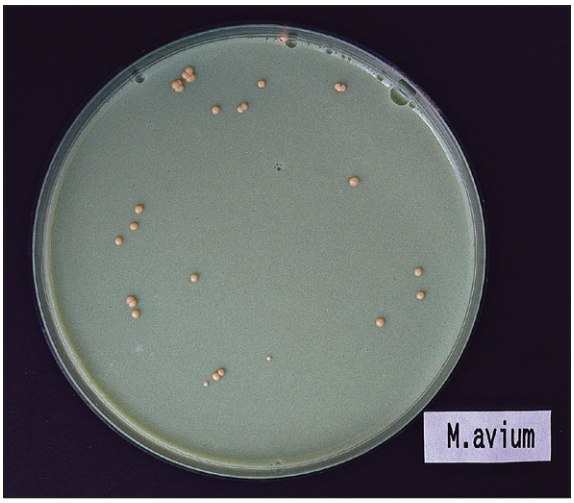


Figure 11.6 *Mycobacterium avium* colonies on Lowenstein–Jensen medium.

Figure 11.7 *Mycobacterium bovis* colonies on Lowenstein–Jensen medium with pyruvate.

term photochromogenic is applied to those mycobacteria that produce pigment only if exposed to light. The scotochromogenic mycobacteria produce pigment when incubated either in the light or in the dark. Pigment formation is tested with young, well-developed colonies on Lowenstein–Jensen medium. The cultures are exposed to a 100 watt, clear electric light bulb, at a distance of 50 cm, for at least an hour and then incubated again in darkness for a further one to three days. After this treatment the photochromogens will develop pigment. Older colonies of the mycobacteria in the tuberculosis group often have a yellowish hue but they are described as non-chromogenic.

Figure 11.8 Cultures of *M. tuberculosis* (left) and *M. bovis* (right) on Lowenstein–Jensen medium.

Figure 11.9 Cultures of *M. avium* (left) and *M. fortuitum* (right) on Lowenstein–Jensen medium. *M. fortuitum* is an example of a rapidly growing atypical mycobacterium that often produces pigment.

Microscopic appearance

The mycobacteria are acid-fast from cultures and appear as red-staining, thin, fairly long rods in ZN-stained smears.

Biochemical tests

Some of the biochemical reactions for the mycobacteria of the tuberculosis group are given in Tables 11.3 and 11.4, while those for the atypical mycobacteria are shown in Table 11.4. These methods of identification have largely been superseded by molecular methods. However, details

Figure 11.10 *Mycobacterium vaccae* (left) and *M. marinum* (right) on Lowenstein–Jensen medium. They are both photochromogens.

Figure 11.11 *Mycobacterium phlei* (left) and *M. smegmatis* (right) on Lowenstein–Jensen medium. Both are rapidly growing mycobacteria that often produce pigment.

of some of the more commonly performed biochemical tests are as follows:

- **Niacin production test**: commercially available niacin test strips (Becton Dickinson) are easier and safer to use as this avoids employing toxic BrCN solution used in conventional tests. *Mycobacterium tuberculosis* is the positive control while *M. avium* is negative in this test.
- **Nitrate reduction**: place a few drops of sterile distilled water in a screw-capped tube (16 × 125 mm) and add a loopful of a young culture of the mycobacterium. Use an uninoculated tube as a negative control. Add 2 mL of $NaNO_3$ solution (0.01 M solution $NaNO_3$ in 0.022M phosphate buffer, pH7). Shake and incubate in a water bath at 37 °C for two hours. Add:
  - 1 drop of 1 : 2 dilution of conc. HCl
  - 2 drops of 0.2% aqueous solution of sulphanilamide
  - 2 drops of 0.1% aqueous N-(1-naphthyl) ethylenediamine dihydrochloride.

  Examine for the development of a pink to red colour and compare with the negative control. A strong red indicates nitrate reduced to nitrite. Add a pinch of powdered Zn to all negative tubes (converts nitrate to nitrite). The production of a red colour indicates a negative test (nitrate not reduced). The commercial paper strip method can be used but a negative result should be confirmed by the above test.

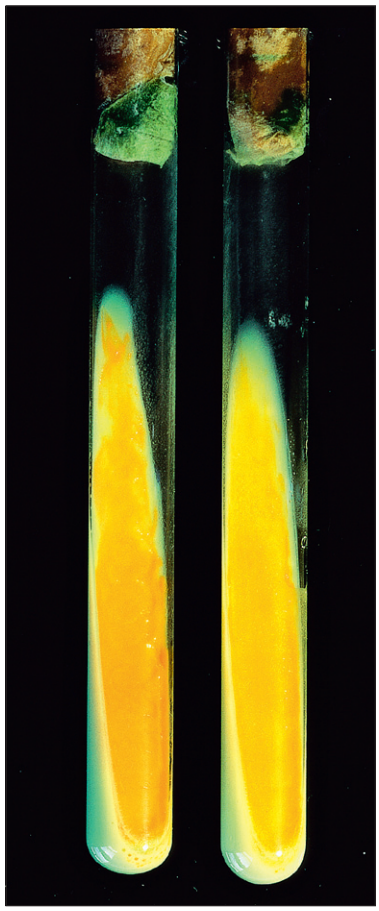


Figure 11.12 Two rapidly growing chromogenic mycobacteria isolated from moss in a wet pasture: *M. aurum* (left) and *M. aichiense* (right).

Table 11.4 In vitro tests for some clinically significant mycobacteria

	Runyon group	Growth within 7 days	Inhibition by glycerol	Colonial morphology	Pigmentation	Niacin production	Tolerance of 5% NaCl	Deamination of pyrazinamide (4 days)	Nitrate reduction	Urease production	Growth on MacConkey agar without crystal violet
Mycobacterium tuberculosis		–	–	R	N	+	–	+	+	v	–
M. bovis		–	+	S(R)	N	–	–	–	–	v	–
M. simiae	I	–	–	S	P	v	–	+	–	+	–
M. kansasii	I	–	–	SR/S	P	–	–	–	+	v	–
M. marinum	I	–	–	S/SR	P	– (+)	–	+	–	+	–
M. scrofulaceum	II	–	–	S	SC	–	–	v	–	v	–
M. avium complex	III	–	–	S/R	N	–	–	+	–	–	– (+)
M. ulcerans	III	–	–	R	N	–	–	–	–	v	–
M. xenopi	III	–	–	S	N (SC)	–	–	v	–	–	–
M. chelonae	IV	+	–	S/R	N	v	+ (–)	+	–	+	+
M. fortuitum	IV	+	–	S/R	N	v	+	+	+	+	+
M. phlei	IV	+	–	R	SC	nd	nd	nd	+	nd	–
M. smegmatis	IV	+	–	R/S	N	nd	nd	nd	+	nd	–

R = rough, S = smooth,SR = intermediate

P = photochromogenic (pigment produced only if culture is exposed to light)

SC = scotochromogenic (pigment produced in the light and in the dark)

N = non-chromogenic (no pigment produced)

v = variable reactions, – (+) = majority negative, + (–) = majority positive, nd = data not available

- **Deamination of Pyrazinamide** (to pyrazinoic acid) in four days: the medium is a Dubos broth base containing 0.1 g pyrazinamide, 2.0 g of pyruvic acid and 15.0 g agar per litre. Dispense in 15 mL amounts in screw-capped tubes. Autoclave at 121°C for 15 minutes and solidify in an upright position. Inoculate the agar with a heavy suspension of a young culture and incubate at 37°C for four days. Add 1 mL of freshly prepared 1% aqueous ferrous ammonium sulphate to the tubes and place in refrigerator for four hours. A positive reaction is given by a pink band in the agar. Use an uninoculated tube and an *M. avium* tube as negative and positive controls, respectively.
- **Urease Test:** mix 1 part of urea-agar base concentrate with 9 parts of sterile water. Dispense in 4 mL amounts in screw-capped tubes (16 × 125 mm). Emulsify a loopful of young culture in the tube of substrate. Incubate at 37°C and a colour change from amber to pink or red is a positive reaction. Discard the test after three days.
- **MacConkey Agar without crystal violet:** inoculate the agar plate with a loopful of a young broth culture of the mycobacterium making a spiral streak from the centre of the agar outwards. Incubate at 37°C and examine for growth after five and 11 days. Only strains of *M. fortuitum* and some subspecies of *M. chelonae* will grow to the end of the spiral streak.

Table 11.5 Inoculation of laboratory animals with mycobacteria that cause tuberculosis

	Mycobacterium tuberculosis	***Mycobacterium bovis***	***Mycobacterium avium* subsp. *avium***
Rabbits (intravenously)	± (pulmonary only)	++ (miliary)	++ (generalized)
Guinea pigs (subcutaneously)	++ generalized	++ generalized	– (+) focal
Chicken (intravenously)	–	–	++ generalized

++ = systemic reaction, ± = comparatively mild reaction, – (+) = localized infection, – = no infection

Other mycobacteria may grow where the inoculum is heaviest.

- **Inhibition and Tolerence Tests:** reagents such as 5% NaCl and thiopen-2-carbonic acid hydrazide (TCH) 10 µg/mL are usually incorporated into media such as Lowenstein–Jensen.

For further details of these and other biochemical tests consult Roberts et al. (1991).

Animal inoculation

This historical method of distinguishing between the tubercle bacilli was based on the variation in the pathogenicity of each for different laboratory animals (Table 11.5). Animal inoculation is now rarely performed because of ethical and economic reasons as well as the risk of infection to laboratory staff.

Field and laboratory immunological tests for tuberculosis

Tests used in national eradication schemes are based on the delayed-type hypersensitivity reaction elicited in animals, infected with tubercle bacilli, after intradermal inoculation of a small amount (usually 0.1 mL) of tuberculin. Tuberculin, a purified protein derivative (PPD) prepared from *M. bovis* or *M. avium*, is a complex mixture of proteins, lipids, carbohydrates and nucleic acids.

Intradermal skin testing using PPD has proved to be an effective diagnostic test for identifying *M.-bovis*-infected cattle. The caudal fold test uses only bovine PPD, whereas the single intradermal comparative test involves the injection of avian and bovine tuberculins simultaneously, but at different sites, into the skin of the neck. Tuberculous cattle show a delayed-type hypersensitivity reaction at the injection site, which is maximal at 72 hours post-inoculation, and characterized by thickening of the skin due to a mononuclear cell infiltration and sometimes oedema (Fig. 11.13). The tuberculin test is used mainly for cattle but it is used occasionally to test pigs, deer and poultry with appropriate modifications in technique for each species.

Cattle can be sensitized to tuberculin not only by infection with *M. bovis* but also by *M. avium*, *M. tuberculosis*, the unidentified acid-fast bacteria responsible for skin tuberculosis and saprophytic mycobacteria. This can lead to false-positive reactions. False-negative reactions can also occur. The term anergy has been used to describe this unresponsive state, which is not well understood. A number of laboratory-based tests have been developed in recent years for the diagnosis of tuberculosis. These include the lymphocyte transformation test, serological tests for circulating antibodies (such as the ELISA) and gamma interferon assays using whole blood. These in vitro tests are usually used in conjunction with the tuberculin test. The gamma interferon assay is approved as a supplementary test for use in several countries and detects infection at an earlier stage than the tuberculin test. The ELISA test is most useful at the later stages of infection and thus is of value in countries at the early stages of eradication programmes in which there are large numbers of chronically infected animals (de la Rua-Domenech et al. 2006).

Figure 11.13 A reactor to the single intradermal comparative tuberculin test. There is no reaction to the avian PPD at the upper site but a marked reaction has occurred to the bovine tuberculin (lower site). Photographed 72 hours after injection.

Laboratory Diagnosis of *Mycobacterium lepraemurium*

Feline leprosy ('acid-fast granuloma') is thought to be caused by *M. lepraemurium* (Fig. 11.14). This mycobacterium cannot be cultured on routine mycobacterial media

Figure 11.14 Lesions of feline leprosy caused by *M. lepraemurium* in a young cat. They present as chronic non-healing ulcers.

Figure 11.15 A South Devon bull with advanced Johne's disease (*M. avium* subspecies *paratuberculosis*). There is pronounced diarrhoea, emaciation and muscle atrophy.

but has been grown on specially adapted media. Numerous long, slender, acid-fast rods are seen in ZN-stained smears of scrapings from non-healing ulcers or from biopsies of the nodules. Histopathological sections from biopsies stained with the ZN stain will also reveal the numerous acid-fast mycobacteria. Culture should be attempted in case the lesions have been caused by one of the atypical mycobacteria, such as *M. smegmatis*, *M. ulcerans* or *M. fortuitum*, which have been recorded as causing lesions in cats.

Laboratory Diagnosis of *Mycobacterium avium* Subspecies *paratuberculosis*

Mycobacterium avium subsp. *paratuberculosis*, formerly called *M. johnei*, causes paratuberculosis (Johne's disease) in cattle (Fig. 11.15), sheep, goats and other ruminants.

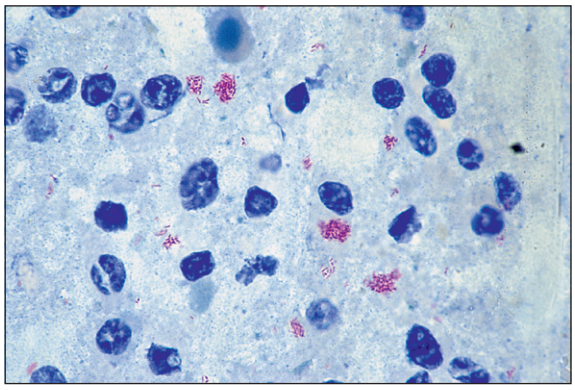


Figure 11.16 *Mycobacterium avium* subsp. *paratuberculosis* in a ZN-stained smear of mucosal scrapings from a bovine ileocaecal valve. The short, acid-fast rods are in clumps indicative of intracellular growth.

Specimens

In live animals with suspected Johne's disease, a small pinch biopsy from the rectum or rectal scrapings are preferred to faecal samples. A biopsy from a mesenteric lymph node, if available, is a useful specimen. Control schemes can be based on the detection of asymptomatic shedder cattle by the detection of *M. avium* subsp. *paratuberculosis* in faecal samples. This can be carried out by culture, which requires a reference laboratory willing to culture a large number of samples, or by using newer techniques based on PCR methods. Usually 15 g faecal samples are submitted, from every adult animal in the herd, at six-month intervals. Specimens from dead animals include a section of ileocaecal valve, washed free of faeces, mesenteric lymph nodes (often the best specimen from sheep and goats) and sections of ileocaecal valve in 10% formalin for histopathology.

Direct microscopy

Faecal or ileocaecal valve mucosal smears from advanced clinical cases, stained by the ZN-stain, usually yield large numbers of short, red-staining rods, that are characteristically in clumps indicative of intracellular growth (Fig. 11.16). Ziehl–Neelsen-stained histopathological sections of ileocaecal valve also reveal large numbers of acid-fast bacilli in clinical cases (Fig. 11.17). Examination of stained faecal smears will only detect about 25% of subclinical excretors. Field observation, however, indicates that those cattle that are excreting sufficient *M. avium* subsp. *paratuberculosis* to detect by faecal smears will eventually become clinical cases of Johne's disease. In cattle this may be six months to three years after the animal has been detected as an asymptomatic shedder.

If only a few, scattered acid-fast bacilli are seen in ZN-stained faecal smears this must be interpreted

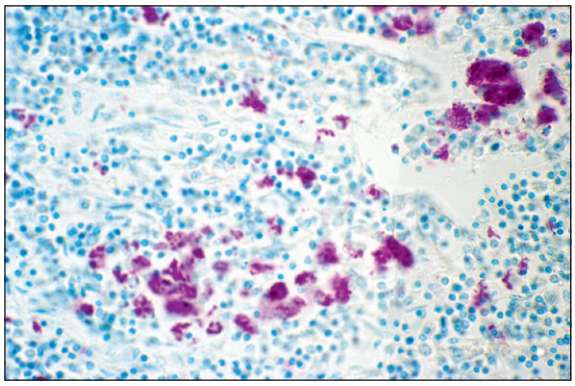


Figure 11.17 A ZN-stained tissue section from the ileocaecal valve area of a bull with paratuberculosis. Large numbers of the acid-fast bacilli are present in animals with clinical disease.

carefully, as harmless atypical mycobacteria can be present in the intestine. A repeat sample should be obtained.

Isolation

This method is far more sensitive than the examination of ZN-stained smears, but is usually only undertaken by reference laboratories. Figure 11.18 summarizes the isolation procedures for *M. avium* subsp. *paratuberculosis.* Benzalkonium chloride is used to decontaminate the specimens and Herrold's egg yolk medium with mycobactin is often used as the culture medium. If contamination by fungi is a problem, amphotericin B can be added to the medium at a final concentration of 5 µg/mL. The egg in Herrold's medium contributes sufficient phospholipids to neutralize the activity of residual benzalkonium chloride in the inoculum. The essential growth factor, mycobactin, can be extracted from *M. phlei* or preferably from a mycobactin-independent strain of *M. avium* subsp. *paratuberculosis* (mycobactin J). The method for preparation of Herrold's egg yolk medium is given in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Gwozdz 2008). Three slants of the Herrold's medium with mycobactin and one slant of the medium without mycobactin are inoculated per sample. The slants are incubated at 37°C and examined once a week for growth for up to 16 weeks. As for *M. bovis,* laboratory systems based on culture in liquid media and detection of growth using radiometric or fluorometric methods, are available. The specificity of culture methods is high, of the order of 98% to 100%. Sensitivity is lower but may be as high as 70% for the detection of clinically affected animals and as low as 25% for detection of infected animals (Nielsen & Toft 2008).

Identification

Identification of isolates using molecular methods is now routine as described below (see Molecular Diagnosis).

Colonial morphology

Primary colonies characteristically appear about seven weeks after inoculation (range 5-14 weeks). The colonies are very small (1 mm diameter), colourless, translucent and hemispherical. The margins are round and even and the surfaces smooth and glistening. The colonies become more opaque and increase in size as incubation is continued, reaching a maximum diameter of about 4 mm. Roughness increases with age.

Microscopic appearance

The cells from primary cultures are highly acid-fast, the cells average 0.5 µm in width and 1.0 µm in length.

Mycobactin dependency

Primary cultures exhibit a strict dependency upon mycobactin for growth. This is somewhat modified on subculture. If growth occurs only on the slants of medium containing mycobactin and not on the slant without mycobactin, this gives evidence of mycobactin-dependency and the culture can be reported as *M. avium* subsp. *paratuberculosis* providing all other tests, such as the microscopic appearance (ZN-smear), produce compatible results. Occasionally if large numbers of *M. avium* subsp. *paratuberculosis* are present in the inoculum, growth may be seen on the slant without mycobactin.

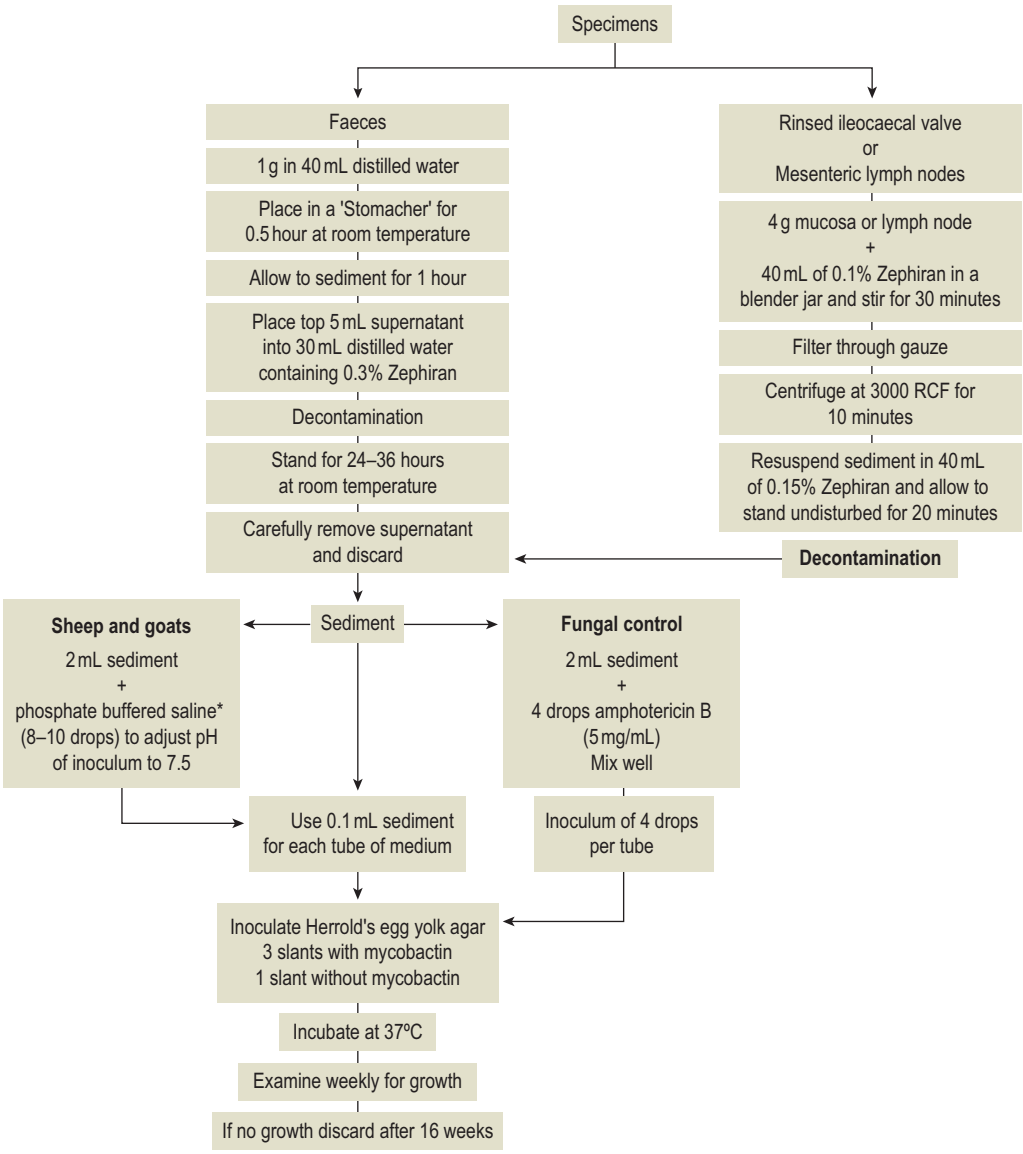
Field and laboratory immunological tests for paratuberculosis

Field diagnostic tests are based on delayed-hypersensitivity reactions to johnin, a purified protein derivative prepared from *Mycobacterium avium* subspecies *paratuberculosis* cultures.

- The intradermal johnin test for cattle is read 48–72 hours after inoculation. A swelling at the injection site indicates a positive reaction. The test may produce up to 75% false-positives.
- The intravenous johnin test is more specific. Cattle developing an elevation in temperature of 1.5°C or more are regarded as positive reactors. This test may detect about 80% of clinical cases but is not useful for asymptomatic shedders. Because of the antigenic relationship between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*, some cattle with clinical Johne's disease react to avian tuberculin. The johnin tests are not diagnostically useful for sheep and goats.

In vitro lymphocyte stimulation test

In this test peripheral blood lymphocytes are collected and exposed to johnin. If the antigens of *M. avium* subsp. *paratuberculosis* elicit a cell-mediated response, the lymphocytes will proliferate. This is measured by the addition



(Growth on mycobactin-enriched slants only: presumptive evidence of *M. avium* subsp. *paratuberculosis)*

* Phosphate buffer: Na_2HPO_4 14.2 g, NaCl 80.0 g and distilled water to 1 litre.
Methods adapted from 'Laboratory Methods in Veterinary Mycobacteriology', Anon Veterinary Services Laboratories, US Department of Agriculture, Ames, Iowa, USA

Figure 11.18 A summary of the isolation procedures for *M. avium* subsp. *paratuberculosis.*

of a radio-labelled nucleic acid precursor. The test is positive only when clinical signs are evident.

Serological tests for antibody detection

Various serological tests have been developed for the diagnosis of paratuberculosis. These include an ELISA, complement fixation test (CFT), agar gel immunodiffusion (AGID), fluorescent antibody test and immunoperoxidase tests. The specificity of ELISA is improved by prior absorption of sera with soluble *M. phlei* antigen. Some ELISAs are commercially available but the sensitivity and specificity of different tests and their usefulness in

detection of subclinically infected cattle is variable (Nielsen & Toft 2008). Both CFT and absorbed ELISA tests are useful for confirmation of infection in clinically affected animals.

Gamma interferon test

This test is widely used for the early detection of subclinically infected animals but few studies independently evaluating the sensitivity and specificity of the test have been published (Nielsen and Toft 2008) and its usefulness for this purpose is uncertain. Indeed, Jungersen et al. (2011) suggest that the test is not useful in determining the infection status of individual animals but can be employed as a herd test.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Treatment of animals affected with tuberculosis or paratuberculosis is not appropriate and antimicrobial susceptibility testing is not usually carried out. Antimicrobial susceptibility testing of *M. bovis* is carried out in some instances and results indicate that organisms are susceptible to most agents tested. In contrast, resistance to multiple antimicrobial agents is a major problem in the treatment of *M. tuberculosis* infections in humans (Koul et al. 2011, WHO, 2011).

Strain Typing

The most frequently employed methods for epidemiological typing of *M. bovis* strains are spoligotyping and variable nucleotide tandem repeat (VNTR) typing. Spoligotyping involves identification of polymorphisms in spacer regions of the direct repeat regions of the chromosome. This technique is easily applied to large numbers of isolates and the patterns obtained are assigned a name through the website http://www.mbovis.org/ (accessed 4 December 2012). Typing using VNTR examines variations in the number of repeats at a number of loci throughout the genome and provides greater discrimination than spoligotyping. Hewinson et al. (2006) reviewed the use of molecular techniques in the typing of *M. bovis* and showed that there is a tendency for particular clones or clonal complexes to predominate in a particular country and even in regions within a country.

Typing of *M. avium* subsp. *paratuberculosis* can be carried out using several different methods including restriction fragment length polymorphisms analysis, analysis of mycobacterial interspersed repetitive units, VNTR and PFGE techniques. These have been reviewed by Castellanos et al. (2011). Strains appear to fall into three types with bovine isolates predominantly classified as Type II, ovine strains as Type I and a third classification of Type III or intermediate strains.

Molecular diagnosis

Molecular methods are frequently used both for identification of cultured isolates and for the detection of mycobacteria in clinical samples. A variety of methods have been developed for the identification of isolates belonging to the *M. tuberculosis* complex. These include PCR or DNA probe detection of targets such as 16S-23S rRNA or insertion sequences IS*6110* and IS*1081*. PCR-based methods based on detection of these genes may also be used for the detection of organisms in clinical specimens including fresh and formalin-fixed tissues. However, it is recommended that culture be carried out in parallel in order to confirm the presence of viable organisms (Cousins 2009, Collins 2011). Specific references for PCR assays for the confirmation of the identity of *M. bovis* isolates through detection of mutations in the *oxy*R, *pncA* gene and the *gyr*B gene and the presence/absence of Regions of Difference are given in Cousins (2009). Spoligotyping may be used for identification as well as for typing of *M. bovis* strains.

PCR detection of IS*900* which is specific for *M. avium* subsp. *paratuberculosis* is used for confirmation of the identity of cultured isolates. Real-time PCR methods have been developed for detection of *M. avium* subsp. *paratuberculosis* in faeces and tissues and are of comparable sensitivity to culture with results available much more rapidly (Alinovi et al. 2009, Imirzalioglu et al. 2011, Sidoti et al. 2011).

REFERENCES

Alinovi, C.A., Ward, M.P., Lin, T.L., et al., 2009. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. paratuberculosis. Veterinary Microbiology 136, 177–179.

Allen, A.R., Minozzi, G., Glass, E.J., 2010. Bovine tuberculosis: the genetic basis of host susceptibility. Proceedings: Biological Sciences/ The Royal Society 277 (1695), 2737–2745.

Castellanos, E., Juan, L.D., Domínguez, L., et al., 2011. Progress in molecular typing of *Mycobacterium avium* subspecies *paratuberculosis*. Research in Veterinary Science (EPUB ahead of print).

Collins, D.M., 2011. Advances in molecular diagnostics for *Mycobacterium bovis*. Veterinary Microbiology 151 (1–2), 2–7.

Corner, L.A., 2006. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk. Veterinary Microbiology 112, 303–312.

Cousins, D.V., 2009. Bovine Tuberculosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE. pp. 1–16. Available from http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/ Accessed 4 December 2012.

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., et al., 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Research in Veterinary Science 81, 190–210.

Gwozdz, J., 2008. Paratuberculosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, 276–291. Available from http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/ Accessed 4 December 2012.

Hewinson, R.G., Vordermeier, H.M., Smith, N.H., et al., 2006. Recent advances in our knowledge of *Mycobacterium bovis:* a feeling for the organism. Veterinary Microbiology 112, 127–139.

Imirzalioglu, C., Dahmen, H., Hain, T., et al., 2011. Highly specific and quick detection of *Mycobacterium avium* subsp. *paratuberculosis* in feces and gut tissue of cattle and humans by multiple real-time PCR assays. Journal of Clinical Microbiology 49 (5), 1843–1852.

Jungersen, G., Mikkelsen, H., Grell, S.N., 2011. Advances in molecular diagnostics for *Mycobacterium bovis*. Veterinary Immunology Immunopathology (EPUB ahead of print).

Khalifeh, M.S., Stabel, J.R., 2004. Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of *Mycobacterium avium* subsp. *paratuberculosis* in monocyte-derived macrophages from naturally infected cattle. Infection & Immunity 72, 1974–1982.

Koul, A., Amoult, E., Lounis, N., et al., 2011. The challenge of new drug discovery for tuberculosis. Nature 469, 483–490.

Nielsen, S.S., Toft, N., 2008. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. Veterinary Microbiology 129, 217–235.

Roberts, G.D., Koneman, E.W., Kim, Y.K., 1991. *Mycobacterium*. In: Balows, A. (Ed.), Manual of Clinical Microbiology, fifth ed. American Society for Microbiology, Washington, DC, USA, pp. 304–339.

Runyon, E.H., 1959. Anonymous mycobacteria in pulmonary disease. Medical Clinics of North America 43, 273–290.

Sidoti, F., Banche, G., Astegiano, S., et al., 2011. Validation and standardization of IS900 and F57 real-time quantitative PCR assays for the specific detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. Canadian Journal of Microbiology 57 (5), 347–354.

Sweeney, R.W., Jones, D.E., Habecker, P., 1998. Interferon-gamma and interleukin 4 gene expression in cows infected with *Mycobacterium paratuberculosis*. American Journal of Veterinary Research 59, 842–847.

WHO, 2011. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. Available at: http://www.who.int/tb/features_archive/m_xdrtb_facts/en/index.html Accessed 4 December 2012.

Chapter 12

Listeria species

Genus Characteristics

The genus *Listeria* belongs to the *Listeria-Brochothrix* family which is a subline within the *Clostridium* subdivision. Six species form the genus *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*; *L. monocytogenes* is the type species. *Listeria ivanovii* has two subspecies: *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*. Based on molecular phylogenetic studies, *Listeria* has also been divided into two distinct descent lines: (i) the closely related *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, L. *innocua* and *L. welshimeri* and (ii) *L. grayi* (Murray et al. 2007).

Listeria species are medium-sized, non-branching, short Gram-positive rods, non-spore-forming and non-acid-fast. They usually occur singly or in a short chain. *Listeria* are facultative anaerobes but growth is enhanced by 10% CO_2. They grow on nutrient agar and blood agar but not on MacConkey agar. Optimal growth temperature is between 30°C and 37°C but they can also grow at 4°C. They are usually catalase-positive, but exceptions are reported (Elsner et al. 1996), oxidase-negative, hydrolyse aesculin, tolerate 10% sodium chloride and are motile at 28°C due to a few (1–5) peritrichous flagella.

Listeria monocytogenes and *L. ivanovii* are both pathogenic for animals. However, *L. monocytogenes* is by far the most significant pathogen causing septicaemia, abortion, mastitis, and central nervous system infection in various animals, mainly ruminants. *L. monocytogenes* also causes disease in man and is an important public health concern. *Listeria grayi*, *L. seeligeri*, *L. innocua* and *L. welshimeri* are considered to be non-pathogenic; however, there have been some reports of *L. seeligeri* causing illness in humans (Rocourt et al. 1986).

Natural Habitat

Listeria species are widely distributed in the environment. Its primary habitat is considered to be soil and decaying vegetation where it survives and grows as a saprophyte. It can also be isolated from sewage, water, animal feed (silage), poultry, various meats, slaughterhouse waste, raw milk, and cheese (Fenlon 1999). *Listeria* species can grow in a temperature range of 3–45°C and within a pH range of 5.6–9.6. Silage is commonly implicated in outbreaks of listeriosis in cattle and sheep as the bacteria can multiply in silage with a pH over 5.5. Human foods frequently associated with listeriosis in man include coleslaw, soft cheeses, delicatessen items, milk, hot dogs, seafood and undercooked poultry meat.

Listeria monocytogenes has been recovered from many species of mammals, fish, birds, crustaceans and insects. Asymptomatic faecal carriers of *L. monocytogenes* occur in man and animals (Fenlon 1999). It is likely that exposure of animals to *L. monocytogenes* is unavoidable because sources of the bacterium are numerous and the organism is hardy and persistent in the environment.

Pathogenesis and Pathogenicity

Listeria monocytogenes can cause septicaemia, abortion, mastitis and central nervous system (CNS) infection (meningoencephalitis in adults and meningitis in young animals) in many animals but primarily in cattle and sheep. The septicaemic form of listeriosis is sometimes referred to as the 'visceral form' while central nervous system infection is known as the 'neural form'. Table 12.1 indicates the hosts and disease syndromes caused by the pathogenic *Listeria* species, while major virulence

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Table 12.1 Main hosts and disease syndromes of the pathogenic *Listeria* species

Species	Host(s)	Disease syndromes
L. monocytogenes	Young animals of many species including lambs, calves and birds	Septicaemia Necrotic foci in liver and other abdominal organs
	Sheep, goats, cattle and occasionally other species	Central nervous system (CNS) infection: neural listeriosis (meningoencephalitis) also called 'circling disease' with microabscesses in the brain stem and perivascular cuffing
	Sheep, goats and cattle	Abortion
	Cattle	Iritis with or without other signs. Often associated with feeding big-bale silage Mastitis
	Human	Cutaneous lesions, meningitis, encephalitis, septicaemia and/or gastroenteritis
L. ivanovii	Sheep and cattle	Abortion

Table 12.2 Main virulence factors of *Listeria monocytogenes*

Virulence determinants (gene)	Functions
Internalin A: 80-kDa surface protein *(inlA)*	Binds to E-cadherin on the basolateral surface of intestinal epithelial cells, a process involved in the invasion of intestinal epithelial cells
Internalin B: surface protein *(inlB)*	Cell invasion
Internalin C: surface protein *(inlc)*	Cell invasion
Internalin related protein *(irpA)*	Unknown role during disseminated infection
Listeriolysin O, LLO*(hly)*: thiol-activated haemolysin	Lysis of phagosome membrane and escape into the cytoplasm
Phospholipase C, PC-PLC	Mediates bacterial cell-to-cell spread Lysis of phagosome membrane and escape into the cytoplasm
Phosphatidylinositol-specific phospholipase C, PIPL-C: lecithinase	Mediates bacterial cell-to-cell spread Lysis of phagosome membrane and escape into the cytoplasm
Actin-polymerizing protein ActA: 90-kDa surface protein *(actA)*	Directs the deposition of host-dependent actin filaments on the end of *Listeria* cells for propulsion into neighbouring cells
Positive regulatory factor A *(prfA)*	A temperature-, pH- and nutrient-regulated factor for *Listeria* virulence determinants
Invasion-associated protein *(iap)*: major extracellular protein (p60)	Invasion
Metalloprotease (*mpl)*	Involved in the proteolytic activation of phospholipase C (PC-PLC)
Flagellin *(fla)*	Flagellar protein with murein-degrading activity
DegU regulator *(DegU)*	Pleiotropic regulator involved in expression of both motility at low temperature and in vivo virulence in mice

factors of *L. monocytogenes* are presented in Table 12.2. The pathogenicity of *L. monocytogenes* is mainly determined by the virulence factors: listeriolysin O, protein ActA, phospholipases C, internalins (In1A and In1B) and a metalloprotease (Popowska & Markiewicz 2004). *Listeria monocytogenesis* is often employed as a model organism in studies on the pathogenesis of intracellular bacteria. It is able to penetrate, multiply and propagate in various eukaryotic cells and is capable of overcoming the three main barriers found in the animal host: the intestinal barrier, the blood–brain barrier and the placenta.

Figure 12.1 *Listeria monocytogenes*: neural form of listeriosis in a silage-fed sheep showing unilateral facial paralysis.

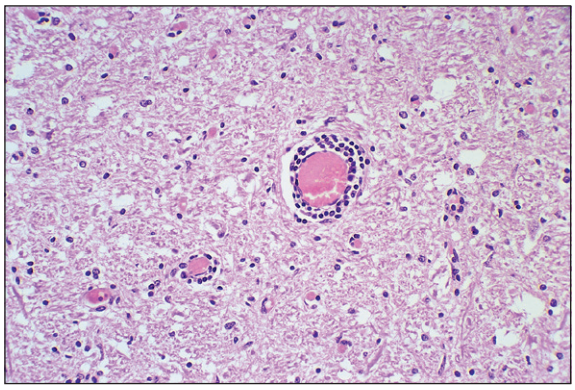

Figure 12.2 Perivascular cuffing in an ovine medulla indicative of the neural form of listeriosis. (H&E stain, ×400)

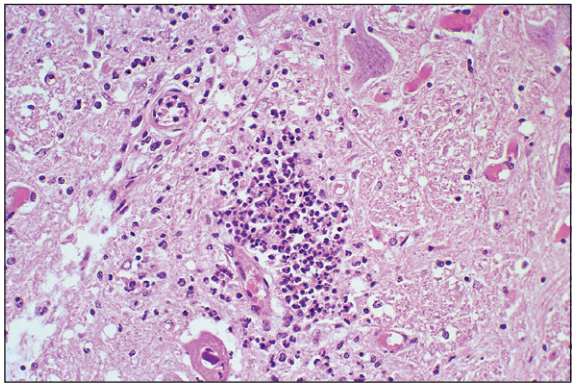

Figure 12.3 A microabscess in the medulla of a sheep with listeriosis. (H&E stain, ×400)

Listeria monocytogenes is a facultative intracellular bacterium. Factors that contribute to the virulence of *L. monocytogenes* can be divided into key events: attachment-invasion, intracytoplasmic growth and cell-to-cell propulsion (Gyles 2004). *Listeria monocytogenes* infections are initiated by its adherence, or attachment, to animal tissue cells. This is followed by invasion and internalization via the internalin A proteins (Cabanes et al. 2002). Both phagocytes and tissue cells can be infected. The bacteria escape from the phagosome via its haemolysin (listeriolysin O, LLO) and the phosphatidylinositol-dependent phospholipase C (Glomski et al. 2002). Once free in the cytoplasm, intracytoplasmic replication occurs leading to invasion of neighbouring cells. The method of cell-to-cell spread of *L. monocytogenes* is particular to this microorganism: deposition of host cell actin filaments on the end of the bacterial cells by the protein ActA promotes propulsion and invagination into neighbouring cells. The invagination process gives a double membrane around the bacterium. After the invasion of new cells, the process of phagosome escape starts again with lysis of the double membrane mediated by LLO and the phosphatidylinositol-dependent phospholipase C. Repetition of the entire process leads to a centripetal distribution of the microorganism.

Listerial meningoencephalitis typically manifests first as depression and confusion followed by drooping of the ears and head tilt (Low & Linklater 1985, Low & Renton 1985) (Fig. 12.1). Salivation and tongue protusion are also usually seen while facial or throat paralysis and twitching may also be observed. The condition is referred to as 'circling disease' since the animal tends to move in one direction. The disease is associated with silage feeding and is seasonal, mostly occurring in winter and early spring when the animals are housed indoors on silage. Most pathogenic bacteria require the availability of iron in the host for their metabolic activities. It has been suggested that high iron levels in silage may predispose cattle and sheep to listeriosis. Entry of the organism is considered to occur via the dental pulp when sheep are cutting or losing teeth (Barlow & McGorum 1985) or, alternatively, through damaged mucosal surfaces and infection of the neural sheath of peripheral nerve endings of trigeminal nerves. The organism travels centripetally along the axons to the central nervous system. Histopathological lesions, usually unilateral, such as perivascular cuffing of mononuclear cells (Fig. 12.2) in the midbrain, pons or medulla oblongata and microabscesses (Fig. 12.3) are characteristic.

Listeria monocytogenes also seems to have a tropism for invading the fetoplacental tissues of a variety of animals. Abortion usually occurs during the third semester of pregnancy. In sheep, abortion can be associated with septicaemia and encephalitis. In ruminants, the organism has been associated with generalized septicaemia and focal necrosis of the spleen and liver as well as mastitis. Cases of subclinical to severe suppurative mastitis have been described and treatment is reported to be difficult to

achieve (Gitter et al. 1980). Because of its common recovery in bovine faecal material, *Listeria* is considered an environmental agent of mastitis (Weber et al. 1995).

Clinical listeriosis is rarely seen in non-ruminant animal species such as pigs, chickens, dogs, cats and horses. It can cause septicaemia or meningitis (Cooper 1989, Schroeder & van Rensburg 1993). Keratitis has been reported in a horse (Sanchez et al. 2001).

Listeria monocytogenes is considered as an opportunistic foodborne pathogen. Cases of human listeriosis may present as meningitis, encephalitis, septicaemia and/or gastroenteritis (Schuchat et al. 1991). *Listeria monocytogenes* infection in humans may also result in an influenza-like syndrome in pregnant women with infection of the foetus resulting in abortion or premature birth. The neural form of the disease can also occur in neonates. Listeriosis is also considered a zoonotic disease since veterinarians and abattoir workers can acquire a primary cutaneous listeriosis that infrequently leads to a generalized form of the disease.

Laboratory Diagnosis

Specimens

For the septicaemic (visceral) form, material from lesions in liver, kidneys or spleen should be submitted. For the CNS (neural) form, spinal fluid, brain stem and tissue from several sites in the medulla oblongata should be forwarded to the diagnostic laboratory. Placenta (cotyledons), amniotic fluid, foetal abomasal contents and foetal tissues (lung, abomasum) are suitable specimens for abortion cases. A full range of specimens should be submitted so that an examination can be made for the other pathogens capable of causing abortion. Specimens for isolation of *Listeria* species should be cultured immediately at 35°C or stored at 4°C for up to 48 hours. To avoid overgrowth of contaminants, freezing at –20°C is recommended. Feed samples such as silage for epidemiological studies should be collected (at least 100 g) as aseptically as possible in sterile containers.

Direct microscopy

Stained smears are not as useful in cases of listeriosis as they are in some other diseases. Smears from lesions may reveal Gram-positive rods (often coccobacillary; Fig. 12.4) but isolation should always be attempted. Histopathological examination of fixed (10% formalin) brain tissue can often give a presumptive diagnosis of neural listeriosis due to the characteristic lesions.

Isolation

Traditional methods of identification involving culture methods based on selective enrichment followed by characterization of plated *Listeria* species on the basis of colony morphology, sugar fermentation and haemolytic activity are considered the gold standard. However, *L. monocytogenes* is widely tested for in food, environmental samples and clinical specimens. As a result, significant developments have occurred in selective enrichment procedures and many new and rapid detection methods using antibody and molecular-based technologies are now available (Gasanov et al. 2005).

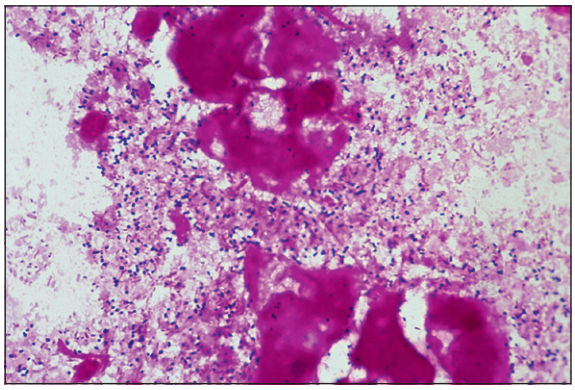

Figure 12.4 *Listeria monocytogenes* in a Gram-stained smear of material from a placenta (bovine abortion). (×1000)

The routine medium for inoculation of clinical specimens is tryptic soy agar with 5% ox or sheep blood. A MacConkey agar plate can be used to detect any Gram-negative pathogens or contaminants. Specimens from septicaemia and abortion cases are inoculated directly onto the laboratory media and incubated aerobically at 35°C for 24–48 hours. Selective media include blood agar with an antibiotic supplement (as described for *Staphylococcus* species) or blood agar containing 0.05% potassium tellurite which is inhibitory to Gram-negative bacteria. A 'cold-enrichment' procedure may be necessary for isolation from brain tissue in cases of neural listeriosis. In this technique, small pieces of spinal cord and medulla are homogenized and a 10% suspension is made in a nutrient broth. The broth suspension is placed in the refrigerator at 4°C and subcultured onto blood agar once weekly for up to 12 weeks. This method selects for *L. monocytogenes*, one of the few pathogens able to grow at refrigerator temperature.

The use of a selective *L. monocytogenes* enrichment method is recommended for non-sterile specimens or foodstuffs to increase sensitivity and the recovery of injured *Listeria* cells. Selective enrichment methods involve the culture of specimens in a selective enrichment broth with inhibitors such as acriflavine and nalidixic acid designed to slow the growth of competitive organisms. A second round of enrichment is sometimes used and referred to as the two-stage enrichment procedure. Following incubation an aliquot of the broth is plated onto selective agar (Fig. 12.5) prior to biochemical

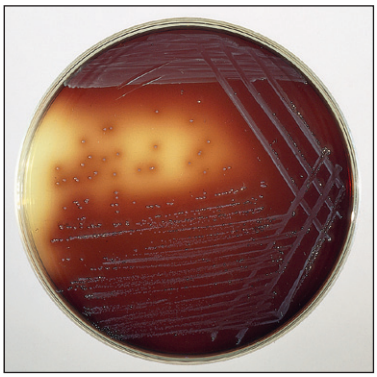

Figure 12.5 *Listeria monocytogenes* on 'Listeria selective agar'. This medium is used for the detection of the bacterium in clinical specimens and foods. Hydrolysis of aesculin results in black zones around the colonies due to the formation of black iron phenolic compounds.

identification of typical colonies. Reference methods are well described in the literature and have been the subject of review (Cassiday & Brackett 1989, Gasanov et al. 2005). A range of selective enrichment broths (*Listeria* Enrichment Broth (LEB), FDA Bacteriological and Analytical Method (BAM) formulation, full strength Fraser, half Fraser, polymyxin B-acriflavine-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM)-egg yolk broth, University of Vermont Medium (UVM)) and selective agars (Oxford (OX), PALCAM, Modified Oxford (MOX), lithium chloride-phenylethanol-moxalactam (LPM)) are available.

Various commercial selective and indicator chromogenic media suitable for differentiating between *L. monocytogenes* and other *Listeria* species are available including CHROMagar *Listeria* (CHROMagar, Paris, France), BCM chromogenic agar test (Biosynth International, Naperville, USA), Chromoagar *Listeria* test (Mast Diagnostics, Reinfeld, Germany), ALOA (Biolife, Milan, Italy) and Rapid'L. Mono agar (BioRad, Marnes de la Coquette, France). Detection is based on the presence or absence of the enzyme phosphatidylinositol-specific phospholipase C (PIPL-C) with positive colonies appearing blue with a white halo. As the enzyme PIPL-C is produced by both *L. monocytogenes* and *L. ivanovii* confirmation testing may be needed. The *Monocytogenes* ID Disc (Biolife, Milan, Italy) uses the alanyl-peptidase enzyme as the basis of identification since this enzyme is produced by all *Listeria* species except *L. monocytogenes*.

Identification

Colonial characteristics

Listeria species produce small transparent colonies with smooth borders that appear on blood agar after 24 hours, becoming greyish-white and 0.5–2.0 mm in diameter after

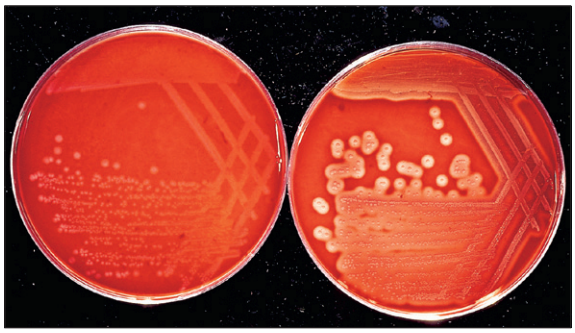

Figure 12.6 *Listeria monocytogenes* (left) and *L. ivanovii* (right) on sheep blood agar. The haemolysis of *L. ivanovii* tends to be more pronounced than that of *L. monocytogenes*.

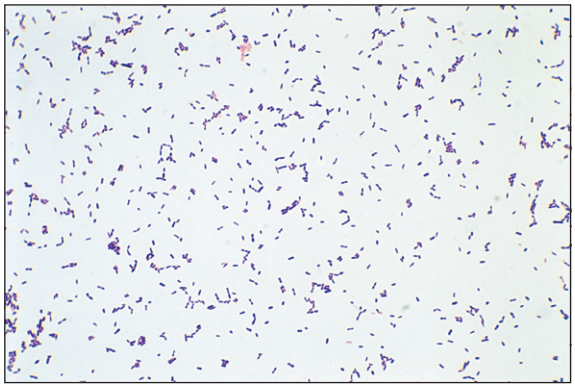

Figure 12.7 *Listeria monocytogenes* in a stained smear from a culture with both Gram-positive rods and cocci present. (Gram stain, ×1000)

one or two days at 35 °C or after a few days at 4 °C. *Listeria ivanovii* produces a comparatively wide zone of haemolysis and is very similar in appearance to a beta-haemolytic *Streptococcus* species. *Listeria monocytogenes* and the non-pathogenic *L. seeligeri* produce narrow zones of beta-haemolysis (Fig. 12.6), often only under the colony itself. Smooth and rough colonial variants occur with *L. monocytogenes*; the rough colonies can yield long filamentous forms. However, filamentous forms can also occur in older cultures.

Microscopic appearance

Listeria species are medium-sized, non-branching, short Gram-positive rods, non-spore-forming and non-acid-fast, measuring about 0.4–0.5 µm in diameter by 0.5–2.0 µm in length (Fig. 12.7). They usually occur singly or in short chains; however, long filaments may be observed in older or rough cultures. There is a tendency for cells from older cultures to decolourize. In smears from rapidly growing cultures or animal tissues the cells can appear coccal. This

fact and their colonial appearance on blood agar can lead to confusion between the pathogenic listeriae and beta-haemolytic streptococci.

Biochemical characteristics

All of the *Listeria* species hydrolyse aesculin in aesculin broths within a few hours. *Listeria monocytogenes*, in particular, shows characteristic 'tumbling motility' when a 2–4-hour broth culture, incubated at 25°C, is examined by the hanging-drop method. This motility is visualized as an end-over-end tumbling of individual cells with periods of quiescence. They are usually non-motile at 35°C. When grown in semisolid motility media *Listeria* species give an unusual umbrella-shaped growth in the sub-surface (Fig. 12.8).

A presumptive identification of *L. monocytogenes* is based on the following tests: colony morphology (small beta-haemolytic colonies at 24 hours on sheep or ox blood agar; *L. innocua* is non-haemolytic), Gram stain (Gram-positive rods tending to show many coccal forms), catalase-positive (beta-haemolytic streptococci, *Erysipelothrix rhusiopathiae* and *Lactobacillus* species are catalase-negative), aesculin hydrolysis in aesculin broth (*Kurthia* spp. are aesculin-negative), tumbling motility in a 2–4-hour broth culture at 25°C, CAMP test positive with *Staphylococcus aureus*, acid production from rhamnose but not from xylose (atypical *L. monocytogenes* may be L-rhamnose negative) and sub-surface umbrella-shaped growth in semi-solid motility media.

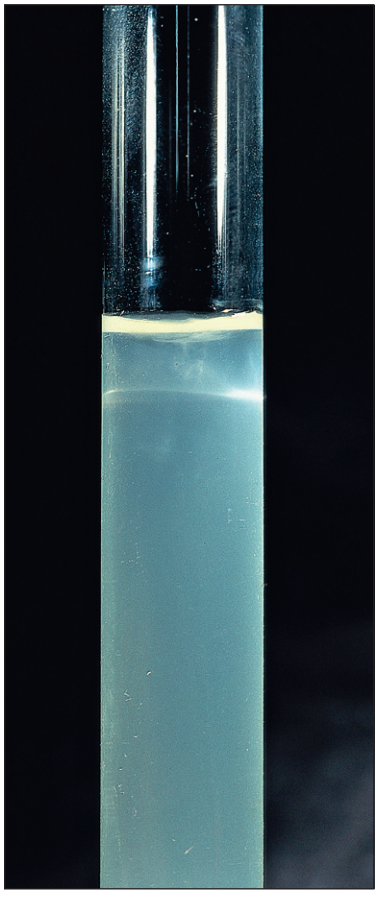

Figure 12.8 Umbrella shaped, sub-surface growth of *L. monocytogenes* in semisolid motility medium.

Table 12.3 presents a short list of tests for the differentiation of species in the genus *Listeria*. Miniaturized biochemical tests for the identification of *L. monocytogenes* are also available such as API-*Listeria* (bioMérieux, Marcy L'Etoile, France) and Micro-ID Listeria (Organon-Teknika, USA). These strips are now incorporated into standard methods. The API Coryne System (bioMérieux) identifies *Listeria* isolates to the genus level (Kerr et al. 1993).

CAMP procedure

Modified CAMP (Christie, Atkins, Munch-Peterson) tests with *Staphylococcus aureus* and with *Rhodococcus equi* are used to differentiate *L. monocytogenes* from *L. ivanovii*. (Figs. 12.9, 12.10 and 12.11). A *S. aureus* or a *R. equi* is streaked horizontally on a sheep blood agar plate and the tested isolate is vertically streaked at a right angle to the *S. aureus* or *R. equi* horizontal line without touching it. Enhancement of haemolysis of *L. monocytogenes* and *L. seeligeri* is seen with the *S. aureus* line; while the same phenomenon is observed between *L. ivanovii* and *R. equi*. Reading must be done with caution since enhancement has been reported between *L. monocytogenes* and *R. equi*. Sometimes the CAMP test does not adequately distinguish *L. monocytogenes* from *L. ivanovii* (Vazquez-Boland et al. 1990). An alternative method using commercially available B-lysin discs has been proposed (USDA 2002) and is now recommended in the USDA method.

Antibody-based tests

Antibody-based testing using enzyme-linked immunosorbent assays (ELISAs) and immuno-capture tests with magnetic beads (Dynal, Melbourne, Australia) or antibody-coated dip sticks (*Listeria* Unique, TECRA International, Frenchs Forest, Australia) can be carried out from enrichment media. Most of these immunoassays are based on antibodies specific to *Listeria* species and are available as commercial kits. Many of them are approved by regulatory authorities. Some of the ELISAs (*Listeria* Unique, TECRA International and VIDAS *Listeria* express, bioMérieux, Marcy L'Etoile, France) are reported to be of equal sensitivity to traditional culture with a result obtained within 30 hours of sample reception.

Pathogenicity Testing

Procedures using laboratory animal inoculation are available to evaluate the virulence of *Listeria* species, but they

Table 12.3 Key tests for rapid identification of *Listeria* species

Species	Tests			Acid from (aerobic incubation for seven days at 35°C):			
		CAMP test reaction					
	Beta-haemolysis	*S. aureus*	*R. equi*	Ribose	L-Rhamnose	Mannitol	D-Xylose
L. monocytogenes	+	+	V	–	+	–	–
L. ivanovii	++[a]	–	+	+	–	–	+
L. ivanovii subsp. *londoniensis*	++	–	+	–	–	–	+
L. seeligeri	+	+	–	–	–	–	+
L. innocua	–	–	–	–	V	–	–
L. welshimeri	–	–	–	–	V	–	+
L. grayi	–	–	–	V	V	+	–

+ = 90% or more strains positive, – = 90% or more strains negative, V = variable; ++[a]= usually a wide zone or multiple zones of beta-haemolysis

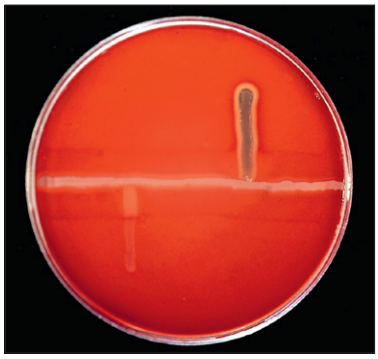

Figure 12.9 CAMP test with *Staphylococcus aureus* (horizontal) showing enhancement of the effect of the staphylococcal beta-haemolysin by *L. monocytogenes* (left) but not by *L. ivanovii* (right).

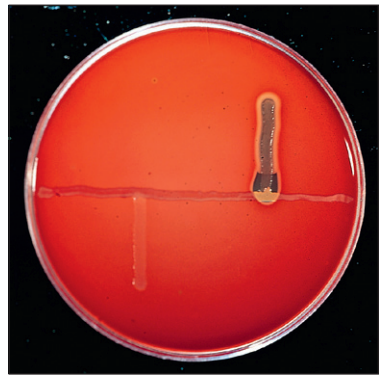

Figure 12.10 CAMP test with *Rhodococcus equi* (horizontal): no reaction by *L. monocytogenes* (left) and enhancement of haemolysis by *L. ivanovii* (right).

are not performed routinely. These tests include (i) inoculation of the conjunctiva of rabbits or guinea pigs (Anton test) where only *L. monocytogenes* causes a purulent keratoconjunctivitis within 24–36 hours of inoculation; (ii) intraperitoneal inoculation of mice with a 24-hour broth culture: both *L. monocytogenes* and *L. ivanovii* are pathogenic for mice, which die within five days with necrotic lesions present in the liver; (iii) inoculation of the chorioallantoic membrane of embryonated eggs. A sensitive immunocompromised-mouse model (Stelma et al. 1987) and a cell culture cytotoxicity assay using a Caco-2 human intestinal epithelial cell line (Pine et al. 1991) have also been developed.

Antimicrobial Susceptibility Testing

Broth microdilution is the recommended Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) method for antimicrobial susceptibility testing of *L. monocytogenes* (document M45-A2, CLSI 2010). Essentially, a 16–18-hour growth of *L. monocytogenes* from a sheep blood agar serves as the inoculum source for a

Figure 12.11 CAMP test with *Rhodococcus equi* streaked across (left to right) a vertical streak of *L. ivanovii* giving an enhanced haemolytic effect.

cation-adjusted Mueller–Hinton broth-lysed horse blood (CAMHB-LHB). Broth microdilutions are incubated for 16–20 hours at 35°C in ambient air.

Antimicrobial resistance

Isolates of *L. monocytogenes* are reported to be usually susceptible to penicillin, ampicillin, gentamicin, erythromycin, tetracycline, rifampin and chloramphenicol (Scheld 1983); and moderately susceptible to fluoroquinolones (Hof et al. 1997). However, plasmids encoding for resistance to chloramphenicol, macrolides, and tetracyclines have been described in clinical human isolates of *L. monocytogenes* (Hadorn et al. 1993). Cephalosporins are reported to be ineffective and should not be used to treat listeriosis (Allerberger & Dierich 1992). Penicillin or ampicillin combined with an aminoglycoside is often recommended for the treatment of human listeriosis since in vitro and in vivo experiments have demonstrated that this combination enhances bactericidal activity of the beta-lactam antibiotic towards *L. monocytogenes* (Moellering et al. 1972).

In one recent case report, cattle with listeriosis were treated with various antibiotics (penicillin G, oxytetracycline, amoxicillin, and amoxicillin/gentamicin combined), but there was no significant difference in the success rate of the different treatments (Schweizer et al. 2006). However, in another report, treatment of sheep and goats with listeriosis showed that gentamicin/ampicillin combination was far better in terms of survival rate than oxytetracycline and chloramphenicol used alone (Braun et al. 2002).

Strain Typing

Multiple typing techniques have been described for *Listeria* and can be divided into early typing methods and new molecular typing procedures. Early typing methods such as serotyping, phage typing, and multilocus enzyme electrophoresis (MLEE) are gradually being replaced by new molecular typing procedures such as ribotyping, PCR-restriction fragment length polymorphism (RFLP), restriction enzyme analysis with pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) or random amplified polymorphic DNA (RAPD) analysis since they are more accurate, reflecting genetic relationships among isolates (Gasanov et al. 2005).

Serotyping is usually conducted in reference laboratories. However, a commercial kit for serotyping *Listeria* is now available (Denka, Seiken, Tokyo, Japan). Serotypes are based on flagellar (H) and somatic (O) antigens and serotyping identifies 13 known serotypes of *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Most human cases are caused by serotypes 1/2a, 1/2b and 4b. Except for serovar 5 (*L. ivanovii*) the serovars are not species-specific. *Listeria innocua*, *L. seeligeri*, *L. monocytogenes* and *L. welshimeri* share one or more common antigens, whereas *L. grayi* does not share antigens with the other species.

There is an international phage typing system for *L. monocytogenes* (Marquet-Van der Mee & Audurier 1995). However, due to an inability to type some strains, the phage system has been replaced by systems based on molecular methods, particularly PFGE analysis. Standardization of methods such as PFGE techniques is of utmost importance for inter-laboratory comparison. Strain examination by MLEE has been useful for taxonomic and evolution studies. However, its discriminatory power is not suitable for epidemiological investigations (Bibb et al. 1990, Boerlin et al. 1991). DNA macrorestriction PFGE pattern analysis is recognized as a highly reproducible and discriminating method for subtyping *L. monocytogenes* isolates (Brosch et al. 1996).The PulseNet laboratories of the Centers for Disease Control and Prevention (CDC) use a standardized PFGE protocol for subtyping *L. monocytogenes* (Graves & Swaminathan 2001) and patterns can easily be compared using the internet (PFGE@cdc.gov; Swaminathan et al. 2001). Direct sequencing of DNA such as the 16S RNA gene (Sallen et al. 1996) or several genes for multilocus sequence typing (MLST) (Cai et al. 2002) has also been employed for *L. monocytogenes*. Sequencing is considered the most accurate method of evaluating genetic relationships between organisms; however, it can be expensive and time-consuming (Gasanov et al. 2005).

Molecular Diagnosis

DNA hybridization tests are used for the differentiation of *L. monocytogenes* from other *Listeria* employing virulence factors genes as target probes. Commercial DNA hybridization tests such as Accuprobe (Gen-probe Inc, San Diego, USA), GeneTrak DNA hybridization kit (NeogenCorporation, Lansing, MI), and the VIT test (VIT, Munich, Germany) are available for the rapid identification or

confirmation of *L. monocytogenes* colonies. PCR-based tests can be used to specifically detect *L. monocytogenes* DNA from fluids, fresh tissues or even paraffin blocks (Jaton et al. 1992). However, direct testing of samples without prior enrichment could give unreliable results (Aznar & Alarcon 2003).

Multiplex PCR assays have also been described for *L. monocytogenes* (Kawasaki et al. 2005, Zhang & Knabel 2005). Commercial PCR assays such as BAX PCR system (Qualicon, Wilmington, DE, USA) and Probelia assay (Sanofi Diagnostic Pasteur, Marne la Coquette, France) are available.

REFERENCES

Allerberger, F.J., Dierich, M.P., 1992. Listeriosis and cephalosporins. Clinical Infectious Diseases 15, 177–178.

Aznar, R., Alarcon, B., 2003. PCR detection of *Listeria monocytogenes*: a study of multiple factors affecting sensitivity. Journal of Applied Microbiology 95, 958–966.

Barlow, R.M., McGorum, B., 1985. Ovine listerial encephalitis: analysis, hypothesis and synthesis. Veterinary Record 116, 233–236.

Bibb, W.F., Gellin, B.G., Weaver, R., et al., 1990. Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Applied and Environmental Microbiology 56, 2133–2141.

Boerlin, P., Rocourt, J., Piffaretti, J.C., 1991. Taxonomy of the genus *Listeria* by using multilocus enzyme electrophoresis. International Journal of Systematic Bacteriology 41, 59–64.

Brosch, R., Brett, M., Catimel, B., et al., 1996. Genomic fingerprinting of 80 strains from the WHO multicenter international typing study of *Listeria monocytogenes* via pulsed-field gel electrophoresis (PFGE). International Journal of Food Microbiology 32, 343–355.

Braun, U., Stehle, C., Ehrensperger, F., 2002. Clinical findings and treatment of listeriosis in 67 sheep and goats. Veterinary Record 150, 38–42.

Cabanes, D., Dehoux, P., Dussurget, O., et al., 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol 10, 238–245.

Cai, S., Kabuki, D.Y., Kuaye, A.Y., et al., 2002. Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. Journal of Clinical Microbiology 40, 3319–3325.

Cassiday, P.K., Brackett, R.E., 1989. Methods and media to isolate and enumerate *Listeria monocytogenes*: A review. Journal of Food Protection 52, 207–214.

Cooper, G.L., 1989. An encephalitic form of listeriosis in broiler chickens. Avian Diseases 33, 182–185.

Clinical and Laboratory Standards Institute (CLSI), 2010. Methods for antimicrobial dilution and disk susceptibility testing for infrequently isolated or fastidious bacteria; approved guideline, 2nd edn, CLSI document M45-MA2, Clinical and Laboratory Standards Institute Wayne, Pennsylvania.

Elsner, H.A., Sobottka, I., Bubert, A., et al., 1996. Catalase-negative *Listeria monocytogenes* causing lethal sepsis and meningitis in an adult hematologic patient. European Journal of Clinical Microbiology and Infectious Diseases 15, 965–967.

Fenlon, D.R., 1999. *Listeria monocytogenes* in the natural environment. In: Ryser, T., Marth, E.H. (Eds.), Listeria, Listeriosis, and Food Safety, second ed, Marcel Dekker, Inc. New York, NY, pp. 30–40.

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. FEMS Microbiology Review 29, 851–875.

Gitter, M., Bradley, R., Blampied, P.H., 1980. *Listeria monocytogenes* infection in bovine mastitis. Veterinary Record 107, 390–393.

Glomski, I.J., Gedde, M.M., Tsang, A.W., et al., 2002. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. Journal of Cell Biology 156, 1029–1038.

Graves, L.M., Swaminathan, B., 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. International Journal of Food Microbiology 65, 55–62.

Gyles, C.L., 2004. Pathogenesis of Bacterial Infections in Animals, third ed. Blackwell Pub, Ames, Iowa.

Hadorn, K., Hachler, H., Schaffner, A., et al., 1993. Genetic characterization of plasmid-encoded multiple antibiotic resistance in a strain of *Listeria monocytogenes* causing endocarditis. European Journal of Clinical Microbiology and Infectious Diseases 12, 928–937.

Hof, H., Nichterlein, T., Kretschmar, M., 1997. Management of listeriosis. Clinical Microbiology Review 10, 345–357.

Jaton, K., Sahli, R., Bille, J., 1992. Development of polymerase chain reaction assays for detection of *Listeria monocytogenes* in clinical cerebrospinal fluid samples. Journal of Clinical Microbioly 30, 1931–1936.

Kawasaki, S., Horikoshi, N., Okada, Y., et al., 2005. Multiplex PCR for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat samples. Journal of Food Protection 68, 551–556.

Kerr, K.G., Hawkey, P.M., Lacey, R.W., 1993. Evaluation of the API Coryne system for identification of *Listeria* species. Journal of Clinical Microbiology 31, 749–750.

Low, C., Linklater, K., 1985. Listeriosis in sheep. In Practice 66–67.

Low, J.C., Renton, C.P., 1985. Septicaemia, encephalitis and

abortions in a housed flock of sheep caused by *Listeria monocytogenes* type 1/2. Veterinary Record 116, 147–150.

Marquet-Van der Mee, N., Audurier, A., 1995. Proposals for optimization of the international phage typing system for *Listeria monocytogenes*: combined analysis of phage lytic spectrum and variability of typing results. Applied Environmental Microbiology 61, 303–309.

Moellering Jr, R.C., Medoff, G., Leech, I., et al., 1972. Antibiotic synergism against *Listeria monocytogenes*. Antimicrobial Agents and Chemotherapy 1, 30–34.

Murray, P.R., Baron, E.J., Jorgensen, J.H., et al., 2007. Manual of Clinical Microbiology, 9th ed. American Society for Microbiology, ASM Press, Washington, DC.

Pine, L., Kathariou, S., Quinn, F., et al., 1991. Cytopathogenic effects in enterocytelike Caco-2 cells differentiate virulent from avirulent *Listeria* strains. Journal of Clinical Microbiology 29, 990–996.

Popowska, M., Markiewicz, Z., 2004. Classes and functions of *Listeria monocytogenes* surface proteins. Polish Journal of Microbiology 53, 75–88.

Rocourt, J., Hof, H., Schrettenbrunner, A., et al., 1986. Acute purulent *Listeria seelingeri* meningitis in an immunocompetent adult. Schweiz Med Wochenschr 116, 248–251.

Sallen, B., Rajoharison, A., Desvarenne, S., et al., 1996. Comparative analysis of 16S and 23S rRNA sequences of *Listeria* species. International Journal of Systematic Bacteriolology 46, 669–674.

Sanchez, S., Studer, M., Currin, P., et al., 2001. *Listeria* keratitis in a horse. Veterinary Ophthalmology 4, 217–219.

Scheld, W.M., 1983. Evaluation of rifampin and other antibiotics against *Listeria monocytogenes* in vitro and in vivo. Reviews of Infectious Diseases 5 (Suppl. 3), S593–S599.

Schroeder, H., van Rensburg, I.B., 1993. Generalised *Listeria monocytogenes* infection in a dog. Journal of the South African Veterinary Association 64, 133–136.

Schuchat, A., Swaminathan, B., Broome, C.V., 1991. Epidemiology of human listeriosis. Clinical Microbiology Review 4, 169–183.

Schweizer, G., Ehrensperger, F., Torgerson, P.R., et al., 2006. Clinical findings and treatment of 94 cattle presumptively diagnosed with listeriosis. Veterinary Record 158, 588–592.

Stelma Jr, G.N., Reyes, A.L., Peeler, J.T.,, 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. Journal of Clinical Microbiology 25, 2085–2089.

Swaminathan, B., Barrett, T.J., Hunter, S.B., et al., 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerging Infectious Diseases 7, 382–389.

US Department of Agriculture (USDA), 2002. Microbiology laboratory Guidebook, Chapter 8, Revision 3: http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/ Accessed 4 December 2012.

Vazquez-Boland, J.A., Dominguez, L., Fernandez, J.F., et al., 1990. Revision of the validity of CAMP tests for *Listeria identification*. Proposal of an alternative method for the determination of haemolytic activity by *Listeria* strains. Acta Microbiologica et Immunologica Hungarica 37, 201–206.

Weber, A., Potel, J., Schafer-Schmidt, R., et al., 1995. Studies on the occurrence of *Listeria monocytogenes* in fecal samples of domestic and companion animals. Zentralblatt für Hygiene und Umweltmedizin 198, 117–123.

Zhang, W., Knabel, S.J., 2005. Multiplex PCR assay simplifies serotyping and sequence typing of *Listeria monocytogenes* associated with human outbreaks. Journal of Food Protection 68, 1907–1910.

Chapter 13

Erysipelothrix species

Genus Characteristics

The genus *Erysipelothrix* is now classified as part of the regular non-spore-forming Gram-positive rods group along with *Lactobacillus* and *Listeria*. *Erysipelothrix* species are mesophilic, facultative anaerobes, non-acid-fast, Gram-positive short rods or long non-branching filaments. They also occur singly or in short chains. *Erysipelothrix* species are non-motile, catalase, methyl red, Voges–Proskauer, indole and oxidase negative, do not hydrolyze aesculin, weakly ferment glucose without gas, do not hydrolyse urea but produce H_2S in triple sugar iron agar. Differentiation of *Erysipelothrix* from related genera is indicated in Table 13.1.

Erysipelothrix rhusiopathiae (previously *E. insidiosa*), the causative agent of erysipelas and a zoonosis, is of clinical significance. *Erysipelothrix tonsillarum*, previously considered to be a serotype of *E. rhusiopathiae* was reclassified as a new species using DNA–DNA hybridization studies (Takahashi et al. 1992). They are the two major species within the genus (Takahashi et al. 2008). *Erysipelothrix tonsillarum* appears to be nonpathogenic for pigs but has been associated with endocarditis in dogs (Eriksen et al., 1987).

Natural Habitat

Erysipelothrix rhusiopathiae is ubiquitous in nature. The microorganism persists in the environment at low temperatures, at alkaline pH and within organic matter. The bacterium can be isolated from many species (mammals, birds, reptiles, amphibians and fish), but it is most commonly associated with pigs which are generally considered the primary reservoir. Healthy carriers can carry the bacteria in their tonsils and other lymphoid tissues of the intestinal tract. Clinically sick pigs as well as asymptomatic animals and healthy carriers can shed the microorganism in their faeces and secretions, representing a significant source of infection and environmental contamination.

Pathogenesis and Pathogenicity

The principal hosts and disease syndromes caused by *E. rhusiopathiae* are indicated in Table 13.2. Infection is usually acquired by ingestion of contaminated food or water. The bacterium may also gain entry via skin wounds.

Swine erysipelas (Pasteur referred to this condition as *rouget*; Fig. 13.1) can be divided into three forms: acute, subacute and chronic (Straw 2006). The acute form is characterized by septicaemia with high temperature, loss of appetite, skin lesions and high mortality. The skin lesions present as diamond-shaped ('diamond-skin' disease) red plaques, which are variable in size and number. A generalized coagulopathy leading to fibrinous thrombosis, invasion of vascular endothelium and deposition of fibrin in perivascular tissues occurs. In severe acute cases, ischaemic necrosis of perivascular tissues can occur. The subacute form is somewhat milder. The chronic form is characterized by endocarditis and polyarthritis. Vascular inflammation, myocardial infarcts and exudation of fibrin followed by destruction of valvular endocardium gives rise to valvular lesions in the heart. Joint lesions result from acute synovitis which gives rise to severe fibrosis leading to erosion of articular cartilage.

Erysipelothrix rhusiopathiae infection is of economic importance for the turkey industry where the infection is generally acute with high mortality (sudden death with or without skin lesions). It only rarely affects chickens.

Infection of humans with *E. rhusiopathiae* is a zoonosis referred to as erysipeloid. It is characterized by a localized cellulitis around the site of inoculation. The lesion is usually violaceous and painful with induration, oedema

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Table 13.1 Differentiation of *Erysipelothrix* from related genera which have regular aerobic Gram-positive non-spore-forming rods

Characteristic	*Lactobacillus*	*Listeria*	*Brochotrix*	*Kurthia*	*E. rhusiopathiae*
H_2S in triple sugar iron	–	–	–	–	+
Catalase	–	+	+	+	–

Table 13.2 Main hosts and disease syndromes of *Erysipelothrix rhusiopathiae*

Host(s)	Disease syndromes
Pigs	Swine erysipelas: **Acute form** Septicaemia (pregnant sows may abort) Skin lesions (diamond skin disease) **Subacute form** Milder septicaemia (pregnant sows may abort) Skin lesions (diamond skin disease) **Chronic form** (follows the acute and subacute forms) Endocarditis Polyarthritis Skin lesions (diamond skin disease)
Turkeys, geese and other birds	Acute septicaemia (sudden death) Endocarditis (chronic form) Arthritis (chronic form)
Sheep (young)	Polyarthritis via umbilicus or wounds (chronic form)
Sheep (adult)	Post-dipping lameness: cellulitis with extension to laminae of feet
Dolphins, cattle, dogs, horses, lambs and rabbits	Occasional infections of varying severity
Humans	Erysipeloid, a localized cellulitis usually on hands and fingers, and rarely endocarditis, arthritis or acute septicaemic disease. Occupational hazard for veterinarians and workers in fish, poultry, and swine and agricultural industries

Figure 13.1 *Erysipelothrix rhusiopathiae*: pathognomonic diamond-shaped, red, urticarial plaques of swine erysipelas (an acute form of the disease).

and inflammation but without suppuration (Fig. 13.2). It is an occupational disease usually contracted via skin abrasions or injuries in individuals handling animals or animal products. Veterinarians, butchers and fish handlers are most frequently infected. Dissemination and endocarditis are rare but can occur in immunocompromised humans. *Erysipelothrix rhusiopathiae* is one of the most common pathogens acquired topically from fish (Lehane & Rawlin 2000).

Erysipelothrix rhusiopathiae virulence factors (Shimoji 2000) and their role in disease pathogenesis are indicated in Table 13.3. The mechanism of invasion of deeper body tissues or the bloodstream is unclear. However, it is reported that neuraminidase plays an important role in the attachment and invasion process into endothelial cells and in the subsequent development of vascular lesions (Nakato et al. 1987). Invasion of other host cells such as synovial cells and chondrocytes from arthritic joints of pigs has been reported (Franz et al. 1995). Strains of *E. rhusiopathiae* vary in virulence. The more virulent strains produce high levels of neuraminidase that can cause vascular damage, thrombus formation and haemolysis. Injury to articular cartilage is thought to be due to an immunological response to persistent antigen or whole bacterial cells in the synovial fluid and it is not known whether superimposed autoimmune reactions are involved. The capsule provides resistance to phagocytosis

Figure 13.2 *Erysipelothrix rhusiopathiae*: erysipeloid, localized cellulitis which develops around the inoculation site. The lesion is violaceous with enduration, oedema and inflammation but without suppuration.

Table 13.3 Main virulence factors of *Erysipelothrix rhusiopathiae*

Virulence determinants	Functions
Capsule (appears as slime or glycocalyx)	Resistance to opsonin-mediated phagocytosis and protection against intracellular killing by macrophages
SpaA protein	Surface protein, a major protective antigen
Surface proteins of 220 kDa (*rspA* gene) and of 85 kDa (*rspB* gene)	Participate in biofilm formation, adhesive surface proteins which bind to polystyrene, fibronectin, and type I and IV collagens
Neuraminidase (sialidase)	Extracellular enzyme which releases terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides of host cells
Hyaluronidase	Extracellular enzyme which is a spreading factor that facilitates the dissemination of the bacteria into tissues by damaging hyaluronic acid, a polysaccharide of the extracellular matrix of connective tissues
Superoxide dismutase	Potential virulence factor, quench oxidative metabolites of phagocytic cells (aid in protecting from intracellular killing)

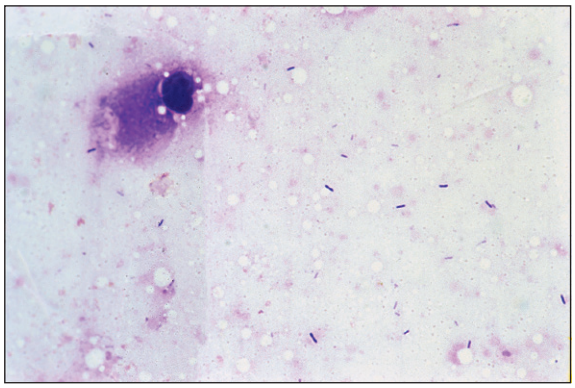

Figure 13.3 *Erysipelothrix rhusiopathiae* in an impression smear from the liver of a pig with the acute septicaemic form of swine erysipelas. The small Gram-positive rods represent the smooth form of the bacterium. (Gram stain, ×1000)

by mononuclear phacocytes (macrophages) and polymorphonuclear leukocytes (Shimoji et al., 1994, 1996). Other virulence factors such as catalase, superoxide dismutase, hyaluronidase, SpaA1 protein and surface proteins of 85 kDa (RspB) and 220 kDa (RspA) also play important roles in the disease process.

Laboratory Diagnosis

Specimens

Liver, spleen, kidney, heart and synovial tissue can be taken at necropsy examination of animals. Recovery of the organism from skin lesions and from lesions associated with chronic forms of the disease may be difficult. In human erysipeloid cases the organism is located deep in the subcutaneous layer and at the edge of the lesion; therefore a biopsy of the site is recommended.

Direct microscopy examination

The morphology of *E. rhusiopathiae* may vary with the disease syndrome:

- Acute cases of the disease: Gram-positive slender, straight or slightly curved rods occurring singly or in short chains (Fig. 13.3).
- Chronic forms of the disease: Gram-positive long filaments (unbranching) that tend to decolourize (Fig. 13.4).

Isolation

Routine isolation is carried out on blood agar along with inoculation of a MacConkey plate to aid in the detection of any Gram-negative pathogens or contaminants. Selective media contain sodium azide (0.1%) and crystal violet

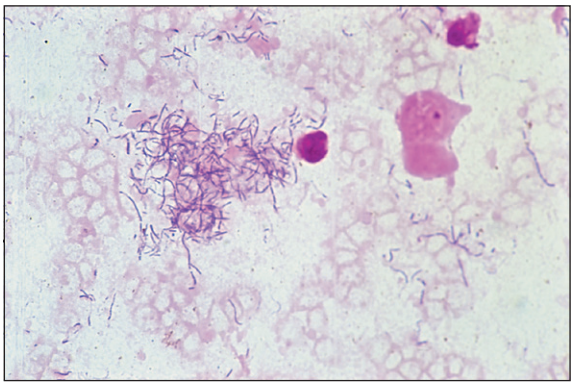

Figure 13.4 The rough form of *E. rhusiopathiae* showing Gram-positive filaments in vegetative lesions on the heart valves of a pig with one of the chronic forms of swine erysipelas. (Gram stain, ×1000)

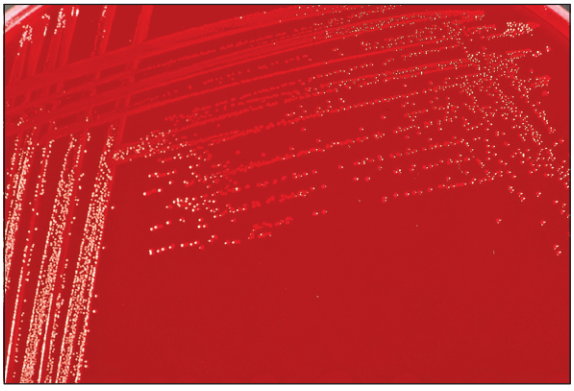

Figure 13.5 Close-up of *E. rhusiopathiae* showing non-haemolytic colonies after 24 hours' incubation on sheep blood agar.

(0.001%). The plates are usually incubated aerobically for 24–48 hours at 35°C although growth is enhanced by 5–10% CO_2. *Erysipelothrix rhusiopathiae* is able to grow in a temperature range of 5°C–42°C (optimum, 30 to 37°C), within a pH range 6.7–9.2 and at an 8.5% sodium chloride concentration. Growth occurs on nutrient agar but is improved by the addition of serum or blood. Growth does not occur on MacConkey agar.

Identification

Colonial characteristics

Non-haemolytic pinpoint colonies (0.1 to 0.5 mm in diameter) appear after 24 hours' incubation (Fig. 13.5). After 48 hours' incubation, a zone of alpha-haemolysis (partial haemolysis on blood agar) often develops under and just around the colonies (Fig. 13.6). Colonial variation becomes obvious at this stage: S-form (smooth)

Figure 13.6 *Erysipelothrix rhusiopathiae* colonies, showing alpha-haemolysis, after 48 hours' incubation on sheep blood agar.

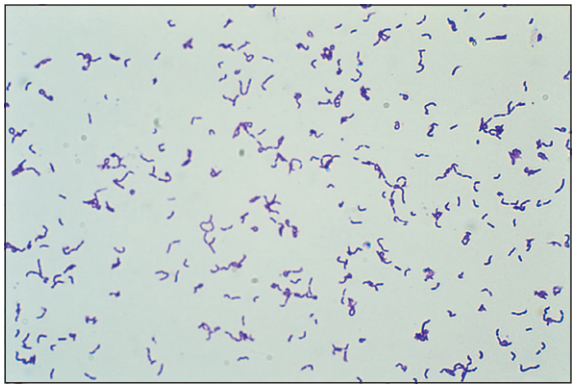

Figure 13.7 *Erysipelothrix rhusiopathiae* in a Gram-stained smear from a culture. There are small Gram-positive rods but also some short filaments indicating that the colonies are changing to the rough form.

colonies are 0.3-1.5 mm in diameter, convex, transparent and circular with an entire edge; R-form (rough) colonies are large, flat, more opaque and have an irregular edge. Rough colonies do not appear to induce alpha-haemolysis.

Microscopic appearance

Smears of the organism from acute disease and from S-form colonies yield medium-sized Gram-positive rods 0.2–0.4 µm by 0.8–2.5 µm in size, while smears from chronic forms of the disease and from R-form colonies reveal Gram-positive filaments of varying lengths up to 60 µm (Fig. 13.7). Decolourization of smears and an appearance similar to a Gram-negative organism may occur.

Biochemical characteristics

Erysipelothrix rhusiopathiae is a facultative anaerobe but growth is enhanced by 10% CO_2. The bacterium is catalase-negative and oxidase-negative. It is non-motile,

Figure 13.8 *Erysipelothrix rhusiopathiae* stab inoculated into a tube of TSI agar. It characteristically produces a small amount of H_2S confined to the stab line.

non-spore-forming, non-acid-fast and does not hydrolyze aesculin or produce urease. A characteristic reaction is produced when TSI agar is stab inoculated and incubated at 35 °C for 24 hours. H_2S is produced, appearing as a thin, black line just along the inoculation stab (Fig. 13.8). *Erysipelothrix rhusiopathiae* is lactose-positive but nitrate-, gelatin-, xylose-, mannose-, maltose- and sucrose-negative. *Erysipelothrix tonsillarum* is usually sucrose positive. The R-forms of *E. rhusiopathiae* give a 'bottle-brush' or 'pipe cleaner' type of growth in stab cultures of nutrient gelatin incubated at room temperature (22 °C). As this reaction takes about five days it is not particularly useful as a diagnostic test. API Coryne (bioMérieux) and VITEK automated systems can be used to identify *E. rhusiopathiae* adequately.

Erysipelothrix rhusiopathiae usually ferments lactose, glucose, levulose and dextrin, but acid production is poor or inconsistent when the bacterium is tested in 1% (w/v) peptone water. Carbohydrate tests can be carried out in peptone water with added sterile horse serum (5–10%) or in nutrient broth plus the test carbohydrate (0.5–1%) with phenol red as the indicator. However, the fermentation pattern varies with the basal medium used. It is advisable to establish the fermentation pattern of known strains in the basal medium of choice.

Pathogenicity testing

This is not carried out routinely. Both mice and pigeons will die within four days after intraperitoneal inoculation with 0.1–0.4 ml of a broth culture from a virulent strain. A mouse protection test, using commercial antiserum, can be used to confirm identification.

Antimicrobial Susceptibility Testing

The susceptibility of *E. rhusiopathiae* isolates is usually determined using a recommended agar dilution procedure and recommended breakpoints approved by the Clinical and Laboratory Standards Institute (document M07-A9; CLSI 2012). Modifications are sometimes applied to this procedure such as the addition of 5% horse blood to Mueller Hinton agar (Fidalgo et al. 2002). Specific guidelines for tiamulin, which is used in veterinary practice for the control and specific therapy of infections in pigs, have been published (Jones et al. 2002).

Antimicrobial resistance

In veterinary medicine, the treatment of choice for acute erysipelas is generally considered to be penicillin alone or with other antibiotics or antiserum (if the outbreak is very severe). The use of antiserum to treat suckling pigs is reported to be a common practice (Straw 2006). Tetracyclines (chlortetracycline and oxytetracycline), lincomycin, and tylosin have also been reported as satisfactory treatment antibiotics for acute erysipelas. However, some isolates have been reported to be resistant to tetracyclines. The presence of the *tet*(M) gene in isolates of tetracycline-resistant *E. rhusiopathiae* has been reported (Yamamoto et al. 2001). Erythromycin, streptomycin, dihydrostreptomycin, chloramphenicol, bacitracin, polymyxin B, neomycin, and sulfonamides seem to be ineffective against erysipelas. There is no effective antibiotic treatment for chronic erysipelas.

In human medicine, penicillin is also the treatment of choice for erysipeloid. *Erysipelothrix rhusiopathiae* is also susceptible to cephalosporins, clindamycin, tetracycline, erythromycine, fluoroquinolone, and imipenem but resistant to aminoglycosides, sulfonamides and vancomycin (Gorby and Peacock 1988).

Erysipelothrix rhusiopathiae is relatively resistant to adverse conditions, can survive in pig faeces for several months and is quite resistant to salting, pickling, and smoking. As a result the effective disinfection of working surfaces and equipment is important in reducing the risk

of transmission and of possible occupational infection. The microorganism is killed by common disinfectants, heat (15 minutes at 60°C) and gamma irradiation. Quaternary ammonium, phenolic, alkali and hypochlorite disinfectants are effective against *E. rhusiopathiae* when applied to clean surfaces such as walls and floors.

Strain Typing

Serotyping is carried out in reference laboratories using a double agar-gel precipitation test with specific hyperimmune rabbit sera and antigen recovered by hot aqueous extraction (Kucsera 1973). Strains of *Erysipelothrix* were determined as belonging to serotypes 1–26 and group N (Kucsera 1977). Serotypes 1 and 2 account for 75–80% of all isolates from pigs. In general, serotyping has been found unreliable in epidemiological studies. *Erysipelothrix tonsillarum* (formerly avirulent serovar 7) can be distinguished from other *E. rhusiopathiae* strains by DNA homology comparisons (Takahashi et al. 1992).

Molecular methods such as restriction fragment length polymorphisms (ribotyping) (Ahrne et al. 1995) and multilocus enzyme electrophoresis (MLEE) (Chooromoney et al. 1994) analysis have shown significant genetic diversity among isolates of *E. rhusiopathiae.* Pulsed-field gel electrophoresis (PFGE) can be used to characterize field isolates and vaccine strains and should be considered as the best method for epidemiological studies (Opriessnig et al. 2004). Also, a new strain-typing method based on nucleotide sequencing of a hypervariable region in the *spa*A gene can be used for discrimination of the live vaccine strain from field isolates (Nagai et al. 2008).

Molecular Diagnosis

Molecular diagnostic procedures for *E. rhusiopathiae* have been used for rapid and direct detection to genus or species level from culture, meat samples and formalin-fixed, paraffin-embedded tissue sections (Makino et al. 1994, Wang et al. 2002). The use of an enrichment broth cultivation-PCR combination assay for rapid diagnosis of swine erysipelas has been described (Shimoji et al. 1998). Molecular methods have also been described for identification of antimicrobial resistance genes, genetic characterization of isolates and nucleotide sequences comparisons. Identification of the tetracycline resistance gene, *tet*(M), in *E. rhusiopathiae* can be performed by PCR (Yamamoto et al. 2001). The nucleotide sequences of *E. rhusiopathine* and *E. tonsillarum* were determined using 16S rRNA genes. The sequences are very similar (99.8%) with only three nucleotides mismatched (Kiuchi et al. 2000). More recently, multiplex PCR assays have been described and can be used to identify and discriminate between all species of *Erysipelothrix* (Pal et al., 2009, Yamazaki, 2006).

REFERENCES

Ahrne, S., Stenstrom, I.M., Jensen, N.E., et al., 1995. Classification of *Erysipelothrix* strains on the basis of restriction fragment length polymorphisms. International Journal of Systematic Bacteriology 45, 382–385.

Chooromoney, K.N., Hampson, D.J., Eamens, G.J., 1994. Analysis of *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* by multilocus enzyme electrophoresis. Journal of Clinical Microbiology 32, 371–376.

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 9th edition, – CLSI document M07-MA9. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Eriksen, K., Fossum, K., Gamlem, H., 1987. Endocarditis in two dogs caused by *Erysipelothrix rhusiopathiae*. Journal of Small Animal Practice 28, 117–123.

Fidalgo, S.G., Longbottom, C.J., Riley, T.V., 2002. Susceptibility of *Erysipelothrix rhusiopathiae* to antimicrobial agents and home disinfectants. Pathology 34, 462–465.

Franz, B., Davies, M.E., Horner, A., 1995. Localization of viable bacteria and bacterial antigens in arthritic joints of *Erysipelothrix rhusiopathiae*-infected pigs. FEMS Immunology and Medical Microbiology 12, 137–142.

Gorby, G.L., Peacock Jr, J.E., 1988. *Erysipelothrix rhusiopathiae* endocarditis: microbiologic, epidemiologic, and clinical features of an occupational disease. Reviews of Infectious Diseases 10, 317–325.

Jones, R.N., Pfaller, M.A., Rhomberg, P.R., et al., 2002. Tiamulin activity against fastidious and non-fastidious veterinary and human bacterial isolates: initial development of in vitro susceptibility test methods. Journal of Clinical Microbiology 40, 461–465.

Kiuchi, A., Hara, M., Pham, H.S., et al., 2000. Phylogenetic analysis of the *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* based upon 16S rRNA. DNA Sequence 11, 257–260.

Kucsera, G.Y., 1973. Proposal for standardization of the designations used for serotypes of *Erysipelothrix rhusiopathiae* (Migula) Buchanan. International Journal of Systematic Bacteriology 23, 184–188.

Kucsera, G., 1977. Serological typing of *Erysipelothrix rhusiopathiae* strains and the epizootiological significance of the typing. Acta Microbiologica et Immunologica Hungarica 27, 19–28.

Lehane, L., Rawlin, G.T., 2000. Topically acquired bacterial zoonoses from fish: a review. Medical Journal of Australia 173, 256–259.

Makino, S., Okada, Y., Maruyama, T., et al., 1994. Direct and rapid detection of *Erysipelothrix rhusiopathiae* DNA in animals by PCR. Journal of Clinical Microbiology 32, 1526–1531.

Nagai, S., To, H., Kanda, A., 2008. Differentiation of *Erysipelothrix rhusiopathiae* strains by nucleotide sequence analysis of a hypervariable region in the spaA gene: discrimination of a live vaccine strain from field isolates. Journal of Veterinary Diagnostic Investigation 20, 336–342.

Nakato, H., Shinomiya, K., Mikawa, H., 1987. Adhesion of *Erysipelothrix rhusiopathiae* to cultured rat aortic endothelial cells. Role of bacterial neuraminidase in the induction of arteritis. Pathology, Research and Practice 182, 255–260.

Opriessnig, T., Hoffman, L.J., Harris, D.L., 2004. *Erysipelothrix rhusiopathiae*: genetic characterization of midwest US isolates and live commercial vaccines using pulsed-field gel electrophoresis. Journal of Veterinary Diagnostic Investigation 16, 101–107.

Pal, N., Bender, J.S., Opriessnig, T., 2009. Rapid detection and differentiation of *Erysipelothrix* spp. by a novel multiplex real-time PCR assay. Journal of Applied Microbiology 108, 1083–1093.

Shimoji, Y., 2000. Pathogenicity of *Erysipelothrix rhusiopathiae*: virulence factors and protective immunity. Microbes and Infection 2, 965–972.

Shimoji, Y., Yokomizo, Y., Sekizaki, T., 1994. Presence of a capsule in *Erysipelothrix rhusiopathiae* and its relationship to virulence for mice. Infection and Immunity 62, 2806–2810.

Shimoji, Y., Yokomizo, Y., Mori, Y., 1996. Intracellular survival and replication of *Erysipelothrix rhusiopathiae* within murine macrophages: failure of induction of the oxidative burst of macrophages. Infection and Immunity 64, 1789–1793.

Shimoji, Y., Mori, Y., Hyakutake, K., et al., 1998. Use of an enrichment broth cultivation-PCR combination assay for rapid diagnosis of swine erysipelas. Journal of Clinical Microbiology 36, 86–89.

Straw, B.E., 2006. Diseases of Swine, ninth ed. Blackwell Pub., Ames, Iowa, pp. 629–638.

Takahashi, T., Fujisawa, T., Tamura, Y., 1992. DNA relatedness among *Erysipelothrix rhusiopathiae* strains representing all twenty-three serovars and *Erysipelothrix tonsillarum*. International Journal of Systematic Bacteriology 42, 469–473.

Takahashi, T., Fujisawa, T., Umeno, A., et al., 2008. A taxonomic study on *Erysipelothrix* by DNA–DNA hybridization experiments with numerous strains isolated from extensive origins. Microbiology and Immunology 52, 469–478.

Wang, Q., Fidalgo, S., Chang, B.J., et al., 2002. The detection and recovery of *Erysipelothrix* spp. in meat and abattoir samples in Western Australia. Journal of Applied Microbiology 92, 844–850.

Yamamoto, K., Sasaki, Y., Ogikubo, Y., et al., 2001. Identification of the tetracycline resistance gene, *tet*(M), in *Erysipelothrix rhusiopathiae*. Journal of Veterinary Medicine B: Infectious Diseases and Veterinary Public Health 48, 293–301.

Yamazaki, Y., 2006. A multiplex polymerase chain reaction for discriminating *Erysipelothrix rhusiopathiae* from *Erysipelothrix tonsillarum*. Journal of Veterinary Diagnostic Investigation 18, 384–387.

FURTHER READING

Wang, Q., Chang, B.J., Riley, T.V., 2010. *Erysipelothrix rhusiopathiae*. Veterinary Microbiology 140 (3–4), 405–417.

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Chapter 14

Bacillus species

Genus Characteristics

Bacillus species are large, Gram-positive, endospore-forming rods. They are known to produce resistant endospores in the presence of O_2 with one exception, *B. infernus*. This large genus accommodates approximatively 100 species including the well-known zoonotic agent *B. anthracis*. The bacilli can be divided into three main groups: *B. subtilis* group (*B. subtilis*, *B. licheniformis*, *B. pumilis*, and *B. amyloliquefaciens*), *B. cereus* group (*B. anthracis, B. cereus*, *B. mycoides* and *B. thuringiensis*) and *B. circulans* group (*B. circulans, B. firmus, B. coagulans* and *B. lentus*). Most species are mesophilic.

Although *Bacillus* species are Gram-positive in young cultures, they can appear Gram-variable or even Gram-negative on account of their spores. They are usually catalase-positive, aerobic or facultatively anaerobic and motile with the exception of *B. anthracis* and *B. mycoides*. Most will grow on nutrient agar but not on MacConkey agar. *Bacillus anthracis*, the agent of anthrax, is an important pathogen of animals (mainly herbivores) and humans and a potential biological weapon.

Natural Habitat

Most of the numerous *Bacillus* species are saprophytes widely distributed in air, soil and water. *Bacillus* spores can survive in a wide variety of habitats since they are resistant to heat, radiation, disinfection and desiccation. Spores can remain dormant in soil for decades. The main habitats are considered to be all sorts of soils and waters. Some *Bacillus* species are opportunistic pathogens (*B. cereus*, *B. licheniformis*) while *B. anthracis* is considered an obligate pathogen of animals and humans.

Bacillus anthracis spores are geographically ubiquitous in soils. This bacillus most commonly infects ungulate herbivores. It has been reported that in nature the spores are associated with fairly heavy particles and are not likely to become airborne at carcass sites, despite rain, wind or some soil agitation (Turnbull et al. 1998). In nature, the vegetative form of *B. anthracis* seems to exist only in a host and is not found in the environment.

Pathogenesis and Pathogenicity

Anthrax

Bacillus anthracis is considered the major animal pathogen in the *Bacillus* genus (Table 14.1) causing anthrax in herbivores and other mammals, including humans. In general, three main clinical forms have been described to date in the literature: cutaneous, gastrointestinal and respiratory (Hanna 1998). However, this classification is less useful in veterinary medicine, where anthrax is viewed as a singular disease that rapidly progresses to death without any specific clinical signs. It is often characterized by a massive septicaemia where hypotension, shock and sudden death are mainly attributed to the lethal toxin (LeTX). In contrast, in human cases of anthrax the symptoms precede death over many days. Animal anthrax is therefore difficult to treat because of the rapid onset of the condition. A mortality rate of approximatively 100% is reported for animal systemic infections. Susceptibility to the disease is variable among different animal species. Cattle, sheep and goats are most susceptible to infection, horses and humans occupy an intermediate position, while pigs, birds and carnivores are comparatively resistant, but can succumb if the infective dose is high. Disease

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Table 14.1 Main diseases caused by the major pathogenic bacilli in veterinary medicine

Species	Host(s)	Diseases
Bacillus anthracis	Cattle and sheep	Septicaemic form of anthrax; usually presents as sudden death
	Carnivores	Comparatively resistant to disease; pattern similar to that in pigs; a massive dose from eating anthrax-infected carcasses can lead to septicaemia
	Horses	Oral route: septicaemia with colic and enteritis Wound infections: localized oedema and lymphadenitis
	Pigs	Subacute anthrax with oedematous swelling in pharyngeal tissues and regional lymphadenitis or intestinal form with a higher mortality
	Human	Intestinal, cutaneous and inhalational forms
Bacillus cereus	Cattle	Rarely mastitis
	Dogs and cats	Rarely food poisoning
	Human	Food-borne diarrhoeal illness
Bacillus licheniformis	Cattle and sheep	Abortion and mastitis
	Human	Food-borne diarrhoeal illness
Bacillus subtilis	Cattle and sheep	Ovine abortion and bovine mastitis

has also occurred in bison, white-tailed deer, ostriches, mink and moose. Rats and some strains of mice are considered resistant.

In cattle, sheep, and goats anthrax is considered a peracute disease characterized by septicaemia with high fever and sudden death (within one or two days). In some cases, the disease may last for about a week. Postmortem findings include exudation of tarry blood from body orifices, failure of the blood to clot, incomplete rigor mortis and splenomegaly in cattle. Indeed, a typical characteristic of anthrax is the failure of blood to clot following death. In the less susceptible species inflammatory subcutaneous oedema of face, throat and neck is a common finding and colic can occur in horses and gastroenteritis in carnivores. Sporadic disease and outbreaks have been observed in pigs where the condition was characterized by swelling of the throat and/or digestive disturbances with a low mortality rate (Edginton 1990, Williams et al. 1992). Research has suggested that meat from healthy pigs killed 21 days after the last case following an outbreak of anthrax should not pose a public health risk (Redmond et al. 1997).

Anthrax occurs when endospores of *B. anthracis* gain entry to a host through ingestion, from soil when grazing or in contaminated food, through abrasions of the skin or following inhalation. Inhalation occurs to a lesser extent in animals than in humans. Transmission by biting insects may be important especially during an outbreak. Following entry, the endospores are rapidly phagocytosed by macrophages and then germinate inside the macrophages. Vegetative bacteria are released into the blood in which they rapidly multiply to high numbers. Virulence of most *B. anthracis* strains is associated with two megaplasmids (Table 14.2). Strains lacking either plasmid are avirulent or significantly attenuated. Plasmid pXO1 carries the genes for the anthrax tripartite protein toxin complex (Okinaka et al., 1999), while plasmid pXO2 carries the biosynthetic genes for the antiphagocytic poly-D-glutamic acid capsule. The anthrax tripartite toxin comprises three components: a protective antigen, a lethal factor and an oedema factor. These proteins act in binary combinations to produce the two anthrax toxins (Leppla 1995): oedema toxin (protective antigen and oedema factor) and lethal toxin (protective antigen and lethal factor). Capsule and toxin virulence factors seem to be regulated by host-specific signals such as CO_2 concentration.

Humans can incidentally acquire the disease by contact with endospores from infected animals or their contaminated products or from a bioterrorism source. About 95% of human anthrax cases are the cutaneous form, 5% respiratory, while the gastrointestinal form is very rare. There are no known cases of human-to-human transmission. Only a few endospores are required to cause cutaneous anthrax, while the infectious doses in gastrointestinal and respiratory forms are usually very high (50% lethal dose > 10,000 spores). Cutaneous anthrax cases are readily treated and become life-threatening only on exceptional occasions. The skin lesion starts with a pruritic papule which evolves to a painless black eschar (Dixon et al. 1999). The respiratory and gastrointestinal (GI) forms are both highly fatal forms of the disease if not treated. The gastrointestinal form usually occurs two to five days after the ingestion of spores from contaminated meat or food products. The

Table 14.2 Main virulence factors of *Bacillus anthracis*

Genes	Virulence determinants	Functions
Plasmid pXO2		
capB, *capC* and *capA*	Poly-D-glutamic acid capsule	Inhibits the phagocytosis of vegetative cells by macrophages
dep	Capsule degradation factor (dep)	A gene associated with the depolymerization of the capsular polymer: capsule degradation factor
acpA	Regulator of encapsulation (acpA)	A *trans*-acting regulatory gene: involved in the regulation of encapsulation
Plasmid pXO1		
Lethal toxin (LeTx) made up of:		Triggers the oxidative burst pathway of macrophages and the release of cytokines (TNF-α and IL-1β)
lef	Lethal factor (LF)	Zinc-metalloprotease: cleaves various substrates and inactivates the mitogen-activated protein kinase kinase (MAPKK)
pagA	Protective antigen (PA)	Host-cell-binding moiety: facilitates the entry of LF into the host cell cytoplasm
Edema toxin (EdTx) made up of:		Increases cellular CAMP and disrupts water homeostasis
cya	Edema factor (EF)	Calmodulin-dependent adenylate cyclase
pag	Protective antigen (PA)	Host-cell-binding moiety: facilitates the entry of EF into the host cell cytoplasm
Others:		
atxA	Activator of transcription (atxA)	*Trans*-acting regulatory genes: activates transcription of the anthrax toxin genes
pagR	pagR	*Trans*-acting regulatory genes
topA	topoisomerase (topA)	Type I topoisomerase

respiratory form occurs after the inhalation of endospores usually by workers handling contaminated animal products or hides (Woolsorter's disease). Endospores can remain dormant for more than 60 days in the lungs (Barakat et al. 2002).

Sporulation process

Bacillus anthracis will sporulate in an opened (aerobic conditions) carcass under decomposition where conditions of nutrient deprivation result in mature endospores. The sporulation process can be simplified into four steps:

1. asymetric septation of the vegetative bacilli (mother cell)
2. maturation of the forespore compartment
3. death of the mother cell surrounding the forespores
4. production of one endospore per mother cell.

Bacillus anthracis endospores are basically made of four structures: the core, the cortex, the spore coat, and the exosporidium. They contain protein, DNA, calcium, dipicolinic acid (DPA), a paracrystaline lipid bilayer, a thick cross-linked peptidoglycan and a collagen-like surface protein named BclA (*Bacillus* collagen-like protein of *anthracis*). Endospores are inert with no measurable metabolism and will not divide like vegetative cells. They have no ATP, no synthesis and very little or no water. The germination process occurs within the host and is triggered by various nutrient germinants which contact receptors on the spore itself. This causes the release of chelated Ca^{2+}-dipicolinic acid from the spore core and hydration as water floods the core. Expulsion of DPA activates cortex lytic enzymes that degrade the spore cortex allowing germination to progress.

Other bacilli

The majority of the other *Bacillus* species have little or no pathogenic potential but can occur commonly as contaminants on laboratory media. Table 14.1 summarizes the main hosts and diseases of the pathogenic bacilli. *Bacillus cereus* is best known as a cause of food-borne illness in humans and occasionally affects animals, mainly dogs and cats (Chastain & Harris 1974). *Bacillus cereus* can produce two types of food-poisoning: the diarrhoeal type and the emetic type. *Bacillus licheniformis* is an important cause of bovine abortion and occasionally causes bovine mastitis. *Bacillus subtilis* has also been implicated in cases of food-poisoning and in cases of bovine mastitis and ovine abortion (Logan 1988). *Bacillus coagulans* has been isolated from bovine abortion cases whereas *B. pumilus* has been isolated from cases of bovine mastitis (Logan 1988). Some *Bacillus* species cause disease in insects, such as *B. thuringiensis*, which is widely exploited in agriculture as an insecticide by virtue of its plasmid-borne crystal toxin genes

(Romeis et al. 2006), and *B. larvae*, recently reclassified *Paenibacillus larvae*, which causes American foulbrood in honeybees. '*B. piliformis*' has been reclassified *Clostridium piliforme* on the basis of 16S rRNA sequence analysis and is responsible for Tyzzer's disease (see Chapter 16) in laboratory mice and foals (Duncan et al. 1993).

Laboratory Diagnosis

Specimens

Bacillus anthracis

Safety considerations in relation to anthrax should be discussed with laboratory workers and veterinarians. Great care should be exercised when dealing with specimens from suspected cases of anthrax. During sample collection, disposable gloves, overalls, and boots should be worn and disinfected after use. If dusty samples are handled, headgear and dust mask should be considered. All procedures should be carried out in a biohazard safety cabinet and infective and contaminated materials subsequently autoclaved followed by incineration. Stained smears, that have been heat-fixed, are potentially dangerous as they may contain viable spores. It should be remembered that the endospores of *B. anthracis* can remain viable for many years or even several decades.

A postmortem animal examination is usually unnecessary and should never be carried out unless the carcass can be taken to a place where the surrounding area can be thoroughly decontaminated following the examination. Because of the risk of human infection, personnel carrying out a postmortem should take adequate safety precautions. Endospores are not formed in the animal body but sporulation is triggered when vegetative cells are exposed to air. In an unopened carcass, the vegetative forms do not survive the putrefaction process. If anthrax is suspected in cattle or sheep, thin blood smears should be made from blood taken from ear or tail veins as soon as possible after death, for both culture (blood agar) and direct examination to demonstrate the capsule (M'Fadyean-stained smear). In horses and pigs oedematous fluid can be collected from localized sites instead of blood. As pigs usually do not suffer from the overwhelming bacteraemia that occurs in herbivores, the large rods may not be visible in blood smears. Peritoneal fluid is often more useful diagnostically than blood smears in this species. If the animal has been opened, spleen or lymph node samples should be taken. In the case of very old, putrefied carcasses, it is recommended that swabs of the nostrils and eye sockets should be taken along with samples of contaminated soil underneath the animal's nose and anus.

Direct microscopy examination

Bacillus anthracis is a Gram-positive, rod-shaped, nonmotile, spore-forming bacterium which needs to be differentiated from other *Bacillus* species. *Bacillus anthracis* produces a capsule in vivo and either Giemsa, Wright's or M'Fadyean's polychrome methylene blue stains can be used to demonstrate the capsule which is of diagnostic importance. Polychrome methylene-blue-stained smears reveal square-ended, blue rods arranged singly, in pairs or in short chains surrounded by pink to purplish capsular material (M'Fadyean's reaction) which is characteristic for *B. anthracis* (Fig. 14.1).

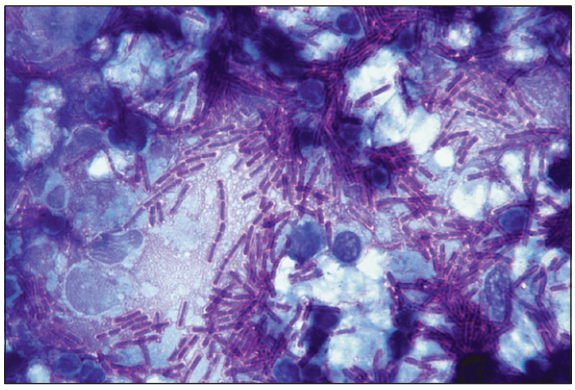

Figure 14.1 *Bacillus anthracis* in a bovine blood smear collected from a peripheral blood vessel showing square-ended, blue bacilli in short chains surrounded by a pink capsule. (Polychrome methylene blue stain, ×1000)

Isolation

Isolation and identification of *B. anthracis* can be performed safely in a diagnostic veterinary bacteriology laboratory with good laboratory practices. If there is minimal handling of the organism, vaccination may not be required (Murray et al. 2007). *Bacillus* species in the *B. cereus* group grow well on sheep or ox blood agar, aerobically at 35°C in 24–48 hours. Contaminated specimens such as old carcasses, hair, bonemeal and other animal feeds should be ground finely, steeped in saline and then heated at 65°C for 10 minutes (or 62.5°C for 15 minutes) to heat shock the spores and destroy non-spore-forming contaminants. Direct plating can be carried out on blood, nutrient or selective agars. A selective medium for *B. anthracis*, polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar (Appendix 2), has been described (Knisely 1966). For visualization of spores, it is helpful to grow the organism for a few days on nutrient agar with 5 mg of manganese sulphate per litre.

For *B. cereus* isolation from specimens with mixed microflora, nutrient or tryptic soy broth or agar with the addition of polymyxin (100,000 U/L) may be used. Commercial selective media, such as '*Bacillus cereus* selective agar base' (Oxoid) with a polymyxin supplement are also available (Fig. 14.2).

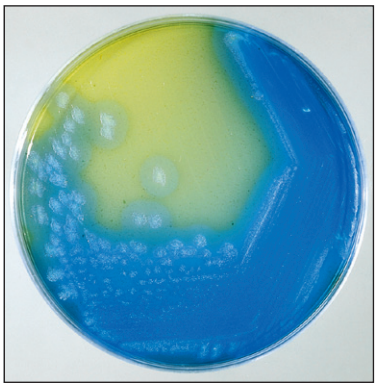

Figure 14.2 *Bacillus cereus* on '*Bacillus cereus* selective agar' developed for the isolation and enumeration of this bacterium from foods. The typical colonies are crenated and have a distinctive peacock-blue colour surrounded by precipitation of the egg yolk in the medium giving a hazy turquoise zone. The medium is rendered selective by the addition of polymyxin B. The pH indicator is bromothymol blue.

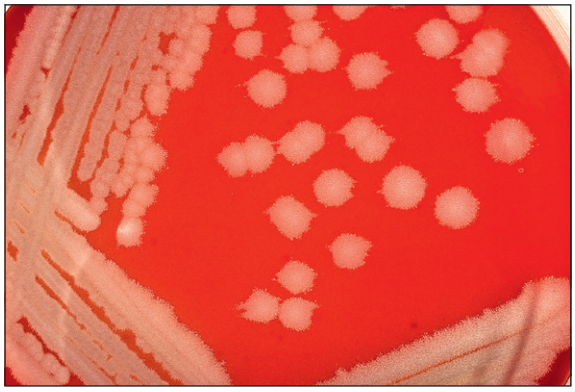

Figure 14.3 *Bacillus anthracis* on sheep blood agar illustrating non-haemolytic, flat, 'ground-glass', dry colonies with irregular edges.

Identification

Colonial morphology

Bacillus anthracis has a unique colony morphology on blood agar and this helps in differentiation from other members of the *B. cereus* group. *Bacillus anthracis* is almost always non-haemolytic (rarely strains show weak haemolysis). After 48 hours' incubation the colonies are about 5 mm in diameter, flat, dry, whitish to greyish often with a granular 'ground-glass' appearance (Fig. 14.3). Under low magnification, curved and curled peripheral projections at the edge of the colonies give rise to a 'Medusa head' appearance, which is due to long chains of rods growing out and back into the colony. *Bacillus anthracis* forms a capsule when grown on nutrient agar with 0.7% of bicarbonate and incubated at 37°C with 5–20% CO_2 (Green et al. 1985); colonies will then appear mucoid.

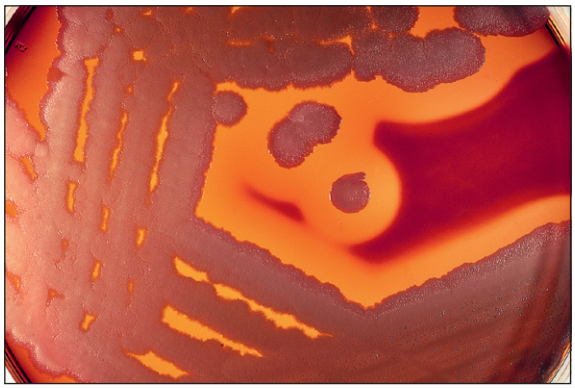

Figure 14.4 *Bacillus cereus* on sheep blood agar. The morphology resembles that of *B. anthracis* but the colonies are usually strongly haemolytic.

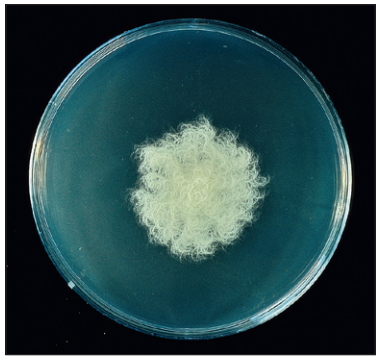

Figure 14.5 *Bacillus mycoides* inoculated centrally on nutrient agar and incubated at 25°C for three days. It has a rhizoid type colony.

B. cereus produces colonies similar to those of *B. anthracis* but they tend to be slightly larger (2–7 mm in diameter) with irregular edges, have a slightly greenish hue and most strains are surrounded by a wide zone of complete haemolysis (Fig. 14.4). The colonies of *B. thuringiensis* have a similar appearance to those of *B. cereus* but tend to have slightly more regular edges.

B. mycoides has markedly rhizoid or hairy-looking colonies that can have an almost fungal appearance. This is best seen if a nutrient agar plate is inoculated centrally and incubated at 25–30°C for a few days (Fig. 14.5). Most strains are weakly haemolytic, adherent and readily cover the agar surface.

Microscopic appearance

All four species in the *B. cereus* group appear as Gram-positive rods from young cultures, about 1 × 3–5 µm in size. However, these organisms do not always Gram stain

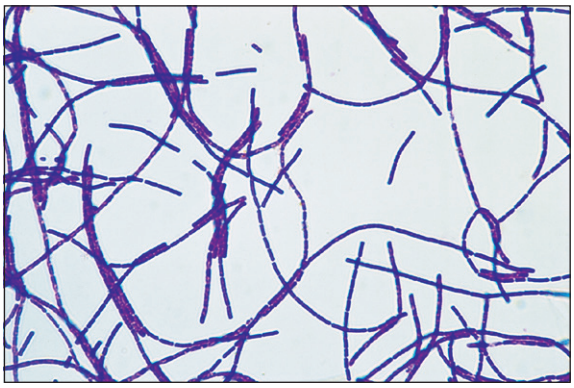

Figure 14.6 *Bacillus anthracis* from a culture: Gram-positive rods in long chains with incipient sporulation. (Gram stain, ×1000)

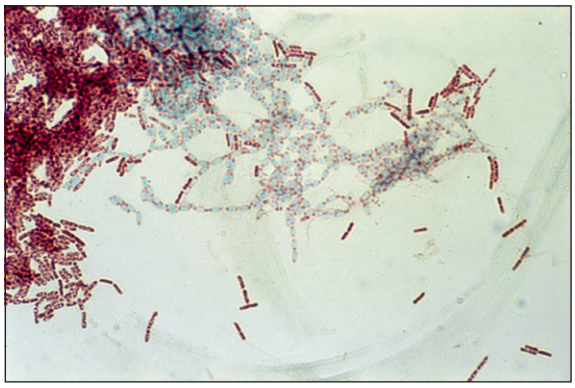

Figure 14.7 Spores of *Bacillus cereus* from culture: the endospores are stained by malachite green while the cytoplasm of the mother cells is counterstained by safranin (red). (×1000)

Table 14.3 Characteristics for differentiating members of the *Bacillus cereus* group

Characteristic	*B. anthracis*	*B. cereus*	*B. mycoides*	*B. thuringiensis*
Motility	–	+	–	+
Penicillin susceptibility (10-unit disc)	S	R	R	R
Gamma phage	S (lysis)	R	V (lysis may occur)	R
Hemolysis	– (or weak)	+	+ (weak)	+
Nutrient agar with 0.7% NaOH under 10% CO_2	mucoid colonies	unchanged	unchanged	unchanged
Gelatin stab culture	Inverted fir tree type of growth	Rapid liquefaction	Rapid liquefaction	Rapid liquefaction
Parasporal crystals (three-day culture)	–	–	–	+
Egg yolk reaction (lecithinase activity)	+ (weak)	+	+	+
Pathogenicity for mice or guinea pigs (sc or iv)	+ (death in 24–48 hours)	+ if large dose (non-invasive)	–	–

R = resistant, S = susceptible, + = >85% positive, – = 0 to 15% positive, V = variable (26 to 74% positive)

positively due to the presence of endospores. Specifically, *B. anthracis* measures 1.2–10 µm × 0.5–2.5 µm and can often be in long chains (Fig. 14.6). Central or terminal endospores may be produced in older cultures and appear as oval, non-stained areas within the mother cell. Other types of inclusions should not be mistaken for spores. Phase-contrast microscopy can be used to visualize spores which then appear larger, more phase-bright and more regular than inclusions such as polyhydroxybutyrate granules or storage vacuoles. A Gram-stained smear with unstained areas can be stripped of oil with acetone-alcohol, washed with water and then stained for spores by flooding with 10% aqueous malachite green for about 45 minutes with no heating. The smear is then washed and counterstained with 0.5% aqueous safranin for 30 seconds. Spores will appear green within pink to red bacterial cells at a magnification of ×1000 (Fig. 14.7). When available, phase-contrast microscopy gives better results and is more convenient than spore staining. *B. thuringiensis* characteristically has cuboid or diamond-shaped parasporal crystals in the cells. These glycoprotein crystals can be best seen in phase-contrast preparations from cultures over two days old.

Biochemical and other tests

All species in the *B. cereus* group are closely related, but they can be differentiated based on colonial morphology and on a few other characteristics as shown in Table 14.3.

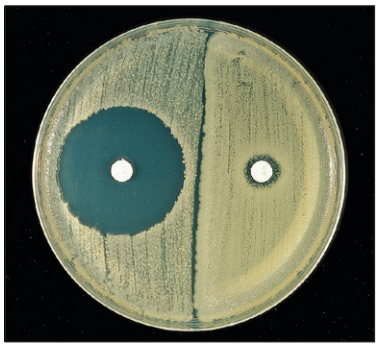

Figure 14.8 *Bacillus anthracis* (left) and *B. cereus* (right) on Isosensitest agar demonstrating the susceptibility of *B. anthracis* to penicillin (10-unit disc) compared to the resistance of *B. cereus*.

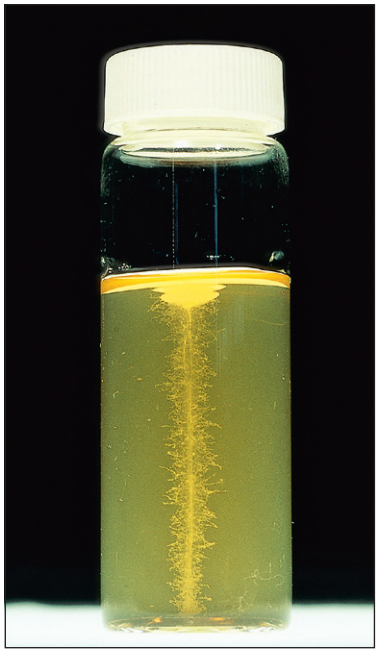

Figure 14.9 *Bacillus anthracis* stab inoculated into nutrient gelatin giving the characteristic 'inverted fir-tree' type growth after eight days at 25°C.

Most *B. anthracis* isolates are susceptible to penicillin while *B. cereus* and the other two species are resistant (Fig. 14.8). *Bacillus cereus*, *B. mycoides* and *B. thuringiensis* rapidly liquefy nutrient gelatin while *B. anthracis* slowly produces an inverted fir-tree type of liquefaction with side-shoots radiating from the stab line (Fig. 14.9). All show lecithinase activity on egg yolk agar (Fig. 14.10) but that of *B. anthracis* is weak. *Bacillus anthracis* and the majority of the *Bacillus* species do not normally produce capsules in or on laboratory media and the colonies have a dry appearance. However, *B. anthracis* can be induced to produce a capsule

Figure 14.10 Strong lecithinase activity by *B. cereus* (top) on egg yolk agar after 24 hours' incubation. *B. anthracis* (left) gives a weak opaque zone after 48 hours and *B. licheniformis* (right) is unreactive on this medium.

as described above, whereupon the colonies will appear quite mucoid.

The API 50CHB test strip can be used with the API 20E test strip (bioMérieux, Marcy l'Etoile, France) to identify most *Bacillus* species (Logan et al. 1985). A *Bacillus* card for VITEK automated identification system and a Biolog Inc. (Hayward, CA) *Bacillus* database are also available. Capsule and cell-wall antigens can be detected by direct fluorescent antibody assays. Gamma phage lysis can be used to confirm the identification of *B. anthracis* (Barakat et al. 2002). Other more sophisticated and less convenient diagnostic tools include fingerprinting by fatty acid methyl ester profiling, polycrylamide gel electrophoresis, pyrolysis mass spectrometry and Fourier transform infrared spectroscopy (Murray et al. 2007).

Determination of pathogenicity

For diagnostic purposes, experimental determination of pathogenicity and virulence is only carried out if any doubt remains about the identity of *B. anthracis* and molecular techniques have largely replaced this procedure. Virulent *B. anthracis* strains are much more pathogenic than other *Bacillus* species and are highly invasive. A light suspension of *B. anthracis* placed on a scarified area at the base of a mouse's tail can cause death. Large doses of *B. cereus*, given to a mouse or guinea pig subcutaneously or intraperitoneally, are needed before the bacterium proves fatal.

Ascoli test

This is an antigen detection test, more specifically a thermoprecipitation test dating from 1911, and used when viable *B. anthracis* could no longer be demonstrated in tissues. About 2–3 g of homogenized material in a little saline is briefly boiled and passed through filter paper. This filtrate is used as the antigen in a ring precipitation

or gel diffusion test with known *B. anthracis* precipitating hyperimmune serum. The test provides rapid retrospective evidence of anthrax infection in an animal using its tissues. However, molecular techniques have largely replaced this procedure.

Serology

Protective antigen, lethal factor and oedema factor and the antibodies produced against them can be used as the basis of enzyme immunoassay systems suitable for routine confirmation of anthrax infection, for monitoring responses to anthrax vaccines and for epidemiological investigations in humans and animals. In these tests, antibodies against protective antigen alone appear to be satisfactory. For retrospective diagnosis of anthrax or to evaluate immune status following vaccination, a skin test utilizing Anthraxin (a heat-stable extract from a non-capsulated *B. anthracis*) has been used in humans and animals in the former Soviet Union (Shlyakhov & Rubinstein 1994) where cell-mediated immunity to anthrax is expressed by a delayed-type hypersensitivity.

Other *Bacillus* species

Clinical specimens for isolation of other *Bacillus* species can be handled safely without special measures on the open bench. They normally survive transport in fresh samples or in standard transport medium. A definitive identification of the numerous *Bacillus* species, other than *B. anthracis*, requires a range of tests that are usually carried out in reference laboratories (Murray et al., 2007). The API 20E/50CHB test strips (bioMérieux) can be used to presumptively identify *Bacillus* species and subspecies such as *B. subtilis*, *B. licheniformis* and *B. circulans* which are commonly seen on diagnostic culture plates as contaminants. All these species are mesophilic and grow well between 30 and 37 °C. The colonial morphology is comparatively distinctive in most cases but their identity should be confirmed by biochemical tests.

Bacillus subtilis has round to irregular colonies with a dull, granular, cream to brownish surface (Fig. 14.11). It is variably haemolytic on blood agar. Active spreading of the colonies can occur on agar with a moist surface. *Bacillus subtilis* is Gram-positive in smears made from young colonies. The cells are 0.6–0.8 × 2–3 µm in size and the endospores, which are widespread in the environment, are ellipsoidal, central and do not bulge the sporangium. *Bacillus licheniformis* colonies are opaque, dull, rough, wrinkled, strongly adherent to the agar and commonly display hair-like outgrowths (Fig. 14.12). It is named for the similarity between the colonies and the appearance of lichen. Some strains are haemolytic. The cells and endospores are similar to those of *B. subtilis*.

Bacillus circulans is a species that is genetically heterogeneous. Some strains are unusual in that the colonies themselves are motile and move over the surface of an agar plate in a circular manner. As a parent colony moves, cells are left behind and these in turn form colonies that are also motile (Figs 14.13 and 14.14). This bacillus is described as Gram-variable and the cells are often Gram-negative even in smears from young cultures. The cells are 0.5–0.7 × 2–5 µm in size and the spores are ellipsoidal, variable in position and bulge the mother cell. *Bacillus coagulans* and *Bacillus pumilus* do not display distinctive colony features.

Figure 14.11 *Bacillus subtilis* on sheep blood agar showing the dull, wrinkled, irregular colonies. Some strains are haemolytic.

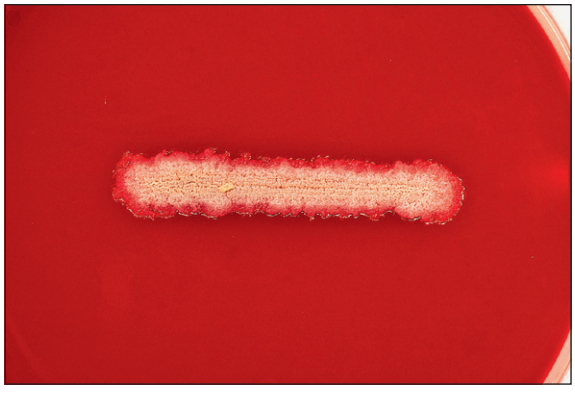

Figure 14.12 *Bacillus licheniformis* on sheep blood agar inoculated as a streak to show the heaped, wrinkled, lichen-like appearance. Some strains are haemolytic.

Antimicrobial Susceptibility Testing

Susceptibility of *B. anthracis* to antimicrobial agents can be determined using the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) broth microdilution reference method, the Etest strips (AB BIODISK, Solna, Sweden) agar gradient diffusion method or the agar dilution method (Coker et al. 2002, Mohammed et al. 2002). However, CLSI breakpoints for staphylococci may have to be used for some antimicrobials.

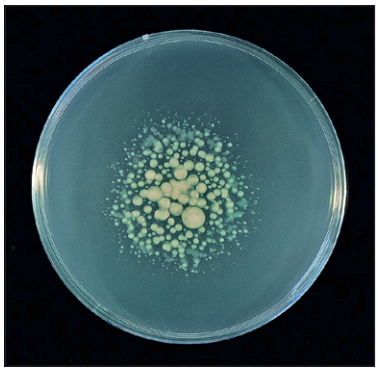

Figure 14.13 *Bacillus circulans* centrally inoculated to show the motile colonies moving outwards over the agar surface. This plate was incubated at 25°C for 72 hours.

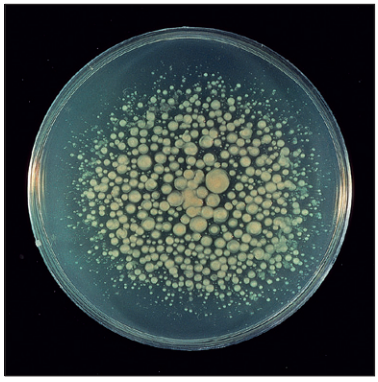

Figure 14.14 *Bacillus circulans*: the same plate as Fig. 14.13 to show the further progress of the motile colonies after a further 48 hours' incubation at 25°C.

Antimicrobial resistance

Historically, susceptibility to penicillin and other β-lactam agents was often a defining trait of *B. anthracis* because all other members of the *B. cereus* group are resistant to penicillin. However, recent studies on antimicrobial susceptibility profiles of clinical and non-clinical isolates of *B. anthracis* have revealed penicillin G-resistant strains (Chen et al. 2004) although they are considered as uncommon. *Bacillus anthracis* is normally susceptible to penicillin and also to gentamicin, erythromycin, chloramphenicol, ciprofloxacin, doxycycline, and streptomycin. It is resistant to cephalosporins due to production of constitutive or inducible β-lactamases (Frean et al. 2003).

Bacillus cereus strains commonly exhibit resistance to penicillin and other β-lactam agents due to chromosomally encoded β-lactamases. *Bacillus thuringiensis* and most *B. mycoides* strains are resistant to penicillin.

Strain Typing

Bacillus anthracis is reported to display little genetic diversity between strains (Keim & Smith 2002), making strain discrimination particularly difficult. The underlying genetic basis for this plasticity is not known. However, subtle morphological and biochemical differences exist. Multiple-locus variable-number tandem repeat (VNTR) analysis can be performed to type isolates against various strains of *B. anthracis*. The differences between the VNTR patterns have been used to suggest phylogenetic relationships among worldwide isolates (Keim & Smith 2002). Generally isolates fall into one of three phylogenetic branches A, B and C, with lineage A widely distributed throughout the world (Kolstø et al. 2009, Simonson et al. 2009).

Molecular Diagnosis

New molecular diagnostic techniques have mainly focused on the use of the polymerase chain reaction (PCR) to amplify specific markers of *B. anthracis* for rapid diagnosis (Rantakokko-Jalava & Viljanen 2003). Various real-time PCR assays for rapid detection of *B. anthracis* have been described and are considered the most sensitive DNA detection assays available (Ellerbrok et al. 2002, Patra et al. 2002). Primers are usually based on specific plasmid-borne and chromosomal genes in the *B. anthracis* genome such as the lethal factor (*lef*), edema factor (*cya*), and protective antigen gene A (*pagA*) located on the pXO1 plasmid, the capsular protein genes A, B, and C (*capA*, *capB*, and *capC*) located on the pXO2 plasmid, and the chromosomal SASP (small acid soluble protein) gene (Jones et al. 2005). Chromosomal gene-based real-time PCR assays such as the DNA gyrase A gene (*gyrA*) or other chromosomal loci are helpful for detecting the presence of *B. anthracis* DNA and also in determining gene copy numbers in a specimen. However, they are not as sensitive as the plasmid-based assays due to the presence of more than one copy per *B. anthracis* of the pXO1 and/or pXO2 plasmid (Jones et al. 2005). PCR-based detection of *B. anthracis* in formalin-fixed tissue (Levine et al. 2002) and of spores in heat-treated specimens has been described (Fasanella et al. 2003).

Sequencing of 16S rRNA gene as a rapid tool for identification of *B. anthracis* has been described (Sacchi et al. 2002). The technique can be used for rapid identification and differentiation of *B. anthracis* from other *Bacillus* species but not for strain individualization (Jones et al. 2005). Identification of *B. anthracis* by *rpoB* sequence analysis and multiplex PCR has also been described (Ko et al. 2003). The use of multiplex PCR on a DNA chip has been described for the identification and characterization of *B. anthracis* (Wang et al. 2004). More recently, a rapid-viability PCR method for detection of live, virulent *B. anthracis* in environmental samples has been described (Letant et al., 2011). These new rapid molecular diagnostic tools are increasingly becoming useful in the veterinary clinical setting.

REFERENCES

Barakat, L.A., Quentzel, H.L., Jernigan, J.A., et al., 2002. Fatal inhalational anthrax in a 94-year-old Connecticut woman. Journal of the American Medical Association 287, 863–868.

Chastain, C.B., Harris, D.L., 1974. Association of *Bacillus cereus* with food poisoning in dogs. Journal of the American Veterinary Medical Association 164, 489–490.

Chen, Y., Tenover, F.C., Koehler, T.M., 2004. Beta-lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. Antimicrobial Agents and Chemotherapy 48, 4873–4877.

Coker, P.R., Smith, K.L., Hugh-Jones, M.E., 2002. Antimicrobial susceptibilities of diverse *Bacillus anthracis* isolates. Antimicrobial Agents and Chemotherapy 46, 3843–3845.

Dixon, T.C., Meselson, M., Guillemin, J., et al., 1999. Anthrax. New England Journal of Medicine 341 (11), 815–826.

Duncan, A.J., Carman, R.J., Olsen, G.J., et al., 1993. Assignment of the agent of Tyzzer's disease to *Clostridium piliforme* comb. nov. on the basis of 16S rRNA sequence analysis. International Journal of Systematic Bacteriology 43, 314–318.

Edginton, A.B., 1990. An outbreak of anthrax in pigs: a practitioner's account. Veterinary Record 127, 321–324.

Ellerbrok, H., Nattermann, H., Ozel, M., et al., 2002. Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. FEMS Microbiology Letters 214, 51–59.

Fasanella, A., Losito, S., Adone, R., et al., 2003. PCR assay to detect *Bacillus anthracis* spores in heat-treated specimens. Journal of Clinical Microbiology 41, 896–899.

Frean, J., Klugman, K.P., Arntzen, L., et al., 2003. Susceptibility of *Bacillus anthracis* to eleven antimicrobial agents including novel fluoroquinolones and a ketolide. Journal of Antimicrobial Chemotherapy 52, 297–299.

Green, B.D., Battisti, L., Koehler, T.M., et al., 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infection and Immunity 49, 291–297.

Hanna, P., 1998. Anthrax pathogenesis and host response. Current Topics in Microbiology and Immunology 225, 13–35.

Jones, S.W., Dobson, M.E., Francesconi, S.C., et al., 2005. DNA assays for detection, identification, and individualization of select agent microorganisms. Croatian Medical Journal 46, 522–529.

Keim, P., Smith, K.L., 2002. *Bacillus anthracis* evolution and epidemiology. Current Topics in Microbiology and Immunology 271, 21–32.

Knisely, R.F., 1966. Selective medium for *Bacillus anthracis*. Journal of Bacteriology 92, 784–786.

Ko, K.S., Kim, J.M., Kim, J.W., et al., 2003. Identification of *Bacillus anthracis* by *rpoB* sequence analysis and multiplex PCR. Journal of Clinical Microbiology 41, 2908–2914.

Kolstø, A.B., Tourasse, N.J., Økstad, O.A., 2009. What sets *Bacillus anthracis* apart from other *Bacillus* species? Annual Review of Microbiology 63, 451–476.

Leppla, S.H., 1995. Anthrax toxins. In: Moss, JIB., Vaughn, M., Tu, AT. (Eds.), Bacterial Toxins and Virulence Factors in Disease. Marcel Dekker, New York, NY, pp. 543–572.

Letant, S.E., Murphy, G.A., Alfaro, T.M., et al., 2011. Rapid-viability PCR method for detection of live, virulent *Bacillus anthracis* in environmental samples. Applied Environnemental Microbiology 77, 6570–6578.

Levine, S.M., Perez-Perez, G., Olivares, A., et al., 2002. PCR-based detection of *Bacillus anthracis* in formalin-fixed tissue from a patient receiving ciprofloxacin. Journal of Clinical Microbiology 40, 4360–4362.

Logan, N.A., 1988. *Bacillus* species of medical and veterinary importance. Journal of Medical Microbiology 25, 157–165.

Logan, N.A., Carman, J.A., Melling, J., et al., 1985. Identification of *Bacillus anthracis* by API tests. Journal of Medical Microbiology 20, 75–85.

Mohammed, M.J., Marston, C.K., Popovic, T., et al., 2002. Antimicrobial susceptibility testing of *Bacillus anthracis*: comparison of results obtained by using the National Committee for Clinical Laboratory Standards broth microdilution reference and Etest agar gradient diffusion methods. Journal of Clinical Microbiology 40, 1902–1907.

Murray, P.R., Baron, E.J., Jorgensen, J.H., et al., 2007. Manual of clinical microbiology, 9th ed. American Society for Microbiology, ASM Press, Washington, D.C.

Okinaka, R.T., Cloud, K., Hampton, O., et al., 1999. The sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. Journal of Bacteriology 181, 6509–6515.

Patra, G., Williams, L.E., Qi, Y., et al., 2002. Rapid genotyping of *Bacillus anthracis* strains by real-time polymerase chain reaction. Annals of the New York Academy of Sciences 969, 106–111.

Rantakokko-Jalava, K., Viljanen, M.K., 2003. Application of *Bacillus anthracis* PCR to simulated clinical samples. Clinical Microbiology and Infection 9, 1051–1056.

Redmond, C., Hall, G.A., Turnbull, P.C., et al., 1997. Experimentally assessed public health risks associated with pigs from farms experiencing anthrax. Veterinary Record 141, 244–247.

Romeis, J., Meissle, M., Bigler, F., 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nature Biotechnology 24, 63–71.

Sacchi, C.T., Whitney, A.M., Mayer, L.W., et al., 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerging Infectious Diseases 8, 1117–1123.

Shlyakhov, E.N., Rubinstein, E., 1994. Human live anthrax vaccine in the former USSR. Vaccine 12, 727–730.

Simonson, T.S., Okinaka, R.T., Wang, B., et al., 2009. Bacillus anthracis in China and its relationship to worldwide lineages. BMC Microbiology 9, 71. doi: 10.1186/1471-2180-9-71.

Turnbull, P.C., Lindeque, P.M., Le Roux, J., et al., 1998. Airborne movement of anthrax spores from carcass sites in the Etosha National Park, Namibia. J Applied Microbiology 84, 667–676.

Wang, S.H., Wen, J.K., Zhou, Y.F., et al., 2004. Identification and characterization of *Bacillus anthracis* by multiplex PCR on DNA chip. Biosensors and Bioelectronics 20, 807–813.

Williams, D.R., Rees, G.B., Rogers, M.E., 1992. Observations on an outbreak of anthrax in pigs in north Wales. Veterinary Record 131, 363–366.

Chapter 15

Non-spore-forming anaerobes

The non-sporing obligate anaerobes constitute a large, diverse group of Gram-positive and Gram-negative bacteria that exist in the environment but also as commensals on mucous membranes of animals and humans, particularly in the intestinal tract as part of the normal flora. Phenotypic characteristics are highly variable depending on the genus and species in question and are described under Laboratory Diagnosis and associated tables. Although knowledge of these bacteria is incomplete as they can be nutritionally demanding and require strict anaerobic conditions for isolation, more information is becoming available through the use of advanced molecular techniques. They are commonly implicated in necrotic and suppurative conditions, often as mixed infections with facultative anaerobic bacteria. Figure 15.1 briefly summarizes the more important genera of these non-spore-forming anaerobes.

Pathogenicity

The infections are often endogenous from normal flora at the site or may be wounds contaminated by nearby flora. For these strict anaerobes to multiply at a focus in animal tissue the redox potential of the tissues must be lowered. This can occur through trauma and necrosis, ischaemia, parasitic invasion or concomitant multiplication of facultative anaerobes. The conditions caused by these non-sporing anaerobes include soft tissue abscessation and cellulitis, post-operative wound infections, periodontal abscesses, aspiration pneumonia, lung and liver abscesses, peritonitis, pleuritis, myometritis, osteomyelitis, mastitis and footrot. Some of the more common infections are shown in Table 15.1. The major pathogens of veterinary significance described in this chapter are *Fusobacterium necrophorum* and *Dichelobacter nodosus*, with organisms such as *Prevotella* spp. and *Porphyromonas* spp. being isolated from ruminant foot conditions and other necrotic or suppurative lesions. *Brachyspira* and *Treponema* spp. are described in Chapter 31 and *Actinomyces bovis* and *Actinobaculum suis* in Chapter 10.

Fusobacterium necrophorum is a normal inhabitant of the gastrointestinal tract and is a major cause of necrobacillosis in animals, particularly calf diphtheria, liver abscesses in cattle and necrotic and suppurative conditions of the foot in ruminants, pigs and horses (Table 15.1). There are two subspecies, *F. n. necrophorum* and *F. n. funduliforme*, with *F. n. necrophorum* being the more pathogenic. Its major virulence factor is a leukotoxin. This toxin, encoded by the gene *lktA*, is particularly effective against ruminant leukocytes and induces activation and apoptosis of these cells (Narayanan et al. 2002). Other important virulence attributes include the production of high levels of endotoxin, haemagglutinin production, and dermotoxic activity which may be due to a collagenolytic cell wall component (Okamoto et al. 2005, Tadepalli et al. 2009).

Virulence of *Dichelobacter nodosus*, the primary causal agent in footrot of sheep, is dependent on the presence of Type IV fimbriae and the production of proteases (Kennan et al. 2011). The fimbriae are highly immunogenic and are classified into 10 major serogroups, designated A to I and M. In addition, strains of *D. nodosus* may be classified as virulent, benign or of intermediate virulence according to the clinical lesions and proteases produced. The *fimA* gene is essential for virulence in sheep and antigenic diversity of the fimbriae is based on variation in the carboxy-terminal of this gene. It has been suggested that serogroup conversion, possibly due to recombination following natural transformation, may occur in the field (Kennan et al. 2003). Such events would explain some of the difficulties encountered in control of footrot by vaccination

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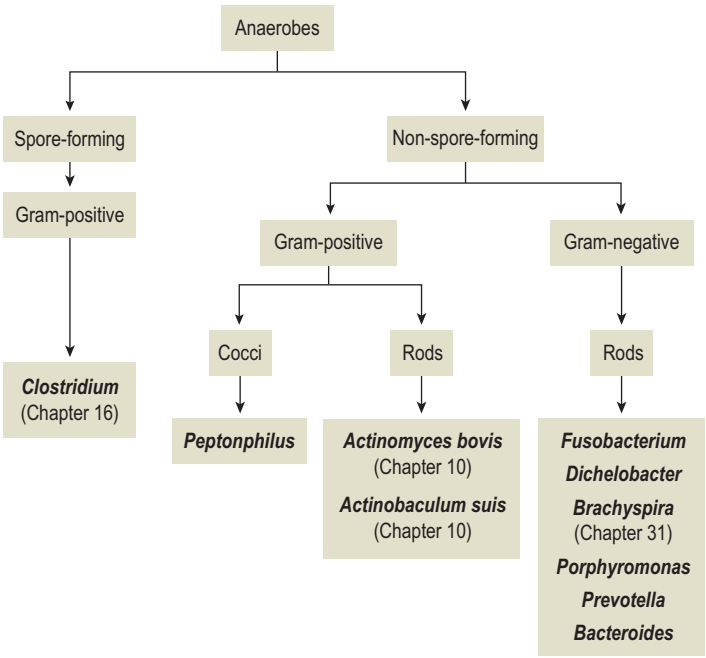

Figure 15.1 Anaerobes of veterinary importance.

Table 15.1 Diseases caused by non-spore-forming anaerobes

Non-spore-forming anaerobe	Associated pathogens	Host(s)	Disease
Peptoniphilus indolicus	*Trueperella (Arcanobacterium) pyogenes*	Cattle	Summer mastitis
Actinobaculum suis		Sows	Pyelonephritis (normal flora in preputial diverticulum of boars)
Actinomyces bovis		Cattle	Lumpy jaw
Actinomyces spp. (unclassified)		Horses	Fistulous withers and poll evil
		Sows	Granulomatous mastitis
Brachyspira hyodysenteriae		Pigs	Swine dysentery
Dichelobacter nodosus	*Fusobacterium necrophorum, Trueperella (Arcanobacterium) pyogenes*, unclassified spirochaete	Sheep	Contagious (virulent) footrot
Dichelobacter nodosus		Goats, cattle, pigs	Infections of the interdigital skin
Prevotella melaninogenica	*Porphyromonas levii, Fusobacterium necrophorum, Trueperella (Arcanobacterium) pyogenes*	Cattle	Interdigital necrobacillosis (foul-of-the-foot, footrot)
Prevotella melaninogenica		Cattle, sheep, dogs and cats	Suppurative conditions

Table 15.1 Diseases caused by non-spore-forming anaerobes—cont'd

Non-spore-forming anaerobe	Associated pathogens	Host(s)	Disease
Prevotella heparinolytica		Horses, cats	Lesions in the buccal cavity
Bacteroides fragilis		Calves, lambs, foals, piglets	Occasional diarrhoeal disease (enterotoxigenic strains)
		Cattle	Mastitis
		Pigs	Abscesses
Porphyromonas asaccharolytica		Dogs, cats, horses, cattle	Osteomyelitis
Porphyromonas macacae		Cats	Subcutaneous abscesses and emphysema
Porphyromonas leviii	*Fusobacterium necrophorum*, *Trueperella (Arcanobacterium) pyogenes* *Prevotella melaninogenica*	Cattle	Interdigital necrobacillosis Associated with summer mastitis
Fusobacterium necrophorum	*Trueperella (Arcanobacterium) pyogenes*	Cattle	Calf diphtheria (necrotic foci in larynx, trachea and buccal cavity) Liver abscesses (feed-lot cattle) Metritis, cellulitis, mastitis, foot lesions
		Sheep	Foot abscess Ovine interdigital dermatitis ('scald') Lip and leg ulcerations
		Pigs	'Bull-nose' (following injury from fitting nose rings) Necrotic enteritis Liver abscess
		Horses	'Thrush' of the frog Necrobacillosis of lower limbs
		Chickens	Avian diphtheria (often secondary to fowl pox)
		Rabbits	Necrobacillosis of lips and mouth
Fusobacterium nucleatum		Several animal species	Non-specific infections
Fusobacterium russii		Cats	Soft tissue infections

and suggest that benign strains of *D. nodosus* may have importance as a source of alternative fimbrial antigens. A summary of major virulence factors of *F. necrophorum* and *D. nodosus* is given in Table 15.2.

Virulence factors of the Gram-positive anaerobic cocci have been little studied in animals but some species have been shown to produce a number of exoenzymes and induction of capsule formation is thought to be an important virulence mechanism.

Laboratory Diagnosis: General

Choice of specimens

As the non-sporing anaerobes constitute a major portion of the normal flora, the specimens must be collected with care to avoid contamination from the normal anaerobic flora, situated mainly on mucous membranes and in the intestinal tract. Unacceptable specimens include those

Table 15.2 Major virulence factors of the non-spore-forming anaerobes *Fusobacterium necrophorum* and *Dichelobacter nodosus*

Pathogen	Virulence factor	Function
Fusobacterium necrophorum	Leukotoxin	Low concentrations induce apoptosis, high concentrations lyse bovine leukocytes. Also cytotoxic for ruminant hepatocytes. Moderately toxic for equine neutrophils
	Haemagglutinin	Adherence to ruminal epithelium
	Haemolysin	Damages erythrocytes leading to impaired oxygen transport which contributes to anaerobic environment
	Dermonecrotic toxin	Lysis of collagen
Dichelobacter nodosus	Type IV fimbriae	Twitching motility, which is essential for disease production Adherence to host cells
	Serine proteases	Degrade fibronectin, elastin and other hoof proteins

from the gastrointestinal tract, throat, buccal cavity, voided urine, tracheal washings, and swabs from the surface of the urogenital tract and nasopharynx. The following samples are suitable for culture of the non-spore-forming anaerobes:

- Pus from abscesses
- Discharges from wounds (surgical and traumatic)
- Direct pleural aspirates
- Peritoneal aspirates
- Joint fluids
- Urine if taken by suprapubic puncture
- Tissue specimens (biopsy, necropsy and postoperative surgical).

Collection of specimens

Specimens for the isolation of these strict anaerobes should be placed immediately in an oxygen-free container, especially small pieces of tissue or material taken on swabs. Appendix 2 describes the preparation of a modified Cary–Blair medium to be used with swabs sterilized and stored in an oxygen-free atmosphere.

Larger pieces of tissue (over 2 cm^3) usually maintain an anaerobic microenvironment deep in the tissue and can be placed in an air-tight jar for transportation. Fluid specimens can be collected by aspiration and injected into an anaerobic transport vial. All specimens for anaerobic culture should be cultured within a few hours of collection. It is best to keep the specimens at ambient temperature rather than in the refrigerator as oxygen absorption is greater at lower temperatures.

Direct examination

Gram-stained smears of the specimens are useful as a screening process, although many of these anaerobes are not morphologically distinctive. Dilute carbol fuchsin (4–8 minutes) stained smears are more useful for many of the Gram-negative species as they tend to stain faintly with the Gram-stain. *Fusobacterium necrophorum* in clinical specimens is long and filamentous (about 1 µm in diameter) and stains in a characteristically irregular manner (Fig. 15.2). *Fusobacterium nucleatum* appears as thin rods (3–10 µm long) with tapered ends, often occurring in pairs. *Dichelobacter nodosus* is a large rod characterized by the presence of terminal enlargements at one or both ends. Table 15.3 summarizes the microscopic appearance of some of the non-spore-forming anaerobes.

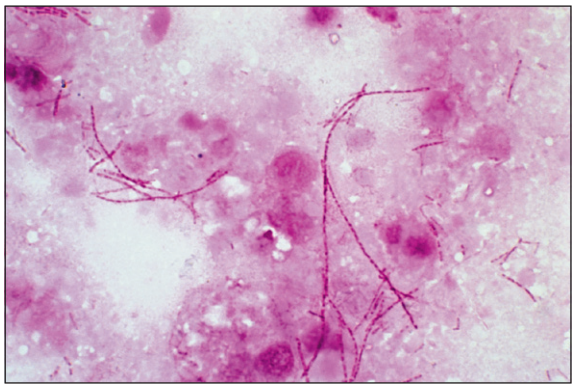

Figure 15.2 *Fusobacterium necrophorum* in long, non-branching filaments that stain in a characteristically irregular manner (soft tissue abscess in cow). (DCF stain, ×1000)

Isolation

A general and brief description of the culture, media and identification of these anaerobic bacteria is presented. The

Table 15.3 Summary of the colonial and cellular morphology of the non-spore-forming anaerobes

Colonial morphology	Microscopic appearance
Actinobaculum suis	
Grey, smooth, circular, 2–3 mm with a shiny centre and dull edge. Slightly raised at centre and gives poor beta-haemolysis on sheep blood agar	Pleomorphic Gram-positive rods in palisade and Chinese-letter formation. Size 0.5–1.0 × 1.0–3.0 μm
Peptoniphilus indolicus	
Greyish to yellow, shiny, circular, entire colonies, 0.5–1.0 mm in diameter. On freshly prepared media the colonies are viscous; on stored media they may be friable. Some strains are surrounded by a small zone of complete haemolysis	Gram-positive cocci, 0.5–0.6 μm in diameter, occurring singly, in pairs or in short chains
Dichelobacter nodosus	
Three basic colonial types are described: B-type: papillate or beaded (most pathogenic) from ovine footrot. M-type: mucoid (less pathogenic) from non-invasive infections of sheep and cattle. C-type: circular (non-pathogenic), results from repeated passage in media. The colonies generally are greyish-white and 0.5–3.0 mm in diameter in three–seven days	Gram-negative, fairly large (1.7 × 3–6 μm), slightly curved, non-motile rods. Often swollen at one or both ends. They occur singly or occasionally in pairs
Prevotella melaninogenica	
Circular, entire, convex and shiny colonies, 0.5–2.0 mm in diameter. Colonies become darker after five to 14 days, being black in the centre with a grey-brown periphery. Haematin pigment is seen best on media containing laked blood. A few strains are haemolytic on rabbit blood agar. The colonies fluoresce under ultra-violet light	Gram-negative rods (0.5–0.8 × 0.9–2.5 μm) with an occasional cell of 10 μm or longer
Prevotella asaccharolytica	
Colonies are 0.5–1.0 mm in diameter, round, convex, opaque and light-grey after 48 hours' incubation. In six to 14 days the colonies may become black. Some strains are haemolytic on rabbit blood agar	Gram-negative rods (0.8–1.5 × 1.0–3.5 μm). Cells from solid media tend to be shorter than those from broth cultures
Bacteroides fragilis	
Colonies are circular, entire, low convex, translucent to semi-opaque. They tend to have concentric rings of growth. Less than 1% of strains are haemolytic. *Bacteroides fragilis* will grow on bile aesculin agar with 5% sheep blood. Aesculin is hydrolyzed	Gram-negative tods (0.8–1.3 × 1.6–8.0 μm). Occur singly or in pairs and have rounded ends. Vacuoles are often present
Porphyromonas levii	
Colonies are minute, circular, entire and low-convex. After two to three days, colonies are buff or light-brown and dark-brown after five to seven days' incubation	Gram-negative rods (0.6–1.2 × 2.0–7.0 μm). Occur in pairs or short chains
Fusobacterium necrophorum	
Grey to yellowish, shiny colonies, 2–3 mm in 48 hours. Haemolysis is variable. Many strains are lipase-positive on egg yolk agar. Does not produce lecithinase	Gram-negative, long and filamentous but does not branch. Filaments can be up to 100 μm in length and 0.5–0.7 μm in diameter. May have tapered or rounded ends. Irregular staining is characteristic

Continued

Table 15.3 Summary of the colonial and cellular morphology of the non-spore-forming anaerobes—cont'd

Colonial morphology	Microscopic appearance
Fusobacterium nucleatum	
Circular to slightly irregular, convex, translucent colonies (1–2 mm) often with a 'flecked' appearance. Usually non-haemolytic except occasionally just under the colony	Long, thin Gram-negative rods with tapered to pointed ends (0.4–0.7 × 3–10 μm). Central swellings and intracellular granules may occur
Fusobacterium russii	
Circular, smooth, shiny, entire, convex, translucent colonies, 0.5–1.0 mm in diameter. Clear haemolysis occurs on horse blood agar	Gram-negative rods (0.3–0.7 × 1.5–4.0 μm) with some thick filaments about 10–15 μm in length. Palisade arrangement of cells is often seen. Beaded forms with pointed ends are common in thioglycollate medium

techniques can be arduous and expensive unless a laboratory specializes in this area.

Methods for anaerobic culture

The three main methods for achieving an anaerobic atmosphere for the culture of these strict anaerobes are:

- Anaerobic jars with a catalyst, an anaerobic indicator and an atmosphere free of oxygen:
  - Anaerobic jars with vents. These can be evacuated (to 20–24 inches of mercury), flushed twice with commercial grade nitrogen gas (N_2) and then filled with an anaerobic gas mixture (10 % hydrogen (H_2), 5 % carbon dioxide (CO_2) and 85 % nitrogen (N_2)). This mixture can be ordered in cylinders from a commercial gas supplier.
  - Anaerobic jar without vents. These are used with commercially available envelopes that deliver an H_2–CO_2 atmosphere.
- Anaerobic bags or pouches are commercially available products suitable for culturing small numbers of samples (e.g. Bio-Bag™ Type A, GasPak™ Pouch, Becton-Dickinson). Plates are placed in the bags and an oxygen-removal system is activated. These bags can be used for transport of specimens also.
- Anaerobic chambers or anaerobic glove-boxes. These are usually large plastic chambers kept constantly under an anaerobic atmosphere. They may contain temperature control devices and other equipment for culturing anaerobes. The media and specimens are introduced through a chamber lock and manipulations inside are conducted with the operator's hands and arms in gloves that are an integral part of the tent wall.

The latter method is usually used only in laboratories specializing in anaerobic culture work. Anaerobic jars or anaerobic bags give satisfactory results for laboratories culturing small numbers of anaerobic samples. However, for culture of strict anaerobes, they should be used in conjunction with an anaerobic holding system.

Media for anaerobic bacteria

Agar media: enriched blood agar is used for these fastidious anaerobes. To a nutritious agar base such as Eugon, Columbia, trypticase soy or Schaedler brain-heart, is added 0.5 % yeast extract, vitamin K (10 μg/mL) and haemin (5 μg/mL). The preparation is given in Appendix 2. The media can be made selective for the Gram-negative anaerobes by the addition of an antibiotic supplement, either paromomycin (100 mg/mL) and vancomycin (7.5 μg/mL) or kanamycin (100 μg/mL) and vancomycin (7.5 μg/mL). *Bacteroides* spp. (except *B. ureolyticus*) are resistant to kanamycin but the *Fusobacterium* spp. are sensitive to this antibiotic. A 'Fastidious Anaerobe agar' (Lab M) is available commercially with various antibiotic supplements, depending on the anaerobe that is being sought.

Specific media have been recommended for *Dichelobacter nodosus* such as one described by Gradin & Schmitz (1977) that consists of Eugon agar base (BBL) with 0.2 % (w/v) yeast extract, l0 % defibrinated horse blood and 1 μg/mL lincomycin. Members of the *B. fragilis* group will grow on bile aesculin medium with 5 % sheep blood and hydrolyze the aesculin. A medium containing nalidixic acid, colistin sulphate and metronidazole for the primary isolation of *Actinobaculum suis* has been described by Dagnall & Jones (1982).

Agar media should be used immediately after preparation or stored and then pre-reduced in an anaerobic jar for 6–24 hours before use. The plates are streaked with the specimens and placed as quickly as possible under an anaerobic atmosphere for incubation at 35–37°C. Plates should not be discarded until the eighth day of incubation.

Liquid media: these are useful adjuncts to agar media if the initial sample contains very small numbers of the required anaerobe and also for growing and maintaining

pure cultures. Cooked meat broth with 0.4 % glucose or thioglycollate medium are suitable with the addition of the vitamin K-haemin supplement (Appendix 2).

Liquid media must be placed in a boiling water bath for 10 minutes, to expel absorbed oxygen, and rapidly cooled to 37°C immediately before inoculation. The media should be inoculated near to the bottom of the tube or bottle with as little disturbance as possible. The inoculated tubes or bottles are incubated anaerobically, with loose caps, at 35–37°C and not discarded until after seven days of incubation. Liquid media can be used together with agar media in plates but not alone.

Identification

Colonial morphology and microscopic appearance

The cellular morphology, and sometimes the colonial morphology, can be very variable depending on the strain, medium and cultural conditions. *Fusobacterium necrophorum* produces grey to yellowish colonies on blood agar, that are about 2–3 mm in diameter after 48 hours' incubation (Fig 15.3). A Gram-stained smear from the colonies shows long Gram-negative filaments (Fig 15.4) that are less characteristic in appearance than those from direct microscopic examination of specimens. Lipase, but not lecithinase, activity is exhibited by *F. necrophorum* on egg yolk agar (Fig 15.5). *Dichelobacter nodosus*, in a Gram-stained smear from enriched blood agar (Fig 15.6), appears as straight or slightly curved rods with the characteristic terminal knobs on one or both ends of the cells (Fig 15.7). Table 15.3 summarizes the colonial and cellular morphology of some of the pathogenic anaerobic non-sporing bacteria but, with a few exceptions, these characteristics are too variable to be used for identification purposes.

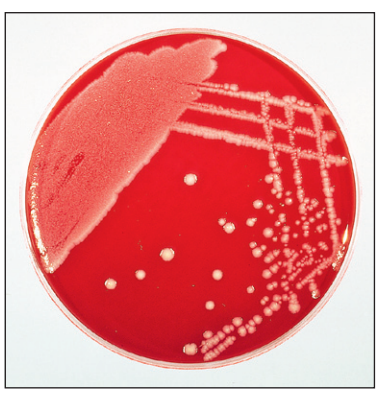

Figure 15.3 *Fusobacterium necrophorum* on sheep blood agar (72 hours' incubation at 37°C).

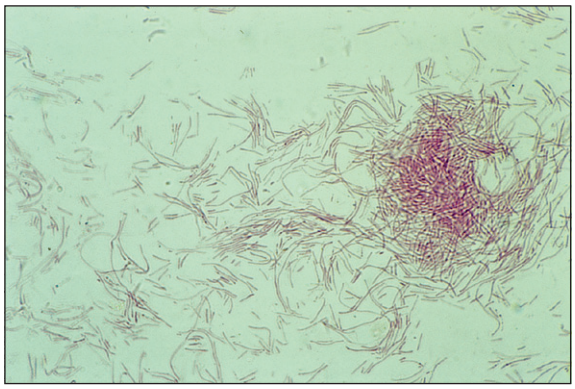

Figure 15.4 Long, non-branching Gram-negative filaments of *Fusobacterium necrophorum* from a culture. (Gram stain, ×1000)

Figure 15.5 *Fusobacterium necrophorum* on egg yolk medium with a pearly zone around the colonies due to lipase activity.

Figure 15.6 Close-up of *Dichelobacter nodosus* on enriched sheep blood agar.

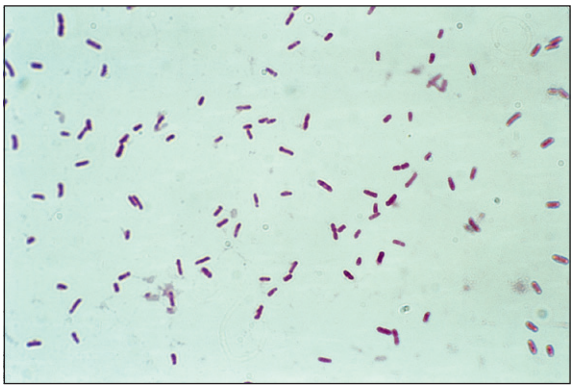

Figure 15.7 *Dichelobacter nodosus* from a culture: straight or slightly curved large Gram-negative rods with terminal enlargements at one or both ends. (Gram stain, ×1000)

Commercial anaerobic identification systems

These systems have been designed for human medical microbiology and not all have been evaluated for use with veterinary isolates. However, they include many of the veterinary pathogens in their identification systems. Examples include API 20A and Rapid ID 32A (bioMérieux, Marcy L'Etoile, France); RapID ANA II (Remel Inc., Lenexa, Kansas, USA) and BBL Crystal Identification (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA). These kits include tests for a range of saccharolytic and proteolytic enzymes, tests for indole production and nitrate reduction and others. Although these are 'rapid' kits, a heavy inoculum is required and prior subculture onto one or several plates for 48 hours is frequently necessary in order to obtain an adequate inoculum. Interpretation of the saccharolytic and proteolytic reactions can be difficult and some experience in reading these tests is required.

Conventional biochemical tests in tubed media

The preparation and use of conventional tubed media for biochemical tests is described by Dowell & Hawkins (1977).

Gas-liquid chromatographic analysis

This method for the detection of the end products of metabolism is most reliable and reproducible for identification of these anaerobic species. The volatile fatty acids and non-volatile fermentation products are characteristic for each species. Holeman et al. (1977) gives a detailed description of the gas chromatography procedures for anaerobes. The Capco Anaerobic Identification System (Capco Instruments) has proved to be a satisfactory, commercially available system.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Current Clinical and Laboratory Standards Institute (2012) guidelines specify the use of the agar dilution or broth microdilution methods for testing antimicrobial susceptibility of anaerobes. Unfortunately these techniques are not practical for use in many diagnostic laboratories. Good agreement between results obtained using the agar dilution method and the Etest (AB Biodisk, bioMérieux) are reported but the Etest is relatively expensive. The disk diffusion technique is unsatisfactory for use with anaerobic organisms. Thus, routine susceptibility testing for anaerobes is frequently not carried out. However, some surveillance studies carried out for human pathogens suggest that in common with aerobic organisms, anaerobes are showing a trend towards increased resistance (Koeth et al. 2004, Dubreuil & Odou 2010).

Molecular Methods for Detection and Identification

Molecular methods are increasingly used for the detection and identification of the non-spore-forming anaerobes, particularly those associated with diseases of major economic importance in animals, such as *Dichelobacter nodosus* infection in sheep. La Fontaine et al. (1993) described a PCR-based method to detect *D. nodosus* without the need for culture and this technique has been used in India also (Wani et al. 2004). In addition, single and multiplex PCR techniques can be used to identify strains of *D. nodosus* to serogroup level (Dhungyel et al. 2002, Wani and Samanta 2006). However, the latter cannot be used to distinguish virulent from benign strains and thus additional tests are required to address this aspect of identification. Virulence can be assessed based on conventional tests for protease characteristics or PCR-based tests using primers for virulence-associated genetic elements (Rood et al. 1996; Wani and Samanta 2006).

The two subspecies of *F. necrophorum* can be differentiated by haemagglutination and by random amplified polymorphic DNA polymerase chain reaction (Narongwanichgarn et al. 2001). Considerable research has been completed in the use of molecular techniques for the identification of anaerobes of clinical significance in human medicine, including Gram-positive anaerobic cocci and *Bacteroides* species (Song 2005). However, the application of molecular techniques for the identification of anaerobes is still prohibitively expensive for many clinical diagnostic laboratories and thus biochemical identification remains of primary importance.

REFERENCES

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, Approved Standard - Eighth Edition (M11-A8). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Dagnall, G.J.R., Jones, J.C.T., 1982. A selective medium for the isolation of *Corynebacterium suis*. Research in Veterinary Science 32, 389–390.

Dhungyel, O.P., Whittington, R.J., Egerton, J.R., 2002. Serogroup specific single and multiplex PCR with pre-enrichment culture and immuno-magnetic bead capture for identifying strains of *D. nodosus* in sheep with footrot prior to vaccination. Molecular and Cellular Probes 16 (4), 285–296.

Dowell, V.R. Jr., Hawkins, T.M., 1977. Laboratory Methods in Anaerobic Bacteriology. CDC Laboratory Manual, DHEW Publications, No. 78-8272, Centers for Disease Control, Atlanta, Georgia, USA.

Dubreuil, L., Odou, M.F., 2010. Anaerobic bacteria and antibiotics: What kind of unexpected resistance could I find in my laboratory tomorrow? Anaerobe 16 (6), 555–559.

Gradin, J.L., Schmitz, J.A., 1977. Selective medium for isolation of *Bacteroides nodosus*. Journal of Clinical Microbiology, 6, 298–302.

Holeman, L.V., Cato, E.P., Moore, W.E.C., 1977. Anaerobe Laboratory Manual, fourth ed. Anaerobe Laboratory, VPI, Blacksburg, Virginia 24061, USA.

Kennan, R.M., Han, X., Porter, C.J., et al., 2011. The pathogenesis of ovine footrot. Veterinary Microbiology 153, 59–66.

Kennan, R.M., Dhungyel, O.P., Whittington, R.J., et al., 2003. Transformation-mediated serogroup conversion of *Dichelobacter nodosus*. Veterinary Microbiology 92 (1–2), 169–178.

Koeth, L.M., Good, C.E., Appelbaum, P.C., et al., 2004. Surveillance of susceptibility patterns in 1297 European and US anaerobic and capnophilic isolates to co-amoxiclav and five other antimicrobial agents. Journal of Antimicrobial Chemotherapy 53 (6), 1039–1044.

La Fontaine, S., Egerton, J.R., Rood, J.I., 1993. Detection of *Dichelobacter nodosus* using species-specific oligonucleotides as PCR primers. Veterinary Microbiology 35 (1–2), 101–117.

Narayanan, S., Stewart, G.C., Chengappa, M.M., et al., 2002. *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. Infection and Immunity, 70 (8), 4609–4620.

Narongwanichgarn, W., Kawaguchi, E., Misawa, N., et al., 2001. Differentiation of *Fusobacterium necrophorum* subspecies from bovine pathological lesions by RAPD-PCR. Veterinary Microbiology 82 (4), 383–388.

Okamoto, K., Kanoe, M., Inoue, M., et al., 2005. Dermotoxic activity of a collagenolytic cell wall component from *Fusobacterium necrophorum* subsp. *necrophorum*. Veterinary Journal 169 (2), 308–310.

Rood, J.I., Howarth, P.A., Haring, V., et al., 1996. Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of *Dichelobacter nodosus*. Veterinary Microbiology 52 (1–2), 127–141.

Song, Y., 2005. PCR-based diagnostics for anaerobic infections. Anaerobe February–April 2005 11 (1–2), 79–91.

Tadepalli, S., Narayanan, S.K., Stewart, G.C., et al., 2009. *Fusobacterium necrophorum*: a ruminal bacterium that invades liver to cause abscesses in cattle. Anaerobe 15, 36–43.

Wani, S.A., Samanta, I., Bhat, M.A., et al., 2004. Molecular detection and characterization of *Dichelobacter nodosus* in ovine footrot in India. Molecular and Cellular Probes October 2004 18 (5), 289–291.

Wani, S.A., Samanta, I., 2006. Current understanding of the aetiology and laboratory diagnosis of footrot. Veterinary Journal 171 (3), 421–428.

BIBLIOGRAPHY

Engelkirk, P.G., Duben-Engelkirk, J., Dowell, V.R. Jr., 1992. Principles and Practice of Clinical Anaerobic Bacteriology. Star Publishing Company, Belmont, California, USA.

Jousimies-Somer, H., Summanen, P., Citron, D., et al., 2002. Wadsworth-KTL Anaerobic Bacteriology Manual, sixth ed. Star Publishing Company, Belmont, California, USA.

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Chapter 16

Clostridium species

Genus Characteristics

The *Clostridium* species are large (0.3–1.3 × 3–10 μm), Gram-positive, anaerobic, endospore-producing rods (the spores usually bulge the mother cell). All the pathogenic species are straight rods except *C. spiroforme* which is curved or spiral. The cells from older cultures or when producing endospores have a tendency to decolourize. *Clostridium perfringens* is the only species that produces a capsule in animal tissues and it is non-motile. Most of the other species are motile by peritrichate flagella. The clostridia are fermentative, oxidase-negative and catalase-negative. The strictness of anaerobic requirements varies among the species but they all prefer an atmosphere containing between 2 and 10% CO_2. Most clostridia require enriched media that include amino acids, carbohydrates, vitamins and blood or serum. Optimum growth of the pathogenic clostridia occurs at 37°C. There are over 200 *Clostridium* species of which about 14 are of veterinary importance. Most of the pathogenic species produce one or more exotoxins of varying potency.

Natural Habitat

The clostridia have a wide distribution in soil, freshwater and in marine sediments throughout the world, although some species or types are present only in localized geographical areas. Many of the pathogenic clostridia are normal inhabitants of the intestinal tract of animals and man, and often cause endogenous infections. Other clostridia are more commonly present in the soil and cause exogenous infections from wound contamination or by ingestion.

Pathogenicity

Although exotoxins are important in most of the clostridial diseases, the potency of the toxin(s) produced and the invasive ability of the clostridia varies. This allows a generalized, but convenient, division of the pathogenic *Clostridium* species into the following groups:

- Neurotropic clostridia (*C. tetani* and *C. botulinum*) that produce potent neurotoxins but are non-invasive and colonize the host to a very limited extent.
- Histotoxic clostridia (*C. chauvoei*, *C. septicum*, *C. novyi* types A and B, *C. haemolyticum*, *C. sordellii* and *C. perfringens* type A) that produce less potent toxins than the first group but are invasive. This includes the gas-gangrene-producing clostridia.
- Clostridia that are enteropathogenic or produce enterotoxaemias (*C. perfringens* types A-E, *C. difficile*, *C. colinum* and *C. spiroforme*). Enterotoxins are formed in the intestines and absorbed into the blood stream producing a generalized toxaemia.
- The atypical clostridium, *C. piliforme*, which causes Tyzzer's disease in foals and laboratory animals.

Table 16.1 summarizes the hosts and diseases of the pathogenic clostridia.

Laboratory Diagnosis (General)

Specimens

Specimens should be taken from recently dead animals as bacteria such as *C. perfringens*, *C. septicum* and enteric facultative anaerobes are rapid postmortem invaders. For isolation, blocks of affected tissue (4 cm^3) or fluids in

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Table 16.1 Summary of the hosts and diseases caused by pathogenic clostridia

Clostridium species	Hosts	Diseases
Neurotoxic clostridia		
Clostridium tetani	Horses, ruminants, humans and other animals	Tetanus
Clostridium botulinum (types A to F)	Many animal species and humans	Botulism
Clostridium argentinense (*Clostridium botulinum* type G)	Humans	Botulism
Histotoxic clostridia		
Clostridium chauvoei	Cattle, sheep (pigs)	Blackleg (black quarter)
Clostridium septicum	Cattle, sheep and pigs	Malignant oedema
	Sheep	Braxy
	Chickens	Necrotic dermatitis
Clostridium novyi		
Type A	Sheep	Big head of rams
	Cattle and sheep	Gas gangrene
Type B	Sheep (cattle)	Black disease (necrotic hepatitis)
Clostridium sordellii	Cattle, sheep, horses	Gas gangrene
Enterotoxigenic and enteropathogenic clostridia		
Clostridium perfringens		
Type A	Humans	Food poisoning, gas gangrene
	Lambs	Enterotoxaemic jaundice
	Dogs	Haemorrhagic gastroenteritis
	Pigs	Necrotizing enterocolitis (mild)
	Chickens	Necrotic enteritis (occasional cases)
Type B	Lambs (under three weeks old)	Lamb dysentery
	Neonatal calves and foals	Enterotoxaemia
Type C	Piglets, lambs, calves, foals	Haemorrhagic enterotoxaemia
	Adult sheep	Struck
	Chickens	Necrotic enteritis
Type D	Sheep (all ages except neonates) (goats, calves)	Pulpy kidney disease
Type E	Calves	Haemorrhagic enteritis
	Rabbits	Enteritis
Clostridium spiroforme	Rabbits and guinea pigs	Spontaneous and antimicrobial-induced diarrhoea
Clostridium difficile	Foals, pigs, dogs, hamsters, rabbits (calves)	Spontaneous and antimicrobial-induced diarrhoea
	Humans	Antimicrobial-induced diarrhoea, important nosocomial infection

Table 16.1 Summary of the hosts and diseases caused by pathogenic clostridia—cont'd

Clostridium species	Hosts	Diseases
Clostridium colinum	Game birds, young chickens and turkey poults	Quail disease (ulcerative enteritis)
Atypical clostridia		
Clostridium piliforme	Foals, laboratory animals (other wild and domesticated animals)	Tyzzer's disease, hepatic necrosis

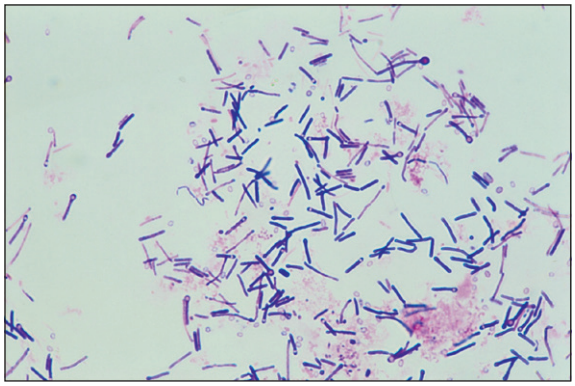

Figure 16.1 Sporing rods of *Clostridium tetani* in Gram-stained smear of necrotic material from a penetrating wound. The spores are spherical, terminal and bulge the mother cell giving the typical 'drum-stick' appearance.

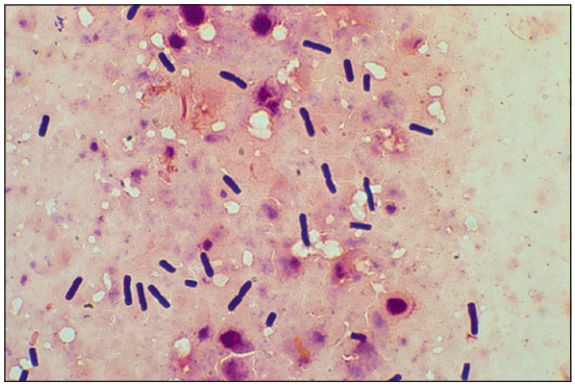

Figure 16.2 *Clostridium perfringens*: large Gram-positive rods in a mucosal scraping from the small intestine of a lamb that had recently died from pulpy kidney disease. (Gram stain, ×1000)

air-free containers, should be collected when possible rather than swab-taken samples that expose the clostridia to the lethal action of atmospheric oxygen. Commercial systems are satisfactory where the swab is in an oxygen-free gas and after use the swab is placed in Cary–Blair transport medium or other commercially available transport system. In some clostridial diseases, such as the enterotoxaemias, the demonstration of toxin is required for diagnosis. The contents of the small intestine are collected from a recently dead animal and submitted to the laboratory as soon as possible, as the toxins are labile.

Direct microscopy

Gram-stained smears from specimens are used to observe the morphological types of organisms present. The fluorescent antibody (FA) technique is widely employed for specific identification, particularly for the histoxic clostridial diseases.

- Gram-stained smears from affected tissues may reveal large Gram-positive rods that tend to decolourize easily when sporing. *Clostridium spiroforme* is an exception being curved or helical. The characteristic 'drumstick' forms of *C. tetani*, (Fig. 16.1) due to the spherical spores being terminal and bulging the cell, may be seen in necrotic material from wounds associated with tetanus. This is suggestive, but by no means conclusive, as other clostridia, such as *C. tetanomorphum* have a similar morphology. In cases of suspected enterotoxaemia, the presence of large numbers of fat Gram-positive rods in a smear of the small intestinal mucosa, from a recently dead animal (Fig. 16.2), is presumptive evidence of the condition.
- Fluorescent antibody (FA) technique is used routinely for diseases associated with *C. chauvoei* (Fig. 16.3), *C. septicum*, *C. novyi* and *C. sordellii* as fluorescent labelled antisera can be obtained commercially. Affected tissue as well as a piece of rib containing bone marrow (about 14 cm long) are useful specimens. A bacteraemia usually occurs with these clostridial diseases so the bacteria would be expected to be present in bone marrow. This tissue has the added advantages of giving low background autofluorescence and being one of the last tissues to be invaded, postmortem, by bacteria such as *C. septicum*.

General isolation procedures

In general, freshly prepared or pre-reduced blood agar is suitable for the isolation of clostridia. Media for the more

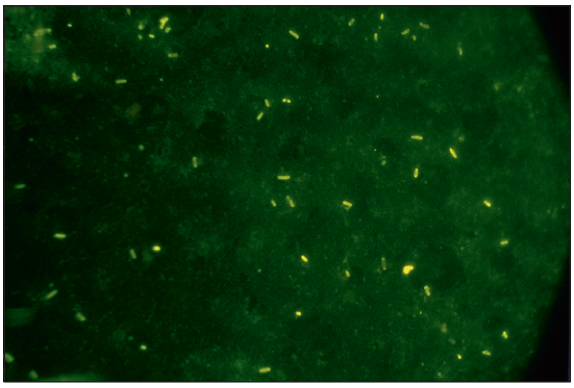

Figure 16.3 Direct fluorescent antibody technique showing *C. chauvoei* in muscle tissue from a case of blackleg in a heifer. (×400)

fastidious anaerobes such as *C. chauvoei*, *C. haemolyticum* and *C. novyi* types B and C are given in Appendix 2. Stored agar media gradually absorb oxygen from the atmosphere, so it is important to use either freshly prepared blood agar or pre-reduced plates that have been stored under anaerobic conditions soon after preparation. A blood agar and a MacConkey agar plate should be inoculated and incubated aerobically. These plates will detect any aerobic pathogens that may be present and also indicate the degree of contamination of the specimen by facultative anaerobes. Liquid and semisolid media with a low redox potential such as cooked meat broth and thioglycollate medium (Fig. 16.4) can be used to grow and maintain pure cultures of the clostridia. They are of limited use for primary inoculation as any fast-growing anaerobes or facultative anaerobes will outgrow the *Clostridium* species of interest. Immediately before inoculating cooked meat broth or thioglycollate medium, they should be boiled to expel absorbed oxygen and then rapidly cooled to 37°C.

Most of the clostridia pathogenic for animals are strict anaerobes, the exception being *C. perfringens* which is relatively aerotolerant. However, all should be grown under strict anaerobic conditions with the atmosphere containing 2–10% CO_2 as this enhances their growth. An anaerobic jar with a catalyst, an anaerobic indicator and an envelope delivering $H_2 + CO_2$ is usually satisfactory.

Biochemical reactions

Some of the biochemical reactions of the pathogenic clostridia are given in Table 16.2. On egg yolk agar the clostridia with lecithinase activity produce an opalescent change around the colonies due to enzymatic action on the lecithin in the medium. Those producing a lipase cause a pearly layer or iridescent film that can cover the colonies and in some cases extend into the surrounding agar (Fig. 16.5). *Clostridium perfringens* inoculated into

Figure 16.4 Growth of *C. perfringens* in thioglycollate medium.

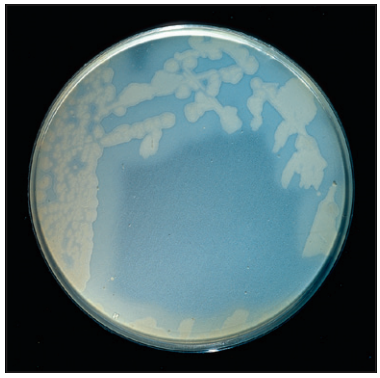

Figure 16.5 *Clostridium botulinum* type C on egg yolk medium giving a pearly layer around the colonies due to lipase activity. Lecithinase is not produced by this bacterium.

litmus milk medium produces the classical 'stormy-clot' or 'stormy-fermentation' reaction (Fig. 16.6). The lactose in the medium is fermented by *C. perfringens* producing acid which coagulates the casein and induces a colour change from blue to pink (litmus pH indicator). The acid clot is then broken up by gas formation. Miniaturized

Table 16.2 Biochemical reactions of the clostridia pathogenic for animals

Clostridium species	Egg yolk agar					Acid from				
	Lecinthase	Lipase	Hydrolysis of gelatin	Digestion of casein	Indole production	Glucose	Lactose	Sucrose	Maltose	Additional characteristics
C. tetani	–	–	+	–	V	–	–	–	–	Terminal, spherical endospores
C. botulinum I	–	+	+	+	–	+	–	–	+	Toxin types A, B, F*
II	–	+	+	–	–	+	–	–	+	Toxin types B, E, F*
III	V	+	+	–	V	+	–	–	V	Toxin types C, D*
C. argentinense IV	–	–	+	+	–	–	NA	–	NA	Toxin type G
C. chauvoei	–	–	+	–	–	+	+	+	+	
C. septicum	–	–	+	+	–	+	+	–	+	
C. novyi A	+	+	+	–	–	+	–	–	+	
B	+	–	+	+	V	+	–	–	+	
C	–	–	+	–	+	+	–	–	NA	No toxin produced
C. haemolyticum	+	–	+	+	+	+	–	–	–	
C. sordellii	+	–	+	+	+	+	–	–	+	Urease positive
C. colinum	–	–	–	–	–	+	–	+	+	
C. perfringens	+	–	+	+	–	+	+	+	+	Non-motile, 'Stormy-clot' in litmus milk
C. spiroforme	–	–	–	–	–	+	NA	+	NA	Spiral and curved
C. difficile	–	–	+	–	–	+	–	–	–	

+ = positive reaction, – = negative reaction, V = variable reaction, NA = data not available

*Most strains of *C. botulinum* produce one toxin only

commercial systems such as API 20A (bioMérieux) or Rapid ID 32A (bioMérieux) are available for the identification of many of the clostridia. The principal fermentation products can also be used to identify the *Clostridium* species by gas chromatography.

Animal inoculation

With the current availability of ELISA, molecular and other techniques it is no longer necessary to use laboratory animals for the diagnosis of clostridial diseases in many cases. However, in some circumstances these tests are not sufficiently sensitive or specific and tests involving laboratory animals are required. Laboratory animals, usually young guinea pigs or mice, can be used in one of two ways:

- As 'biological filters' for contaminated specimens or for material judged to contain small numbers of the pathogenic *Clostridium* species.
- In neutralization or protection tests to specifically identify the toxin(s) present and hence the clostridial pathogen involved in the disease. These procedures are most commonly used in tetanus, botulism and in the enterotoxaemias caused by *C. perfringens*.

Figure 16.6 The 'stormy clot' reaction of three isolates of *C. perfringens* in litmus milk medium. The tube on the left is uninoculated.

Figure 16.7 *Clostridium tetani*: advanced tetanus in a young calf showing rigidity of limbs, opisthotonos and raised tail-head. Note pyogenic infection of umbilicus, the probable portal of entry of *C. tetani* in this case.

NEUROTOXIC CLOSTRIDIA

Clostridium tetani

Clostridium tetani is a straight, slender (0.4–0.6 × 2–5 μm), Gram-positive rod that characteristically produces a terminal, spherical endospore that bulges the cell giving the characteristic 'drumstick' appearance to the bacterium (Fig. 16.1). The endospores are highly resistant and although boiling kills the spores of most strains in 15 minutes, autoclaving at 121 °C for 15 minutes is completely sporicidal. There are 10 serological types of *C. tetani*, based on flagellar antigens, but the neurotoxin is antigenically uniform.

Natural habitat

Soil, especially that contaminated by animal faeces, is the natural habitat. *Clostridium tetani* is often transient in the intestines of horses and other animals.

Pathogenesis

Clostridium tetani produces the exotoxins tetanolysin (a haemolysin) which may enhance tissue invasion and tetanospasmin (a neurotoxin) which is plasmid-coded and responsible for the signs of tetanus. The endospores enter traumatic or surgical wounds, especially after castration or docking, via the neonatal umbilicus (Fig. 16.7) or into the uterus following dystocia in cattle and sheep. The presence of facultative anaerobes and necrotic tissue create anaerobic conditions and the *C. tetani* spores germinate. The vegetative cells multiply at the entry site and produce the potent tetanospasmin. This travels via peripheral nerves or blood stream to ganglioside receptors of the motor nerve terminals to which it binds irreversibly. The toxin travels to the nerve cell body and its dendritic processes in the central nervous system by retrograde intra-axonal flow. The toxin then undergoes transcytosis from the motor neuron to its site of action in the inhibitory neurons. Tetanus toxin is a bipartite toxin with the light chain being the toxic moiety. The heavy chain mediates attachment and internalization of the toxin. Following internalization which takes place through endocytosis, the low pH of the endosome induces a conformational change in the heavy chain, causing it to form a pore through which the light chain translocates into the cytosol. The disulphide bridge joining the two chains is reduced in the cytosol. The light chain is a zinc endopeptidase which cleaves synaptobrevin, a vesicle-associated membrane protein. Cleavage of this protein prevents the vesicles containing inhibitory neurotransmitters from releasing their contents, resulting in spastic paralysis and the characteristic tetanic spasms. Tetanospasmin binds specifically to gangliosides in nerve tissue and once bound cannot be neutralized by antitoxin. When toxin travels up a regional motor nerve in a limb, tetanus develops first in the muscles of that limb, then spreads to the opposite limb and moves upwards. This is known as ascending tetanus and is usually seen only in the less susceptible animals such as dogs and cats. Descending tetanus is the common form in susceptible species such as humans and horses. In this form toxin circulating in the blood stream affects the susceptible motor nerve centres that serve the head and neck first and later the limbs. Once established, signs of tetanus are similar in all animal species.

Laboratory diagnosis

In tetanus the diagnosis is often based on the history and on the characteristic clinical signs.

Direct microscopy

Gram-stained smears of material from a wound may reveal the characteristic 'drumstick' sporing forms of *C. tetani* (Fig. 16.1). This is not completely diagnostic as other clostridia such as *C. tetanomorphum* have a similar morphology.

Isolation

Necrotic tissue from a wound or wound exudate can be heated to 80°C for 20 minutes and used to inoculate a blood agar plate and another blood agar plate containing 3% agar ('stiff agar'). A tube of thioglycollate medium or cooked meat broth could also be inoculated and subcultured onto blood agar after two to three days' incubation. The blood agar plates are incubated at 37°C for three to four days under an atmosphere of H_2 and CO_2.

Identification

Colonial morphology

Clostridium tetani is haemolytic and on normal blood agar tends to have a spreading, swarming growth (Figs 16.8 and 16.9) while on 'stiff agar' (3%) individual rhizoid colonies are formed (Fig. 16.10).

Biochemical reactions

Clostridium tetani liquefies gelatin but does not ferment the usual range of carbohydrates. Other reactions are given in Table 16.2. Alternatively, PCR-based procedures can be used to identify *C. tetani* colonies (Akbulut et al. 2005). Demonstration and identification of the toxin is more important than the isolation and identification of the bacterium.

Toxin identification

The toxin present in an animal's serum or in filtrate from cooked meat broth or thioglycollate medium, can be demonstrated in laboratory animals and identified by neutralization or protection tests using specific antitoxin. In the protection test the animals are protected with antitoxin at least two hours before inoculation with the material containing toxin. The control mice show typical signs of tetanic spasm in the region of inoculation (Fig. 16. 11).

Clostridium botulinum

Clostridium botulinum is a straight rod (0.9–1.2 × 4–6 μm) and at a pH near or above neutrality produces oval, subterminal spores. The spores are very resistant but are killed at 121°C for 15 minutes while the toxins are destroyed at 100°C for 20 minutes. Four phenotypically distinct groups of *C. botulinum* are recognized (Sharma & Whiting 2005) as shown in Table 16.2. In addition, seven types of *C.*

Figure 16.9 Spot inoculation of *C. tetani* to illustrate the characteristic spreading growth on normal blood agar containing 1.5% agar. Oblique illumination.

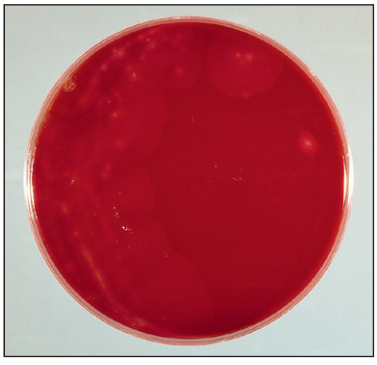

Figure 16.8 *Clostridium tetani* colonies on sheep blood agar showing spreading growth and a narrow zone of beta-haemolysis.

Figure 16.10 *Clostridium tetani* on stiff sheep blood agar (3% agar) which prevents spreading and gives individual rhizoid colonies.

Figure 16.11. Demonstration of the activity of tetanospasmin (*Clostridium tetani*) in a mouse.

botulinum are distinguishable based on the antigenicity of the toxin produced. Eight different neurotoxins are produced by *C. botulinum* types A–G (two types of C toxin). *Clostridium botulinum* Type G has been renamed *C. argentinense*. The toxins are identical in action but differ in potency, distribution and antigenicity and those of types C and D are known to be bacteriophage-coded. The optimum pH for *C. botulinum* is neutral to slightly alkaline (pH 7.0–7.6) and the optimal temperature lies between 30–37°C.

Natural habitat

The endospores are widely, but unevenly, distributed in soils and aquatic environments throughout the world. Germination of the endospores, with growth of vegetative cells and production of toxin, occurs in anaerobic situations such as contaminated cans of meat, fish or vegetables, carcasses of invertebrate and vertebrate animals, rotting vegetation and baled silage. Table 16.3 indicates the toxins produced, source of toxin and animals susceptible to each of the *C. botulinum* types.

Pathogenesis

Botulism is an intoxication usually caused by ingestion of preformed toxin in foodstuffs. The toxin is absorbed from the intestinal tract and is transported via the bloodstream to peripheral nerve cells where it acts at the neuromuscular junctions of cholinergic nerves and also at peripheral autonomic synapses. Botulinum toxin has a similar structure and mode of action to tetanus toxin with differences in clinical signs in the two diseases attributable to the different sites of action of the toxins. The heavy chain binds to susceptible cells and the toxin enters the cell through endocytosis. Following entry into the cytosol the zinc metalloprotease acts on synaptobrevin and other SNARE (SNAP (Soluble NSF Attachment Protein) Receptor) proteins which prevent the release of acetylcholine at the myoneural junctions. This results in flaccid paralysis, death being caused by circulatory failure and respiratory paralysis. Less common methods of acquisition of toxin are wound botulism (toxicoinfection) and infant botulism (intraintestinal toxicoinfection). In wound botulism the spores are introduced into wounds where they germinate. Toxin is formed at this localized site and spreads through the body. The 'shaker foal' syndrome is thought to be caused in this way. In humans, wound botulism is increasingly seen in drug addicts following use of contaminated needles. Infant botulism occurs when spores germinate in the intestines when the normal flora has not yet been fully established. This form is seen in human infants ('floppy baby' syndrome) and as rare epidemics of type C in broiler chickens and turkey poults. The toxin is one of the most potent known: one milligram of the neurotoxin contains more than 120 million mouse lethal doses. A comparison of the toxins of *C. tetani* and *C. botulinum* is shown in Table 16.4. Botulism is most common in water birds (Fig. 16.12), ruminants, horses, mink and poultry. Carnivores are relatively resistant to all types and pigs are susceptible

Table 16.3 Toxins of *Clostridium botulinum*, susceptible animals and sources of toxin

Toxin	Most susceptible animals	Sources of toxin
Type A	Humans, chickens, mink	Vegetables, fruit, meat, fish
Type B	Humans, horses (cattle, chicken)	Meat and meat products, vegetables, fish
Type C	Birds,	Dead invertebrates, maggots, rotting vegetation and refuse material
	Cattle, horses, mink, dogs (pigs, humans)	Carcasses, ensiled poultry litter, chicken manure as feed supplement, poor quality baled silage
Type D	Cattle, sheep (horses, humans)	Carcasses, bones
Type E	Humans, birds, fish	Fish, fish products, sludge in earth-bottomed ponds (farmed fish)
Type F	Humans	Meat (liver paste), fish
Type G	Humans	Soil

Table 16.4 Comparison of the toxins of *Clostridium tetani* and *Clostridium botulinum*

	Clostridium tetani	*Clostridium botulinum*
Site of toxin production	Wounds	Carcasses, decaying vegetation and occasionally wounds and intestine
Location of encoding genes	Plasmid	Chromosome, phage (types C and D)
Structure of toxin	Bipartite, heavy chain (involved in binding), light chain is a protease (toxic moiety)	Bipartite, heavy chain (involved in binding), light chain is a protease (toxic moiety)
Mode of action	Toxin acts centrally. Cleaves proteins that mediate fusion of neurotransmitter vesicles with presynaptic membrane of inhibitory interneurons	Toxin acts peripherally. Cleaves proteins that mediate fusion of neurotransmitter vesicles with presynaptic membrane of cholinergic nerves
Type of paralysis	Spastic	Flaccid
Antigenic types of toxin	Tetanospasmin (one antigenic type)	Eight different toxins produced by types A to G

Figure 16.12 *Clostridium botulinum* type C: botulism in a herring gull (*Larus argentatus*) showing flaccid paralysis of wings and legs.

to the toxin of type A but resistant to those of B, C and D. The oral toxicity of type D toxin is high for cattle and type C toxins are more readily absorbed through the intestinal wall of chickens and pheasants.

Laboratory diagnosis

The diagnosis of botulism is based on history, clinical signs and demonstration and identification of toxin in serum of moribund or recently dead animals as well as the detection of toxin and/or *C. botulinum* in the suspect foodstuff. Demonstration of toxin in animals that have been dead for some time may not be significant. *Clostridium botulinum* spores can be transient in the intestines of normal animals and the death of the animal creates an anaerobic environment suitable for the germination of the spores and toxin production. Great care must be taken when working with materials containing *C. botulinum* toxins because of their high potency.

Toxin demonstration

Serum or centrifuged serous exudates from animals can be directly inoculated intraperitoneally (0.5 mL) into mice. If toxin is present the characteristic 'wasp waist' appearance (Fig. 16.13) in the mice will be seen in a few hours or up to five days. The appearance is due to abdominal breathing because of paralysis of respiratory muscles. Cattle are extremely susceptible to botulism and detection of toxin in serum is difficult. Thus detection of toxin in gastrointestinal contents, which have been frozen immediately after collection to prevent postmortem multiplication of *C. botulinum* organisms, may be a more rewarding approach (Hogg et al. 2008).

Extraction of toxin in foodstuffs is accomplished by macerating the product in saline overnight. The suspension is centrifuged and the supernatant filtered through a 0.45 µm bacteriological filter. As the toxin can be in a protoxin form, nine parts of filtrate are treated with one part of 1% trypsin solution and incubated at 37°C for 45 minutes. Mice or guinea pigs can be inoculated intraperitoneally.

ELISA can be used for the detection of toxin but none of the currently available assays are as sensitive as mouse bioassay (Cai & Singh 2007).

Figure 16.13 Mouse inoculated with serum containing the toxin of *C. botulinum* . Note the characteristic 'wasp-waist' appearance.

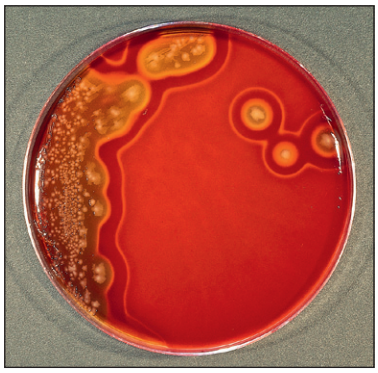

Figure 16.14 *Clostridium botulinum* on sheep blood agar.

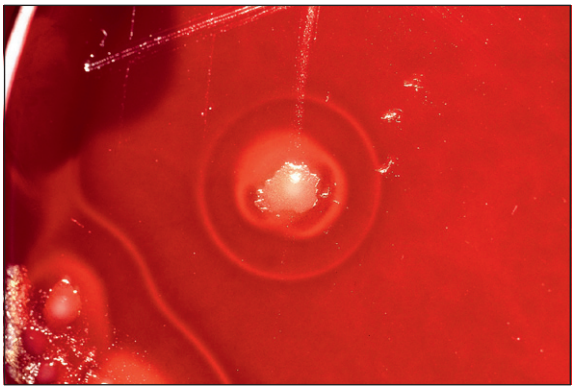

Figure 16.15 Close-up of *C. botulinum* type C on sheep blood agar showing beta-haemolysis and an irregular heaped colony with a granular surface.

Toxin identification

Mouse (or guinea pig) neutralization tests using a polyvalent antitoxin initially, followed by monovalent antitoxins, if they are available, are used to identify the toxin and the type of *C. botulinum* involved.

Isolation of *C. botulinum* from foodstuffs

Several samples of the foodstuffs are macerated in a small amount of physiological saline. The suspension is heated at 65–80°C for 30 minutes to kill most of the contaminant organisms and to induce the *C. botulinum* spores to germinate. Blood agar plates are inoculated with the suspension and incubated under $H_2 + CO_2$ at 35°C for up to five days. Type E spores require treatment with lysozyme to aid germination.

The colonies on blood agar are usually haemolytic (Fig. 16.14) and vary in appearance from slightly domed with a ragged edge (Fig. 16.15), flat and rough or a film-like growth. The suspect colonies are identified by biochemical tests and, as seen in Table 16.2, there are four cultural types. To determine whether the isolate is a toxin-producing strain, a cooked meat broth is inoculated and incubated at 30°C for five to 10 days. Filtrates are prepared and laboratory animals or ELISA can be used for demonstration and identification of the toxin.

HISTOTOXIC CLOSTRIDIA

Gas-Gangrene Clostridia

The clostridia commonly causing gas gangrene are summarized in Table 16.5. Occasionally other clostridia, present in soils and in the intestines of animals, are capable of causing a similar syndrome. Diseases caused by these clostridia are distributed worldwide.

Pathogenesis

The toxins produced by the gas-gangrene clostridia are not as potent as those of *C. tetani* and *C. botulinum* but the gas-gangrene bacteria are invasive. The disease syndrome can vary from simple wound infections, anaerobic cellulitis to severe and fatal gas gangrene. The infections can be either endogenous or exogenous in origin.

Endogenous infections often occur with blackleg in calves caused by *C. chauvoei*. Endospores are ingested and normally pass harmlessly through the intestinal tract but occasionally the spores pass from the intestine via the lymphatics and bloodstream to muscle masses, usually in the hindquarters but sometimes in cardiac muscle. Trauma to the area where the spores are lodged causes tissue necrosis and hence anaerobic conditions favouring germination of the spores and a supply of amino acids and other nutrients for vegetative cells. Toxin is produced followed by localized damage and finally a terminal toxaemia and bacteraemia. In exogenous infections, spores are introduced into wounds where they may germinate in the anaerobic necrotic material and toxin is produced by the vegetative cells. Although *C. chauvoei* is known to produce a number of different toxins (Table 16.5), the exact role of these toxins in the pathogenesis of infection is unclear. However, Frey et al. (2012) demonstrated that *C. chauvoei* toxin A (CCtA) is responsible for much of the cytotoxicity and haemolytic activity of this organism. This toxin is a pore-forming toxin belonging to the leucocidin superfamily. In braxy, the mucosa of the abomasum is thought to become damaged due to cold conditions from an adjacent rumen filled with frozen food. Any *C. septicum* spores present can germinate and replication of the bacterium leads to toxin production, toxaemia and rapid death. The toxins produced by the histotoxic clostridia and their mode of action are presented in Table 16.5.

Table 16.5 Summary of the histotoxic clostridia, including their major toxins

Clostridium species	Route of entry	Disease	Toxin		Clinical and postmortem signs
			Name	**Mode of action**	
C. chauvoei	Endogenous, from spores in muscles (cattle)	Blackleg	CCtA	Cytotoxic haemolytic, major toxin contributing to virulence	Usually sudden death, especially if heart muscle is involved. Fever, swelling of muscle masses of hind quarters. Muscles dry and spongy with small gas bubbles. Sweet, rancid odour and muscles are dark red to black. Crepitation can be felt
	Exogenous, through wounds (sheep)		α	Haemolysin	
			β	Deoxyribo-nuclease	
			γ	Hyaluronidase	
			δ	Haemolysin	
			NanA	Neuraminidase / sialidase	
C. septicum	Exogenous, through wound	Malignant oedema,	α	Pore-forming toxin, haemolysin, major toxin contributing to virulence	Fever. Soft swelling around wound in malignant oedema with much exudate and gas. Muscles dark red to black
	Endogenous, from spores in abomasum	Braxy	β	Deoxyribo-nuclease	
			γ	Hyaluronidase	
			δ	Haemolysin	
C. novyi type A	Exogenous through wounds	Big-head in young rams. Gas gangrene	α	Cytotoxin, a cholesterol-dependent cytolysin	Lesions similar to those of malignant oedema
C. sordellii	Usually exogenous, through wounds	Gas gangrene	α	Lecithinase, haemolytic	Similar syndrome to malignant oedema, lesions of the abomasal wall in some animals
			β	Cytotoxin, a cholesterol-dependent cytolysin, major toxin contributing to virulence	
C. perfringens, type A	Exogenous in gas gangrene and via wounds in necrotic dermatitis	Gas gangrene. Necrotic enterocolitis of pigs	α	A phospholipase, lethal, necrotizing, haemolytic, major toxin contributing to virulence	Oedema, tissue necrosis and gangrene. Mild lesions of enterocolitis and villous atrophy in pigs.
	Endogenous (part of intestinal flora) in necrotic enteritis in chickens and necrotic enterocolitis of pigs	Gangrenous dermatitis and necrotic enteritis in chickens	θ	Perfringolysin O, a cholesterol-dependent cytolysin	Sudden increase in mortality and necrotic lesions in intestinal mucosa of chickens with necrotic enteritis
			NetB	Role unclear, essential to virulence in some strains causing necrotic enteritis in chickens	

Continued

Table 16.5 Summary of the histotoxic clostridia, including their major toxins—cont'd

Clostridium species	Route of entry	Disease	Toxin		Clinical and postmortem signs
			Name	Mode of action	
C. novyi type B	Endogenous + liver fluke damage	Black disease (infectious necrotic hepatitis)	α	Cytotoxin, a cholesterol-dependent cytolysin	Sudden death with grey-yellow foci in the liver. Excess fluid in body cavities. Venous congestion occurs that darkens the skin
			β	Lecithinase, necrotizing, haemolytic,	
C. haemolyticum	Endogenous + liver fluke damage	Bacillary haemoglobinuria	β	Lecithinase, necrotizing, haemolytic	Sudden death with signs of abdominal pain and port-wine-coloured urine. Infarcts in liver

Table 16.6 Microscopic and colonial appearance of the gas-gangrene clostridia

Clostridium species	Gram-stained impression smears	Colonial appearance
C. chauvoei	Oval, subterminal or central spores with typical lemon-shaped forms. Cells 0.6–0.8 × 3–8 μm	Colonies with large zone of clear haemolysis
C. septicum	Characteristic long filamentous forms, Spores oval and subterminal. Individual cells 0.6–0.8 × 3–8 μm but filamentous forms are much longer	Swarming, spreading, haemolytic growth on normal agar. On 'stiff' agar the colonies are irregular with a rhizoid edge. Some strains produce smooth, round colonies
C. novyi type A	Large Gram-positive rods with oval to cylindrical, subterminal spores. There is little or no swelling of the mother cell. Cells are 0.8–1.0 × 3–10 μm	Large, irregular colonies with a rhizoidal edge and a large zone of clear haemolysis
C. sordellii	Gram-positive rods with cylindrical spores that do not bulge the mother cell	Irregular, translucent colonies on 'stiff' agar which become white on ageing
C. perfringens	Short, fat, Gram-positive rods that do not commonly produce spores. The spores, if present, are oval, subterminal and bulge the mother cell. Chains of cells can occur. Cells are 0.6–0.8 × 2–4 μm	Smooth, round, glistening colonies surrounded by 'target' or double-haemolysis (theta toxin giving a clear zone and partial haemolysis by the alpha toxin)

Laboratory diagnosis

Fluorescent antibody technique

Commercial fluorescein-labelled specific antisera are available for *C. chauvoei* (Fig. 16.3), *C. septicum*, *C. novyi* and *C. sordellii*. The FA technique is a rapid and convenient method for identifying these clostridia. The technique is carried out on acetone-fixed smears of affected tissue or bone marrow from a rib.

Gram-stained impression smears

Gram-stained impression smears on affected tissue can yield some useful information. The morphology of the gas-gangrene organisms are given in Table 16.6 and illustrated by Figures 16.16 to 16.20.

Isolation and colonial appearance

Sheep blood agar with liver extract is used to isolate the rather fastidious *C. chauvoei*. 'Stiff' blood agar (3% agar) and normal blood agar are used when attempting to isolate *C. septicum* and *C. sordellii*. *Clostridium perfringens* (Fig. 16.21) and *C. novyi* type A grow well on normal blood agar. The inoculated plates are incubated under strict anaerobic conditions with 10% CO_2 at 37°C for two to four days. The colonial appearance of each is given in Table 16.6.

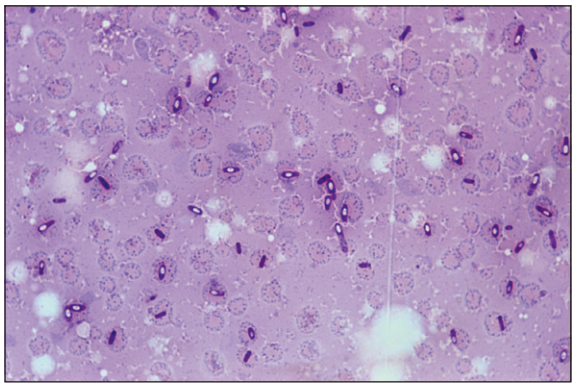

Figure 16.16 *Clostridium chauvoei* spores in a tissue smear. They are oval, central to subterminal and bulge the mother cell. The citron (lemon-shaped) forms are characteristic. (Gram stain, ×1000)

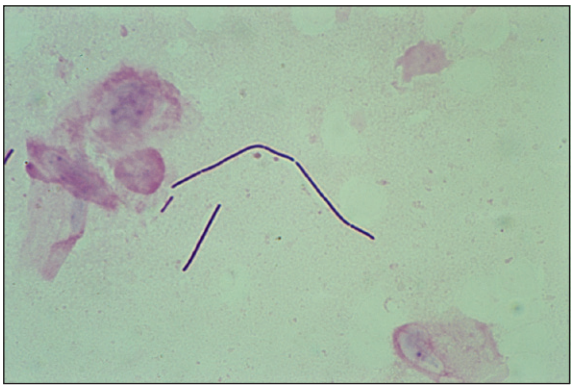

Figure 16.17 *Clostridium septicum* in characteristic long forms in a Gram-stained smear of affected muscle. (×1000)

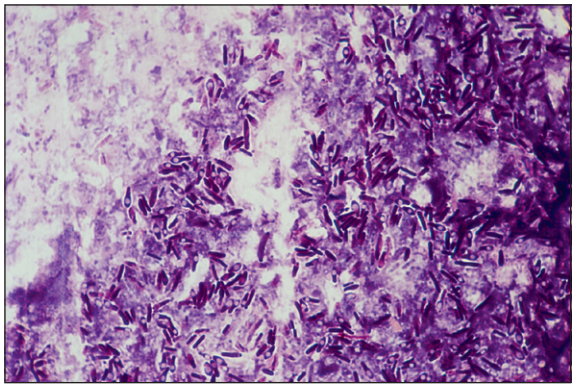

Figure 16.18 *Clostridium novyi* spores in a tissue smear. They are oval, subterminal and slightly bulge the mother cell. (Gram stain ×1000)

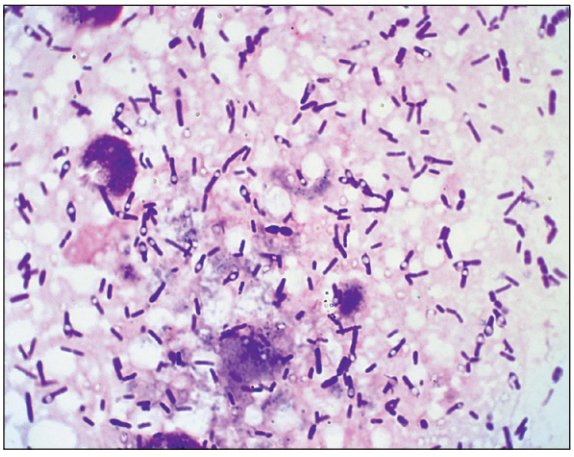

Figure 16.19 Spores of *C. perfringens*: not commonly seen but usually subterminal, large, oval and bulge the mother cell. (Gram stain, ×1000)

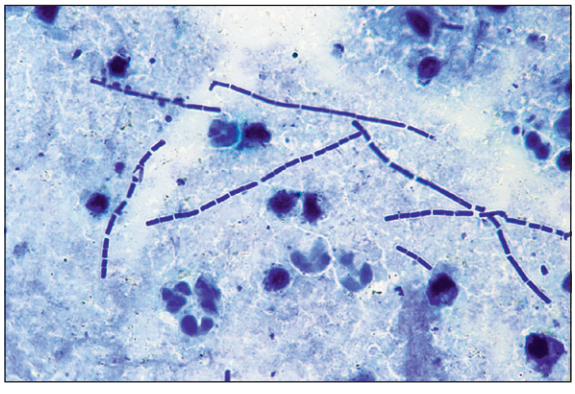

Figure 16.20 Chains of *C. perfringens* cells. (Methylene blue stain, ×1000)

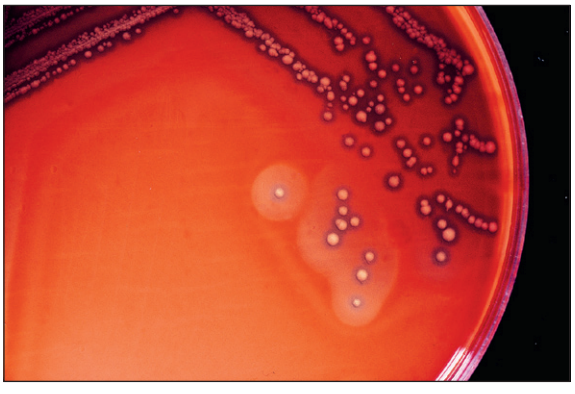

Figure 16.21 *Clostridium perfringens* on sheep blood agar showing the characteristic 'target' haemolysis.

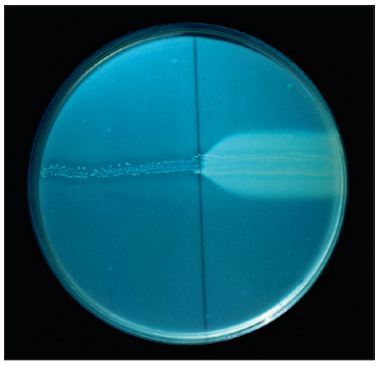

Figure 16.22 Nagler test for *C. perfringens* alpha toxin. The toxin is a lecithinase and attacks the lecithin in egg yolk agar (right). This reaction is neutralized on the left by specific antitoxin.

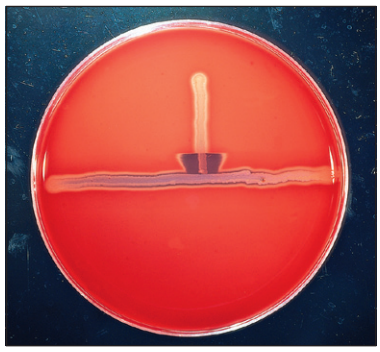

Figure 16.23 CAMP test with *Streptococcus agalactiae* (vertical streak) enhancing the partial haemolysis produced by the alpha toxin of *C. perfringens*.

Biochemical reactions

Clostridium chauvoei ferments sucrose but rarely salicin, while *C. septicum* ferments salicin but not sucrose. Other biochemical reactions are given in Table 16.2.

Nagler reaction of C. perfringens

Type A antitoxin (alpha antitoxin) is spread over half of an egg yolk agar plate and allowed to dry. The suspect *C. perfringens* is streaked across both halves of the plate. All the types of *C. perfringens* produce the alpha toxin which is a lecithinase, a type of phospholipase. On the half of the plate without the antitoxin the lecithin in the medium is attacked causing opalescence around the streak. The lecithinase reaction is neutralized on the half of the plate with the antitoxin but the growth of *C. perfringens* is unaffected (Fig. 16.22).

CAMP reaction of C. perfringens

A diffusible factor produced by *Streptococcus agalactiae* enhances the partial haemolysis of the alpha toxin of *C. perfringens* (Fig. 16.23). The complete zone of haemolysis seen immediately around the *C. perfringens* colonies is caused by the theta toxin.

Histotoxic Clostridia Affecting the Liver

Clostridium novyi type B is common in soil and in the normal intestinal tract of herbivores. It produces black disease (necrotic hepatitis) in sheep. *Clostridium haemolyticum* (*C. novyi* type D) can be found in the ruminant digestive tract, liver and in the soil, and is the cause of bacillary haemoglobinuria in cattle. *Clostridium novyi* type A is associated with gas gangrene. *Clostridium colinum*, the cause of quail disease, is excreted in faeces of birds with the chronic form of the condition. A toxin has not been identified for this clostridial species. Hepatic necrosis is present in some instances in addition to intestinal lesions.

Table 16.7 Toxins of histotoxic clostridia

	Toxins	
	Alpha	**Beta**
C. novyi type A	+	–
C. novyi type B	+	+
C. haemolyticum	–	+++

Pathogenesis

Alpha toxin is produced by *C. novyi* type A and type B (Table 16.7). This toxin is a cholesterol-dependent cytolysin and belongs to a family of clostridial toxins known as the large clostridial cytotoxins. Perfringolysin produced by *C. perfringens* and the alpha and beta toxins of *C. difficile* and *C. sordellii* also belong to this group. The toxin inactivates low molecular weight GTP-binding proteins by glucosylation which disrupts the cytoskeleton, resulting in death of the cell. It is phage encoded. The beta toxin is a phospholipase and is produced by *C. novyi* type B as well as by *C. haemolyticum* in greater amounts. This may account for the haemolytic crisis and death in cases of bacillary haemoglobinuria.

In black disease and bacillary haemoglobinuria, the spores, normally present in the intestine, may reach the liver and remain dormant in the Kupffer cells. Traumatic damage to the liver, especially due to migrating liver fluke, produces tissue damage and anaerobic conditions suitable for spore germination. There is replication of the clostridia resulting in toxaemia, bacteraemia and often death.

Laboratory diagnosis

Direct Gram-stained smears

Presence of characteristic liver lesions together with large numbers of Gram-positive rods in liver impression smears, from a recently dead animal or bird, is suggestive for the diseases. *Clostridium novyi* type B and *C. haemolyticum* are large Gram-positive rods (0.8–1.0 × 3–10 µm) that produce oval to cylindrical, subterminal spores with little bulging of the mother cell.

Fluorescent antibody technique is useful for the identification of *C. novyi* type B and *C. haemolyticum* in acetone-fixed liver impression smears.

Isolation

Clostridium novyi and *C. haemolyticum* are very demanding in both their anaerobic and nutritional requirements. Very strict anaerobic procedures are necessary and media containing cysteine (Moore's medium), described in Appendix 2, should be used. These clostridia can die within 15 minutes of being exposed to atmospheric oxygen. The colonies are haemolytic, small and usually rhizoidal in nature.

Biochemical reactions

These are shown in Table 16.2.

Animal inoculation

Toxins in the liver can be demonstrated by intramuscular injection of homogenates into guinea pigs. The guinea pigs die in one to two days. Specific antitoxin is not readily available for neutralization tests.

ENTEROPATHOGENIC AND ENTEROTOXIGENIC CLOSTRIDIA

Clostridium perfringens

Clostridial enterotoxaemias are acute, highly fatal intoxications that affect sheep, lambs, calves, piglets and occasionally foals. The diseases are caused by the major exotoxins (enterotoxins) of *Clostridium perfringens* types A, B, C and D, and, occasionally type E such as in *C. perfringens* type E-associated enterotoxaemia in calves.

Clostridium perfringens is relatively aerotolerant, nonmotile, has a polysaccharide capsule in tissue and is a short, fat Gram-positive rod (0.6–0.8 × 2–4 µm). The spores are oval, subterminal and bulge the mother cell. They are rarely produced, one exception being in the intestinal tract of humans in food poisoning cases when the enterotoxin is released at the completion of sporulation upon lysis of the mother cell. Characteristic reactions of the bacterium are the double-zoned haemolysis on blood agar (Fig. 16.21), stormy-clot (stormy-fermentation) in litmus milk medium (Fig. 16.6) and the Nagler reaction (Fig. 16.22). The five types (A–E) are based on the different combinations of the toxins elaborated by the organism.

Natural habitat

Type A occurs in the intestinal tract of humans and animals as well as in most soils. Types B to E are more adapted to survival in the intestines but in outbreaks of disease they survive long enough in soil to infect other animals. Type C does not appear to be a commensal in all instances or species and true infections occur.

Pathogenesis

Minor toxins are produced such as theta (haemolysin), kappa (collagenase), mu (hyaluronidase) and nu (DNase) and these may contribute to tissue damage. However, the major toxins, alpha, beta, epsilon and iota are of greatest importance. The major toxin(s) produced by each *C. perfringens* type are shown in Table 16.8.

- ***Alpha toxin.*** This is a lecithinase (phospholipase) that attacks cell membranes causing cell death and destruction. The alpha toxin is produced by all types and gives a partial zone of haemolysis on blood agar. The Nagler reaction is based on the neutralization of the lecithinase activity of this toxin on egg yolk medium.
- ***Beta toxin***, a pore-forming toxin, is lethal and necrotizing. It binds to vascular endothelial cells causing degeneration, thrombosis and subsequent necrosis (Uzal & McClane 2011). It is sensitive to trypsin and this explains the predilection of types B and C for neonates as colostrum has anti-trypsin activity. It is a labile toxin and may be destroyed if there is a delay in small intestinal contents, containing the toxin, reaching the laboratory. The beta toxin is the most important factor in the enterotoxaemias caused by type B.

Table 16.8 The major toxins of the five types of *Clostridium perfringens*

Clostridium perfringens	Major toxin			
Type	α	β	ε	ι
A	+	–	–	–
B	+	+	(+)	–
C	+	+	–	–
D	+	–	+	–
E	+	–	–	+

- ***Epsilon toxin*** is secreted as a protoxin (prototoxin) and is activated in the intestines by proteases such as trypsin. Pulpy kidney disease is not usually seen in neonatal lambs as colostrum contains an antitrypsin factor that can prevent the epsilon toxin being activated. The toxin itself is a pore-forming toxin, similar in structure to the enterotoxin of *C. perfringens* and the alpha-toxin of *C. septicum*. It acts by disrupting the energy producing pathways of the cell, resulting in depletion of ATP and programmed cell necrosis (Fennessey et al. 2012). Gut permeability is increased, assuring absorption of the toxin into the bloodstream. It damages vascular endothelium (including blood vessels in the brain) leading to fluid loss and oedema. The epsilon toxin can be regarded as an enterotoxin and a neurotoxin.
- ***Iota toxin*** is also produced as a protoxin and is not unique to *C. perfringens* type E, the C2 toxin of *C. botulinum* belongs to the same family while iota-like toxins are also formed by *C. spiroforme* and *C. difficile*. Iota toxin is a binary toxin with enzyme and binding components. It ADP-ribosylates cellular actin which blocks its polymerization and ultimately results in cell death (Nagahama et al. 2011).

The enterotoxaemias are often precipitated by certain husbandry and environmental factors such as abrupt changes in feeding, usually to a richer diet, and overeating on high protein and energy-rich foods. This tends to lead to slowing of peristalsis with retention of bacteria in the intestines and absorption of toxins. The bacterium inhabits the large intestine in normal animals but if overgrowth occurs (inadequately digested carbohydrate and the provision of a rich medium for the proliferation of the bacterium) *C. perfringens* can spill-over into the small intestine with the production of a large amount of toxin and enterotoxaemia. Table 16.9 summarizes the clostridial enterotoxaemias.

Laboratory diagnosis

The definitive diagnosis of the enterotoxaemias is based on the demonstration and identification of the toxins in the small intestine using ELISA or a mouse or guinea-pig neutralization test. Other tests can be useful adjuncts particularly for pulpy kidney disease.

- Gram-stained smears can be made from the mucosa of the small intestine of a recently dead animal. Large numbers of fat Gram-positive rods are highly suggestive of an enterotoxaemia as very few clostridia are normally present in the small intestine.
- Histopathology on brain sections to demonstrate focal symmetrical encephalomalacia in pulpy kidney disease. The lesion is not always observed as its development depends on the time from the first clinical signs to death. If present, the lesion is characteristic for the disease.
- Glycosuria (glucose in urine) is suggestive of pulpy kidney disease.
- Demonstration of toxin in the small intestine. ELISA techniques are now available for demonstration of toxin in small intestinal contents and their sensitivity is comparable to that of *in vivo* tests (Uzal & Songer 2008). Therefore, tests employing laboratory animals are now seldom necessary. Where used, a suitable specimen is 20–30 mL of ileal contents from a recently dead animal. The toxins are labile so the specimen should reach the laboratory as soon as possible after collection. The ileal contents are centrifuged and the clear supernatant is tested for toxin. If the ileal contents are very mucoid, it may be difficult to obtain a supernatant. Placing a small piece of cotton wool at the top of the centrifuge tube, before centrifuging, helps to take the mucus into the deposit. In ileal contents, the epsilon and iota toxins are usually in the active form. To demonstrate the toxin 0.4 mL of the clarified ileal contents can be inoculated intravenously into each of two mice. If a mouse dies within five minutes this is probably due to shock; deaths from toxin usually occur within 10 hours.
- Identification of the toxin in the clarified ileal contents is carried out by a neutralization test. Intravenous inoculation in mice or intradermal injection into white, shaved, young guinea pigs is used with commercial antitoxins to *C. perfringens* types A to E. The mixtures and dose for inoculation are as follows:
  - Test: 0.5 mL supernatant + 0.2 mL sterile saline + 0.1 mL antitoxin
  - Control: 0.5 mL supernatant + 0.3 mL sterile saline
  - Dose: 0.2 mL intradermally in guinea pig and 0.4 mL intravenously for a mouse.

Prior to inoculation the mixtures are allowed to stand at bench temperature for one hour to allow neutralization of the toxin by the antitoxin.

If pulpy kidney disease is suspected, and is the common form of enterotoxaemia in lambs in the area, it is acceptable to conduct a neutralization test using the type D antitoxin only and omitting the initial step of demonstrating the toxin. Two mice for the test and two mice for control should be used. The test is positive if the two control mice die in 10–12 hours but the test mice remain alive and well. If all four mice remain well, no toxin was present in the ileal contents and if all four mice die, either another toxin (other than the alpha and epsilon toxins) was present or the ileal contents contained an excessive amount of epsilon toxin and complete neutralization was not attained. The test could be repeated using 0.2 mL type D antitoxin in the above mixture or the full set

Table 16.9 The clostridial enterotoxaemias

Clostridium perfringens type	Hosts	Disease	Major toxins	
			Name	**Mode of action**
A	Humans	Food poisoning	Enterotoxin	Pore-forming toxin (similar to ε toxin) which is released on cell lysis following completion of sporulation
	Chickens	Necrotic enteritis	netB	Pore-forming toxin, appears to be essential virulence factor in some strains
			α	A phospholipase, lethal, necrotizing, haemolytic
	Pigs	Necrotizing enterocolitis	α	
	Horses	Neonatal enteritis, antibiotic-induced diarrhoea in adults	α	
	Dogs	Haemorrhagic gastroenteritis	α	
B	Lambs	Lamb dysentery	α	A phospholipase, lethal, necrotizing, haemolytic
	Calves and foals	Haemorrhagic enteritis	β	Pore-forming toxin, targets vascular endothelial cells, lethal, major virulence factor
			ε	Pore-forming toxin, activated by proteolytic enzymes
C	Piglets (usually one to three days old), foals, calves and lambs	Haemorrhagic enterotoxaemia in young farm animals	α	A phospholipase, lethal, necrotizing, haemolytic
			β	Pore-forming toxin, targets vascular endothelial cells, lethal, major virulence factor
		'Struck'	TpeL	Large clostridial cytotoxin, glucosylating toxin, induces apoptosis in target cells
	Adult sheep and goats		Enterotoxin	Pore-forming toxin
			β2	Probably a pore-forming toxin, pathogenic role uncertain
			θ	Perfringolysin O, a cholesterol-dependent cytolysin
D	Sheep (all ages except neonates), rare cases in calves and goats	Pulpy kidney disease, enterotoxaemia	α	A phospholipase, lethal, necrotizing, haemolytic
			ε	Pore-forming toxin, activated by proteolytic enzymes, major virulence factor
E	Calves	Enterotoxaemia	α	A phospholipase, lethal, necrotizing, haemolytic
			ι	Requires activation by proteases, blocks polymerization of cellular actin resulting in cell death, major virulence factor

Table 16.10 Neutralization of *Clostridium perfringens* toxins by antitoxin

	Toxins of *Clostridium perfringens* types A to E				
	A α	B α β ε	C α β	D α ε	E α ι
Antitoxin					
Type A: anti-α	–	×	×	×	×
Type B: anti-α anti-β anti-ε	–	–	–	–	×
Type C: anti-α anti-β	–	×	–	×	×
Type D: anti-α anti-ε	–	×	×	–	×
Type E: anti-α anti-ι	–	×	×	×	–

× = death of mouse or lesion in skin of guinea pig

– = antitoxin has neutralized the specific toxin and the mouse or guinea pig is unaffected

of neutralization tests carried out using the antitoxins to *C. perfringens* types A to E.

Table 16.10 gives a simplified version of the expected neutralization results of toxins in ileal contents by antitoxins to *C. perfringens* types A to E. The test is often carried out in duplicate using untreated and trypsin-treated (1% trypsin solution for one hour at 37°C) supernatant containing toxin. This is to ensure that the epsilon and iota toxins, produced in protoxin form, are converted into the active form. However, the trypsin treatment will destroy any beta toxin that may be present. This procedure is particularly necessary if a pure culture of *C. perfringens*, grown in cooked meat broth, is being typed based on the toxins that have been produced in the broth because the epsilon and iota toxins will be in the protoxin form.

OTHER ENTEROPATHOGENIC CLOSTRIDIA

Clostridium spiroforme

Clostridium spiroforme occurs as a loosely coiled, spiral Gram-positive form in smears from cultures on blood agar. However, in smears from faeces or caecal contents the spiral morphology is not so marked and it has a semicircular form. The spores are terminal. It is non-haemolytic and produces convex, circular, shiny, whitish to grey colonies on blood agar under anaerobic conditions at 37°C. *Clostridium spiroforme* produces an exotoxin which is cytotoxic and similar structurally and functionally to the iota toxin of *C. perfringens* type E It has been shown to be the cause of spontaneous diarrhoea in weanling rabbits. Diarrhoea can also be induced in adults by the administration of antibiotics, especially clindamycin. *Clostridium spiroforme* has been frequently isolated from rabbits with 'mucoid enteritis' and may, with other microorganisms, play a part in the disease. Naturally occurring enterocolitis has been reported in foals and pigs.

The presence of semicircular Gram-positive bacteria in faeces or caecal contents is not sufficient for a diagnosis, the toxin should be demonstrated in mice or guinea pigs and identified by a neutralization test. Alternatively, PCR-based procedures for detection of the toxin-encoding genes may be carried out as an aid to diagnosis (Drigo et al. 2008).

Clostridium difficile

Clostridium difficile is a large (0.5 × 3–6 μm) Gram-positive rod that forms oval, subterminal spores. On blood agar the colonies are non-haemolytic and raised with a rhizoid edge. Special blood agar is required for isolation containing yeast extract, haemin, vitamin K, cysteine and antimicrobial agents (Borriello &Honour 1981). It produces an enterotoxin (designated 'A') and a cytotoxin (B), both of which belong to the group of large clostridial cytotoxins. A third toxin, *C. difficile* binary toxin, is produced by some strains; its role in pathogenesis is currently unclear. *Clostridium difficile* has been found to be a cause of human, hamster, rabbit and guinea pig enterocolitis following antibiotic therapy, particularly clindamycin, which profoundly alters the intestinal microbial population. However, natural diarrhoeal diseases have been described in dogs, foals and neonatal pigs. The exact mechanism of disease production remains unclear as *C. difficile* and its toxins can be detected in the faeces of normal animals. Nevertheless, more severe disease was recorded by Ruby et al. (2009) in diarrhoeic horses with *C. difficile* than in those animals in which the organism was not detected.

Both *C. difficile* and its toxins can be detected in the faeces and intestinal contents of affected animals. A definitive diagnosis is made by enzyme immunoassays for the detection of both toxins A and B in faecal specimens (Laughton et al 1984). ELISA kits are commercially available for toxin detection in humans; nosocomial *C. difficile* infections are of major concern worldwide. It is uncertain whether these kits are reliable for testing specimens from animals as few studies have been completed. Chouicha & Marks (2006) conducted a study on canine faecal samples,

the results of which suggested that kit sensitivity was poor for this type of sample.

Clostridium colinum

Clostridium colinum is a Gram-positive rod, about 1 µm in diameter and 3–4 µm long. It has oval, subterminal spores but sporulation is infrequent. *Clostridium colinum* is fastidious and primary isolation is difficult. Success has been reported in tryptose-phosphate-glucose broth with 8% sterile citrated horse plasma; thioglycollate broth with 3–10% horse serum; or in five to eight day fertile chicken eggs. Several passages are needed after which the clostridium can be grown on blood agar. Polymyxin B (25 mg/mL) can be added to the isolation media to suppress contaminants.

In quail disease, *C. colinum* passes from the intestine via the portal circulation and lodges in the liver where diffuse liver necrosis is produced. The intestine becomes ulcerated and in some birds extensive necrosis of the spleen occurs. Affected birds are inactive, sluggish and anorexic. They may die within one to two days but occasionally linger for a longer period.

Atypical Clostridia

Clostridium piliforme

Clostridium piliforme is an obligate intracellular pathogen. It is a filamentous Gram-variable spore-forming organism which cannot be grown on conventional media but can be cultured in fertile eggs or tissue culture. It causes Tyzzer's disease, which is characterized by severe hepatic necrosis, in foals, laboratory animals and occasionally in other species. Subclinical infections also occur. The organism can be demonstrated in hepatocytes using the Warthin–Starry silver impregnation technique.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

The agar dilution method as described in CLSI (2012a, 2012b) is the reference method for testing of anaerobic bacteria, including the clostridia. Broth microdilution is frequently employed also. For all of the clostridial diseases in animals, prevention of disease (usually through vaccination) is the best course of action. Antimicrobial agents are used for the prevention and treatment of clostridial disease in intensive production systems. Recent studies suggest that although the pathogenic clostridia in animals remain broadly susceptible to many antimicrobial classes, resistance is becoming more common. Tetracycline and metronidazole resistance has been recorded in *C. perfringens* isolates from dogs (Kather et al. 2006, Gobeli et al. 2012). Tetracycline resistance in *C. perfringens* in broilers varies depending on geographical location with high levels recorded in Belgium where lincomycin resistance levels were also high (Gholamiandehkordi et al. 2009). In contrast, Gharaibeh et al.(2010) recommended treatment of necrotic enteritis with tetracyclines in Jordan. All isolates in their study showed low MICs against this agent whereas MICs against tilmicosin, lincomycin and erythromycin were high. *Clostridium perfringens* from poultry have shown some levels of resistance to bacitracin (Chalmers et al., 2007, Slavic et al., 2011, Watkins et al., 1997). The genes responsible for this resistance have recently been identified (Charlebois et al., 2012). Several reports are available on resistance levels in animal isolates of *C. difficile*. Ciprofloxacin resistance was detected in 99% of strains from pigs in a US study (Susick et al. 2012) although the prevalence of tetracycline and erythromycin resistant isolates was low. In another US study, the multidrug resistant profile of ciprofloxacin-tetracycline-erythromycin was detected in more than 20% of young pigs tested (Thakur et al. 2010). High levels of resistance to many antimicrobial agents were recorded in *C. spiroforme* isolates by Agnoletti et al. (2009) in a study of intensively farmed rabbits.

Molecular Diagnosis and Strain Typing

Clostridium perfringens

The complete genome sequence of a number of *C. perfringens* strains is available and reveals a high degree of genomic diversity in this organism. Over 300 unique genomic islands were identified with features that may contribute to the various disease phenotypes and virulence patterns of pathogenic strains. These features include mobile elements, metabolic capabilities, extracellular capsules, toxins and other secreted enzymes (Myers et al. 2006). Many multiplex PCR assays have been developed to detect the genes encoding the four major toxins produced by *C. perfringens* types A to E (Erol et al. 2008). In addition, detection of the genes encoding the enterotoxin (*cpe)* and the beta2-toxin (*cpb2*) allows subtyping of the bacteria. Multiplex PCR is reported to be an effective and rapid method for typing of *C. perfringens* isolates from a variety of animals, including foals, piglets or lambs (Garmory et al. 2000). A dual-labelled fluorescence hybridization probe (TaqMan®) based real-time multiplex PCR assay was developed for detection of toxin genes alpha (*cpa*), beta (*cpb*), iota (*ia*), epsilon (*etx*), beta2 (*cpb2*) and enterotoxin (*cpe*) directly from cattle faeces (Gurjar et al. 2007). The pore-forming toxin netB has been reported as a virulence factor in strains capable of causing necrotic enteritis (Keyburn et al. 2010). The *netB* gene can be identified by PCR.

Amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE) were found to be equally suitable for subtyping of *C. perfringens* isolates of poultry origin (Engstrom et al. 2003). Multiple-locus variable-number tandem repeat analysis (MLVA) has also been described as an efficient tool for *C. perfringens* strain typing and could be used in epidemiological studies (Sawires & Songer 2005). The assay uses five variable tandem repeat (VNTR) loci. The agreement between MLVA and PFGE methods was good. A multilocus sequence typing (MLST) scheme was developed for *C. perfringens* with the objective of classifying strains according to disease presentation and/or host preference (Jost et al. 2006). In this MLST scheme, sequence data from one virulence and seven housekeeping genes of *C. perfringens* were obtained which comprised all five toxin types from various host species. Three clonal complexes with 80 sequence types (STs) in total were identified. Typing of intestinal *C. perfringens* isolates from outbreaks of avian necrotic enteritis using a MLST technique with an additional locus, *pfoS* has also been described (Chalmers et al. 2008).

Clostridium difficile

Many publications are available on the molecular detection and typing of *C. difficile* but the majority relate to samples and isolates from humans. A multiplex PCR toxigenic culture approach has been described for simultaneous identification and toxigenic typing of human and animal *C. difficile* intestinal infections (Lemee et al. 2004). Three pairs of primers are used for amplification of a species-specific internal fragment of the *tpi* (triose phosphate isomerase) gene, an internal fragment of the *tcdB* (toxin B) gene, and an internal fragment of the *tcdA* (toxin A) gene. This assay allows distinction between toxin A-positive, toxin B-positive (A+B+) strains and toxin A-negative, toxin B-positive (A-B+) variant strains. More recently, a multiplex real-time PCR has been developed as a rapid screening assay to detect the presence of the *tcdA* and *tcdB* genes of *C. difficile* directly in faecal samples (de Boer et al. 2010) although the test has a low positive predictive value and should be used in conjunction with other tests. A multiplex real-time PCR has also been described for the detection of *C. difficile* genes encoding toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtA* and *cdtB*) in faecal samples from preweaned calves, in retail minced meat samples, and in pasteurized milk samples (Houser et al. 2010). Real-time PCR and pyrosequencing analysis were described for the rapid identification of hypervirulent *C. difficile* strains in human faecal samples and results correlated well with cultural results (Wroblewski et al. 2009).

Clostridium difficile is represented by multiple strain types as determined by restriction endonuclease analysis (REA) and by PCR ribotyping, two well-characterized typing systems (Cheknis et al. 2009). Today, *C. difficile* genotyping is most commonly done by PFGE or PCR ribotyping (Noren 2010). Comparison of these techniques with an automated repetitive extragenic palindromic sequence-based PCR (rep-PCR) method (bioMérieux) revealed that the automated rep-PCR could be used for first-line molecular typing in local clinical microbiology laboratories. The method is reported as easy to use as well as rapid, requiring less 'hands-on' time than PCR ribotyping or PFGE typing (Pasanen et al. 2011). Killgore et al. (2008) recently compared seven techniques for typing international epidemic strains of *C. difficile:* MLVA, amplified fragment length polymorphism (AFLP), surface layer protein A gene sequence typing (slpAST), PCR-ribotyping, restriction endonuclease analysis (REA), MLST and PFGE. All isolates were typable by all techniques and a current epidemic strain of *C. difficile* (BI/027/NAP1) was differentiated from other strains. The discrimination index scores ranged from 0.964 to 0.631 in the following order: MLVA, REA, PFGE, slpAST, PCR-ribotyping, MLST, and AFLP. All techniques were able to detect outbreak strains. However, only REA and MLVA showed sufficient discrimination to differentiate between strains from different outbreaks.

Clostridium botulinum

The mouse bioassay is the current gold standard by which *C. botulinum* neurotoxins (BoNTs) are confirmed. However, this method is expensive, slow and labour-intensive. Thus, PCR-based assays have been used extensively for the detection of BoNT-producing bacteria in food, animals and faecal samples (Fach et al. 2009). Single (Dahlsten et al. 2008), multiplex (Umeda et al. 2010), seminested PCR (Shin et al. 2007) and real-time (Lindberg et al. 2010) PCR assays have been described in the literature.

Histotoxic clostridia

Clostridium chauvoei conventional PCR assays have been described to amplify specific segments of the 16S ribosomal RNA gene (Bagge et al. 2009) or a 516-bp fragment of the structural flagellin gene (Kojima et al. 2001) in both cultures and clinical samples. The clinical signs of blackleg are very similar to those of malignant oedema and assays that offer the detection of both *C. chauvoei* and *C. septicum* in clinical samples are of diagnostic value. A multiplex PCR system based on the flagellin gene *fliC* sequence has been described to rapidly identify *C. chauvoei, C. haemolyticum, C. novyi* types A and B, and *C. septicum* (Sasaki et al. 2002). More recently, a multiplex real-time PCR based on the detection of the *spo0A* gene allowed the simultaneous identification of *C. chauvoei* and *C. septicum* (Lange et al. 2010). The assay was successfully tested on tissue samples from clinical blackleg cases. A quantitative real-time PCR assay was developed to measure the levels of *C. septicum* in healthy birds as well as in samples from gangrenous dermatitis cases in poultry. The assay

specifically targets the *C. septicum* alpha toxin gene, *csa* (Neumann et al. 2010).

Other clostridia

PFGE and PCR based techniques have both been described for the molecular characterization of *C. tetani* (Plourde-Owobi et al. 2005). *C. sordellii* DNA was directly detected by a broad-range 16S rRNA PCR in clinical human samples (Valour et al. 2010). A PCR for the detection of *C. spiroforme* and its binary toxin encoding genes has been described (Drigo et al. 2008). *Clostridium piliforme* is impossible to cultivate on media, thus its diagnosis is based on typical gross lesions and histological demonstration of intracellular bacteria at the periphery of the necrotic foci. A nested PCR assay was developed for detection of this organism by Niepceron & Licois (2010).

REFERENCES

Agnoletti, F., Ferro, T., Guolo, A., et al., 2009. A survey of *Clostridium spiroforme* antimicrobial susceptibility in rabbit breeding. Veterinary Microbiology 136 (1–2), 188–191.

Akbulut, D., Grant, K.A., McLauchlin, J., 2005. Improvement in laboratory diagnosis of wound botulism and tetanus among injecting illicit-drug users by use of real-time PCR assays for neurotoxin gene fragments. Journal of Clinical Microbiology 43 (9), 4342–4348.

Bagge, E., Lewerin, S.S., Johansson, K.E., 2009. Detection and identification by PCR of *Clostridium chauvoei* in clinical isolates, bovine faeces and substrates from biogas plant. Acta Veterinaria Scandinavica 51, 8.

Borriello, P.S., Honour, P., 1981. Simplified procedure for the routine isolation of *Clostridium difficile* from faeces. Journal of Clinical Pathology 34, 1126–1127.

Cai, S.W., Singh, B.R., 2007. Botulism diagnostics: from clinical symptoms to in vitro assays. Critical Reviews in Microbiology 33 (2), 109–125.

Chalmers, G., Bruce, H.L., Hunter, D.B., et al., 2008a. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chickens. Journal of Clinical Microbiology 46 (12), 3957–3964.

Chalmers, G., Martin, S.W., Hunter, D.B., et al., 2008. Genetic diversity of *Clostridium perfringens* isolated from healthy broiler chickens at a commercial farm. Veterinary Microbiololy 127, 116–127.

Charlebois, A., Jalbert, L.A., Harel, J., et al., 2012. Characterization of genes encoding for acquired bacitracin resistance in *Clostridium perfringens*. PLoS ONE 7, e44449.

Cheknis, A.K., Sambol, S.P., Davidson, D.M., et al., 2009. Distribution of *Clostridium difficile* strains from a North American, European and Australian trial of treatment for *C. difficile* infections: 2005–2007. Anaerobe 15, 230–233.

Chouicha, N., Marks, S.L., 2006. Evaluation of five enzyme immunoassays compared with the cytotoxicity assay for diagnosis of *Clostridium difficile*-associated diarrhea in dogs. Journal of Veterinary Diagnostic Investigation 18 (2), 182–188.

Clinical and Laboratory Standards Institute (CLSI), 2012a. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard, eighth ed, CLSI document M11-MA8. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2012b. Performance Standards for Antimicrobial Susceptibility Testing; 22nd informational supplement, CLSI document M100-MS22. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Dahlsten, E., Korkeala, H., Somervuo, P., et al., 2008. PCR assay for differentiating between Group I (proteolytic) and Group II (non-proteolytic) strains of *Clostridium botulinum*. International Journal of Food Microbiology 124, 108–111.

de Boer, R.F., Wijma, J.J., Schuurman, T., et al., 2010. Evaluation of a rapid molecular screening approach for the detection of toxigenic *Clostridium difficile* in general and subsequent identification of the tcdC Delta117 mutation in human stools. Journal of Microbiological Methods 83, 59–65.

Drigo, I., Bacchin, C., Cocchi, M., et al., 2008. Development of PCR protocols for specific identification of *Clostridium spiroforme* and detection of sas and sbs genes. Veterinary Microbiology 131, 414–418.

Engstrom, B.E., Fermer, C., Lindberg, A., et al., 2003. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Veterinary Microbiology 94, 225–235.

Erol, I., Goncuoglu, M., Ayaz, N.D., et al., 2008. Molecular typing of *Clostridium perfringens* isolated from turkey meat by multiplex PCR. Letters in Applied Microbiology 47, 31–34.

Fach, P., Micheau, P., Mazuet, C., et al., 2009. Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum, Clostridium baratii* and *Clostridium butyricum*. Journal of Applied Microbiology 107, 465–473.

Fennessey, C.M., Ivie, S.E., McClain, M.S., 2012. Coenzyme depletion by members of the aerolysin family of pore-forming toxins leads to diminished ATP levels and cell death. Molecular BioSystems 11 June 2012 (EPUB ahead of print).

Frey, J., Johansson, A., Bürki, S., et al., 2012. Cytotoxin CctA, a major virulence factor of *Clostridium chauvoei* conferring protective immunity against myonecrosis. Vaccine 27 June 2012 (EPUB ahead of print).

Garmory, H.S., Chanter, N., French, N.P., et al., 2000. Occurrence of *Clostridium perfringens* beta2-toxin

amongst animals, determined using genotyping and subtyping PCR assays. Epidemiology & Infection 124, 61–67.

Gharaibeh, S., Al Rifai, R., Al-Majali, A., 2010. Molecular typing and antimicrobial susceptibility of *Clostridium perfringens* from broiler chickens. Anaerobe 16 (6), 586–589.

Gholamiandehkordi, A., Eeckhaut, V., Lanckriet, A., et al., 2009. Antimicrobial resistance in *Clostridium perfringens* isolates from broilers in Belgium. Veterinary Research Communications 33 (8), 1031–1037.

Gobeli, S., Berset, C., Burgener, I., et al., 2012. Antimicrobial susceptibility of canine *Clostridium perfringens* strains from Switzerland. Schweizer Archiv für Tierheilkunde 154 (6), 247–250.

Gurjar, A.A., Hegde, N.V., Love, B.C., et al., 2007. Real-time multiplex PCR assay for rapid detection and toxin typing of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. Molecular and Cellular Probes 22 (2), 90–95.

Hogg, R., Livesey, C., Payne, J., 2008. Diagnosis and implications of botulism. In Practice, 30 (7), 392–397.

Houser, B.A., Hattel, A.L., Jayarao, B.M., 2010. Real-time multiplex polymerase chain reaction assay for rapid detection of *Clostridium difficile* toxin-encoding strains. Foodborne Pathogens and Disease 7, 719–726.

Jost, B.H., Trinh, H.T., Songer, J.G., 2006. Clonal relationships among *Clostridium perfringens* of porcine origin as determined by multilocus sequence typing. Veterinary Microbiology 116, 158–165.

Kather, E.J., Marks, S.L., Foley, J.E., 2006. Determination of the prevalence of antimicrobial resistance genes in canine *Clostridium perfringens* isolates. Veterinary Microbiology 113 (1–2), 97–101.

Keyburn, A.L., Yan, X.X., Bannam, T.L., et al., 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Veterinary Research 41, 21.

Killgore, G., Thompson, A., Johnson, S., et al., 2008. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile:* restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. Journal of Clinical Microbiology 46431–46437.

Kojima, A., Uchida, I., Sekizaki, T., et al., 2001. Rapid detection and identification of *Clostridium chauvoei* by PCR based on flagellin gene sequence. Veterinary Microbiology 78, 363–371.

Lange, M., Neubauer, H., Seyboldt, C., 2010. Development and validation of a multiplex real-time PCR for detection of *Clostridium chauvoei* and *Clostridium septicum*. Molecular and Cellular Probes 24, 204–210.

Laughton, B.E., Viscidi, R.P., Gdovin, S.L., et al., 1984. Enzyme immunoassays for detection of *Clostridium difficile* toxins A or B in faecal specimens. Journal of Infectious Diseases 149, 781–788.

Lemee, L., Dhalluin, A., Testelin, S., et al., 2004. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of *Clostridium difficile*. Journal of Clinical Microbiology 42, 5710 –5714.

Lindberg, A., Skarin, H., Knutsson, R., et al., 2010. Real-time PCR for *Clostridium botulinum* type C neurotoxin (BoNTC) gene, also covering a chimeric C/D sequence-Application on outbreaks of botulism in poultry. Veterinary Microbiology 146, 118–123.

Myers, G.S., Rasko, D.A., Cheung, J.K., et al., 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Research 16, 1031–1040.

Nagahama, M., Umezaki, M., Oda, M., et al., 2011. *Clostridium perfringens* iota-toxin b induces rapid cell necrosis. Infection and Immunity 79 (11), 4353–435360.

Neumann, A.P., Dunham, S.M., Rehberger, T.G., et al., 2010. Quantitative real-time PCR assay for *Clostridium septicum* in poultry gangrenous dermatitis associated samples. Molecular and Cellular Probes 24, 211–218.

Niepceron, A., Licois, D., 2010. Development of a high-sensitivity nested PCR assay for the detection of *Clostridium piliforme* in clinical samples. Veterinary Journal 185, 222–224.

Noren, T., 2010. *Clostridium difficile* and the disease it causes. Methods in Molecular Biology 646, 9–35.

Pasanen, T., Kotila, S.M., Horsma, J., et al., 2011. Comparison of repetitive extragenic palindromic sequence-based PCR with PCR ribotyping and pulsed-field gel electrophoresis in studying the clonality of *Clostridium difficile*. Clinical Microbiology and Infection 17, 166–175.

Plourde-Owobi, L., Seguin, D., Baudin, M.A., et al., 2005. Molecular characterization of *Clostridium tetani* strains by pulsed-field gel electrophoresis and colony PCR. Applied and Environmental Microbiology 71, 5604–5606.

Ruby, R., Magdesian, K.G., Kass, P.H., 2009. Comparison of clinical, microbiologic, and clinicopathologic findings in horses positive and negative for *Clostridium difficile* infection. Journal of the American Veterinary Medical Association 234, 777–784.

Sasaki, Y., Kojima, A., Aoki, H., et al., 2002. Phylogenetic analysis and PCR detection of *Clostridium chauvoei, Clostridium haemolyticum, Clostridium novyi types* A and B, and *Clostridium septicum* based on the flagellin gene. Veterinary Microbiology 86, 257–267.

Sawires, Y.S., Songer, J.G., 2005. Multiple-locus variable-number tandem repeat analysis for strain typing of *Clostridium perfringens*. Anaerobe 11, 262–272.

Sharma, S.K., Whiting, R.C., 2005. Methods for detection of *Clostridium botulinum* toxin in foods. Journal of Food Protection 68, 1256–1263.

Shin, N.R., Yoon, S.Y., Shin, J.H., et al., 2007. Development of enrichment semi-nested PCR for *Clostridium botulinum* types A, B, E, and F and its application to Korean environmental samples. Molecules and Cells 24, 329–337.

Slavic, D., Boerlin, P., Fabri, M., et al., 2011. Antimicrobial susceptibility of

Clostridium perfringens isolates of bovine, chicken, porcine, and turkey origin from Ontario. Can J Vet Res 75, 89–97.

Susick, E.K., Putnam, M., Bermudez, D.M., et al., 2012. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter. Veterinary Microbiology 157 (1–2), 172–178 (EPUB 21 December 2011).

Thakur, S., Putnam, M., Fry, P.R., et al., 2010. Prevalence of antimicrobial resistance and association with toxin genes in *Clostridium difficile* in commercial swine. American Journal of Veterinary Research 71 (10), 1189–1194.

Umeda, K., Seto, Y., Kohda, T., et al., 2010. A novel multiplex PCR method for *Clostridium botulinum* neurotoxin type A gene cluster typing. Microbiology and Immunology 54, 308–312.

Uzal, F.A., McClane, B.A., 2011. Recent progress in understanding the pathogenesis of *Clostridium perfringens* type C infections. Veterinary Microbiology 153 (1–2), 37–43.

Uzal, F.A., Songer, J.G., 2008. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. Journal of Veterinary Diagnostic Investigation 20 (3), 253–265.

Valour, F., Boisset, S., Lebras, L., et al., 2010. *Clostridium sordellii* brain abscess diagnosed by 16S ribosomal DNA sequencing. Journal of Clinical Microbiology 48, 3443–3444.

Watkins, K.L., Shryock, T.R., Dearth, R.N., et al., 1997. In-vitro antimicrobial susceptibility of *Clostridium perfringens* from commercial turkey and broiler chicken origin. Veterinary Microbioloy 54, 195–200.

Wroblewski, D., Hannett, G.E., Bopp, D.J., et al., 2009. Rapid molecular characterization of *Clostridium difficile* and assessment of populations of *C. difficile* in stool specimens. Journal of Clinical Microbiology 47, 2142–2148.

FURTHER READING

Jiu-Cong, Zhang, Li, Sun, Qing-He, Nie, 2010. Botulism, where are we now? Clinical Toxicology 48, 867–879.

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Chapter 17

Enterobacteriaceae

Most members of the family *Enterobacteriaceae* share the following characteristics: Gram-negative, medium-sized rods (0.4–0.6 × 2–3 μm; Fig. 17.1); peritrichate arrangement of flagella, if motile; facultatively anaerobic and ferment, rather than oxidize, glucose; catalase-positive and oxidase-negative; reduce nitrate to nitrite and are able to grow on non-enriched media such as nutrient agar. There are a few exceptions to these general properties, for example, *Shigella dysenteriae* is catalase-negative; *Tatumella ptyseos* is motile by polar, subpolar or lateral flagella and *Photorhabdus* species do not regularly reduce nitrate.

Nomenclature

There are, at present, more than 40 genera and over 180 well-defined species in the *Enterobacteriaceae*. Traditionally the genera and species of the family have been distinguished biochemically and this is convenient for identification of clinical isolates. However, genetic means of defining species, based on DNA–DNA homology, has led to the recognition of numerous new species, some previously regarded as aberrant biotypes, and also to the recognition of genetically closely related members as single genomic species.

The terms 'coliform' or 'coliform bacteria' have no taxonomic significance but are used to refer to those members of the *Enterobacteriaceae* that usually ferment lactose, such as *Escherichia coli*, *Klebsiella* and *Enterobacter* species and sometimes to describe other members of the family.

Habitat

The members of the *Enterobacteriaceae* are geographically widespread and many are widely distributed throughout the environment in soil, water, on plants as well as in the intestines of animals and humans. However, a few species occupy a limited ecological niche, such as *Salmonella* Typhi, that causes typhoid fever in man and is found only in humans.

Differentiation of the *Enterobacteriaceae*

Conventional microbiology

All enterobacteria will grow on blood and MacConkey agars and these are used routinely to isolate them in diagnostic laboratories. Although MacConkey agar is a selective medium, it is relatively permissive and allows the growth of some other Gram-negative bacteria as well as the enterobacteria. Brilliant green agar and xylose-lysine-deoxycholate (XLD) medium are more selective and used for the isolation of salmonellae, although some other enterobacteria are able to grow on them. A number of other selective agars are frequently used for the isolation of salmonellae and these are detailed under the relevant section. Table 17.1 gives the reactions of some members of the *Enterobacteriaceae* on MacConkey agar, brilliant green agar and XLD medium. The uninoculated media (Fig. 17.2) are illustrated together with the appearance of 11 enterobacteria and *Pseudomonas aeruginosa* on these selective/indicator media (Figs 17.3 to 17.14 inclusive). A summary of isolation methods for the detection and presumptive identification of important members of the *Enterobacteriaceae* is given in Figure 17.15.

MacConkey agar

Fermentable sugar: lactose.

pH indicator: neutral red (pale-straw at pH 8 and pink at pH 6.8).

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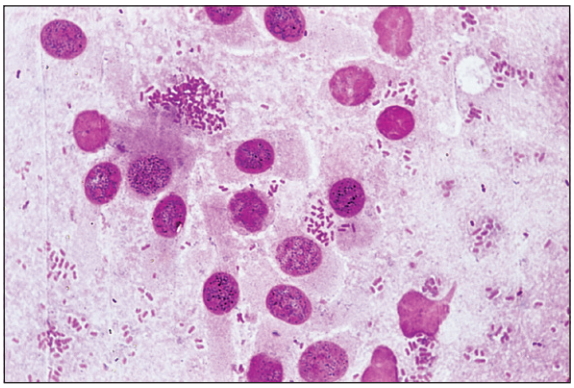

Figure 17.1 Gram-negative medium-sized rods of *Escherichia coli* in a tissue smear. The morphology is typical of most members of the *Enterobacteriaceae*. (Gram stain, ×1000)

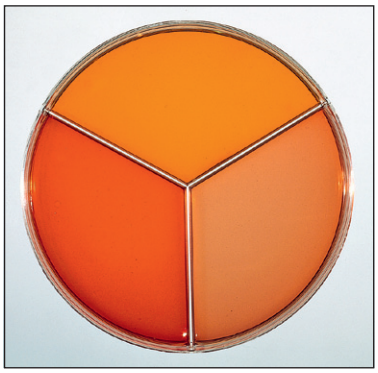

Figure 17.2 Uninoculated selective media used for the isolation of members of the *Enterobacteriaceae*: XLD medium (left), brilliant green agar (top) and MacConkey agar (right).

Figures. 17.3–17.13 Reactions of some members of the *Enterobacteriaceae* on XLD medium (left), brilliant green agar (top) and MacConkey agar (right).

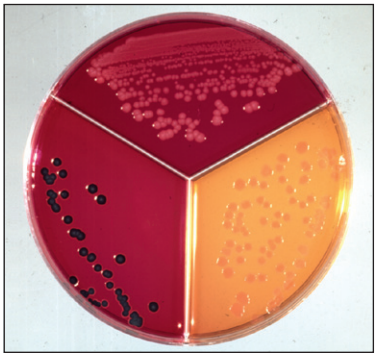

Figure 17.3 *Salmonella* Enteritidis.

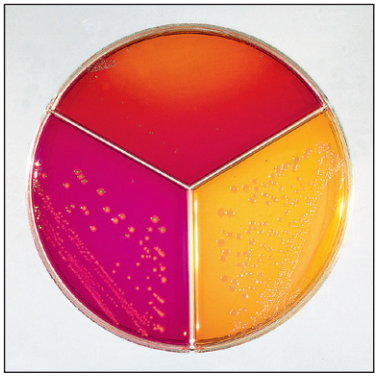

Figure 17.5 *Edwardsiella tarda*.

Figure 17.4 *Proteus mirabilis*.

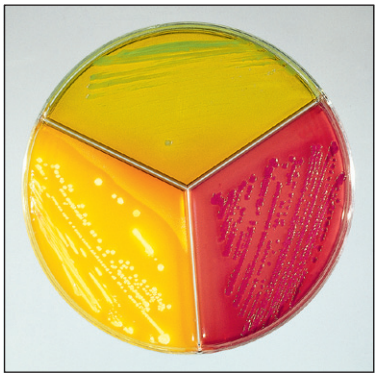

Figure 17.6 *Escherichia coli*.

Table 17.1 Reactions of some members of the *Enterobacteriaceae* on selective/indicator media

Figure number	Bacterium	Lactose	Sucrose	Xylose	H_2S	Lysine	MacConkey agar (lactose, neutral red)	Brilliant green agar (lactose, sucrose, phenol red)	XLD agar (lactose, sucrose, xylose, lysine, H_2S, phenol red)
17.3	*Salmonella* Enteritidis	–	–	+	+	+	Pale colonies (alk)	Red (alk)	Red/black centre (alk)
17.4	*Proteus mirabilis*	–	(–)	+	+	–	Pale (alk)	Red (alk)	Yellowish/black centre (alk)
17.5	*Edwardsiella tarda*	–	–	–	+	+	Pale (alk)	No growth	Reddish/black centre (alk)
17.6	*Escherichia coli*	+	d	+	–	(+)	Bright pink (acid)	[Yellow-green] (acid)	Yellow (acid)
17.7	*Klebsiella pneumoniae*	+	+	+	–	+	Pink (acid)	Yellow-green (acid)	Yellow (acid)
17.8	*Enterobacter aerogenes*	+	+	+	–	+	Pink (acid)	Yellow-green (acid)	Yellow (acid)
17.9	*Providencia stuartii*	–	d	–	–	–	Pale (alk)	[Yellow-green] (acid)	Yellow (acid)
17.10	*Citrobacter diversus*	d	(–)	+	–	–	Pink (acid)	[Green] (acid)	Yellow (acid)
17.11	*Serratia marcescens*	–	+	–	–	+	Pale + pigment	Red-yellow (acid)	Red (alk)
17.12	*Yersinia enterocolitica*	–	+	d	–	–	Pale pink	[Green] (acid)	Yellow (acid)
17.13	*Yersinia pseudotuberculosis*	–	–	+	–	–	Pale (alk)	[Red] (alk)	Red (alk)

[ ] = poor growth, alk = alkaline reaction, H_2S = hydrogen sulphide, + = 90–100% strains positive, (+) = 76–89% positive, d = 26–27% positive, (–) = 11–25% positive, – = 0–10% positive

Figure 17.7 *Klebsiella pneumoniae.*

Figure 17.8 *Enterobacter aerogenes.*

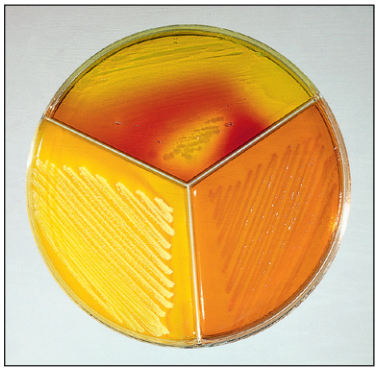

Figure 17.9 *Providencia stuartii.*

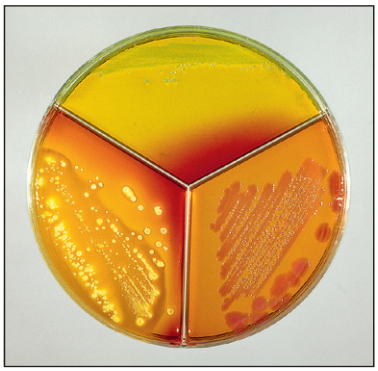

Figure 17.10 *Citrobacter diversus.*

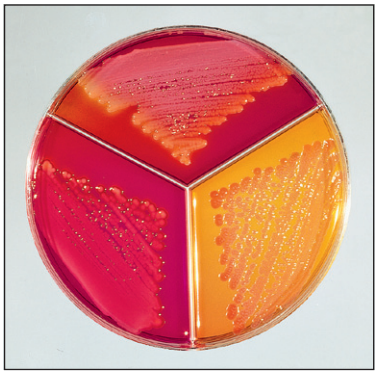

Figure 17.11 *Serratia marcescens.*

Figure 17.12 *Yersinia enterocolitica.*

Inhibitors: bile salts and crystal violet (anti-Gram-positive bacteria).

Reactions: if the bacterium can ferment lactose, acid metabolic products are produced and the medium and colonies appear pink (lactose-positive). If the organism is unable to use the lactose, then it attacks the peptone (nitrogen source) in the medium with resulting alkaline metabolic products and the medium and colonies appear pale/straw-coloured (lactose-negative).

Brilliant green agar

Fermentable sugars: lactose and sucrose

pH indicator: phenol red (red at pH 8.2 and yellow at pH 6.4)

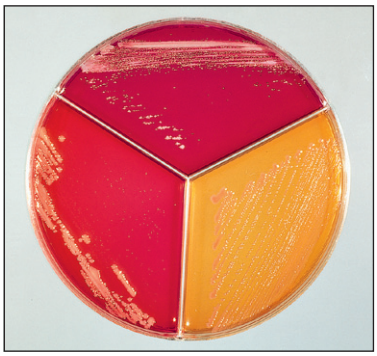

Figure 17.13 *Yersinia pseudotuberculosis.*

Figure 17.14 Reactions of *Pseudomonas aeruginosa* on XLD medium (left), brilliant green agar (top) and MacConkey agar (right) for comparison with the reactions of the *Enterobacteriaceae*.

Inhibitor: brilliant green dye inhibits the growth of most enterobacteria to some extent, except *Salmonella* species.

Reactions: similar to those occurring on MacConkey agar except that the bacteria may ferment one or both of the sugars with an acid reaction (yellowish-green). Certain enterobacteria, such as *Salmonella* species, are unable to ferment either sugar and attack the peptone instead, with an alkaline reaction (red colonies and medium).

XLD medium

Fermentable sugars: lactose, sucrose and xylose.

pH indicator: phenol red (red at pH 8.2 and yellow at pH 6.4).

Other substrates: lysine and chemicals for detecting hydrogen sulphide (H_2S) production.

Inhibitor: bile salts (sodium deoxycholate).

Reactions: salmonellae will first ferment the xylose creating a temporary acid reaction but this is reversed by the subsequent decarboxylation of lysine with alkaline metabolic products. Superimposed on the red (alkaline) colonies is the production of hydrogen sulphide, so most salmonellae have red colonies with a black centre (Fig. 17.3). *Edwardsiella tarda* also gives this reaction although the H_2S production is less marked and the periphery of the colonies tends to be a yellowish-red colour. The large amount of acid produced by enterobacteria that can ferment either lactose or sucrose, or both, prevents the reversion to alkaline conditions even if the bacterium is able to decarboxylate the lysine.

Triple sugar iron (TSI) agar

This is an indicator medium only and does not contain an inhibitor. A brief description of the medium and technique for inoculation is given in Chapter 2. It is prepared in tubes with a slant.

Fermentable sugars: glucose 0.1%, lactose 1.0% and sucrose (or saccharose) 1.0%.

Other substrates: chemicals to indicate hydrogen sulphide (H_2S) production.

pH indicator: phenol red (red at pH 8.2 and yellow at pH 6.4).

Reactions: all members of the *Enterobacteriaceae* are capable of fermenting glucose and the small amount (0.1%) will be attacked preferentially and rapidly. At this early stage both the butt and slant will be yellow due to acid production from the glucose fermentation. Some enterobacteria attack the lactose and/or sucrose (each at a 1.0% concentration) in the medium and in this case sufficient acid is produced to maintain both the butt and the slant in an acid (yellow) condition.

Bacteria that are unable to ferment either lactose or sucrose, after the depletion of the limited amount of glucose, will use the peptone in the medium. This is a less efficient method of producing energy and occurs mainly at the surface of the slant in the presence of atmospheric oxygen. The metabolites of peptone are alkaline and this causes the slant to revert back to the original red colour. Some members of the *Enterobacteriaceae,* including most *Salmonella* spp., are able to produce hydrogen sulphide. This reaction is superimposed over the sugar fermentations and is seen as a blackening of the medium.

The general interpretation of the reactions is as follows:

- Alkaline (red) slant and acid (yellow) butt: glucose fermentation only.
- Acid (yellow) slant and acid (yellow) butt: lactose and/or sucrose attacked as well as the glucose.
- Blackening of the medium: hydrogen sulphide production.

The reactions are illustrated in Figure 17.16, while Figure 17.17 gives a summary of the differentiation of some of the enterobacteria by their reactions in TSI agar and lysine decarboxylase broth.

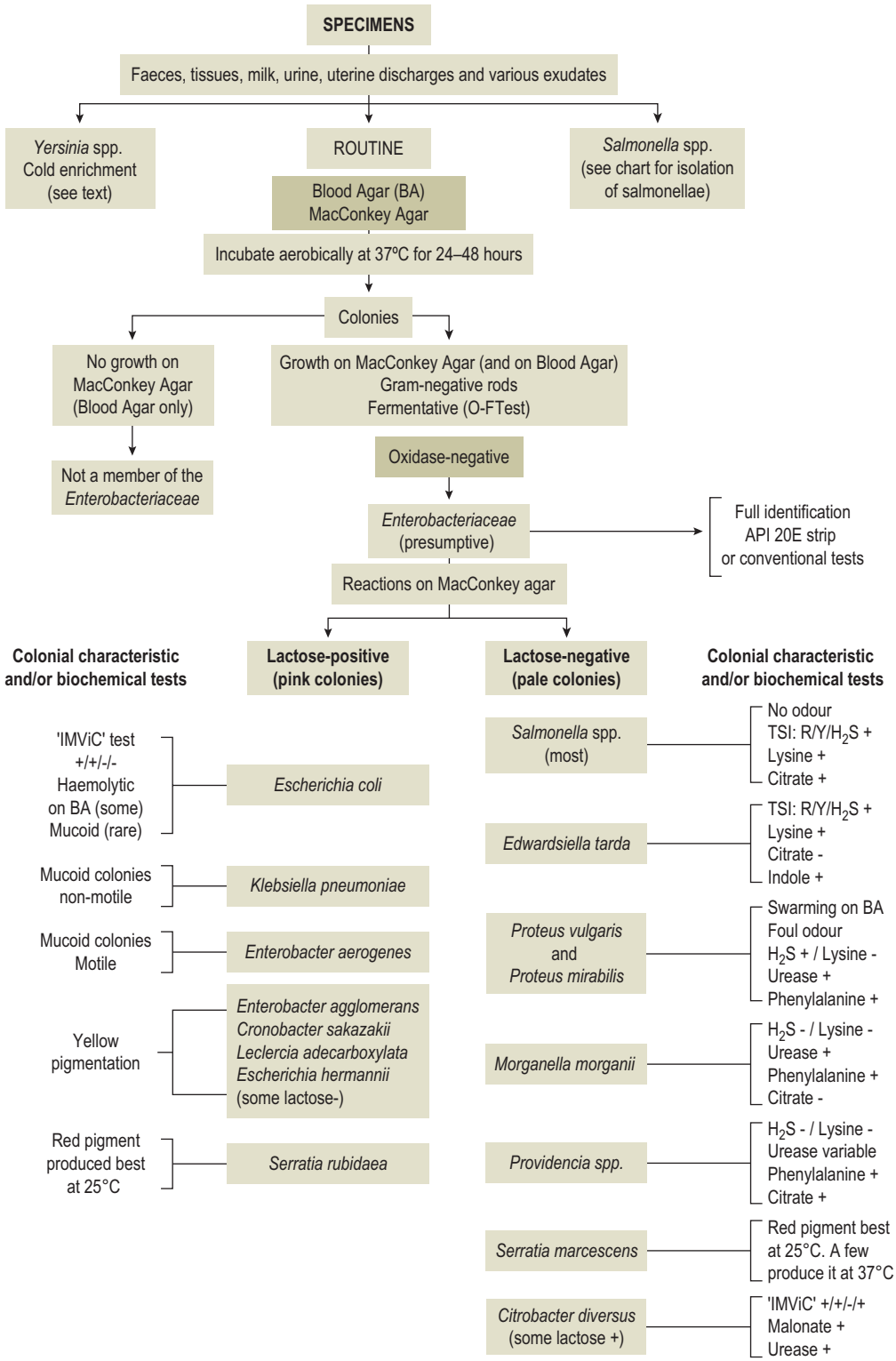

Figure 17.15 Routine isolation of important members of the *Enterobacteriaceae* and their presumptive identification on colonial morphology and /or biochemical tests. + = positive reaction, – = negative, 'IMViC' = indole, methyl red, Voges–Proskauer and citrate tests, TSI = triple sugar iron agar, lysine = lysine decarboxylase test

Figure 17.16 TSI agar slopes showing the range of reactions from the left, uninoculated, R/Y/H_2S+, R/Y/H_2S–, Y/Y/H_2S+, Y/Y/H_2S–. R = red (alkaline), Y = yellow (acid), H_2S+ = hydrogen sulphide produced, H_2S– = hydrogen sulphide not produced

Pathogenicity

The *Enterobacteriaceae* can be divided into three groups based on their pathogenicity for animals:

- Major pathogens of animals such as *Salmonella* species, *Escherichia coli* and three of the *Yersinia* species.
- Opportunistic pathogens that are known to occasionally cause infections in animals. These include species within the genera *Klebsiella, Enterobacter, Proteus, Serratia, Edwardsiella, Citrobacter, Morganella* and *Shigella*. *Shigella* species cause disease in humans and other primates.
- Organisms of uncertain significance for animals. These include species from 17 genera of the *Enterobacteriaceae* and they are summarized in Table 17.2. As some of them may be isolated from clinical specimens a range of their biochemical reactions is given in Table 17.3.

All Gram-negative bacteria, including the members of the *Enterobacteriaceae*, have lipopolysaccharides in the outer membrane of the cell wall that are potent endotoxins, the main endotoxic principle being lipid A. The bacteria must die and lyse before the endotoxin is released. The effects of endotoxin in the animal body include fever, leukopaenia followed by leukocytosis and hyperglycaemia with a subsequent fall in blood sugar and lethal shock after a latent period. The more pathogenic members of the *Enterobacteriaceae* have other virulence factors such as adhesins to attach to host cells, capsules that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron and exotoxins that include enterotoxins and cytotoxins. These will be reviewed in the relevant sections on each genus/organism.

ESCHERICHIA COLI

Natural Habitat

Escherichia coli is a natural inhabitant of the large intestine and lower small intestine of all mammals. It is usually present in larger numbers in carnivores and omnivores than in herbivores. *Escherichia coli* is excreted in faeces and can survive in faecal particles, dust and water for weeks or months. The presence of *E. coli* in water samples, being tested for potability, is taken as evidence of faecal pollution.

Pathogenesis and Pathogenicity

Predisposing causes

Although *E. coli* strains possess a number of virulence factors which assist them in colonizing the intestine, invading the host and producing disease, predisposing causes are of paramount importance and largely determine whether or not clinical signs of illness will occur:

- Neonates obtaining insufficient passive immunity (antibodies) from colostrum. This might be due to either a quantitative or qualitative deficiency.
- Intensive husbandry practices lend themselves to rapid transmission of the pathogenic *E. coli* strains.
- Poor hygiene that allows a build-up of pathogenic strains in the environment of the young animal. A large dose of pathogenic *E. coli* may overcome colostral immunity.
- Young neonates, under one week of age, are particularly susceptible because:
  - The normal flora of the intestines is not fully established
  - They have a naive immune system
  - Receptors for the adhesins of enterotoxigenic *E. coli* are present for the first week of life only in calves and for the first six weeks of life in piglets.
- Recently weaned pigs are subject to stress factors such as altered surroundings, companions and diet. Heavy grain diets in particular can lead to a massive colonization of the anterior small intestine by enterotoxigenic strains of *E. coli*.
- Oedema disease occurs most commonly in young weanling pigs but the disease can occur in older pigs. The following factors are often present prior to the occurrence of oedema disease in pigs:
  - Recent change in feed
  - The pigs are thriving and growing rapidly
  - Mild diarrhoea noticed a few days before the signs of oedema disease appear.

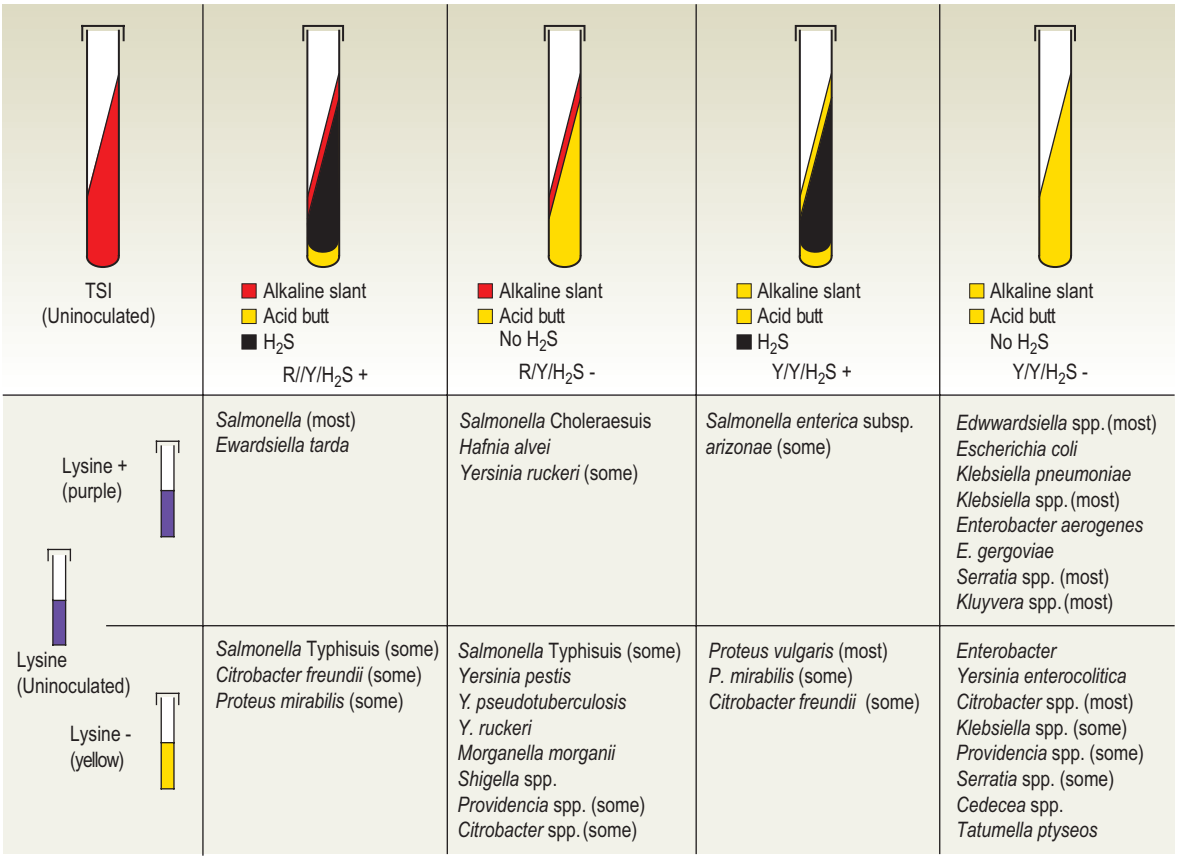

Figure 17.17. Reactions of the *Enterobacteriaceae* in triple sugar iron agar and lysine decarboxylase broth. R = red (alkaline), Y = yellow (acid), H_2S+ = hydrogen sulphide produced, H_2S– = hydrogen sulphide not produced.

Types of pathogenic *E. coli*

Escherichia coli strains, normally regarded as non-pathogenic, can cause opportunistic infections in various sites of the body such as mammary glands (mastitis) and uterus (metritis). Pathogenic *E. coli* strains are classified according to the type of disease that they produce and according to the virulence determinants which they possess. However, as more is learnt about the virulence attributes of all strains of *E. coli*, it is becoming increasingly clear that the possession of particular virulence genes may not be the only feature that differentiates pathogenic and non-pathogenic strains; the level of expression of those genes is also likely to be of crucial importance.

Escherichia coli strains can be divided into those causing extraintestinal disease and those causing enteric infections. Extraintestinal diseases result from infection with strains causing invasive conditions such as septicaemia (SEPEC), and also include uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC). These strains may be collectively referred to as extraintestinal pathogenic *E. coli* or ExPEC. There are also suggestions for two new animal pathogenic groups: those causing infections of the mammary gland, mammary pathogenic *E. coli* (MPEC) and those affecting the uterus, endometrial pathogenic *E. coli* (EnPEC) (Köhler & Dobrindt, 2011). The types of *E. coli* causing enteric disease include enterotoxigenic strains (ETEC) and attaching and effacing *E. coli* (AEEC). The latter group includes the enteropathogenic *E. coli* (EPEC) and Vero- or Shiga-toxin producing strains (VTEC or STEC). Enterohaemorrhagic *E. coli* and strains of *E coli* producing oedema disease are subgroups of STEC. Of the disease syndromes caused by these pathogenic types, enteric disease produced by ETEC, oedema disease of pigs and extraintestinal diseases, including septicaemia, are the best characterized types in animals. In contrast to ETEC strains, ExPEC and EPEC strains form part of the normal flora in animals and are considered opportunistic pathogens (Gyles & Fairbrother, 2010).

Table 17.2 Genera of the *Enterobacteriaceae* whose species are of uncertain significance for animals

Genus (species)	Site of isolation and possible pathogenicity
Budvicia aquatica	Water and human faeces
Buttiauxella agrestis	Water
Cedecea species	Rare isolates from human clinical specimens (most commonly from the respiratory tract). Bacteraemia in humans has been reported
Ewingella americana	Rare isolate from human clinical specimens
Erwinia herbicola	This and other *Erwinia* species are associated with plants as pathogens or saprophytes
Kluyvera species	Water, sewage, soil and milk. Occasionally isolated from human clinical specimens
Yokenella regensburgei (Koserella trabulsii)	Human respiratory tract, wounds, urine and faeces but pathogenicity uncertain
Leclercia (Escherichia) adecarboxylata	Environment, food and water. It has been isolated from human clinical specimens
Lemiorella species	Isolated from human faeces and urine
Moellerella wisconsensis	Human faeces
Obesumbacterium proteus	Found only in contaminated beer. Biogroup 1 is thought to be a brewery-adapted biochemical variant of *Hafnia alvei*. Unlikely to be pathogenic for animals
Photorhabdus species	Pathogenic for nematodes
Pragia fontium	Isolated from water
Providencia species	Urinary tract of compromised or catheterized human patients, patients suffering from burn infections and diarrhoea. Rarely isolated from faeces of healthy humans
Rahnella aquatilis	Water and from human burn wounds
Tatumella ptyseos	Occasionally isolated from human clinical specimens, mainly from the respiratory tract

Enterotoxigenic E. coli

These strains cause the majority of cases of neonatal colibacillosis in calves, lambs and piglets. They do not appear to be an important cause of diarrhoea in other domestic animals, for reasons which are as yet unclear. Pathogenicity is correlated with the presence of adhesins and the production of enterotoxins. The toxins function by reducing absorption and increasing secretion without damaging the intestinal epithelium. The first step in the production of disease is the adherence of the ETEC to the intestinal epithelium. The structures by which the ETEC adhere are most commonly fimbriae and these are classified according to properties such as their amino acid composition and their ability to agglutinate red blood cells in the presence or absence of D-mannose. The fimbriae described in pigs and calves include F4 (K88), F5(K99), F6 (987P), F17, F18 and F41 (Nagy & Fekete 1999). ETEC strains are host-specific and usually cause disease in the first week of life only. Host specificity can be explained by the presence or absence of genes encoding for fimbrial receptors in the intestinal lining of the host. Age-related resistance may be due to the degree of expression of host receptors. It appears that some of the receptors are over-expressed as the age of the animal increases, thus leading to shedding of receptors into the intestinal lumen. These free receptors coat the ETEC strains in the intestinal contents, thus preventing their adherence to the intestinal lining (Dean et al. 1989). F18 receptors are not produced in newborn pigs but are increasingly expressed up to approximately four weeks of age, thus helping to explain why ETEC are a cause of diarrhoea in pigs up to this age.

Following adherence, ETEC produce protein enterotoxins. The toxins can be subdivided into two main groups: Large heat-labile toxins of approximately 88 kDa in size (LT) and smaller, heat-stable toxins (ST) containing 11–48 amino acids. Both toxin types have two subgroups. Porcine strains of ETEC produce mostly LT1 and Sta whereas strains of ETEC occurring in calves usually produce Sta.

Table 17.3 Biochemical reactions: members of the *Enterobacteriaceae* of uncertain significance

	Indole production	Methyl red	Voges–Proskauer	Citrate	Urease	Phenylalanine deaminase	Hydrogen sulphide	Lysine decarboxylase	Ornithine decarboxylase	Motility (36°C)	Gelatin liquefaction	Growth in KCN broth	ONPG (beta-galactosidase)	Acid from: Dulcitol	Inositol	Lactose	Maltose	Mannitol	Mannose	Rhamnose	Sorbitol	Sucrose	Xylose	Yellow pigment
Budvicia aquatica	–	+	–	–	d	–	(+)	–	–	d	–	–	+	–	–	(+)	–	d	–	+	–	–	+	–
Buttiauxella agrestis	–	+	–	+	–	–	–	–	+	+	–	(+)	+	–	–	+	+	+	+	+	–	–	+	–
Cedecea species	–	+	d	+	–	–	–	–	d	+	–	(+)	+	–	–	v	+	+	+	–	v	v	(+)	–
Ewingella americana	–	(+)	+	+	–	–	–	–	–	d	–	–	(+)	–	–	d	(–)	+	+	(–)	–	–	(–)	–
Erwinia herbicola	(–)	d	d	d	(–)	(–)	–	–	–	(+)	–	d	+	(–)	(–)	d	(+)	+	+	(+)	d	(+)	+	(+)
Hafnia alvei	–	d	(+)	–	–	–	–	+	+	(+)	–	+	+	–	–	–	+	+	+	+	–	–	+	–
Kluyvera species	+	+	–	+	–	–	–	v	+	+	–	+	+	(–)	–	+	+	+	+	+	d	+	+	–
Yokenella regensburgei	–	+	–	+	–	–	–	+	+	+	–	+	+	–	–	–	+	+	+	+	–	–	+	–
Leclercia adecarboxylata	+	+	–	–	d	–	–	–	–	(+)	–	+	+	(+)	–	+	+	+	+	+	–	d	+	d
Lemiorella species	–	v	–	v	–	–	+	–	–	–	–	–	–	v	–	–	–	–	–	–	–	–	+	–
Moellerella wisconsensis	–	+	–	(+)	–	–	–	–	–	–	–	d	+	–	–	+	d	d	+	–	–	+	–	–
Obesumbacterium proteus																								
Biogroup 1	–	(+)	d	–	–	–	–	+	d	–	–	–	d	–	–	–	–	d	+	–	–	–	–	–
Biogroup 2	–	(–)	–	–	–	–	–	+	+	–	–	–	–	–	–	–	d	–	(+)	(–)	–	–	(–)	–
Photorhabdus species	v	–	–	v	v	–	–	–	–	+	d	(–)	–	–	–	–	v	–	+	–	–	–	–	d
Pragia fontium	–	+	–	(+)	–	(–)	(+)	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Providencia species	+	+	–	+	v	+	–	–	–	+	–	+	–	–	v	(–)	–	v	+	v	–	v	–	–
Rahnella aquatilis	–	(+)	+	+	–	+	–	–	–	–	–	–	+	(+)	–	+	+	+	+	+	+	+	+	–
Tatumella ptyseos	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–

+ = 90–100% strains positive, (+) = 76–89% positive, d = 26–75% positive, (–) = 0–10% positive, v = reaction variable among species

STb is associated with porcine strains of ETEC although it may be produced by some strains of ETEC isolated from calves, chickens and humans. LT has a similar mechanism of action to the heat-labile toxin produced by *Vibrio cholerae*. It consists of an A domain and five B subunits. The B subunits bind to the cell and part of the A domain then enters the endoplasmic reticulum of the cell and activates the adenylate cyclase system. The resulting increase in cAMP levels leads to increased fluid and electrolyte secretion and decreased absorption. STa acts by increasing guanylate cyclase activity leading to elevated levels of cGMP in the cell. Increased levels of cAMP or cGMP activate protein kinases which induce phosphorylation of the cystic fibrosis transmembrane regulator (CFTR). This in turn causes secretion of chloride and bicarbonate ions. Protein kinases also inhibit reabsorption of sodium ions (Dubreuil 2012). There is a resultant reduction in absorption of water and electrolytes at the villus tips and an elevated secretion of chloride and water in crypt cells. The mechanism of action of STb differs from that of LT and STa as it does not activate adelyate or guanylate cyclases but phosphorylates CFTR through a different mechanism. Interference with the enteric nervous system may also be important in the secretory diarrhoea induced by *E. coli* enterotoxins (Dubreuil 2012).

Enteropathogenic E.coli

These strains are included in the attaching and effacing *E. coli* (AEEC) because of the nature of the lesions they produce in the intestinal lining. Classical or typical EPEC strains were first described as a cause of diarrhoea in infants and these strains are strict human pathogens and possess a specific (EPEC) adherence factor plasmid (EAF) which is not found in animal isolates of EPEC. Atypical EPEC cause diarrhoea in piglets, lambs, calves and pups. The virulence factors of EPEC are encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE). The genes on this island encode a number of components including the outer membrane protein intimin and its receptor, known as translocated intimin receptor (Tir). Tir is inserted into a host cell membrane where it functions as a receptor for intimin and thus allows close attachment of the *E. coli* cell to the host cell. A characteristic pedestal formation and effacement of microvilli then follow. In addition, other effectors produced by the bacterium cause increased levels of intracellular calcium, secretion of chloride ions, impairment of tight junctions and recruitment of neutrophils.

Shigatoxigenic E. coli

Oedema disease in pigs is often associated with *E. coli* O139 and O141 and these strains are usually haemolytic and produce Shiga toxin. These toxins are similar in activity to the Shiga toxin (cytotoxin) of *Shigella* species and inhibit protein synthesis in host cells following interaction with the 60S ribosomal subunit resulting in death of the cell. There are two types of Shiga toxins, Stx1 that is neutralized by antibody specific for Shiga toxin and Stx2 which contains a number of subtypes. Subtype Stx2e is associated with oedema disease in pigs whereas strains producing Stx1, Stx2, Stx2c and Stx2d are associated with haemorrhagic enteritis in humans. The toxin Stx2e is produced in the intestine but is absorbed and carried via the bloodstream to the target cells, usually endothelial cells of the small arteries. These oedema disease strains of *E. coli* are normally present in the large intestine of pigs, but they appear to multiply rapidly under conditions of stress, particularly a change of diet.

Enterohaemorrhagic E. coli

This group of *E. coli* strains is primarily of importance as a cause of food poisoning in humans, but may sometimes be associated with disease in animals including cases of haemorrhagic enteritis in calves. In addition to the LEE as described for EPEC, they contain other virulence factors including enterohaemolysin (Ehx) and phage-encoded Shiga toxin.

Septicaemic E. coli

Septicaemic (SEPEC) strains are responsible for septicaemia in their hosts. These strains may possess a wide range of virulence factors but few of these virulence factors are common to all strains (Mokady et al. 2005). It appears that each step of the disease process can be mediated by a number of alternative virulence factors and a particular invasive strain may have a unique combination of pathogenic attributes. Adherence to the intestinal lining is the first step in the invasion process. Adherence may be mediated by fimbrial adhesins, for example, F5 as in ETEC strains, by other fimbriae such as long polar fimbriae or by non-fimbrial adhesins. Septicaemic strains carry a plasmid encoding Colicin V (Col V plasmid). This plasmid encodes for type IV pili which have been shown to be important for adherence and invasion in *Salmonella* Typhi. In addition it encodes for serum resistance and the aerobactin iron uptake system, both important virulence factors for systemic survival of *E. coli* strains. Recently the presence of another iron uptake system has been demonstrated, similar to that found in *Yersinia* species, and dependent on the biosynthesis of the siderophore yersiniabactin. Different capsular types are present depending on strain and these are important for survival of the organisms following invasion. The immune response of the host leads to the death of some organisms and endotoxin is released, with resultant clinical signs of pyrexia, weakness, depression and tachycardia.

The diseases caused by *E. coli* in domestic animals are summarized in Table 17.4. Major virulence factors are given in Table 17.5.

Table 17.4 Diseases caused by *Escherichia coli*

Animals involved	Disease	Clinical signs and pathogenesis
Pigs		
Piglets less than one week old	Neonatal diarrhoea (colibacillosis) Colisepticaemia	Profuse watery diarrhoea and severe dehydration, mortality 90–100%. Enterotoxins involved (STa, STb and LT)
	Piglet meningitis	Occasionally septicaemia with invasive strains and death of a piglet within 48 hours of birth
Pigs about two weeks after weaning	Weanling enteritis (colibacillosis)	Diarrhoea, anorexia and fever. Mortality lower than in neonatal pigs. Enterotoxins involved (STa, STb and LT) Acute meningitis and fibrinous polyserositis in piglets has been reported
Weaned pigs	Oedema disease	Often sudden death. Oedema of forehead, eyelids, stomach wall and larynx (hoarse squeal). Nervous signs such as ataxia, convulsions and paralysis may be seen. Shiga toxin (Stx2e) involved often associated with *E. coli* O139 and O 141
Sows after farrowing	Coliform mastitis	One or more mammary glands affected
Gilts after farrowing	Mastitis-metritis-agalactia (MMA) syndrome	Complex syndrome involving stress, hormonal imbalance and coliform infection (often *E. coli*)
Cattle		
Calves less than one week old	'White scours' (colibacillosis)	The appetite is normal at first but decreases as the faeces become more fluid. White pasty faeces around rectum. Dehydration and emaciation occur. Death usually within four to five days if untreated. Enterotoxins involved (STa)
Calves less than one week old	Colisepticaemia	Sudden death due to endotoxic shock. May be signs of diarrhoea, depression, respiratory distress followed by death. Endotoxin is the principal toxin involved although other toxins such as cytotoxic necrotizing factor and cytolethal distending toxin also produced
Calves surviving septicaemia/ bacteraemia	Joint ill	*E. coli* localized in joints and/or kidneys ('white spot'). Entry can be via the umbilicus
Dairy cows soon after parturition	Coliform mastitis	Classically a peracute disease: fever, anorexia, depression and sunken eyes. Death due to endotoxic shock. Less severe form of disease becoming more common. Most frequent in housed cows
Sheep		
Neonatal lambs	Colibacillosis and colisepticaemia	Syndromes similar to those that occur in calves but less common. Enterotoxigenic and enteropathogenic strains involved in colibacillosis
Neonatal lambs	'Watery mouth'	The lamb is dull and anorectic, saliva drools over the muzzle and there is abdominal tympany. There are splashing sounds within the abomasum and death within six to 24 hours. Associated with *E. coli* endotoxaemia
Ewes	Coliform mastitis	Peracute and similar to the condition in cows. Most commonly seen in ewes housed during lambing
Dogs (cats)		
Neonatal pups	Colisepticaemia	Septicaemia, often fatal

Table 17.4 Diseases caused by *Escherichia coli*—cont'd

Animals involved	Disease	Clinical signs and pathogenesis
Bitches	Pyometra	Associated with the progesterone-stimulated endometrium. A vaginal discharge may occur four to eight weeks after oestrus. In a closed-cervix pyometra the bitch is toxaemic and ill Endotoxin involved
Adult dogs	Urinary tract infection	Cystitis (often of bitches) is most common but *E. coli* can ascend higher in the urinary tract. A bacteriuria occurs with greater than 10^5 *E. coli*/mL urine
Poultry		
Young chicks	Omphalitis	Infection of the vestigial yolk sac. The contents are dark, fluid and evil-smelling. Common name is 'mushy-yolk disease'
All ages	Colisepticaemia	Primary or secondary infection via the intestines or respiratory tract. Many body organs are affected with air-sacculitis, peritonitis and ovarian infection
All ages	Coligranuloma	Chronic condition, possibly following a colisepticaemia. There are nodular lesions in the liver and intestines
Other animals		
Neonatal animals such as foals and rabbits	Colibacillosis and colisepticaemia	Diarrhoea and septicaemia. Less common than in calves and piglets

Laboratory Diagnosis

Diagnosis of the opportunistic infections caused by *E. coli*

In this case it is sufficient to isolate *E. coli* in an almost pure growth from carefully taken samples such as cervical swabs, mastitic milk samples and midstream urine. The culture and presumptive identification methods are shown in Figure 17.15. Pathogenic strains of *E. coli* are often haemolytic (Fig. 17.18) and as they are strong lactose-fermenters the colonies on MacConkey agar are bright-pink (Fig. 17.19). Eosin methylene blue (EMB) agar is occasionally used in diagnostic laboratories and on this medium *E. coli* colonies have a unique and characteristic metallic sheen (Fig. 17.20). Other chromogenic agars for the easy identification of *E. coli* are available commercially. The 'IMViC' test (indole+/ MR+/ VP–/ citrate–) is a quick presumptive method of identifying *E. coli* (Fig. 17.21) as almost no other lactose-positive member of the *Enterobacteriaceae* gives this combination of results. Biochemical reactions of some clinically significant members of the *Enterobacteriaceae*, including *E. coli*, are given in Table 17.6. Molecular virulence typing can be used for isolates such as UPEC which possess defined virulence attributes.

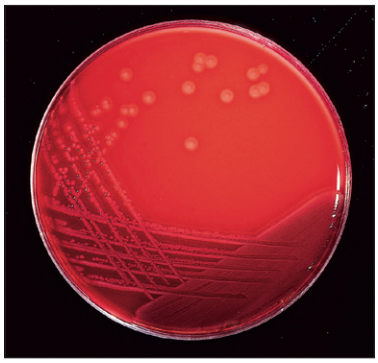

Figure 17.18 Haemolytic *Escherichia coli* on sheep blood agar.

Diagnosis of the septicaemic strains

The diagnosis, in this case, must be based on the isolation and identification of *E. coli* from normally sterile sites in the body such as bone marrow, joints, spleen or blood. Liver specimens should be avoided as there can be movement of enteric bacteria to the liver in the agonal stage of a disease.

Table 17.5 Major virulence factors of pathogenic *Escherichia coli* in animals

Pathotype	Virulence attribute	Function
ETEC	Fimbriae, including F4, F5, F6, F18, F41 depending on host species and strain	Adherence
	Enterotoxins, STa, STb, LT-I, LT-II, depending on host species and strain	Disruption of fluid and ion homeostasis (chloride, bicarbonate and sodium ions)
EPEC (atypical)	Locus of enterocyte effacement (LEE) encodes a number of components including:	
	Type III secretion system	Secretion of effector proteins
	Eae (intimin)	Adherence
	Translocated intimin receptor	Receptor for intimin adhesin
	Other effector proteins	Cytoskeletal reorganization resulting in typical attaching and effacing lesion
STEC (strains causing oedema disease)	Fimbriae, primarily F18	Adherence
	Stx2e	Absorbed into the bloodstream, binds to vascular endothelial cells in the nervous system and elsewhere, causing oedema and haemorrhage
	Haemolysin	Haemolysis of red blood cells
SEPEC	Different virulence factors are produced depending on strain	
	Fimbriae	Adherence; some fimbriae also help in avoidance of phagocytosis
	Capsule	Prevention of phagocytosis
	Iron acquisition systems such as aerobactin	Iron scavenging which allows multiplication in the iron-restricted environment of the host
	Endotoxin	Endotoxic shock
	Colicin V	Serum resistance
	Cytotoxic necrotizing factor	Alterations in cytoskeleton
	Cytolethal distending factor	Cell distention and death by apoptosis
UPEC	Fimbriae including P fimbriae, FimH	Adherence; P fimbriae help protect against phagocytosis
	Flagella	Motility, facilitates ascent from the bladder to the kidney
	Iron acquisition systems including siderophores	Iron scavenging
	Alpha haemolysin	Pore-forming toxin
	Cytotoxic necrotizing factor	Alterations in cytoskeleton

Demonstration of the enterotoxigenic strains

The enterotoxigenic strains of *E. coli* are present in large numbers in the small intestine, and in this case it is insufficient to merely isolate and identify the *E. coli*. Demonstration of the significant fimbrial antigens (F4, F5, F6, F17, F18 and F41) or the enterotoxin itself is necessary. Alternatively, molecular detection of virulence genes can be employed.

- Fimbrial antigens. Fimbriae are expressed poorly on selective and some types of non-selective laboratory

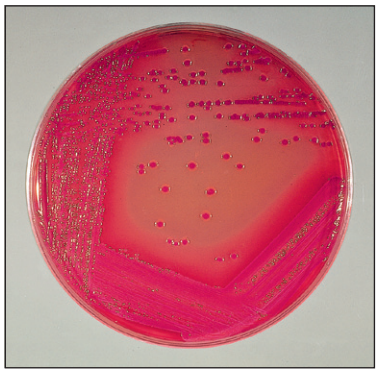

Figure 17.19 *Escherichia coli* on MacConkey agar. Bright pink colonies indicating acid production as a result of the fermentation of lactose. (Neutral red indicator)

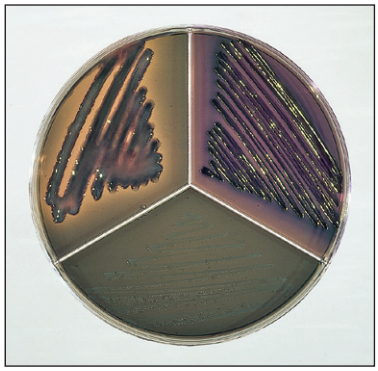

Figure 17.20 *Escherichia coli* (right) giving a distinctive metallic sheen on EMB agar distinguishing it from other members of the *Enterobacteriaceae* such as *Salmonella* sp. (bottom) and *Klebsiella pneumoniae* (left).

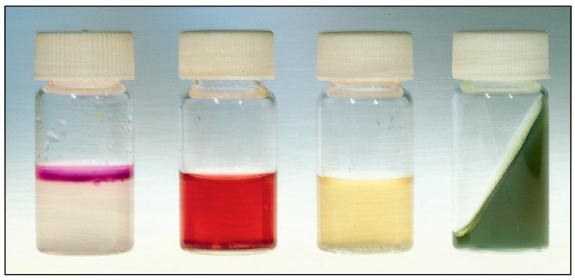

Figure 17.21 The 'IMViC' test for *E. coli*: Indole+/MR+/VP–/Citrate–.

media. E medium (Francis et al. 1982) is advised for F4, F5 and F41. Minca medium (BBL) has been found satisfactory for F6, F5 and F41. Commercial test kits are available for the detection of fimbrial antigens such as a latex agglutination test

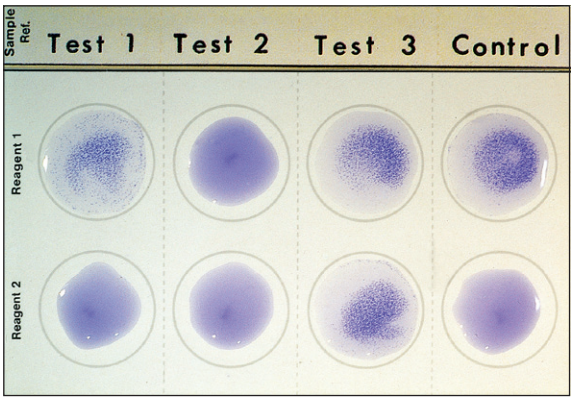

Figure 17.22 A latex plate agglutination test for detecting the K99 pili antigens of enterotoxigenic *E. coli*. Suspensions of three *E. coli* isolates (tests 1, 2 and 3) and a control antigen have each been placed in a top and a bottom well. Reagent 1 is latex particles coated with monospecific antibody to the K99 antigen and has been placed in all the wells in the top row. Reagent 2 is a suspension of latex particles only and has been placed in all the wells in the bottom row. *E. coli* (test 1) is positive for the K99 antigen; *E. coli* (test 2) is negative and *E. coli* (test 3) has autoagglutinated in the bottom well indicating that the test is invalid for this isolate.

(Fig. 17.22). Specific antiserum can be obtained for use in a slide agglutination test. Enzyme-linked immunosorbent assays (ELISA) are available for directly measuring the presence of fimbriae-expressing *E. coli* in faecal extracts. The fluorescent antibody technique, using conjugates prepared against each of the common colonizing-associated antigens, can be used on smears made from scrapings from the ileum of a fresh carcass.

- Demonstration of enterotoxins. The ST and LT toxins can be detected using an ELISA which employs monoclonal antibodies (Carroll et al. 1990).

Diagnosis of the enteropathogenic strains of E. coli

Most of the *E. coli* isolates causing this syndrome have been shown to produce urease. This is an unusual characteristic as generally less than 1% of *E. coli* strains produce this enzyme. Histopathological examination of sections of the ileum should demonstrate the characteristic distortion of the microvilli and the effacement of the mucosal surface.

Diagnosis of oedema disease

Oedema disease is usually diagnosed based on clinical and postmortem findings. The *E. coli* isolates are typically haemolytic and the strains commonly have O antigens

Table 17.6 Biochemical reactions for some clinically significant members of the *Enterobacteriaceae*

	Indole production	Methyl red	Voges–Proskauer	Citrate	Urease	Phenylalanine deaminase	Hydrogen sulphide	Lysine decarboxylase	Ornithine decarboxylase	Motility (36°C)	Gelatin liquefaction	Growth in KCN broth	ONPG (beta-galactosidase)	Acid from									Red pigment	Swarming (blood agar)	Mucoid colonies
														Dulcitol	Inositol	Lactose	Maltose	Mannitol	Mannose	Rhamnose	Sorbitol	Sucrose	Xylose			
Citrobacter diversus	+	+	–	+	(+)	–	–	–	+	+	–	–	+	d	–	d	+	+	+	+	+	(–)	+	–	–	–
Edwardsiella tarda	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–
Enterobacter aerogenes	–	–	+	+	–	–	–	+	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	–	–	+
Enterobacter cloacae	–	–	+	+	d	–	–	–	+	+	–	+	+	(–)	(–)	+	+	+	+	+	+	+	+	–	–	–
Escherichia coli	+	+	–	–	–	–	–	(+)	d	(+)	–	–	+	d	–	+	+	+	+	(+)	+	d	+	–	–	(–)
Klebsiella pneumoniae	–	(–)	+	+	+	–	–	+	–	–	–	+	+	d	+	+	+	+	+	+	+	+	+	–	–	+
Morganella morganii	+	+	–	–	+	+	–	–	+	+	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Proteus mirabilis	–	+	(–)	d	+	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	(–)	+	–	+	–
Proteus vulgaris	+	+	–	(–)	+	+	+	–	–	+	+	+	–	–	–	–	+	–	–	–	–	+	+	–	+	–
Salmonella enterica																										
subspecies *arizonae*	–	+	–	+	–	–	+	+	+	+	–	–	–	+	d	–	+	+	+	+	+	–	+	–	–	–
subspecies *enterica*	–	+	–	+	–	–	+	+	+	+	–	–	+	–	+	d	+	+	+	+	+	–	+	–	–	–
Serratia marcesens	–	(–)	+	+	(–)	–	–	+	+	+	+	+	+	–	(+)	–	+	+	+	–	+	+	–	+	–	–
Serratia rubidea	–	(–)	+	+	–	–	–	d	–	(+)	+	(–)	+	–	(–)	+	+	+	+	–	–	+	+	+	–	–
Shigella species	v	+	–	–	–	–	–	–	v	–	–	–	v	–	–	–	v	v	+	v	v	–	–	–	–	–
Yersinia enterocolitica	d	+	–	–	(+)	–	–	–	+	–	–	–	+	–	d	–	d	+	+	–	+	+	d	–	–	–
Y. pestis	–	(+)	–	–	–	–	–	–	–	–	–	–	(+)	–	–	–	(+)	+	+	–	–	–	+	–	–	–
Y. pseudotuberculosis	–	+	–	–	+	–	–	–	–	–	–	–	d	–	–	–	+	+	+	+	–	–	+	–	–	–

+ = 90–100% strains positive, (+) = 76–89% positive, d = 26–75% positive, (–) = 0–10% positive, v = reaction variable among species. Tests read after 48 hours at 37°C

139 and 141 although the prevalent serogroups may vary depending on geographical region. Formerly, the Stx2e toxin was detected using Vero cell assays but these have been superseded by molecular methods.

Antimicrobial Resistance and Antimicrobial Susceptibility Testing

All *E. coli* isolates considered to be significant should be tested for antimicrobial susceptibility according to CLSI guidelines (CLSI 2008). The disc diffusion method can be used or, for MIC values, broth dilution methods. Resistance to at least two classes of antimicrobial agents is now a common finding in veterinary medicine, as in human medicine. Worldwide data suggest that resistance to broad-spectrum penicillins and trimethoprim is frequently high but relatively low for the third-generation cephalosporins and nitrofuratoin (von Baum & Marre 2005). However, there is increasing concern due to the emergence of fluoroquinolone resistance and the production of extended-spectrum β-lactamases (ESBLs) by multidrug-resistant *E. coli*. Chromosomal and plasmid-borne integrons are important in the development of multidrug resistance in the *Enterobacteriaceae*, in particular class 1 integrons. These often confer resistance to quaternary ammonium compounds and sulphonamides and contain gene cassettes which encode resistance to β-lactams, streptomycin-spectinomycin and trimethoprim. Extended-spectrum β-lactamases inactivate many β-lactams, including third-generation cephalosporins and monobactams. Stains producing ESBLs can be difficult to identify in clinical microbiology laboratories but guidelines for their detection are incorporated in CLSI guidelines (CLSI 2008). The recent emergence of New Delhi metallo-beta-lactamases in members of the *Enterobacteriaceae* including *E. coli* is a major threat to public health as organisms carrying these enzymes are resistant to the carbapenam antibiotics in addition to many other antimicrobial classes. Resistance to fluoroquinolones is based on either inhibition of bacterial DNA topoisomerases or decreased drug uptake and increased efflux. Tetracycline resistance usually results from acquisition of genes encoding efflux pumps. The mechanism of aminoglycoside resistance in the *Enterobacteriaceae* is modification of the functional groups of the drugs leading to reduced affinity for the ribosomal targets.

Surface Antigens of *E. coli*

The capsular (K) antigens are polysaccharides and the cell wall or somatic (O) antigens are determined by the sugar side-chains on the lipopolysaccharide molecules of the outer membrane. The flagellar (H) and fimbrial (F) antigens are proteins. Some of the well-known fimbrial antigens, F4 (K88) and F5 (K99), are adhesins that allow pathogenic *E. coli* strains to adhere to intestinal cells and colonize the small intestine. The O, H and K antigens can be used to serotype strains of *E. coli*, each serotype is designated by the numbers of the antigens that it bears, for example, O157:K85:H19.

Molecular Diagnosis and Typing

Many different molecular methods for the detection of pathogenic *E. coli* in animals have been described. DNA probes specific for the base sequences of *E. coli* genes encoding enterotoxin (LT and ST) are available. Such probes have been used to detect enterotoxigenic strains in cultures and in faecal extracts. Molecular methods used for confirmation of oedema disease strains include detection of virulence attributes by PCR such as assays for the detection of genes encoding F4, F18, intimin, Stx2e and *E. coli* heat-stable enterotoxin 1 (EAST 1). PCR methods for the detection of virulence genes found in the LEE of AEEC have been described (Fröhlicher et al. 2008). Many of the molecular methods used for the identification of virulence factors of *E. coli* have been outlined by DebRoy and Maddox (2001).

Although serotyping is still widely used for epidemiological investigation of *E. coli* disease, a number of molecular methods for *E. coli* strain characterization are now available and increasingly employed. However, no single method currently available is capable of definitively distinguishing between all pathogenic and non-pathogenic subtypes. Multilocus enzyme electrophoresis and PCR-based methods can be used to assign strains to major phylogroups, A, B1, B2, D and E (Boyd & Hartl 1998, Clermont et al. 2000). In general ExPEC strains belong to phylogroups B2 or D whereas intestinal pathogenic strains and commensals belong to groups A and B1. Multilocus VNTR analysis (MLVA) methods are used for epidemiological analysis of intestinal disease caused by *E. coli*, in particular outbreaks of EHEC in humans (Köhler & Dobrindt 2011). A number of MLST schemes are also available.

SALMONELLA

Nomenclature

The nomenclature of this genus has been the subject of much debate and has frequently encompassed several nomenclatural systems that inconsistently divided the genus into species, subspecies, subgenera, groups, subgroups and serotypes. In 1987, it was proposed that the type species for *Salmonella* be changed to *S. enterica*, a name coined by Kauffmann and Edwards in 1952, because no serotype shares its name (Le Minor & Popoff 1987). Although this recommendation was accepted by the Center for Disease Control and Prevention (CDC), the

Table 17.7 Characteristics of *Salmonella* species and subspecies

***Salmonella* species**	**bognori**	**enterica**	**enterica**	**enterica**	**enterica**	**enterica**	**enterica**
Salmonella subspecies	–	*arizonae*	*diarizonae*	*enterica*	*houtenae*	*indica*	*salamae*
Flagella usually mono- or diphasic	Mono	Mono	Di	Di	Mono	Di	Di
Habitat of majority of strains							
Warm-blooded animals	–	–	–	+	–	–	–
Cold-blooded animals and the environment	+	+	+	–	+	+	+
Differential tests							
Beta-galactosidase	+	+	+	–	–	d	–
Lactose	–	(–)	(+)	–	–	(–)	–
Dulcitol	+	–	–	+	–	d	+
Malonate utilization	–	+	+	–	–	–	+
Gelatin hydrolysis	–	+	+	–	+	+	+
Growth in KCN medium	+	–	–	–	–	–	–

+ = 90–100% strains positive, (+) = 76–89% positive, d = 26–75% positive, (–) = 0–10% positive, v = reaction variable among species

World Health Organization Collaborating Centre for Reference and Research on *Salmonella* and by many laboratories worldwide, the request was denied by the Judicial Commission of the International Committee on Systematic Bacteriology. This decision was largely based on the concern that if *S. enterica* were adopted as the type species, *Salmonella* serotype Typhi would be referred to as *S. enterica* subsp. *enterica* serotype Typhi and therefore may be considered as one of the >2000 less dangerous serovars and overlooked by physicians (Brenner et al. 2000). In 2005, the Judicial Commission of the International Committee on Systematics of Prokaryotes accepted the system in which two species are recognized within the genus, *S. bongori* and *S. enterica* (Anon. 2005). *S. bongori* has 21 serotypes that are usually isolated from cold-blooded animals and the environment but rarely from mammalian sources. *S. enterica* contains six subspecies: *S. enterica* subsp. *enterica, S. enterica* subsp. *salamae, S. enterica* subsp. *arizonae, S. enterica* subsp. *diarizonae,* and *S. enterica* subsp. *houtenae, S. enterica* subsp. *indica*. The characteristics and usual habitats of *Salmonella* species and subspecies are presented in Table 17.7. Subspecies classification is primarily based upon molecular methods.

There are currently more than 2500 serotypes of *Salmonella*. The antigenic formulae of *Salmonella* serotypes are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*. The number of serotypes continues to rise, for example, 26 new serotypes were identified in 1999, 12 in 2000 and 22 in 2001 (Popoff et al. 2000, 2001, 2003). These new serotypes are listed in annual updates of the Kauffmann–White scheme. Epidemiologically, it is becoming increasingly important to be able to distinguish *Salmonella* isolates, as definitive typing can assist in tracing the source of an outbreak and also in monitoring trends in antimicrobial resistance.

Natural Habitat

The reservoir for salmonellae is the intestinal tract of warm-blooded and cold-blooded animals, the majority of which are subclinical excretors. However, salmonellae can survive for nine months or more in the environment in sites such as moist soil, water, faecal particles and animal feeds, especially in blood-and-bone and fish meals.

Pathogenesis and Pathogenicity

Salmonella serotypes may be classified according to the type of disease they produce and/or their host range. Strictly host-adapted serotypes such as *S.* Typhi in humans and *S.*Choleraesuis in pigs can produce severe systemic disease in healthy immunocompetent adults. Host-restricted serotypes such as *S.* Dublin in cattle produce disease primarily in cattle but cause a small number of infections in other animals also. Disease is frequently systemic, involving the reproductive tract in pregnant animals

and the production of severe enteritis in very young animals. Serotypes such as *S.* Typhimurium cause enteritis in a wide range of species. Transmission of salmonellae is usually by the faecal–oral route but infection via mucous membranes of the conjunctivae or upper respiratory tract can occur also.

Colonization of the intestinal tract and enteric disease

Salmonellae need to colonize the distal small intestine or colon to initiate enteric disease. Volatile organic acids produced by the indigenous normal anaerobic flora inhibit the growth of salmonellae and the normal flora normally block access to attachment sites required by *Salmonella* species. Disruption of the normal intestinal flora by factors such as antibiotic therapy, diet and water deprivation, increases a host's susceptibility to infection. Reduced peristalsis, stress due to transportation and over-crowding also predispose to colonization of the intestine by salmonellae. *Salmonella* serotypes preferentially bind to lymph follicles and the membranous epithelial (M) cells, which reside in the specialized follicle-associated epithelium (FAE) overlying Peyer's patches, and serve as a major port of entry for *Salmonella* serotypes. However, other cells such as enterocytes may also represent a potential target for invasion by salmonellae in vivo (Jepson & Clark 2001). Colonization of these cells contributes to the development of disease during both localized and systemic infection. Fimbrial adhesins appear to mediate this initial contact between bacterium and cell surface, including long polar fimbriae and curli. Following adhesion, salmonellae may penetrate the intestinal epithelium, but in the case of localized gastrointestinal disease, penetration of epithelium is not always observed. Ability to invade is mediated by large clusters of virulence genes designated *Salmonella* Pathogenicity Islands (SPI). Seventeen of these pathogenicity islands have been described to date although their exact role in the production of disease is unknown in some instances (Barrow et al. 2010). SPI1 genes control infiltration and establishment of infection in the epithelium and intestinal lymphoid tissues whereas SPI2 genes are necessary for the systemic phase of infection. Penetration of the intestinal epithelium occurs through a process of macropinocytosis. A type III secretion system (TTSS), which is a specialized protein secretion system, translocates bacterial effector proteins in response to bacterial contact with epithelial cells. This induces endocytosis that is accompanied by membrane ruffling, activation of various transcription factors and bacterial internalization. Once inside epithelial cells, salmonellae replicate within the vesicles while translocating towards the basal side. They escape from the basal side of epithelial cells into the lamina propria, below the columnar epithelium. *Salmonella*-infected epithelial cells release chemokines and prostaglandins that act to recruit inflammatory cells to foci of infection. At the same time, salmonellae enhance the inflammatory response through secretion of proinflammatory cytokines (Wallis & Galyov 2000). Both processes provoke a massive migration of neutrophils through the epithelium into the intestinal lumen. Neutrophils are the primary inflammatory cells involved and play a very important role in the pathogenesis of *Salmonella*-induced diarrhoea. Given the inflammatory nature of diarrhoea, bacterial lipopolysaccharide (endotoxin) may contribute to this process also. In the lamina propria neutrophils and macrophages phagocytose the organisms present. The toxic oxidative effects of free radicals produced by phagocytes are minimized by bacterial catalase and supeoxide dismutase activities. Survival factors are mainly encoded on SPI 2, 3 and 4 (Hensel 2004). Subsequently, salmonellae move to regional lymph nodes, where they are taken up by macrophages whose actions usually stop the infection and limit it to the intestine.

Invasive strains of salmonellae that produce septicaemia, are able to escape destruction by the host and to multiply within the macrophages of the liver and spleen as well as intravascularly. Survival within macrophages is critical for invasion by *Salmonella* serotypes as it enables the bacteria to evade the immune system and facilitates dissemination to deeper tissues. Studies indicate that the function of a TTSS encoded by SPI2 plays a central role in systemic infections and intracellular pathogenesis (Hensel 2004). Hours after entering macrophages, following replication, a virulence protein secreted by *Salmonella* specifically induces apoptotic cell death. Bacteria are then transported via the lymphatics to mesenteric lymph nodes prior to dissemination through the blood. Over subsequent days the bacteria reside and replicate intracellularly in phagocytic cells of the spleen and liver (Brumell et al. 2002).

Siderophores, that remove iron from the iron-binding proteins of the host, are secreted by invasive salmonellae. Multiplication of the organisms in the body leads to a severe endotoxaemia. Some serotypes seem to be more commonly invasive than others. Invasive strains occur frequently in *S.* Typhi, *S.* Dublin and *S.* Typhimurium.

The diseases and conditions caused by some *Salmonella* serotypes are summarized in Table 17.8 and major virulence factors are presented in Table 17.9. *Salmonella* Dublin is host-adapted to cattle, although infections can occur in other animal species. This serotype occurs in Europe, western USA and South Africa. Both subclinical excretors and latent adult carriers occur. The latent carriers may excrete *S.* Dublin in faeces if stressed by factors such as parturition or a concurrent infection. *Salmonella* Dublin can cause an unusually wide range of clinical syndromes including ischaemic necrosis of the tips of the ears, tail and limbs (Fig. 17.23). This terminal dry gangrene usually appears a few weeks after the recovery of calves from acute

Table 17.8 Diseases caused by selected *Salmonella* serotypes

Host	*Salmonella* serotypes	Disease
Humans	*S.* Typhi*	Typhoid fever
	S. Paratyphi A*	Paratyphoid fever
	S. Schottmuelleri*	Paratyphoid fever
	S. Enteritidis, *S.* Typhimurium and others	Food poisoning
Cattle	*S.* Dublin	Subclinical excretors, latent carriers, enteritis, septicaemia, meningitis in calves, abortion (with or without other apparent clinical signs), osteomyelitis, joint ill, terminal dry gangrene in calves
	S. Typhimurium, *S.* Bovismorbificans and others	Enteritis or septicaemia
Pigs	*S.* Choleraesuis*	Severe outbreaks clinically similar to swine fever (hog cholera); in addition, swine fever can be followed by a secondary infection with *S.* Choleraesuis, earning it the name of 'hog cholera bacillus'. Rectal stricture is sometimes a sequel of the disease. This serotype is now rarely isolated in many parts of Europe but is still prevalent in parts of North America and Asia
	S. Typhisuis*	Chronic enteritis in young pigs. Far less virulent than *S.* Choleraesuis
	S. Typhimurium, *S.* Derby and others	Subclinical carriers, enteritis or septicaemia
Sheep	*S.* Abortusovis* *S.* Montevideo *S.* Dublin	Abortion and diarrhoea in ewes
	S. enterica subspecies *diarizonae*	Abortion and diarrhoea in ewes, subclinical carriers
	S. Typhimurium and others	Enteritis or septicaemia
Horses	*S.* Abortusequi*	Abortion in mares. This serotype is becoming rare in many parts of the world
	S. Typhimurium and others	Enteritis or septicaemia, especially in foals and stressed adults
Poultry and other birds	*S.* Pullorum*	Pullorum disease (bacillary white diarrhoea) in chicks. Transovarian transmission
	S. Gallinarum*	Fowl typhoid in all ages, mainly adults. Egg transmitted
	S. enterica subspecies *arizonae*	Severe infections (enteritis and septicaemia) in chicks and turkey poults. Egg transmitted. Serotype associated with reptiles. Occasional infections in other animals
	S. Enteritidis, *S.* Typhimurium and many other serotypes	Collectively known as 'fowl paratyphoid'. Inapparent infections, enteritis and septicaemia. *S.* Enteritidis may be egg-transmitted. *S.* Typhimurium can cause sudden deaths (septicaemia) in pigeon squabs, or if they survive, swollen wing joints

*= host-adapted serotypes

disease. There is evidence that endotoxin damages the endothelium of blood vessels and also activates the alternate pathway of complement as well as the blood clotting mechanism. This probably leads to a localized form of disseminated intravascular coagulation (DIC) resulting in the terminal ischaemia.

Laboratory Diagnosis

Isolation

Figure 17.24 indicates the steps for the isolation of salmonellae from clinical specimens. The host-adapted serotypes from pigs and poultry are more fastidious than most

Table 17.9 Major virulence factors of *Salmonella* serotypes (possession of virulence factors may vary between serotypes)

Virulence attribute	Function
Fimbriae	Adherence and intestinal colonization
Lipopolysaccharide	Adherence and intestinal colonization Induction of inflammatory response
Type III secretion system-1 (encoded on *Salmonella* Pathogenicity Island 1)	Transfer of effector proteins into host cells during invasion
Effector proteins SopE/E2, SopB, SipA, SipC and others	Invasion of intestinal cells through membrane ruffling and micropinocytosis, disruption of fluid and electrolyte metabolism
Type III secretion system-2 (encoded on *Salmonella* Pathogenicity Island 2)	Transfer of effector proteins involved in intracellular survival
SPI-2 effectors	Prevention of phagosome–lysosome fusion
Salmonella virulence plasmids	Important in the production of systemic disease

Figure 17.23 Terminal dry gangrene in a three-week-old calf following a *Salmonella* Dublin infection. Typically one or both hind limbs, tip of tail and ears are affected (compare with Fig. 44.6, ergotism in a cow).

of the other commonly isolated serotypes. They do not tolerate selenite broth, tetrathionate broth or brilliant green agar, although most strains of *S.* Choleraesuis will grow on modified brilliant green agar (Oxoid). If *Proteus* species are a problem, the enrichment broths can be incubated at 43°C, or sodium sulphathiazole added to the broths at 0.125 mg/100 mLs. Some laboratories add sodium sulphadiazine to brilliant green agar (80 mg/L) to make it more selective.

Water, environmental and feed samples

Occasionally veterinary diagnostic laboratories are asked to examine water or environmental samples and animal feedstuffs for the presence of salmonellae. In such samples, salmonellae may be present in low numbers and there may be many competing bacterial organisms. Pre-enrichment is conventionally recommended for materials that are likely to have low numbers of bacteria that may have been injured or stressed. Several pre-enrichment media have been developed (e.g. lactose broth, buffered peptone water). Buffered peptone water (BPW) is the pre-enrichment broth of choice in conjunction with Rappaport–Vassiliadis (RV) selective enrichment medium (Vassiliadis et al. 1987). Incubation for 18–24 hours is recommended. The temperature for pre-enrichment should range between 35°C and 37°C, which is the optimal growth temperature of *Salmonella* spp. This step assures the resuscitation of 'sublethally injured' or 'damaged' bacteria and their multiplication.

- Water samples. The bacterium can be isolated from water in one of three ways:
  - Add about 100 mL of a water sample to an equal amount of double-strength enrichment broth. Incubate for 48 hours and subculture onto selective/indicator media.
  - If the water sample does not contain particulate matter about 100 mL can be passed through a sterile 0.45 mm membrane filter. Any salmonellae present will be concentrated on the surface of the filter (Fig. 17.25). The filter can be placed on a selective medium (Fig. 17.25) or if few salmonellae are present, the membrane filter can be placed in 10 mL of enrichment broth to increase the number of salmonellae during incubation at 37°C for 24–48 hours. Subsequently a subculture can be made on selective/indicator media.
  - A very effective method for isolating salmonellae from running water, such as a stream, involves the immersion of a sterile pad in the water for 48 hours. The pad consists of cotton wool wrapped in a square of surgical gauze, and securely tied with one end of a long piece of strong string (Fig. 17.26). The device can be placed in a paper envelope and sterilized in the autoclave. The

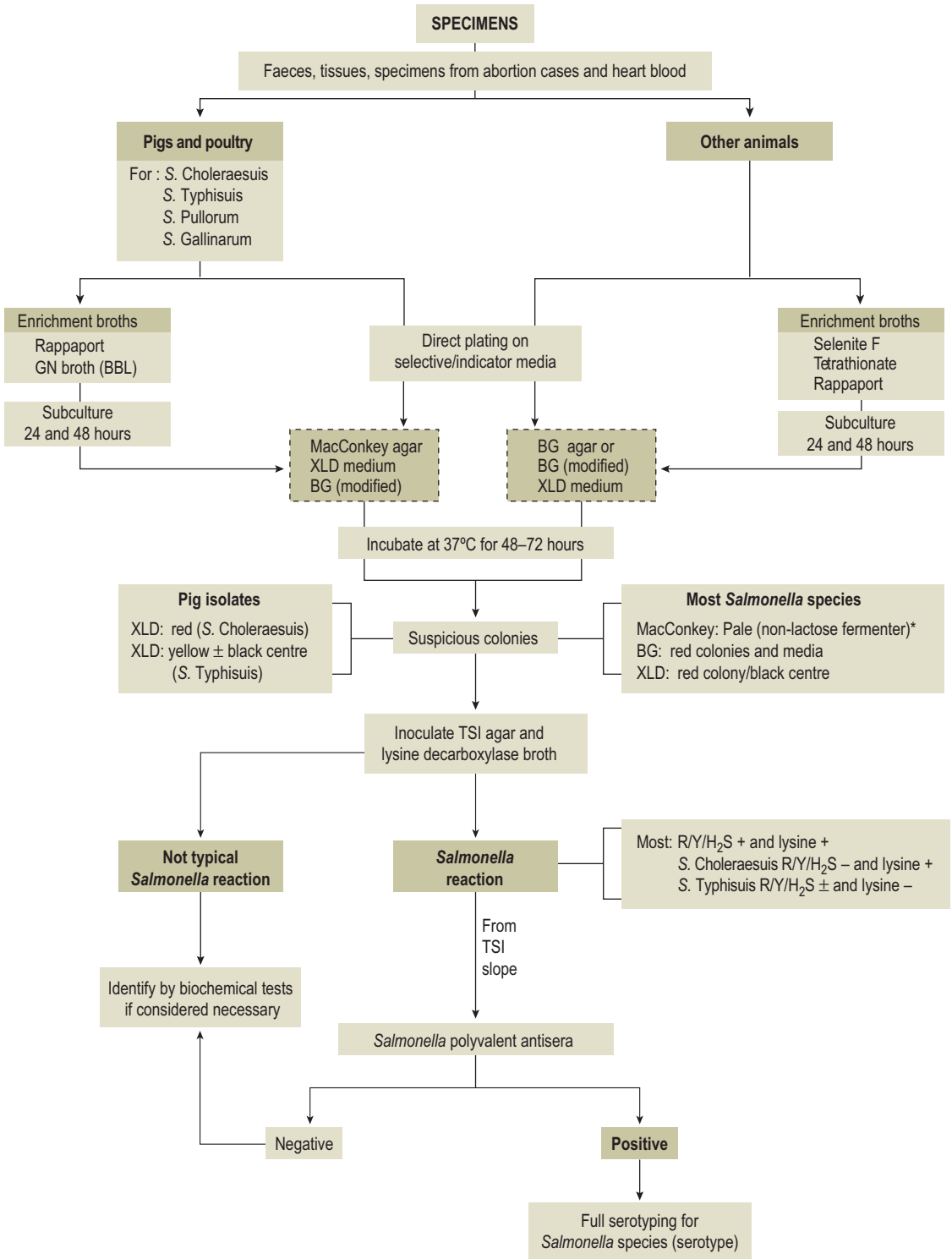

Figure 17.24 Isolation of salmonellae from clinical specimens. BG = brilliant green agar, BG (modified) = brilliant green agar, modified (Oxoid), XLD = xylose-lysine-deoxycholate medium, *= very occasionally *Salmonella* strains are lactose-positive, TSI = triple sugar iron agar.

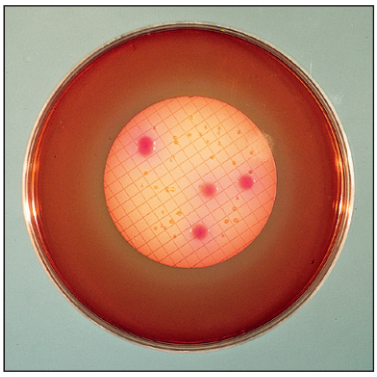

Figure 17.25 Filtration method for isolating salmonellae from water. The membrane filter on MacConkey agar shows lactose-fermenting and non-lactose-fermenting colonies.

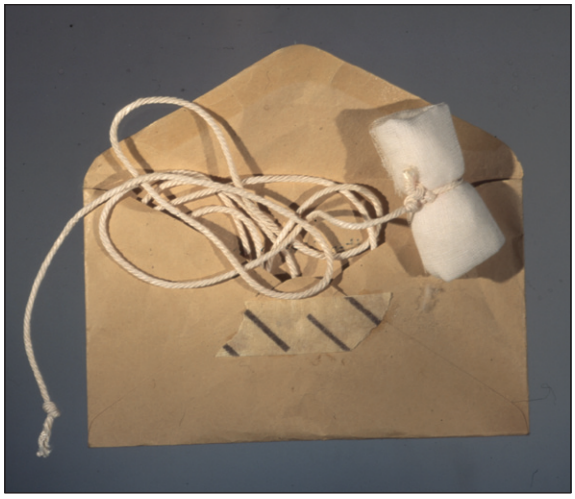

Figure 17.26 An improvised device for recovering salmonellae from ponds or streams.

string is held at the free end and the pad thrown out into the water to be sampled. The end of the string is secured to an object on the bank. After 48 hours the pad is retrieved and carefully placed in a jar containing 50 mL of enrichment broth and the string cut below the point where it was handled. After 24–48 hours' incubation at 37°C, subcultures are made on selective/indicator media.

- Feed samples
  - The salmonellae in feed samples may have been subjected to heat-treatment and will be in a desiccated condition. A representative 25 g sample is broken-up and placed in 225 mL of a non-selective broth such as BPW or lactose broth. Six mL of 105 Tergitol No. 7 is added to samples with a high fat content. The sample is shaken vigorously and incubated at 37°C for 48 hours. The broth can be streaked directly onto brilliant green agar or XLD medium but a 1 mL aliquot should be placed in 10 mL of a selective enrichment broth, such as tetrathionate, or, if RV broth is used, a 0.1 mL aliquot in 10 mL. This is incubated at 37°C for 24 hours and then subcultured onto selective/indicator media.

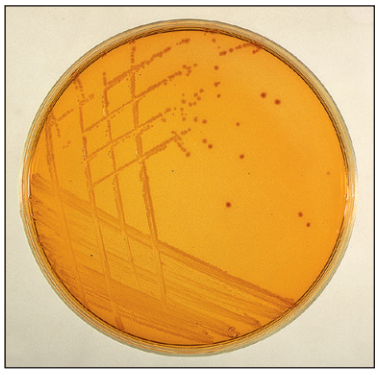

Figure 17.27 Pale, non-lactose-fermenting colonies of *Salmonella* sp. on MacConkey agar.

Procedures for isolation from food are similar to those outlined above but are governed by regulations in many countries and compulsory adherence to certain standard protocols such as EN ISO-6579: 2002 (Anon. 2002) may be required.

Identification

Colonial morphology on selective/indicator media

The majority of salmonellae are non-lactose-fermenters and produce pale colonies on MacConkey agar and an alkaline reaction in the medium (Fig. 17.27). However, it must be remembered that some strains of *S. enterica* subspecies *arizonae* are lactose-positive and strains of *S.* Typhimurium have been encountered carrying plasmids with genes coding for lactose fermentation. Most salmonellae give an alkaline reaction in brilliant green agar and have red colonies (Fig. 17.28). On XLD medium the majority of *Salmonella* serotypes produce hydrogen sulphide and have red colonies with a black (H_2S) centre (Figs 17.29 and 17.30). Colonies characteristic for *Salmonella* on the selective/indicator media are inoculated, singly, into a TSI agar slope and lysine decarboxylase broth. The typical reaction for *Salmonella* in TSI agar is a red (alkaline) slant, yellow (acid) butt and superimposed (black) H_2S production (R/Y/H_2S+); in addition the test for lysine decarboxylation is positive. However, *S.* Choleraesuis does not produce H_2S (Fig. 17.31) although *S.* Choleraesuis biotype Kunzendorf is H_2S-positive. The fastidious *S.* Typhisuis is variable in H_2S production and is lysine-negative. If the

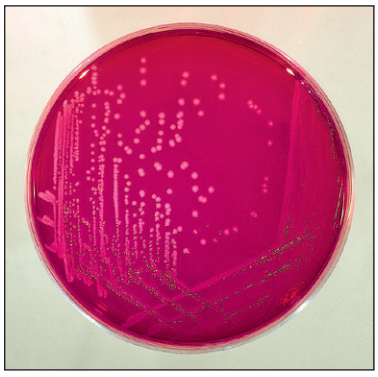

Figure 17.28 *Salmonella* Typhimurium on brilliant green agar. Most salmonellae are unable to ferment either the lactose or sucrose in the medium but instead use the peptone with a consequent alkaline reaction. (Phenol red indicator)

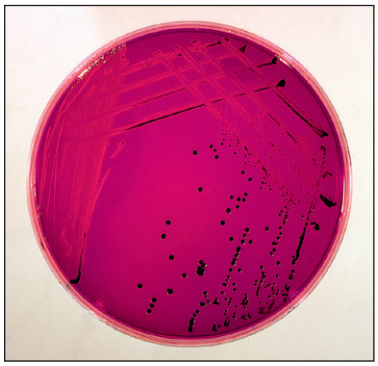

Figure 17.29 *Salmonella* Dublin on XLD medium showing the H_2S production (black centre) and alkaline (red) reaction in the medium and periphery of colony. (Phenol red indicator)

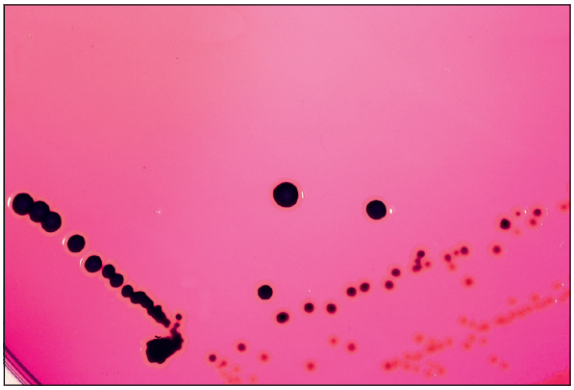

Figure 17.30 Close-up of *S.* Dublin colonies on XLD medium. The colonial appearance is characteristic of most salmonellae on this medium (black centre (H_2S) and red 'skirt').

Figure 17.31 Triple sugar iron (TSI) agar: from left, uninoculated, *S.* Typhimurium with H_2S production (representative of most salmonellae) and *S.* Choleraesuis with no H_2S production.

reaction in TSI agar and lysine decarboxylase broth is equivocal, further biochemical tests should be carried out (Table 17.6) or an identification system used such as API 20E (bioMérieux). Alternatively, molecular methods may be employed.

Salmonella *serotyping*

Bacterial growth for serotyping should be taken from a TSI agar slant or from nutrient agar as colonies from selective media are often unsuitable for typing. Serotyping is based on the O (somatic) and H (flagellar) antigens (Table 17.10) and a slide agglutination test is used (Fig. 17.32). Rare strains of *S.* Dublin have a Vi (virulence) capsular antigen that can mask the cell wall (O) antigens. Boiling a suspension of *S.* Dublin for 10–20 minutes will destroy the Vi antigen. A loopful of culture of the *Salmonella* isolate to be serotyped should be suspended in a drop of saline on a microscope slide and examined for autoagglutination. This can occur with rough strains and will invalidate the serotyping. Smooth–rough dissociation occurs after subculture and most frequently from media containing carbohydrates.

A smooth *Salmonella* to be serotyped is emulsified in a drop of 0.85% saline on a clean microscope slide. A drop

Table 17.10 Antigens of some *Salmonella* serotypes

			Flagella (H) antigens	
Serotype	**Serogroup**	**Somatic (O) antigens**	**Phase 1**	**Phase 2**
S. Paratyphi A	A	1, 2, 12	a	[1, 5]
S. Typhimurium	B	1, 4, [5], 12	i	1, 2
S. Derby	B	1, 4, [5], 12	f, g	[1, 2]
S. Agona	B	4, 12	f, g, s	–
S. Saintpaul	B	1, 4, [5], 12	e, h	1, 2
S. Heidelberg	B	1, 4, [5], 12	r	1, 2
S. Abortusovis	B	4, 12	c	1, 6
S. Abortusequi	B	4, 12	–	e, n, x
S. Typhisuis	C_1	6,7	c	1, 5
S. Choleraesuis	C_1	6,7	c	1, 5
S. Choleraesuis biotype Kunzendorf	C_1	6,7	[c]	1, 5
S. Montevideo	C_1	6, 7, 14	g, m, [p], s	–
S. Oranienburg	C_1	6, 7	m, t	–
S. Newport	C_2	6, 8	e, h	1, 2
S. Bovismorbificans	C_2	6, 8	r	1, 5
S. Kentucky	C_3	8, 20	i	z_6
S. Typhi	D_1	9, 12 [Vi]	d	–
S. Enteritidis	D_1	1, 9, 12	g, m	[1, 7]
S. Dublin	D_1	1, 9, 12, [Vi]	g, p	–
S. Gallinarum	D_1	1, 9, 12	–	–
S. Pullorum	D_1	9, 12	–	–
S. Anatum	E_1	3, 10,	e, h	1, 6
S. Newington	E_2	3, 15	e, h	1, 6
S. Seftenberg	E_4	1, 3, 19	g, [s], t	–
S. Worthington	G_2	1, 13, 23	z	l, w

[ ] = antigen may be present or absent, 1 = O factor whose presence is due to phage conversion

of antiserum is added to, and mixed well with, the *Salmonella* suspension. The slide is rocked gently for about 30 seconds and the antigen–antibody mixture examined for agglutination (Fig. 17.32). The *Salmonella* is first tested against antisera to the O (somatic) antigens and then the H (flagellar) antigens.

Cultures of a *Salmonella* organism that are motile and diphasic will contain cells that have either phase 1 (specific) or phase 2 (non-specific) flagellar antigens. Usually the majority of cells have flagella in one phase but there will be a very few cells with the alternative flagellar antigen. The organism will agglutinate only with antisera to the flagellar antigen that predominates. To obtain a complete antigenic formula for the isolate, the phase must be 'changed'. In reality this involves the selection of the few cells that have the alternative flagellar antigens. This 'phase-changing' can be carried out by the Craigie tube method (Fig. 17.33) or by the ditch-plate method (Fig. 17.34). Some *Salmonella* serotypes are monophasic with the flagellar antigens in one phase. *S.* Pullorum and *S.* Gallinarum are unusual in being non-motile and lacking flagellar antigens.

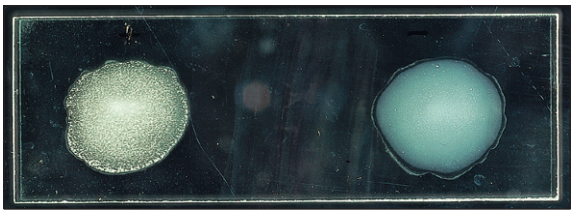

Figure 17.32 *Salmonella* slide agglutination test for serotyping salmonellae showing agglutination with homologous antiserum (left) and a negative reaction (right).

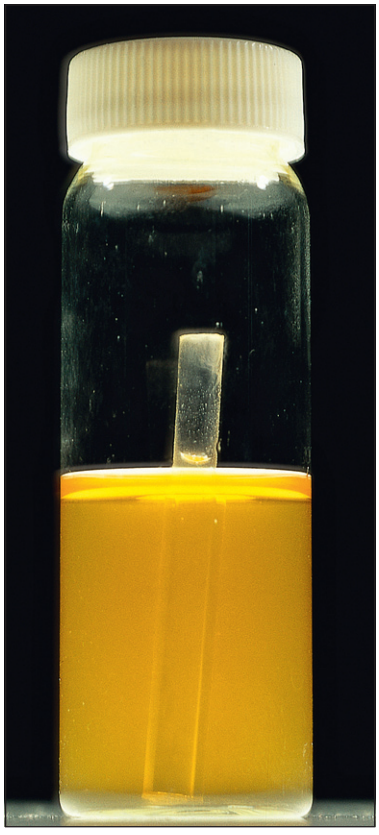

Figure 17.33 The Craigie tube method for 'phase-changing' salmonellae. The semisolid agar contains antiserum specific to the predominating H phase of the *Salmonella* under test. The *Salmonella* culture is inoculated on the agar surface within the central tube. *Salmonella* cells in the alternative H phase are able to move through the agar uninhibited and can be collected, after 24 hours' incubation, from the surface outside the central tube.

A serogroup comprises serotypes with similar O antigens. Lysogenization by certain converting bacteriophages may produce changes in the O antigens. In serogroup E, a phage can alter the O-antigen 3,10 to 3,15 thus changing *S*. Anatum to *S*. Newington. Certain serotypes share an almost identical antigenic formula. These serotypes must be distinguished by biochemical tests (Table 17.11) and are known as biotypes or biovars.

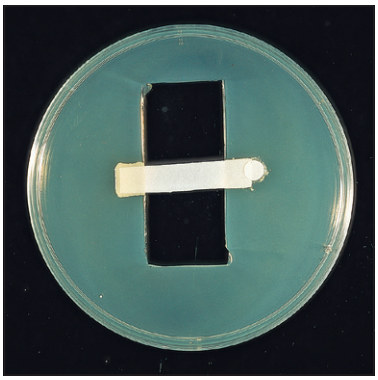

Figure 17.34 The ditch-plate method for 'phase-changing' *Salmonella*. The filter paper strip contains antiserum specific for the predominating H phase of the isolate. The *Salmonella* under test has been inoculated on the left side of the filter paper bridge. *Salmonella* cells in the alternative H phase move across the bridge and can be collected on the filter paper disc on the right.

Phage typing Salmonella *isolates*

Phage typing is based on the sensitivity of a particular isolate to a series of bacteriophages at appropriate dilutions. Phage typing of *S*. Typhi, *S*. Typhimurium and *S*. Enteritidis is carried out at reference laboratories. *S*. Enteritidis phage type (PT) 4 is the cause of a large proportion of the human food poisoning cases in Britain. This phage type, since 1987, has become common in broiler and laying flocks of chicken.

Molecular Diagnosis and Typing Methods

A wide variety of molecular techniques for the detection of salmonellae in clinical samples and in foods have been described. These usually comprise PCR-based procedures including multiplex methods for the detection of different serotypes and 'real-time' methods. Detection in foods frequently involves an initial enrichment step followed by a PCR-based method. Hoorfar (2011) reviewed the detection and enumeration of food-borne pathogens, including salmonellae, in food.

Differentiation of *Salmonella* isolates and identification of sources of foodborne outbreaks can be accomplished using molecular typing techniques. These typing methods utilize restriction endonuclease digestion, nucleic acid amplification (e.g. RAPD-PCR) or nucleotide sequencing techniques (e.g. multi-locus sequence typing). One of the most commonly used techniques is pulsed-field gel

Table 17.11 Biochemical differentiation of *Salmonella* biotypes

	S. Typhisuis	*S.* Choleraesuis	*S.* Choleraesuis biotype Kunzendorf	*Salmonella* (most serotypes)
Hydrogen sulphide (TSI)	v (58%+)	–	+	+
Lysine decarboxylase	–	+	+	+
Citrate (Simmons)	–	+	+	+
Mannitol	–	+	+	+
Inositol	+	–	–	v
Sorbitol	–	(+)	(+)	+
Trehalose	–	–	–	+
Maltose	–	+	+	+
	***S.* Pullorum**	***S.* Gallinarum**	***Salmonella* (most serotypes)**	
Glucose (gas)	(+)	–	+	
Dulcitol	–	+	+	
Maltose	–	+	+	
Ornithine decarboxylase	+	–	+	
Rhamnose	+	–	+	
Motility	–	–	+	

v = variable reactions, (+) = most strains positive

electrophoresis (PFGE). This method separates DNA under conditions of alternating polarity allowing for the resolution of DNA fragments nearly 20 times larger than those separated by traditional agarose gel electrophoresis. PFGE is used in conjunction with restriction enzymes to provide a DNA fingerprint of the bacterial genome. Electronic databases of PFGE profiles now exist (e.g. the PulseNet programme of the CDC), which permits rapid comparison of profiles. These data are useful for the early recognition and effective investigation of outbreaks. Some strains such as *S.* Typhimurium DT104 are not easily differentiated using PFGE and MLVA methods provide superior discrimination of strains for epidemiological purposes (Prendergast et al. 2011).

Antimicrobial Resistance

Multiple resistance (to four or more drugs) is common in many isolates of salmonellae, particularly *S.* Typhimurium, in which it was first identified in the United Kingdom in the 1960s. *Salmonella* Typhimurium DT 29 was associated with numerous infections in cattle and in humans and was resistant to ampicillin, streptomycin, sulphonamides, tetracyclines and furazolidone (Anderson 1968). By 1994 62% of all *S.* Typhimurium isolates were multiresistant (Frost et al. 1995). An important factor in this increase was the epidemic spread of a strain of *S.* Typhimurium DT104 which was resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (ACSSuT). This strain was particularly common in bovine animals in England, Wales, Scotland and Ireland. In contrast to previous multiresistant phage types this strain is also common in poultry, sheep and pigs (Threlfall 2002) although it appears to be declining in prevalence in recent years (Papadopoulou et al. 2009). Antimicrobial resistance in salmonellae is encoded by genes which may be found as part of *Salmonella* genomic islands, integrons and plasmids (Alcaine et al. 2007). For example, Boyd et al. (2000) described the *Salmonella* genomic island 1 and showed that it contained all the resistance genes corresponding to the pentaresistant phenotype ACSSuT. This island is located on the chromosome but can be transmitted to other *Salmonella* serotypes aided by a 'helper plasmid'. Genomic islands confer multiple resistance on serotypes of *Salmonella* such as Agona and Newport. Class 1 integrons are components of genomic islands and have been found on plasmids and transposons in addition to genomic islands. These integrons are thus transferable between *Salmonella* serotypes and can be responsible for different combinations of multidrug resistance. Increases in antimicrobial resistance due to the acquisition of these mobile genetic elements in both animal and human

isolates of *Salmonella* serotypes are a major concern worldwide (Alcaine et al. 2007, Foley & Lynne 2008).

Serology for the Detection of *Salmonella* Antibodies

Agglutination tests, ELISA, antiglobulin and complement fixation tests have been used to detect antibody responses to *Salmonella* infections. For cattle, serological methods of diagnosis are more useful on a herd basis, rather than for individual animals. If testing individual animals, paired acute and convalescent sera should be tested in order to detect a rising antibody titre. Plate agglutination tests, using serum or whole blood, with a stained antigen have been used in national eradication schemes for *S.* Pullorum in chickens. An ELISA developed for the detection of *S.* Enteritidis antibodies in chickens was found to be sensitive and specific (Kim et al. 1991).

YERSINIA SPECIES

There are now 18 species in the genus *Yersinia; Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are responsible for zoonotic infections and *Y. ruckeri* is a pathogen of fish. *Yersinia enterocolitica* is divided into five biogroups and approximately 60 serotypes but only isolates with somatic antigens 2, 3, 5, 8 and 9 appear to cause clinical infections. There are 21 serological groups of *Y. pseudotuberculosis;* types I–V cause disease in animals and humans. *Yersinia pestis* and *Y. pseudotuberculosis* are closely related genetically and it is suggested that *Y. pestis* probably evolved from a serotype of *Y. pseudotuberculosis.*

Natural Habitat

Y. pestis, the cause of bubonic plague in man and a sylvatic cycle in animals, is transmitted mainly by fleas from tolerant rodents, although human infections through cuts, bites, scratches and aerosols can occur. Cats are susceptible to *Y. pestis* and naturally infected cats can pose a health hazard for humans in endemic areas. *Yersinia pseudotuberculosis* persists in wild rodents, lagomorphs and birds as well as in the environment. The intestinal tract of wild and domestic animals appears to be the reservoir for *Y. enterocolitica* but, as with *Y. pseudotuberculosis*, this species of *Yersinia* can replicate in the environment. Pigs, in particular, are carriers of *Y. enterocolitica* strains that are pathogenic for humans.

Pathogenesis

The life cycle of *Y. pestis* is dependent upon replication in a flea vector, classically the Oriental rat flea (*Xenopsylla cheopis*), but fleas of prairie dogs, ground squirrels and some woodrat and mouse fleas can transmit infection also and are important in the USA. Although dog and cat fleas can become infected with *Y. pestis*, they are not efficient transmitters of infection. Fleas feeding on bacteraemic hosts ingest *Y. pestis,* which multiplies and blocks the proventriculus of the flea. This in turn leads to regurgitation of the organism when the blocked flea repeatedly attempts to feed on other animals which then become infected. In addition, infection may occur through contact with infected secretions or tissues, for example, cats may become infected by oral exposure through ingestion of infected rodents. In humans, infection may occur by exposure to infected aerosols from other cases of pneumonic plague either in humans or cats. However, primary pneumonic plague acquired by aerosol infection has not been documented in cats (Orloski et al. 2003).

Infection by the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* occurs by ingestion.

Some virulence factors have been identified that are common to all three pathogenic yersiniae. All three possess a 70-kb virulence plasmid which encodes a type III protein secretion system and *Yersinia* outer proteins or Yops. The type III secretion system exports the Yops from the cell, several of which are delivered directly into phagocytes where they inhibit phagocytosis and proinflammatory cytokine production. There is increasing evidence that all three yersiniae can survive and multiply within macrophages (Pujol & Bliska 2005). A high-pathogenicity island found in more pathogenic strains of yersiniae encodes yersiniabactin, a siderophore which provides the organism with iron and is important for multiplication within the host. Two plasmids which are specific to *Y. pestis* are important for virulence. One of the plasmids encodes the production of a capsule composed of fraction 1 (F1) protein which increases resistance to phagocytosis by both neutrophils and macrophages. It also contains a gene for the production of phospholipase D which is important for multiplication in the midgut of the flea. The other plasmid encodes a plasminogen activator which helps dissemination of the organism from the flea bite area. The invasin gene (*inv*) which is chromosomally encoded in the enteropathogenic yersiniae, assists in translocation of the bacteria across the intestinal wall and colonization of Peyer's patches. The diseases caused by the three *Yersinia* species pathogenic for animals and man are listed in Table 17.12 and virulence factors are summarized in Table 17.13.

Laboratory Diagnosis

Great care must be exercised if a live or dead animal is presented that might be infected with *Y. pestis*. The public health authorities should be notified immediately. The animal, whether alive or dead, should be treated promptly to kill any fleas. It is advisable to wear a gown, mask and gloves when handling the animal. All bacteriological culture work should be conducted in a biohazard cabinet.

Table 17.12 Diseases caused by *Yersinia* species

Yersinia species	Host	Disease(s)	Transmission	Reservoir
Y. pestis	Humans	Bubonic plague ('black death')	Fleas and rat bites	Many rodent species
	Rodents	Sylvatic plague. Infection in rodents usually latent with occasional outbreaks of disease	Occasionally cat bites and scratches	
	Cats	Cats in endemic areas may show mandibular lymphadenitis, fever, depression, anorexia, sneezing and occasionally nervous disturbances. Most infections are fatal		
Y. pseudotuberculosis	Guinea-pigs, other rodents, rabbits, wild and captive birds	Pseudotuberculosis: 1. Septicaemic syndrome 2. Classical syndrome with nodules in internal organs. Seen most commonly in guinea pigs and canaries	Ingestion	Faeces of carrier rodents and wild birds
	Farm animals	Latent infections. Occasional disease such as in captive deer		
	Sheep	Orchitis and epididymitis reported		
	Humans	Mesenteric lymphadenitis, acute terminal ileitis and rare cases of septicaemia. Mainly children and young adults		
Y. enterocolitica	Farm animals	Latent infection with sporadic cases of enteritis or generalized infections. Captive deer particularly susceptible	Ingestion	Carrier state in many animal species, especially pigs
	Humans	Food poisoning (enteritis), mesenteric lymphadenitis (pseudo-appendicitis). Most common in children		Pigs are carriers of strains pathogenic for humans

Specimens

For *Y. pestis*, samples could include oedematous tissue, lymph nodes, nasopharyngeal swabs, transtracheal aspirates, cerebrospinal fluid and blood for culture and serology. Suitable specimens for *Y. pseudotuberculosis* include necrotic internal lesions, lymph nodes and faeces. Faecal samples are usually taken for *Y. enterocolitica*.

Isolation

Yersinia species grow on nutrient, blood and MacConkey agars but the colonies, after 24 hours' incubation, tend to be smaller than those of the other members of the *Enterobacteriaceae*. *Yersinia pestis* grows poorly on agars containing desoxycholate whereas *Y. enterocolitica* and *Y. pseudotuberculosis* grow well on these media. *Yersinia* selective medium (CIN agar) containing the antibiotic supplement cefsulodin (15 mg/L), irgasin (4 mg/L) and novobiocin (2.5 mg/L) is designed for the isolation of *Y. enterocolitica* from faeces, although it should be noted that most strains of biotype 3, serotype O:3 are inhibited by this medium (Fukushima & Gomyoda 1986). A cold-enrichment procedure may be necessary for the isolation of both *Y. enterocolitica* and *Y. pseudotuberculosis* from faecal specimens. A faecal specimen (approximately 5% by volume) is placed in l/15 M phosphate buffered saline (Oxoid) and held in the refrigerator (4°C) for three weeks. Subcultures, at weekly intervals, can be made on MacConkey and *Yersinia* selective medium. *Yersinia* species usually grow faster at 37°C but prefer lower incubation temperatures, particularly on primary isolation. Sometimes additional culture plates, incubated at 22–25°C, can be useful for initial isolation.

Table 17.13 Major virulence factors of *Yersinia* species (possession of virulence factors varies between species)

Virulence attribute	Function
F1 protein (*Y. pestis*)	Capsule associated; prevents opsonization
Adhesins	Adherence, serum resistance
Type III secretion system	Transfer of effector proteins into host cells
Yersinia outer proteins	Evasion of the immune response, prevention of phagocytosis
Yersiniabactin	Iron acquisition
Phospholipase D (*Y. pestis*)	Role in multiplication within the midgut of the flea
Plasminogen activator (*Y. pestis*)	Dissemination from the site of inoculation

Figure 17.35 Comparison of the reactions of *Y. enterocolitica* (right plate) and *Y. pseudotuberculosis* (left plate) on MacConkey agar (top), brilliant green agar (left) and XLD medium (right).

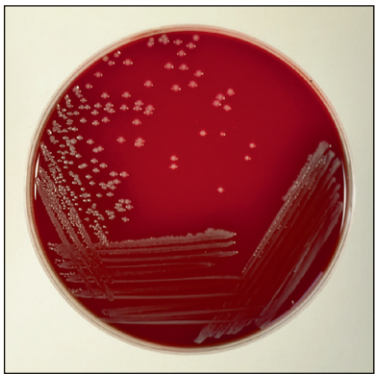

Figure 17.36 *Yersinia pseudotuberculosis* on sheep blood agar. The non-haemolytic, greyish, shiny, discrete colonies are similiar to those of many other members of the *Enterobacteriaceae*.

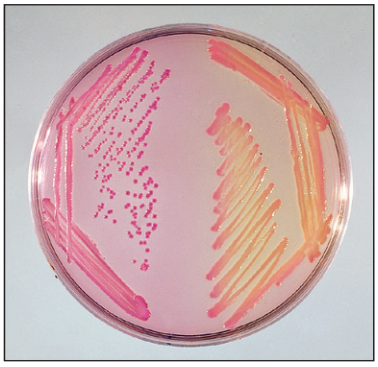

Figure 17.37 *Y. enterocolitica* (left) and *Y. pseudotuberculosis* (right) on '*Yersinia* selective agar' formulated for the isolation and enumeration of *Y. enterocolitica* from clinical specimens and foods. Typical colonies of the bacterium develop dark red 'bullseyes' surrounded by a transparent border. (Neutral red indicator)

Identification

Colonial morphology

The *Yersinia* species are lactose-negative (Fig. 17.35) although lactose-positive strains of *Y. enterocolitica* occur. *Yersinia enterocolitica* will grow well on media, such as brilliant green and XLD, intended for *Salmonella* isolation but *Y. pseudotuberculosis* is less tolerant. *Yersinia enterocolitica* and *Y. pseudotuberculosis* (Fig. 17.36) are non-haemolytic on blood agar. The colonies of *Y. enterocolitica* on *Yersinia* selective medium (Oxoid) have dark red centres with a transparent periphery (Fig. 17.37).

Biochemical tests

The yersiniae are identified by conventional biochemical tests (Table 17.6) or by means of a commercial test strip such as API 20E (bioMérieux). *Yersinia pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are non-motile at 37°C but *Y. pseudotuberculosis* and *Y. enterocolitica* strains can be motile at 28°C. *Yersinia pseudotuberculosis* is almost always urease-positive as are most strains of *Y. enterocolitica*, but *Y. pestis* does not produce this enzyme. Only certain strains of *Y. enterocolitica* cause human infections and these pathogenic serotypes can be detected biochemically (Farmer & Kelly 1991). They are negative for the pyrazinamidase test,

salicin fermentation and aesculin hydrolysis, whereas the non-pathogenic strains are positive to these tests. The pathogenic strains grow as small red colonies on congo red-magnesium oxalate (CR-MOX) agar but non-pathogenic strains are unable to grow on the medium. *Y. pestis* can be confirmed in reference laboratories by the fluorescent antibody technique, bacteriophage-susceptibility and by molecular methods (Gage 1998).

Molecular diagnosis and typing

There are numerous reports published on the detection of *Y. pestis* and *Y. pseudotuberculosis* by PCR, including real-time PCR (Fukushima et al. 2003). Assays capable of differentiating between isolates of these two species have been published also (Chase et al. 2005). Because *Y. pestis* is regarded as one of a number of agents that could be used in bioterrorist acts, recent research has included the development of DNA microarrays and real time PCR methods that can detect multiple agents in different sample types (Tomioka et al. 2005, Matero et al. 2011).

PCR-based detection methods have been developed for the identification of *Y. enterocolitica* in clinical, faecal and food samples (reviewed by Fredriksson-Ahomaa & Kerkeala 2003). Methods targeting genes located on the virulence plasmid and methods based on virulence genes located on the chromosome have been developed. Colony hybridization methods for the identification of isolates can also be used and are based on genes similar to those used for detection of yersiniae in natural samples.

In common with the other major pathogens in the *Enterobacteriaceae*, typing of yersiniae has been carried out using MLST, MLVA and PFGE-based techniques. MLVA was found to have greater discriminatory ability than PFGE methods in the investigation of a major food-borne outbreak of *Y. enterocolitica* in Finland (Sihvonen et al. 2011).

Antimicrobial Susceptibility

Disc diffusion methods can be used for determination of antimicrobial susceptibility of yersiniae while determination of MIC data is by agar dilution techniques. Published data on antimicrobial-resistant patterns of recent isolates of *Y. pestis* were reviewed by Brouillard et al. (2006), who recommended that doxycycline should be considered as a first-line antibiotic for this pathogen. Porcine *Y. enterocolitica* isolates show variable resistance patterns, in some cases reflecting usage of antimicrobial agents on the farm of origin. There is evidence of emerging resistance to fluoroquinolones in isolates of *Y. ruckeri* (Gibello et al. 2004).

Serological Tests to Demonstrate Antibodies

Enzyme immunoassays can be used for the detection of antibodies to the pathogenic *Yersinia* species.

ENTEROBACTERIA THAT ARE OPPORTUNISTIC PATHOGENS

Natural Habitat and Pathogenicity

The diseases and natural habitat of these members of the *Enterobacteriaceae* are summarized in Table 17.14.

Laboratory Diagnosis

Direct microscopy

As the enterobacteria share the property of being Gram-negative, medium-sized rods with many other bacterial genera, direct microscopy is not usually helpful. However, in urinary tract infections a bacterial count (Chapter 2) could be carried out on freshly taken, mid-stream urine. In dogs, 10^5 bacteria/mL urine is taken to indicate a clinical bacteriuria.

Isolation

All the members of the *Enterobacteriaceae,* including the opportunistic pathogens, will grow on the routine diagnostic media, blood and MacConkey agars (Fig. 17.15).

Identification

Colonial morphology and reactions on selective/indicator media are helpful. Reactions on MacConkey agar indicate whether or not the bacterium ferments the lactose in the medium. In several genera, lactose fermentation is late or irregular and in these cases the ONPG test for β-galactosidase (Chapter 2) reveals a potential ability to attack lactose.

Generally, on blood agar, the colonies of most of the members of the *Enterobacteriaceae* are similar. They are usually relatively large, 2–3 mm after 24 hours' incubation, non-haemolytic, shiny, round and greyish in colour. However, a few enterobacteria have distinctive colonial characteristics. Most *Proteus mirabilis* and *P. vulgaris* strains will swarm on blood agar. *Proteus* species grow in a colonial fashion for a period causing a build-up of toxic metabolic products. Long, flagellated swarm cells are formed (Fig. 17.38) that move quickly across the contaminated agar and colonial growth occurs on the fresh agar. Metabolic products again accumulate to a critical level and the swarming cycle is repeated. If a spot inoculation of a *Proteus* species is made on a blood agar or nutrient agar plate, rings of heavy growth interspersed with thin areas of swarming growth will be seen (Fig. 17.39). Normally the bile salts in MacConkey agar prevent the swarming of *Proteus* species, but if the surface of the agar is moist, swarming may even be seen on this medium (Fig. 17.40). On blood agar, in particular, the powerful and foul odour

Table 17.14 Diseases caused by members of the *Enterobacteriaceae* that are opportunistic pathogens

Pathogen	Habitat	Disease(s)
Citrobacter diversus	Faeces of man, animals and in soil and sewage	Meningitis in human neonates and mastitis in cattle have been reported. It is probably capable of opportunistic infections in other mammals
Edwardsiella tarda	Water, mud and reptilian intestines	Fish (eels and catfish) and marine mammals: abscesses in muscle, liver and kidneys Mild diarrhoea reported in pigs, calves and dogs Latent intestinal infections in tortoises
Enterobacter aerogenes	Water, soil, sewage and faeces	Coliform mastitis in cattle Uterine infections in mares Occasionally part of the mastitis-metritis-agalactia (MMA) syndrome in sows
Klebsiella pneumoniae	Intestinal tract of animals and man, soil and sawdust	Coliform mastitis in cattle Cervicitis and metritis in mares Urinary tract infections in dogs Pneumonia and suppurative conditions in foals
Morganella morganii	Faeces of animals	Ear and urinary tract infections in dogs and cats
Proteus mirabilis and *P. vulgaris*	Faeces of mammals and environment	Urinary tract infections of dogs and horses Associated with otitis externa in dogs and cats Diarrhoea in young mink, lambs, calves, goats and pups
Serratia marcescens	Environmental organism	Bovine mastitis Septicaemia in chickens and immunosuppressed mammals Infections in geckos and tortoises
Shigella species	Intestinal tract of humans and other primates	Dysentery/diarrhoea in humans and other primates. Very few reports of infection in other animals. Dogs can be infected from human owners and may excrete the bacteria for short periods, without clinical signs

Figure 17.38 Gram-stained smear of the swarming growth of a *Proteus mirabilis* showing the long 'swarm cells'. (Gram stain, ×1000)

Figure 17.39 Characteristic swarming of a *Proteus* species following spot inoculation in the centre of a blood agar plate.

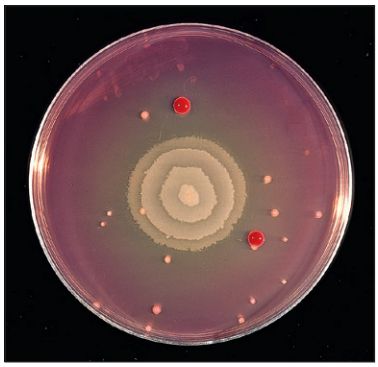

Figure 17.40 A MacConkey agar plate exposed to an aerosol during the spreading of slurry showing a *Proteus* species swarming, red-pigmented colonies of *Serratia rubidaea* and mucoid, pale pink colonies of a *Klebsiella* species.

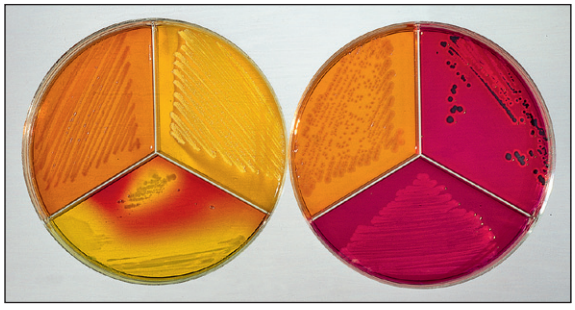

Figure 17.41 Reactions of *P. vulgaris* (left) and *P. mirabilis* (right) on two separate plates: MacConkey agar (left), XLD medium (right) and brilliant green agar (bottom). Note that the *P. mirabilis* strain on the right is giving similiar reactions to that of most salmonellae except that with this strain of *P. mirabilis* the periphery of the colonies on the XLD medium tends to be yellowish.

of *Proteus* species will be noticed and the bacteria tend to turn the blood agar a chocolate-brown colour. Most *P. vulgaris* and *P. mirabilis* strains produce hydrogen sulphide (H_2S) in TSI and XLD media. As they are also lactose-negative, *P. mirabilis* can give a reaction similar to most of the salmonellae (R/Y/H2S+) in TSI. However, *Proteus* species are almost always lysine-decarboxylase-negative. Similarly on XLD medium (Fig. 17.41) some strains of *Proteus* can mimic *Salmonella* colonies by having a black centre (H_2S production) but the periphery of the colony tends to have a yellowish tinge (Fig. 17.4).

Klebsiella pneumoniae (Fig. 17.42) and *Enterobacter aerogenes* have very mucoid colonies on primary isolation indicative of the presence of a large capsule around individual cells. Both are lactose-fermenters but the colonies are pale pink on MacConkey agar. The rare strains of *Escherichia coli* that are mucoid are usually a more vivid pink (Fig. 17.43).

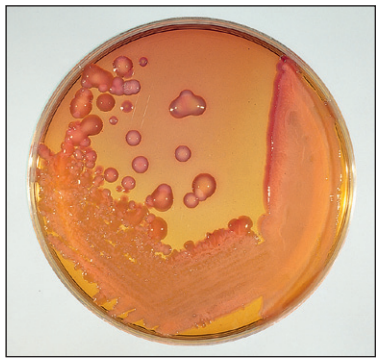

Figure 17.42 *Klebsiella pneumoniae* on MacConkey agar. The bacterium is a lactose-fermenter but the characteristic large, mucoid colonies always tend to be pale pink.

Figure 17.43 A mucoid strain of *E. coli* (left) compared to a non-mucoid *E. coli* strain (right) on MacConkey agar. *Klebsiella pneumoniae* (bottom) is normally mucoid and tends to be pale pink.

A few members of the *Enterobacteriaceae* produce pigments. Both *Serratia marcescens* and *S. rubidaea* form the red pigment called prodigiosin (Fig. 17.44). This pigment is produced best at 25°C but some strains can form the pigment at 37°C. The red pigmentation is superimposed on any colour reaction that occurs in MacConkey agar due to the pH indicator, neutral red (Fig. 17.45). A number of the enterobacteria produce a yellow pigment as demonstrated by *Enterobacter agglomerans* (Fig. 17.46).

Microscopy and tests for primary identification

All the enterobacteria, including the opportunistic pathogens are medium-sized, Gram-negative rods (Fig. 17.47) although occasionally some members show coccobacillary or long forms, such as the swarm cells of *Proteus* species. An O-F test demonstrates that they are facultative

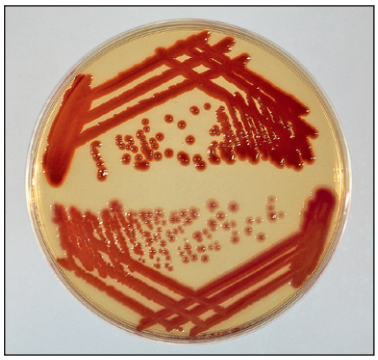

Figure 17.44 *Serratia rubidaea* (top) and *Serratia marcescens* (bottom) on nutrient agar showing the production of red pigment (prodigiosin).

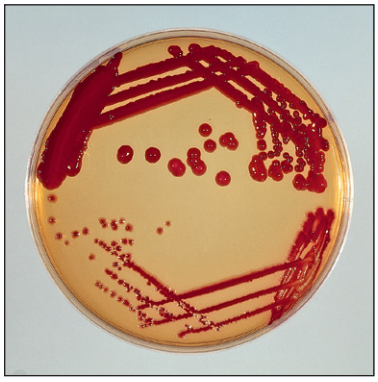

Figure 17.45 *Serratia rubidaea* (top) and *S. marcescens* (bottom) on MacConkey agar. Both are non-lactose-fermenters (pale reaction in the medium). The colony colour is due to the production of the red pigment, not a pH change.

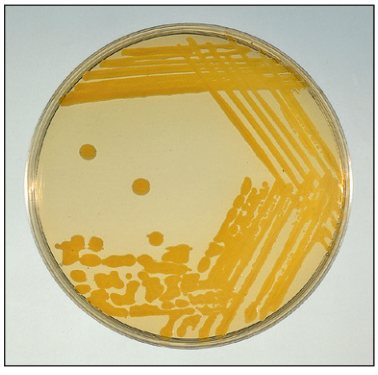

Figure 17.46 *Enterobacter agglomerans* on nutrient agar showing mucoid colonies and yellow pigmentation.

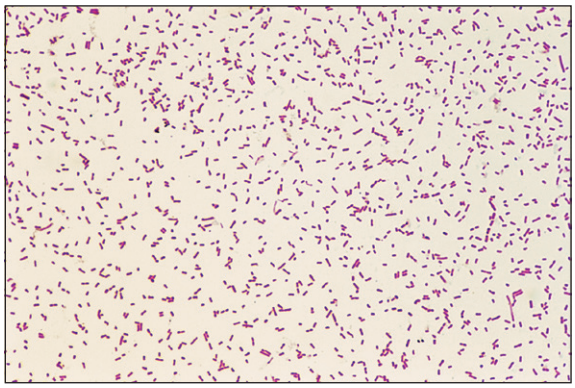

Figure 17.47 Medium-sized Gram-negative rods of *E. coli* from a culture. The morphology is representative of most members of the *Enterobacteriaceae*. (Gram stain, ×1000)

anaerobes, but the fact that all the enterobacteria are oxidase negative is the most useful characteristic as most of the other Gram-negative bacteria are oxidase-positive.

Biochemical tests

Figure 17.15 indicates the short range of biochemical tests that, together with the reaction on MacConkey agar and the colonial morphology, can give a presumptive identification of the opportunistic enterobacteria. The combination of a TSI agar slope and lysine decarboxylase broth, while designed for the presumptive identification of salmonellae, can also be useful for other enterobacteria (Fig. 17.17). If there is doubt about the identification of an isolate, a full range of biochemical tests should be carried out (Table 17.6). The methods and interpretation of the tests are given in Chapter 2. A more expensive, but less time-consuming, method of identification is to use a commercial strip such as the API 20E (bioMérieux) for the enterobacteria and other Gram-negative organisms. The method of inoculation and interpretation of the API 20E is also given in Chapter 2.

Serotyping for Antigen Detection

Klebsiella species are sometimes serogrouped by their capsular (K) antigens in reference laboratories. At least 77 capsular types of *Klebsiella* have been described. K1, K2 and K5 are venereally transmitted and are the predominant types in the isolates from metritis in mares.

Antimicrobial Susceptibility Tests

As with other members of the *Enterobacteriaceae*, multiple antimicrobial resistance is an increasing problem with these opportunistic pathogens. Antimicrobial susceptibility testing should be carried out with any isolate that is considered to be significant, before the treatment of the animal with antimicrobial drugs.

REFERENCES

Alcaine, S.D., Warnick, L.D., Wiedmann, M., 2007. Antimicrobial resistance in non-typhoidal *Salmonella*. Journal of Food Protection 70 (3), 780–790.

Anderson, E.S., 1968. Drug resistance in *Salmonella* Typhimurium and its implications. BMJ 3 (5614), 333–339.

Anon, 2002. EN ISO 6579:2002(E) 4th edn, Microbiology– General Guidance on Methods for the Detection of *Salmonella*. International Organization for Standardization, Geneva, Switzerland.

Anon, 2005. The type species of the genus *Salmonella* Lignieres 1900 is *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987, with the type strain LT2T, and conservation of the epithet enterica in *Salmonella enterica* over all earlier epithets that may be applied to this species. Opinion 80. International Journal of Systematic and Evolutionary Microbiology 55, 519–520.

Barrow, P.A., Jones, M.A., Thomson, N., 2010. *Salmonella*. In: Gyles, C.L., Prescott, J.F., Songer, J.G. (Eds.), Pathogenesis of Bacterial Infections in Animals, fourth ed. Wiley-Blackwell, Iowa, USA, pp. 231–265.

Boyd, D.A., Peters, G.A., Ng, L.K., 2000. Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhimurium DT104. FEMS Microbiology Letters 189, 285–291.

Boyd, E.F., Hartl, D.L., 1998. Chromosomal regions specific to pathogenic isolates of *Escherichia coli* have a phylogenetically clustered distribution. Journal of Bacteriology 180 (5), 1159–1165.

Brenner, F.W., Villar, R.G., Angulo, F.J., et al., 2000. *Salmonella* nomenclature. Journal of Clinical Microbiology 38 (7), 2465–2467.

Brouillard, J.E., Terriff, C.M., Tofan, A., et al., 2006. Antibiotic selection and resistance issues with fluoroquinolones and doxycycline against bioterrorism agents. Pharmacotherapy 26 (1), 3–14.

Brumell, J.H., Perrin, A.J., Goosney, D.L., et al., 2002. Microbial pathogenesis: new niches for salmonella. Current Biology 12 (1), R15–R17.

Carroll, P.J., Woodward, M.J., Wray, C., 1990. Detection of LT and ST1a toxins by latex and EIA tests. Veterinary Record 127, 335–336.

Chase, C.J., Ulrich, M.P., Wasieloski, L.P. Jr, et al., 2005. Real-time PCR assays targeting a unique chromosomal sequence of *Yersinia pestis*. Clinical Chemistry 51 (10), 1778–1785.

Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology 66 (10), 4555–4558.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, third ed. Approved Standard M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Dean, E.A., Whipp, S.C., Moon, H.W., 1989. Age-specific colonization of porcine intestinal epithelium by 987P-piliated enterotoxigenic *Escherichia coli*. Infection and Immunity 57 (1), 82–87.

DebRoy, C., Maddox, C.W., 2001. Identification of virulence attributes of gastrointestinal *Escherichia coli* isolates of veterinary significance. Animal Health Research Reviews 2, 129–140.

Dubreuil, J.D., 2012. The whole Shebang: the gastrointestinal tract, *Escherichia coli* enterotoxins and secretion. Current Issues in Molecular Biology 14 (2), 71–82.

Farmer, J.J., Kelly, M.T., 1991. *Enterobacteriaceae* In: Balows, A., Hausler, W.J., Herrmann, K.L., et al. (Eds.), Manual of Clinical Microbiology, fifth ed. American Society for Microbiology, Washington, DC, pp. 360–383.

Foley, S.L., Lynne, A.M., 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. Journal of Animal Science 86 (14 Suppl), E173–E187.

Francis, D.H., Remmers, G.A., De Zeeuw, P.S., 1982. Production of K88, K99, and 987P antigens by *Escherichia coli* cultured on synthetic and complex media. Journal of Clinical Microbiology 15, 181–183.

Fredriksson-Ahomaa, M., Korkeala, H., 2003. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. Clinical Microbiology Reviews 16 (2), 220–229.

Fröhlicher, E., Krause, G., Zweifel, C., et al., 2008. Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. BMC Microbiology 8, 144.

Frost, J.A., Threlfall, E.J., Rowe, B., 1995. Antibiotic resistance in salmonellas from humans in England and Wales: the situation in 1994. PHLS Microbiology Digest 12 (3), 131–133.

Fukushima, H., Gomyoda, M., 1986. Growth of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* biotype 3B serotype O3 inhibited on cefsulodin-Irgasan-novobiocin agar. Journal of Clinical Microbiology 24 (1), 116–120.

Fukushima, H., Tsunomori, Y., Seki, R., 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. Journal of Clinical Microbiology, 41, 5134–5146.

Gage, K.L., 1998. Plague. In: Colliers, L., Balows, A., Sussman, M., Hausles, W.J. (Eds.), Topley and Wilson's Microbiology and Microbiological Infections, vol 3. Edward Arnold Press, London, pp. 885–903.

Gibello, A., Porrero, M.C., Blanco, M.M., et al., 2004. Analysis of the gyrA gene of clinical *Yersinia ruckeri* isolates with reduced susceptibility to quinolones. Applied and Environmental Microbiology 70 (1), 599–602.

Gyles, C.L., Fairbrother, J.M., 2010. *Escherichia coli*. In: Gyles, C.L., Prescott, J.F., Songer, J.G., et al. (Eds.), Pathogenesis of Bacterial Infections in Animals, fourth ed.

Wiley-Blackwell, Iowa, USA, pp. 267–308.

Hensel, M., 2004. Evolution of pathogenicity islands of *Salmonella enterica*. International Journal of Medical Microbiology 294, 95–102.

Hoorfar, J., 2011. Rapid detection, characterization, and enumeration of foodborne pathogens. APMIS supplement 133, 1–24.

Jepson, M.A., Clark, M.A., 2001. The role of M cells in *Salmonella* infection. Microbes and Infection 3 (14–15), 1183–1190.

Kim, C.J., Nagaraja, K.V., Pomeroy, B.S., 1991. Enzyme-linked immunosorbent assay for the detection of *S. enteritidis* infection in chicken. American Journal of Veterinary Research 52, 1069.

Köhler, C.D., Dobrindt, U., 2011. What defines extraintestinal pathogenic *Escherichia coli*? International Journal of Medical Microbiology 301, 642–647.

Le Minor, L., Popoff, M.Y., 1987. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. Request for an Opinion. International Journal of Systematic Bacteriology 37, 465.

Matero, P., Hemmilä, H., Tomaso, H., et al., 2011. Rapid Field Detection Assays for *Bacillus anthracis, Brucella* spp., *Francisella tularensis* and *Yersinia pestis*. Clinical Microbiology and Infection 17, 34–43.

Mokady, D., Gophna, U., Ron, E.Z., 2005. Virulence factors of septicemic *Escherichia coli* strains. International Journal of Medical Microbiology 295 (6–7), 455–462.

Nagy, B., Fekete, P.Z., 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Veterinary Research 30, 259–284.

Orloski, K.A., Lathrop, S.L., 2003. Plague: a veterinary perspective. Journal of the American Veterinary Medical Association 222, 444–448.

Papadopoulou, C., Davies, R.H., Carrique-Mas, J.J., et al., 2009. *Salmonella* serovars and their antimicrobial resistance in British turkey flocks in 1995 to 2006. Avian Pathology 38 (5), 349–357.

Popoff, M.Y., Bockemühl, J., Brenner, F.W., 2000. Supplement 1999 (no. 43) to the Kauffmann–White scheme. Research in Microbiology 151 (10), 893–896.

Popoff, M.Y., Bockemühl, J., Brenner, F.W., et al., 2001. Supplement 2000 (no. 44) to the Kauffmann–White scheme. Research in Microbiology 152 (10), 907–909.

Popoff, M.Y., Bockemühl, J., Gheesling, L.L., 2003. Supplement 2001 (no. 45) to the Kauffmann–White scheme. Research in Microbiology 154 (3), 173–174.

Prendergast, D.M., O'Grady, D., Fanning, S., et al., 2011. Application of multiple locus variable number of tandem repeat analysis (MLVA), phage typing and antimicrobial susceptibility testing to subtype *Salmonella enterica* serovar Typhimurium isolated from pig farms, pork slaughterhouses and meat producing plants in Ireland. Food Microbiology 28 (5), 1087–1094.

Pujol, C., Bliska, J.B., 2005. Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. Clinical Immunology 114 (3), 216–226.

Sihvonen, L.M., Toivonen, S., Haukka, K., et al., 2011. Multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. BMC Microbiology 11, 42.

Threlfall, E.J., 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. FEMS Microbiology Review 26 (2), 141–148.

Tomioka, K., Peredelchuk, M., Zhu, X., et al., 2005. A multiplex polymerase chain reaction microarray assay to detect bioterror pathogens in blood. Journal of Molecular Diagnostics 7 (4), 486–494.

Vassiliadis, P., Mavromati, C., Trichopoulus, D., et al., 1987. Comparison of procedures based upon Rappaport–Vassiliadis medium with those using Muller–Kauffmann medium containing Teepol for the isolation of *Salmonella* sp. Epidemiology & Infection 99 (1), 143–147.

von Baum, H., Marre, R., 2005. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. International Journal of Medical Microbiology 295 (6–7), 503–511.

Wallis, T.S., Galyov, E.E., 2000. Molecular basis of *Salmonella*-induced enteritis. Molecular Microbiology 36 (5), 997–1005.

Chapter 18

Pseudomonas, Burkholderia and *Stenotrophomonas* species

Genus Characteristics

The genus *Pseudomonas* was originally organized into five major species clusters (rRNA homology groups). However, this classification has undergone revision and *Pseudomonas* species have now been reclassified into many different genera. In addition to *Pseudomonas*, two of these genera are of significance in veterinary medicine: *Burkholderia* and *Stenotrophomonas*. The genus *Pseudomonas* (sensu stricto) represents the rRNA homology group 1 with the type species *Pseudomonas aeruginosa* which is the most important veterinary pathogen in the genus *Pseudomonas* followed by *P. fluorescens*. Two species of the genus *Burkholderia* (formerly rRNA group II pseudomonads), *B. mallei* and *B. pseudomallei*, are generally recognized as important animal or human pathogens. The genus *Stenotrophomonas* has one species of clinical veterinary significance, *S. maltophilia* (formerly *Pseudomonas maltophilia* or *Xanthomonas maltophilia*) (Versalovic 2011). *Pseudomonas* and *Burkholderia* species are medium-sized (0.5–1 μm × 1.5–5 μm) straight or slightly curved Gram-negative rods. *Stenotrophomonas* species tend to be straight and slightly smaller (0.4–0.7 μm × 0.7–1.8 μm). These bacteria are strict aerobes, non-spore-forming, oxidative, catalase-positive, oxidase-positive (except *P. oryzihabitans, P. luteola* and the genus *Stenotrophomonas*) and most are motile by one or several polar flagella (except *B. mallei*). Some species produce soluble pigments and most will grow on MacConkey agar as lactose non-fermenters as well as converting nitrate to nitrite or nitrogen gas.

Natural Habitat

Pseudomonas, *Stenotrophomonas* and *Burkholderia* species have a worldwide distribution. These bacteria are environmental microorganisms typically found in water, soil, on plants, fruits and vegetables. They are opportunistic pathogens of animals, humans and plants. They have a predilection for aqueous environments, surviving well in them. As a result, they can be problematic in hospital settings. Potential sources of *P. aeruginosa* are diverse including disinfectants, ointments, soaps, eye drops, irrigation fluids and equipment. This bacterium is frequently found in aerators and traps of sinks. As *P. aeruginosa* is resistant to many antimicrobials, it frequently causes infection in animals undergoing antibiotic treatment or in immunocompromised hosts. *Pseudomonas aeruginosa* is found infrequently as part of the microbial flora of healthy animals. In these animals, it can be found on skin and mucous membranes, particularly the gastrointestinal tract. *Pseudomonas fluorescens*, present in soil and water, is associated with food spoilage and lesions in reptiles and fish (Sakai et al. 1989; Swain et al. 2007).

Burkholderia mallei, the aetiologic agent of glanders, is a listed disease by the Office International des Épizooties (OIE), also known as the World Organization for Animal Health. Glanders is now rare as there has been considerable success in the global eradication of this disease, principally owing to the fact that *B. mallei* is an obligate parasite with a restricted host range and, in addition, effective tests are available to detect carriers of the infection. Infected *Equidae* are the reservoir for *B. mallei*. This organism is unable to survive in the environment for more than two weeks. It is easily killed by desiccation, sunlight and common disinfectants. *Burkholderia mallei* once had a wide geographical distribution but now is mainly seen in China and Mongolia with pockets of infection in India, Iraq, Turkey and the Philippines. *Burkholderia pseudomallei*, the cause of melioidosis, is found primarily in tropical and subtropical regions; particularly in the rice-growing areas

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of Thailand, Vietnam and India; but also in the Northern Territory of Australia (Edmond et al. 2001). The disease usually occurs in tropical regions between 20° northern and southern latitudes but melioidosis has been reported in localized areas of France, Iran, China and the USA.

Stenotrophomonas maltophilia is readily isolated from water, soil and sewage. It has emerged as an opportunistic pathogen in animals and immunocompromised humans. *Stenotrophomonas maltophilia* is resistant to many antimicrobials (Denton & Kerr 1998) and mainly causes hospital-acquired infections in humans. The respiratory tract is the most common site of infection. This microorganism has also been isolated from the semen of boars and bulls, diminishing semen quality and viability and resulting in impaired fertilization and embryonic development in vitro (Bielanski et al. 2003, Althouse & Lu 2005). *Stenotrophomonas maltophilia* is part of the normal microflora of the mouth and cloacae of healthy snakes (Hejnar et al. 2007).

Pathogenesis and Pathogenicity

Burkholderia mallei is the causative agent of glanders, a disease of livestock that particularly affects horses, mules, and donkeys (Table 18.1). This bacterium is a highly pathogenic microorganism for both humans and animals. *Burkholderia pseudomallei* is the aetiological agent of melioidosis, a disease in which treatment failures and relapses are common, with pneumonia as the most common clinical presentation. Both of these species have been identified as potential agents of bioterrorism (category B biothreat agents). *Pseudomonas aeruginosa* and *S. maltophilia* are both considered opportunistic pathogens and can cause a variety of infections (Table 18.1). A number of saprophytic *Pseudomonas* species and *Burkholderia* species have been implicated in occasional infections of animals (Jackson & Phillips 1996, Berriatua et al. 2001, Matchett et al. 2003) including *P. fluorescens* (septicaemia in rainbow trout and Tilapia and necrotizing hepatitis in pet birds), *P. putida* (endotoxic shock in a cynomolgous macaque) and *B. cepacia*, formerly *P. cepacia* (outbreak of subclinical mastitis in a flock of dairy sheep).

Pseudomonas aeruginosa is rarely involved in primary disease. Predisposing causes include trauma to tissue (burns and wounds), debilitation due to malignancy or immunodeficiency and an imbalance in the normal flora, often caused by antibiotic therapy. *Pseudomonas aeruginosa* possesses cell-associated virulence factors such as pili, flagella, lipopolysaccharide and alginate/biofilm. It also produces a number of extracellular products such as protein exotoxin A, proteases, type III secretion system exoenzymes, rhamnolipid, phospholipase C, and siderophores (pyochelin, pyocyanin, and pyoverdin). These virulence factors all play a role in disease pathogenesis (Table 18.2). Flagella provide motility and act as adhesins to mucin and respiratory epithelial cells. Pili are the major adhesins implicated in the initial attachment phase to host tissues. *P. aeruginosa* occurs in both rough and smooth lipopolysaccharide (LPS) forms (Sadovskaya et al. 2000). LPS is involved in adherence and invasion and its lipid A part mediates inflammation and tissue damage. Under particular conditions, *P. aeruginosa* can produce an alginate structure which is a slime-like, mucoid exopolysaccharide. This structure can form a viscous gel surrounding the bacteria and help in the generation of biofilms involved in adherence. It also protects the bacterium from phagocytosis. The type III secretion system consists of bacterial proteins which act as a syringe to deliver cytotoxins into the cytoplasm of host cells. The toxins are involved in epithelial cell damage and in the inhibition of phagocytic cells. Siderophores are involved in iron acquisition and promote survival in low-iron conditions such as host tissues. Interestingly, pyocyanin can colour pus and stain wool a greenish blue.

Burkholderia mallei is a host-adapted pathogen, causing glanders (pulmonary and nasal forms) or farcy (the skin form) in the *Equidae* population. Humans and members of the cat family are susceptible with occasional infections in dogs, goats, sheep and camels. The disease is characterized by a high fever, with respiratory clinical signs such as swollen nostrils, catarrhal nasal discharge, lymphadenopathy, dyspnea, and pneumonia. Yellow or grey nodules occur on the mucosa of the upper respiratory tract. The skin form is also characterized by the formation of tubercle-like nodules, usually along the cutaneous lymphatics, which frequently ulcerate. Strain variations may determine whether suppurative or granulomatous lesions predominate. The disease can be acute or chronic and many infections are fatal if not treated at an early stage. In horses, the disease is usually chronic and can be carried for many years before clinical signs appear. Transmission occurs from infected animals via contaminated food and water and less commonly from aerosols and infection of wounds. Few microorganisms are necessary to cause this contagious disease. Primary lesions occur at the point of entry (skin or mucosal surfaces) with dissemination via the lymphatic system and dissemination by the bloodstream. Human infection has occurred rarely and sporadically among laboratory workers and also those in direct contact with infected, domestic animals. In addition to animal exposure, cases of human-to-human transmission have been reported. Human disease cases can present as localized, suppurative cutaneous infections, pulmonary infections, bloodstream infections or suppurative chronic infections of the skin.

Virulence factors such as capsular material, LuxI and LuxR quorum-sensing signals, a possible antigenic variation system and a type III secretion system have been reported for *B. mallei*. Its capsular polysaccharide is reported as a major virulence factor (DeShazer et al. 2001) and one of the factors facilitating its persistence in the body. The bacterium is capable of intracellular survival.

Table 18.1 Main diseases caused by the major pathogenic *Pseudomonas, Burkholderia* and *Stenotrophomonas species* in veterinary medicine

Species	Host(s)	Typical Diseases
Pseudomonas aeruginosa	Many animals	Wound infections
	Cattle	Mastitis, uterine infections, enteritis, arthritis, respiratory infections and botryomycosis
	Dogs and cats	Otitis externa, urinary tract infections, corneal ulcer, pneumonia, endocarditis and deep pyoderma
	Chinchillas	Septicaemia, pneumonia, conjunctivitis, enteritis, otitis media and interna with neurological manifestations, sudden death, and abortion
	Horse	Metritis, abortion, corneal ulcer, abscesses
	Mink	Haemorrhagic pneumonia, septicaemia
	Poultry	Septicaemia
	Sheep and goats	Pneumonia, mastitis, fleecerot, lung abscesses and 'green wool' (skin infection)
	Snakes	Necrotic stomatitis and other necrotic lesions
	Swine	Respiratory infections, enteritis, otitis
	Lab. animals	Septicaemia, enteritis
Pseudomonas fluorescens	Fish	Septicaemia, tail/fin rot
	Poultry	Embryonic mortality
	Cattle	Mastitis
Stenotrophomonas maltophilia	Goats, sheep	Lymphadenitis, fleece rot in sheep
	Reptiles	Septicaemia, ulcerative stomatitis
	Cattle, swine	Diminished semen quality and viability, impaired fertilization and embryonic development in vitro
Burkholderia mallei	Horse	Glanders (chronic)
	Donkey	Glanders (acute)
	Dogs and cats	Glanders (acute)
Burkholderia pseudomallei	Cattle, dogs, cats, horse, sheep, goats, and swine	Melioidosis (pseudoglanders)

Many Gram-negative pathogens regulate virulence factor expression by using a cell density mechanism termed quorum sensing. *Burkholderia mallei* produces several acyl-homoserine lactones (acyl-HSLs) which serve as quorum-sensing signals (Ulrich et al. 2004). Genomic analysis of *B. mallei* has identified a number of putative virulence factors. The genome contains numerous insertion sequence elements and a vast number of simple sequence repeats. It is likely that variation in simple sequence repeats in key genes provide a mechanism for generating antigenic variation. This may account for the mammalian host's inability to build a durable adaptive immune response to *B. mallei* (Nierman et al. 2004). Mutagenesis experiments have shown that a functional type III secretion system is required for the full pathogenicity of *B. mallei* in animal models of infection (Ulrich & DeShazer 2004). A major function of this secretion system is to secrete virulence-associated proteins into target cells of the host organism.

Burkholderia pseudomallei is the causative agent of melioidosis or pseudoglanders. This pathogen is a facultative intracellular bacterium which has a wide host range, including humans, horses, sheep, goats, dogs, cats, cattle,

Table 18.2 Main virulence factors of *Pseudomonas aeruginosa*

Virulence determinants	Functions
Exotoxin A (ADP-ribosyl-transferase)	Cytotoxic, invasion of tissue and cellular damage, immunosuppressive action
Flagellum	Motility, adherence to mucin
Elastase (LasB and LasA)	Damage to tissues of the lungs and blood vessels
Alkaline protease	Tissue damage
Phospholipase C (haemolysin)	Tissue damage, stimulation of inflammatory mediators
Siderophores (pyoverdin, pyocyanin, pyochelin)	Iron uptake
Rhamnolipid (haemolysin with lecithinase activity)	Damage to host cell membranes and impaired mucociliary clearance
Type III secretion system (exoenzymes S, T, U and Y)	Damage to host tissues, cytotoxic, implicated in invasion process
Alginate-biofilm	Protection from phagocytosis, adhesin, antimicrobial resistance
LPS	Adherence to epithelial cells and invasion, resistance to phagocytosis, serum resistance, and production of proinflammatory cytokines
Pili	Adherence to epithelial cells and mucin

and pigs. Infections occur via contaminated food or water, from aerosols and contact with contaminated ground via skin abrasions or wounds. Transmission can also occur via arthropod bites. Zoonotic transmission has not been documented. However, cases of human-to-human transmission have been reported. In animals, infections are usually systemic and chronic but acute disease with terminal septicaemia may occur. The typical lesions are nodules which may suppurate and can form in any tissue, including the brain. The manifestations of the disease depend on the extent and distribution of the lesions in the animal. In humans, the clinical signs of melioidosis (also referred to as Whitmore's disease) vary greatly from an asymptomatic presentation to a fatal septicaemia. The incubation period is frequently prolonged and disease signs may appear months or even years after infection.

Burkholderia pseudomallei is motile via its flagella. It also produces several other potential virulent factors such as extracellular proteases, serine metalloprotease, haemolysin, lipase, lecithinase, endotoxin, lethal toxins, and surface capsule-like structures. The toxins include a lethal factor with anticoagulant activity and a skin-necrotizing proteolytic agent. Malleobactin is a siderophore involved in iron acquisition (Alice et al. 2006). Studies have indicated that virulence of selected *B. pseudomallei* isolates is variable and dependent on factors such as iron bioavailability, inoculum size and host risk factors (Ulett et al. 2001).

Stenotrophomonas maltophilia is also considered an opportunistic pathogen which can cause a variety of infections in veterinary medicine. In humans, it is considered as an emerging nosocomial bacterial pathogen which is being isolated with increasing frequency from the airways of cystic fibrosis patients. *Stenotrophomonas maltophilia*-associated infection is problematic because many strains of the bacterium are resistant to multiple antibiotics. Other virulence factors of note include the ability to form a biofilm, adherence and the ability to invade respiratory epithelial cells (Di Bonaventura et al. 2007).

Laboratory Diagnosis

Burkholderia mallei and *B. pseudomallei* are among the most dangerous bacteria to work with in a laboratory. A biohazard cabinet must be used and all other safety procedures employed according to biosafety level (BSL)-3 guidelines. *Pseudomonas* and *Stenotrophomonas* can be handled in a biosafety level-2 laboratory.

Specimens

These will be varied and will depend on the clinical signs and lesions. Standard collection and transport methods are sufficient to ensure the recovery of *Pseudomonas*, *Burkholderia* and *Stenotrophomonas* species.

Direct microscopy

Direct microscopy from specimens is of little diagnostic use as *Pseudomonas*, *Burkholderia* and *Stenotrophomonas* are medium-sized, Gram-negative rods with no other distinctive characteristics. However, direct microscopy of Gram-stained smears with *B. pseudomallei* will often reveal small Gram-negative bacilli with bipolar staining, 'safety pin' appearance. A fluorescent antibody technique may be useful for *B. mallei* and *B. pseudomallei* (Walsh et al. 1994).

Isolation

Pseudomonas, *Burkholderia* and *Stenotrophomonas* species are non-fastidious and will grow on trypticase soy agar, 5% blood agar, chocolate agar and on less complex media. The use of selective media will facilitate the recovery of

these bacteria from specimens with mixed flora. Commercial selective media are available for *P. aeruginosa* and usually contain cetrimide, acetamide, nitrofurantoin, or 9-chloro-9(4-diethylaminophenyl)-9,10-dihydro-10-phenylacridine hydrochloride. *Pseudomonas aeruginosa* will also grow on many of the selective media intended for the *Enterobacteriaceae* such as MacConkey, brilliant green and XLD agars. MacConkey agar is considered a useful selective medium for the recovery of most of the *Pseudomonas* species. Members of the *Pseudomonas*, *Burkholderia* and *Stenotrophomonas* genera grow in broth blood culture systems within the five-day standard incubation guideline. *Burkholderia* species will also grow on MacConkey agar, with the exception of *B. mallei*. However, selective media which inhibit the growth of *P. aeruginosa* is recommanded for the recovery of *B. pseudomallei* (Ashdown agar or broth with colistin) and *B. cepacia* (PC, OFPBL, and BCSA agars). The growth of *B. mallei* is enhanced by 1% glycerol. A selective medium for *B. mallei* can be made by adding 1000 units polymyxin E, 1250 units bacitracin and 0.25 mg actidione to 100 mL of trypticase soy agar. The cultures for *P. aeruginosa*, *B. pseudomallei* and *B. mallei* are incubated aerobically at 35–37°C for 24–48 hours. Some of the saprophytic *Pseudomonas* species, such as *P. fluorescens* grow extremely poorly, or not at all, at 37°C and 30°C is often the upper temperature limit of their growth range.

Identification

Colonial morphology

The colonies of *P. aeruginosa* are large (3–4 mm), flat, spreading, greenish-blue with a serrated edge and a characteristic fruity, grape-like odour of aminoacetophenone. Colonial variation includes smooth, soft and shiny (S-forms), dwarf, dry and granular (R-forms) not unlike some colonies of *Bacillus* species, and mucoid (M-forms) that are frequently biochemically atypical. Most strains give a clear zone of haemolysis on blood agar (Fig. 18.1). Pyocyanin, a bluish pigment unique to *P. aeruginosa*, gives the blue-green colour associated with many cultures. Some strains have colonies with a distinctive metallic sheen (Fig. 18.2). *Pseudomonas aeruginosa* produces large, pale colonies on MacConkey agar (unable to utilize lactose) with greenish-blue pigment superimposed (Fig. 18.3). Red colonies and medium, indicative of an alkaline reaction, are seen on brilliant green (Fig. 18.2) and XLD agars. No H_2S is produced on XLD medium. Strains of *P. aeruginosa* produce the water-soluble diffusible pigments pyocyanin (blue, phenazine pigment), pyoverdin (water-soluble yellow-green or yellow-brown pigment), pyorubin (red) and pyomelanin (dark brown) (Fig. 18.4) in varying combinations and amounts. When pyoverdin is combined with pyocyanin, the bright green colour characteristic of *P. aeruginosa* is expressed. Some strains produce all four pigments. Pyorubin and pyomelanin are less commonly produced, develop slowly and are seen best by growing the

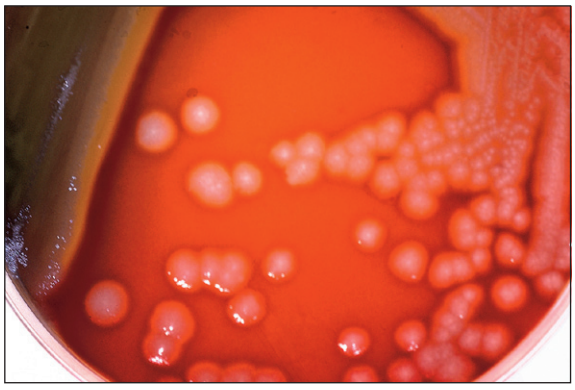

Figure 18.1 *Pseudomonas aeruginosa* on sheep blood agar showing large, flat, irregular-edged colonies resembling those of some *Bacillus* species. The green-blue pyocyanin pigment is most obvious in areas of heaviest growth.

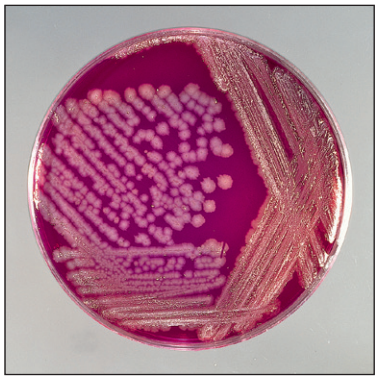

Figure 18.2 *Pseudomonas aeruginosa* on brilliant green agar where the alkaline reaction is similiar to that given by *Salmonella* species. The metallic sheen displayed by this strain is a feature of some isolates.

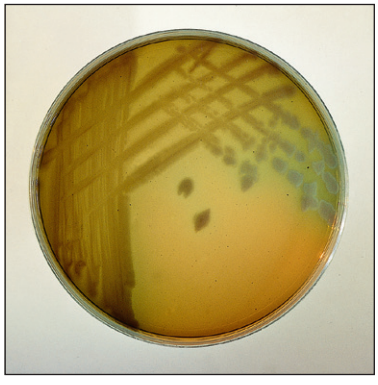

Figure 18.3 *Pseudomonas aeruginosa* on MacConkey agar. It has the pale colonies of a non-lactose-fermenter with green-blue pyocyanin pigment superimposed.

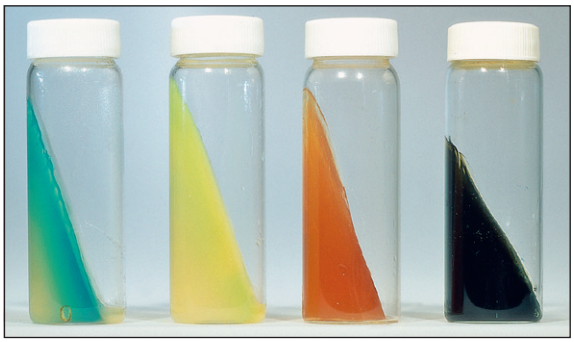

Figure 18.4 Pigments produced by *P. aeruginosa*. The nutrient agar slopes show from left: pyocyanin (blue-green), pyoverdin (greenish-yellow), pyorubin (red) and pyomelanin (dark brown).

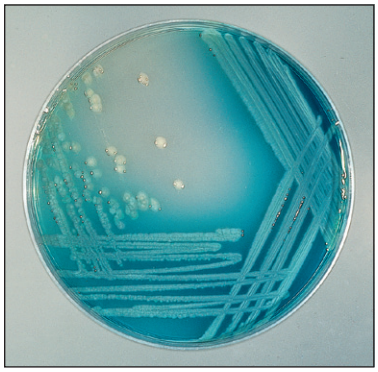

Figure 18.5 *Pseudomonas aeruginosa* on '*Pseudomonas* agar P'. This medium enhances the production of pyocyanin.

strains on nutrient agar slants at room temperature for up to two weeks. As pyocyanin is unique to *P. aeruginosa* this is an important diagnostic characteristic although strains vary in the amount of the pigment they produce. Media such as *Pseudomonas* agar P (BD Diagnostics) (Fig. 18.5) will enhance pyocyanin production and *Pseudomonas* agar F (BD Diagnostics) enhances pyoverdin production (Fig. 18.6). Pyoverdin, once called 'fluorescin', will fluoresce under ultra-violet light. *Pseudomonas aeruginosa* is classified as a member of the fluorescent pseudomonad group which produce pyoverdin. Some strains of *P. aeruginosa* do not produce pigments and are highly mucoid. These may also be atypical in certain biochemical reactions, making them difficult to identify.

Colonies of *Stenotrophomonas maltophilia* appear rough on sheep blood agar with a lavender-green colour and an ammonia-like odour. The colonial growth of *B. pseudomallei* varies from smooth and mucoid (first one to two days of incubation) to rough with a dull, wrinkled, corrugated surface (after a few days' incubation). In the smooth form the colonies are round, low-convex, entire, shiny and greyish-yellow. After several days the colonies become

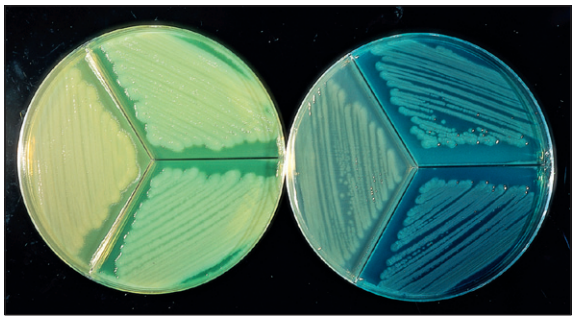

Figure 18.6 Three strains of *P. aeruginosa* on '*Pseudomonas* agar F' (left) and on '*Pseudomonas* agar P' (right). These media are used to enhance pigment production.

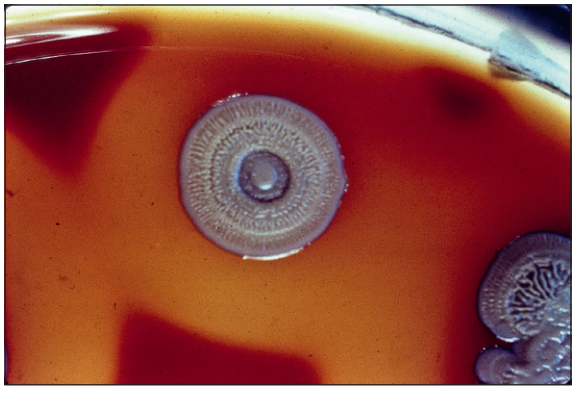

Figure 18.7 *Burkholderia pseudomallei*: smooth colonial form on sheep blood agar after several days' incubation. The colonies are smooth, glistening, opaque, yellowish-brown and umbonate with a zone of clear haemolysis.

opaque, yellowish-brown and umbonate (Fig. 18.7). The growth has a strong characteristic earthy or musty odour. Partial and later complete haemolysis occurs on sheep blood agar. *Burkholderia pseudomallei* grows on MacConkey agar, utilizing lactose (Fig. 18.8), but there is no growth on deoxycholate or Salmonella-Shigella (SS) agars. On Ashdown agar, colonies appear with a pink colour because of their neutral red absorption. Although *B. pseudomallei* is not a true biosafety level 3 microorganism, a biosafety cabinet should be used for manipulation. The growth of *B. mallei* is slower than that of *P. aeruginosa* and *B. pseudomallei* but in 24–48 hours the colonies are 1–2 mm in diameter, smooth and white to cream. As they age they become granular and yellowish or brown in colour. *Burkholderia mallei* is usually unable to grow on MacConkey agar.

Microscopic appearance

All the pseudomonads are medium-sized Gram-negative rods.

Figure 18.8 *Burkholderia pseudomallei* on MacConkey agar (*B. mallei* does not grow on this medium).

Biochemical characteristics

The characteristic colonial appearance, including pyocyanin production (or other pigments) and odour, and a strong oxidase reaction (members of the *Enterobacteriaceae* are oxidase-negative) are sufficient to give a presumptive identification of *P. aeruginosa*. Confident identification can be obtained with the addition of a triple sugar iron (TSI) agar reaction of alkaline over no change and growth at 42°C. Key biochemical tests for *P. aeruginosa* that do not produce pigments also include hydrolysis of acetamide and reduction of nitrates to nitrogen gas. Other characteristics of *Pseudomonas* species, *B. pseudomallei*, *B. mallei* and *S. maltophilia* are given in Table 18.3. *Burkholderia mallei* is the only non-motile species of this genus. Additional tests that can facilitate its identification include arginine dihydrolase activity, oxidation of glucose and failure to oxidize sucrose or maltose. *Stenotrophomonas maltophilia* is a glucose-oxidizer with positive reactions for DNase and lysine decarboxylase. DNase activity is a key test that can

Table 18.3 Presumptive identification of *Pseudomonas, Burkholderia and Stenotrophomonas species* of significance in veterinary medicine

Test[a]	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Stenotrophomonas maltophilia*	*Burkholderia mallei*	*Burkholderia pseudomallei*
Glucose fermented	–	–	–	–	–
Oxidase	+	+	–	v	+
Growth at 42°C	+	–	v	–	+
Motility	+	+	+	–	+
Urease	v	v	–	v	v
Growth on MacConkey	+	+	+	v–	+
Nitrate reduction	+	v	v	+	+
Gas from nitrate	+	–	–	–	+
Odour	Fruity, grape-like	–	Ammonia-like	–	Putrid becoming earthy
Pigment	+ (pyocyanin, pyoverdin, pyorubin, and pyomelanin)	+ (pyoverdin)	– but colonies become brown-tan, soluble	– but colonies are yellow to brown	– but colonies become orange to cream
Haemolysis	+	v	–	–	+
Acid from:					
Maltose	–	–	+	–	+
Mannitol	v	v	–	–	+

[a]+ = greater than or equal to 90% positive; – = less than or equal to 10% positive; v = variable, 11 to 89% positive

Table 18.4 Differentiation of *P. aeruginosa*, *S. maltophilia* and other saprophytic pseudomonads

Test[a]	*P. aeruginosa*	*P. fluorescens*	*P. putida*	*P. cepacia*	*P. stutzeri*	*S. maltophilia*
Pyoverdin produced	+	+	+	–[b]	–	–
Oxidase	+	+	+	+	+	–
Growth at 42°C	+	–	–	+	+	–
Growth at 5°C	–	+	+	–	–	+
Urease	+	+	+	+	+	–
Growth on MacConkey	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	–	+	–
Gelatin	+	+	–	+	–	+
Oxidation of:						
Glucose	+	+	+	+	+	–
Lactose	–	–	–	+	–	–
Maltose	–	–	–	+	+	+

[a]+ = greater than or equal to 90% positive; – = less than or equal to 10% positive; v = variable, 11 to 89% positive, [b]= some strains produce a yellowish non-fluorescent pigment

be detected on DNase plate medium with methyl green as an indicator. A positive result will produce a zone of clearing around the colonies. Commercial identification systems such as API 20NE or API 20E (bioMérieux), Microbact 24E strip (MedVet, Australia), VITEK-1 or VITEK-2 (bioMérieux), Microscan WalkAway (Dade International) can be used to identify some of the *Pseudomonas*, *Burkholderia* and *Stenotrophomonas* species. Table 18.4 highlights the reactions of *P. aeruginosa* and *S. maltophilia* and other saprophytic pseudomonads that may be isolated from clinical specimens.

Determination of pathogenicity

Pathogenicity determination can be achieved by intraperitoneal inoculation of male guinea pigs with infective material containing either *B. pseudomallei* or *B. mallei*. The Strauss reaction, which consists of a localized peritonitis and purulent inflammation of the testicular tunica vaginalis, develops in two to three days.

Serology and immunological tests

Both cell-mediated and antibody-mediated responses are elicited by infection with *B. mallei*. Complement fixation (a prescribed test for international trade), agglutination, enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination, counter-immunoelectrophoresis, Rose Bengal plate agglutination test, and microarray-based tests can be used in the diagnosis of glanders (Neubauer et al. 2005). False-positive reactions may occur in areas where melioidoisis is endemic as the serological tests detect antibodies that cross-react with those of *B. pseudomallei*.

The mallein test, a prescribed test for international trade, is used to demonstrate the hypersensitivity developed after infection with *B. mallei*. Mallein is a glycoprotein extracted from the bacterium. In infected animals, subcutaneous inoculation of mallein (subcutaneous test) results in swelling at the injection site and fever; instillation of mallein into the conjunctival sac (ophthalmic test) is followed in 6–12 hours by an inflammatory and purulent reaction in the eye; inoculation of a small amount of mallein into the skin of the lower eyelid (intrapalpebral test) gives a localized, oedematous swelling and purulent conjunctivitis. Healed lesions of the nasal mucosa are activated in glanderous animals after mallein tests and this can be a useful diagnostic feature. In many countries such as the United States, where the complement fixation test is the official test for glanders for importation of horses, the mallein test is performed on those animals that have anti-complementary sera.

Complement-fixing, solid-phase radioimmunoassay and indirect haemagglutinating (IHA) antibodies are produced after infection with *B. pseudomallei*. The IHA assay can be used in endemic regions and can be adapted for a microtiter plate test. Cross-reactions with other organisms such as *B. cepacia* have been observed. However, the diagnosis of melioidosis depends more on the isolation and

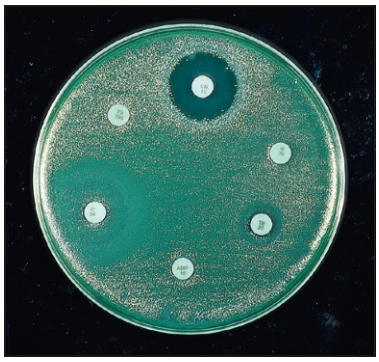

Figure 18.9 *Pseudomonas aeruginosa* on Isosensitest agar demonstrating the characteristic multiple resistance to antibiotics.

identification of the bacterium than on clinical findings and serological tests.

For both *B. mallei* and *B. pseudomallei*, immunohistochemistry performed on fixed tissue or biopsies and indirect fluorescent antibody assays on various specimens may be useful.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is necessary for *P. aeruginosa* isolates as multiple drug resistance (Fig. 18.9) is frequently encountered with this bacterium. Broth microdilution and disk diffusion are the reference methods for antimicrobial susceptibility testing of *P. aeruginosa*. Studies have shown that the results of both the E test (AB BioDisk) and disk diffusion correlate well with those of the microbroth dilution MIC test and the agar dilution MIC test (Burns et al. 2000). A rapid colorimetric assay for determining the susceptibility of *P. aeruginosa* to bactericidal antibiotics which is based on the reduction of a tetrazolium salt has been described (Tunney et al. 2004). There have been concerns about the accuracy of commercial automated systems such as MicroScan WalkAway, VITEK, VITEK 2 and Micronaut Merlin automated broth microtitre system (Balke et al. 2004, Sader et al. 2006). There is no obvious explanation for the discrepancy between the results. Quality control (QC) standards and determination of susceptible/resistance breakpoints should be according to procedures established by the Clinical and Laboratory Standards Institute (CLSI documents M31-A3, M07-A9, M02-A11, M100-S22; CLSI 2008, 2012a, 2012b, 2012c).

Combination antimicrobial susceptibility testing assesses the efficacy of drug combinations in vitro (double-disc diffusion, broth microdilution (checkerboard synergy test) and time-kill testing methods. It may demonstrate antimicrobial efficacy against bacterial isolates of *P. aeruginosa* or *S. maltophilia* even when individual antibiotics have little or no effect (Krueger et al. 2001, Waters & Ratjen 2008).

Specific interpretative guidelines are currently not available for the antimicrobial susceptibility testing of *Burkholderia* and *Stenotrophomonas* species. The guidelines used for *P. aeruginosa* or *Acinetobacter* species are usually applied to those species in accordance with CLSI recommendations (NCCLS Approved standard M7-A5, NCCLS Approved standard M100-S12; Traub et al. 1998). MIC microbroth assays or E tests are reported to give the most reliable antimicrobial susceptibility results for *B. pseudomallei* (Jenney et al. 2001), while the disk diffusion method is not recommended (Lumbiganon et al. 2000). For *B. mallei*, the E test usually gives lower MICs than the broth dilution (Heine et al. 2001). For *S. maltophilia*, the microbroth dilution assay, the E test or the agar dilution method is preferred over the disk diffusion test (Yao et al. 1995). However, difficulties have been reported with all of these methods (Nicodemo et al. 2004).

Antimicrobial resistance

Pseudomonas aeruginosa is usually susceptible (>75% in North America) to anti-pseudomonad penicillins (such as piperacillin and piperacillin-tazobactam), aminoglycosides (such as amikacin and tobramycin), cefepime, ceftazidime, ciprofloxacin, meropenem and imipenem (Versalovic 2011). Studies have shown that both aminoglycosides and fluoroquinolones can be synergistic when tested in combination with beta-lactams or carbapenems (Westbrock-Wadman et al. 1999, Pai et al. 2001). *Pseudomonas aeruginosa* is resistant to penicillins, ampicillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, tetracyclines, macrolides, rifampin, chloramphenicol, trimethoprim-sulfamethoxazole, narrow- and extended-spectrum cephalosporins and oral broad-spectrum cephalosporins such as cefixime and cefpodoxime (Versalovic 2011). A study performed on strains of *P. aeruginosa* isolated from different pathological specimens originating from dogs during the years from 1993 to 2000 revealed that besides imipenem, the quinolone antibiotics, marbofloxacin and ciprofloxacin were the most effective (Seol et al. 2002). It is reported that resistance may occur during treatment, particularly if monotherapy has been used. Resistance to beta-lactams, carbapenems, aminoglycosides and fluoroquinolones can be attributed to many different mechanisms such as mutation in the *gyrA* gene or in genes encoding porins, penicillin-binding proteins, efflux pumps (overexpression of efflux pump) and chromosomal encoded beta-lactamases (Westbrock-Wadman et al. 1999, Pai et al. 2001, Livermore 2002, Teresa Tejedor et al. 2003). *P. aeruginosa* isolates from various animal sources expressed the MexAB-OprM efflux system, the MexEF-OprN or the MexXY-OprM systems (Beinlich et al. 2001). These efflux pumps, including also the MexCD-OprJ, play a major role in antibiotic extrusion and resistance (Sugimura et al. 2008). In addition to antibiotics, these pumps promote export of numerous dyes,

detergents, inhibitors, disinfectants, organic solvents and homoserine lactones involved in quorum sensing (Poole 2001, Papadopoulos et al. 2008).

Burkholderia mallei has a similar antimicrobial susceptibility range to that of *B. pseudomallei* with the exception of its susceptibility to aminoglycosides. Antimicrobial susceptibilities have also been reported to ceftazidime, imipenem, ciprofloxacin, doxycycline and piperacillin while resistance to most penicillins, cephalosporines, rifampin and chloramphenicol is common (Kenny et al. 1999, Heine et al. 2001). *B. mallei* is also usually susceptible to sulphonamides and tetracyclines. However, affected horses are not usually treated since eradication has been found to be most effective.

Burkholderia pseudomallei is usually susceptible in vitro to ceftazidime, cefoperazone, amoxicillin-clavulanic acid, ampicillin-sulbactam, chloramphenicol and doxycycline but resistant to aminoglycosides (Versalovic 2011). Variable antimicrobial susceptibility results can be obtained with fluoroquinolones and trimethoprim-sulfamethoxazole (Kenny et al. 1999 Jenney et al. 2001).

Stenotrophomonas maltophilia is intrinsically resistant to many antimicrobial agents and resistance can develop quickly (Garrison et al. 1996). Trimethoprim-sulfamethoxazole is usually active againts *S. maltophilia* and can be used in combination with ticarcillin-clavulanic acid or minocycline. Aminoglycoside and fluoroquinolone resistances are reported to be due to mutations in the outer membrane proteins. Resistance to beta-lactams is mediated by beta-lactamases.

Strain Typing

Molecular methods, including ribotyping (Rivas et al. 2001) and pulsed-field gel electrophoresis (Las Heras et al. 2002), have replaced the conventional serotyping, antibiograms, phage typing, bacteriocin typing and biotyping methods for strain typing of *P. aeruginosa*. The PCR-based techniques such as randomly amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus PCR are usually sufficiently discriminatory to study the clonal relationship between strains of *P. aeruginosa* (Lau et al. 1995, Pujana et al. 2000, Wolska and Szweda 2008). *Burkholderia mallei* strains have been characterized using RAPD and multilocus sequence typing (MLST). RAPD was considered the best method used for detecting strain differences of *B. mallei*. Pulsed-field gel electrophoresis and ribotyping have been used to study strain relatedness among *B. pseudomallei* (Lew & Desmarchelier 1993, Ko et al. 2007) and *S. maltophilia* isolates (Bingen et al. 1994, Van Couwenbergh and Cohen 1994).

Molecular Diagnosis

Pseudomonas aeruginosa, *B. mallei*, *B. pseudomallei* and *S. maltophilia* can all be identified using molecular-based techniques. The most widely used PCR primers for *P. aeruginosa* are based on *gyrB*, *toxA*, 16S–23S rDNA internal transcribed spacer, 16S rDNA, *oprI*, *oprL*, *algD GDP* mannose and *fliC* genes; some of these PCR assays are also able to detect *P. aeruginosa* directly in clinical samples (Tyler et al. 1995, da Silva Filho et al. 1999, Spilker et al. 2004, Kurupati et al. 2005). A PCR assay targeting the *ecfX* gene was recently developed due to false-positive results obtained with some of the above PCR assays (Lavenir et al. 2007). A diagnostic multiplex PCR assay has been used for the identification of specific epidemic strains of *P. aeruginosa* with excellent specificity and sensitivity (Fothergill et al. 2008). Real-time protocols suitable for the detection of *P. aeruginosa* in clinical samples have been described (Jaffe et al. 2001, Qin et al. 2003).

Two real-time PCR assays for the rapid and specific identification of *B. mallei* have been developed (Tomaso et al. 2006, Ulrich et al. 2006). *Burkholderia pseudomallei* and *B. mallei* can be identified and discriminated using a multiplex PCR (Lee et al. 2005). This assay has been proposed for epidemiological typing of *B. pseudomallei* and *B. mallei* strains. A real-time PCR for the identification and discrimination of *B. pseudomallei*, *B. mallei* and *B. thailandensis* has also been developed which utilized the uneven distribution of type III secretion system genes among these three species (Thibault et al. 2004). A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to differentiate between *B. mallei* and *B. pseudomallei* has also been described (Tanpiboonsak et al. 2004). The assay employs digestion with Sau3AI to facilitate a more reliable and rapid identification of the two species.

Two TaqMan real-time PCR assays to detect the presence of two genes unique to *B. pseudomallei* have been evaluated (Supaprom et al. 2007). Identification of *B. pseudomallei* and related bacteria by multiple-locus sequence typing-derived PCR and real-time PCR has been described as a robust and appropriate assay for general detection as well as for species identification purposes (Wattiau et al. 2007). An evaluation of three PCR-based methods (seminested PCR protocol for the 16–23s spacer region, *lpxO* gene and *phaC* gene) for *B. pseudomallei* identification revealed that single PCR targets should be used with caution (Merritt et al. 2006). Optimized PCR protocols for fast and reliable detection of *B. pseudomallei* DNA in paraffin wax embedded tissues have also been published (Hagen et al. 2002). It has been proposed to use this method for retrospective histopathological investigations.

Identification of *S. maltophilia* can be achieved by species-specific PCR using two primers specific for the 23S rRNA gene (Giordano et al. 2006). The detection of *S. maltophilia* directly from clinical samples by another rRNA-directed PCR assay is based on primers directed against the 23S rRNA gene (Whitby et al. 2000). A multiplex PCR method has been developed to identify *P. aeruginosa*, *B. cepacia* complex, and *S. maltophilia* directly in clinical specimens (da Silva Filho et al. 2004).

REFERENCES

Alice, A.F., Lopez, C.S., Lowe, C.A., et al., 2006. Genetic and transcriptional analysis of the siderophore malleobactin biosynthesis and transport genes in the human pathogen *Burkholderia pseudomallei* K96243. Journal of Bacteriology 188, 1551–1566.

Althouse, G.C., Lu, K.G., 2005. Bacteriospermia in extended porcine semen. Theriogenology 63, 573–584.

Balke, B., Hoy, L., Weissbrodt, H., et al., 2004. Comparison of the Micronaut Merlin automated broth microtiter system with the standard agar dilution method for antimicrobial susceptibility testing of mucoid and non-mucoid *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. European Journal of Clinical Microbiology & Infectious Diseases 23, 765–771.

Beinlich, K.L., Chuanchuen, R., Schweizer, H.P., 2001. Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of *Pseudomonas aeruginosa*. FEMS Microbiology Letters 198, 129–134.

Berriatua, E., Ziluaga, I., Miguel-Virto, C., et al., 2001. Outbreak of subclinical mastitis in a flock of dairy sheep associated with *Burkholderia cepacia* complex infection. Journal of Clinical Microbiology 39, 990–994.

Bielanski, A., Bergeron, H., Lau, P.C., et al., 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 46, 146–152.

Bingen, E.H., Denamur, E., Elion, J., 1994. Use of ribotyping in epidemiological surveillance of nosocomial outbreaks. Clinical Microbiology Reviews 7, 311–327.

Burns, J.L., Saiman, L., Whittier, S., et al., 2000. Comparison of agar diffusion methodologies for antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Journal of Clinical Microbiology 38, 1818–1822.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard, third ed. CLSI document M31-MA3. Clinical and Laboratory Standards Institute, Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Ninth Edition. CLSI document M07-MA9, Clinical and Laboratory Standards Institute, Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard – Eleventh Edition. CLSI document M02-A11, Clinical and Laboratory Standards Institute, Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-MS22, Clinical and Laboratory Standards Institute, Wayne, PA.

da Silva Filho, L.V., Levi, J.E., Oda Bento, C.N., et al., 1999. PCR identification of *Pseudomonas aeruginosa* and direct detection in clinical samples from cystic fibrosis patients. Journal of Medical Microbiology 48, 357–361.

da Silva Filho, L.V., Tateno, A.F., Velloso Lde, F., et al., 2004. Identification of *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia* in respiratory samples from cystic fibrosis patients using multiplex PCR. Pediatric Pulmonology 37, 537–547.

Denton, M., Kerr, K.G., 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clinical Microbiology Reviews 11, 57–80.

DeShazer, D., Waag, D.M., Fritz, D.L., et al., 2001. Identification of a Burkholderia mallei polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microbial Pathogenisis 30, 253–269.

Di Bonaventura, G., Prosseda, G., Del Chierico, F., et al., 2007. Molecular characterization of virulence determinants of *Stenotrophomonas maltophilia* strains isolated from patients affected by cystic fibrosis. International Journal of Immunopathology and Pharmacology 20, 529–537.

Edmond, K.M., Bauert, P., Currie, B.J., 2001. Paediatric melioidosis in the Northern Territory of Australia: an expanding clinical spectrum. Journal of Paediatric Child Health 37, 337–341.

Fothergill, J.L., Upton, A.L., Pitt, T.L., et al., 2008. Diagnostic multiplex PCR assay for the identification of the Liverpool, Midlands 1 and Manchester CF epidemic strains of Pseudomonas aeruginosa. J Cyst Fibros 7, 258–261.

Garrison, M.W., Anderson, D.E., Campbell, D.M., et al., 1996. *Stenotrophomonas maltophilia:* emergence of multidrug-resistant strains during therapy and in an in vitro pharmacodynamic chamber model. Antimicrobial Agents and Chemotherapy 40, 2859–2864.

Giordano, A., Magni, A., Trancassini, M., et al., 2006. Identification of respiratory isolates of *Stenotrophomonas maltophilia* by commercial biochemical systems and species-specific PCR. Journal of Microbiological Methods 64, 135–138.

Hagen, R.M., Gauthier, Y.P., Sprague, L.D., et al., 2002. Strategies for PCR based detection of *Burkholderia pseudomallei DNA* in paraffin wax embedded tissues. Molecular Pathology 55, 398–400.

Heine, H.S., England, M.J., Waag, D.M., et al., 2001. In vitro antibiotic susceptibilities of *Burkholderia mallei* (causative agent of glanders) determined by broth microdilution and E-test. Antimicrobial Agents and Chemotherapy 45, 2119–2121.

Hejnar, P., Bardon, J., Sauer, P., et al., 2007. *Stenotrophomonas maltophilia* as

a part of normal oral bacterial flora in captive snakes and its susceptibility to antibiotics. Veterinary Microbiology 121, 357–362.

Jackson, M.K., Phillips, S.N., 1996. Necrotizing hepatitis in pet birds associated with Pseudomonas fluorescens. Avian Diseases 40, 473–476.

Jaffe, R.I., Lane, J.D., Bates, C.W., 2001. Real-time identification of *Pseudomonas aeruginosa* direct from clinical samples using a rapid extraction method and polymerase chain reaction (PCR). Journal of Clinical Laboratory Analysis 15, 131–137.

Jenney, A.W., Lum, G., Fisher, D.A., et al., 2001. Antibiotic susceptibility of *Burkholderia pseudomallei* from tropical northern Australia and implications for therapy of melioidosis. The International Journal of Antimicrobial Agents 17, 109–113.

Kenny, D.J., Russell, P., Rogers, D., et al., 1999. In vitro susceptibilities of *Burkholderia mallei* in comparison to those of other pathogenic Burkholderia spp. Antimicrobial Agents and Chemotherapy 43, 2773–2775.

Ko, W.C., Cheung, B.M., Tang, H.J., et al., 2007. Melioidosis outbreak after typhoon, southern Taiwan. Emerg Infect Dis 13, 896–898.

Krueger, T.S., Clark, E.A., Nix, D.E., 2001. In vitro susceptibility of *Stenotrophomonas maltophilia* to various antimicrobial combinations. Diagnostic Microbiology and Infectious Disease 41, 71–78.

Kurupati, P., Kumarasinghe, G., Laa Poh, C., 2005. Direct identification of *Pseudomonas aeruginosa* from blood culture bottles by PCR-enzyme linked immunosorbent assay using oprI gene specific primers. Molecular and Cellular Probes 19, 417–421.

Las Heras, A., Vela, A.I., Fernandez, E., et al., 2002. DNA macrorestriction analysis by pulsed-field gel electrophoresis of *Pseudomonas aeruginosa* isolates from mastitis in dairy sheep. Veterinary Record 151, 670–672.

Lavenir, R., Jocktane, D., Laurent, F., et al., 2007. Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species-specific ecfX gene target. Journal of Microbiological Methods 70, 20–29.

Lau, Y.J., Liu, P.Y., Hu, B.S., et al., 1995. DNA fingerprinting of *Pseudomonas aeruginosa* serotype O11 by enterobacterial repetitive intergenic consensus-polymerase chain reaction and pulsed-field gel electrophoresis. Journal of Hospital Infection 31, 61–66.

Lee, M.A., Wang, D., Yap, E.H., 2005. Detection and differentiation of *Burkholderia pseudomallei, Burkholderia mallei* and *Burkholderia thailandensis* by multiplex PCR. FEMS Immunology and Medical Microbiology 43, 413–417.

Lew, A.E., Desmarchelier, P.M., 1993. Molecular typing of *Pseudomonas pseudomallei:* restriction fragment length polymorphisms of rRNA genes. Journal of Clinical Microbiology 31 (3), 533–539.

Livermore, D.M., 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa:* our worst nightmare? Clinical Infectious Diseases 34, 634–640.

Lumbiganon, P., Tattawasatra, U., Chetchotisakd, P., et al., 2000. Comparison between the antimicrobial susceptibility of *Burkholderia pseudomallei* to trimethoprim-sulfamethoxazole by standard disk diffusion method and by minimal inhibitory concentration determination. Journal of the Medical Assocication of Thailand 83, 856–860.

Matchett, C.A., Dillehay, D.L., Goodman, M.M., et al., 2003. Postanesthesia death and suspected peracute endotoxic shock due to *Pseudomonas putida* in a cynomolgous macaque (Macaca fascicularis). Comparitive Medicine 53, 309–312.

Merritt, A., Inglis, T.J., Chidlow, G., et al., 2006. PCR-based identification of *Burkholderia pseudomallei*. Revista do Instituto de Medicina Tropical de São Paulo 48, 239–244.

Neubauer, H., Sprague, L.D., Zacharia, R., et al., 2005. Serodiagnosis of *Burkholderia mallei* infections in horses: state-of-the-art and perspectives. Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health 52, 201–205.

Nicodemo, A.C., Araujo, M.R., Ruiz, A.S., et al., 2004. In vitro susceptibility of *Stenotrophomonas maltophilia* isolates: comparison of disc diffusion, Etest and agar dilution methods. Journal of Antimicrobial Chemotherapy 53, 604–608.

Nierman, W.C., DeShazer, D., Kim, H.S., et al., 2004. Structural flexibility in the *Burkholderia mallei* genome. Proceedings of the National Academy of Sciences USA 101, 14246–14251.

Pai, H., Kim, J., Kim, J., et al., 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrobial Agents and Chemotherapy 45, 480–484.

Papadopoulos, C.J., Carson, C.F., Chang, B.J., et al., 2008. Role of the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* in tolerance to tea tree (Melaleuca alternifolia) oil and its monoterpene components terpinen-4-ol, 1,8-cineole, and alpha-terpineol. Applied and Environmental Microbiology 74, 1932–1935.

Poole, K., 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. Journal of Molecular Microbiology and Biotechnology 3, 255–264.

Pujana, I., Gallego, L., Canduela, M.J., et al., 2000. Specific and rapid identification of multiple-antibiotic resistant *Pseudomonas aeruginosa* clones isolated in an intensive care unit. Diagnostic Microbiology and Infectious Diseases 36 (1), 65–68.

Qin, X., Emerson, J., Stapp, J., et al., 2003. Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other non-fermenting Gram-negative bacilli from patients with cystic fibrosis. Journal of Clinical Microbiology 41, 4312–4317.

Rivas, A.L., Bodis, M., Bruce, J.L., 2001. Molecular epidemiologic features and antimicrobial susceptibility profiles of various ribotypes of *Pseudomonas aeruginosa* isolated from humans and ruminants. American

Journal of Veterinary Research 62, 864–870.

Sader, H.S., Fritsche, T.R., Jones, R.N., 2006. Accuracy of three automated systems (MicroScan WalkAway, VITEK, and VITEK 2) for susceptibility testing of *Pseudomonas aeruginosa* against five broad-spectrum beta-lactam agents. Journal of Clinical Microbiology 44, 1101–1104.

Sadovskaya, I., Brisson, J.R., Thibault, P., et al., 2000. Structural characterization of the outer core and the O-chain linkage region of lipopolysaccharide from *Pseudomonas aeruginosa* serotype O5. European Journal of Biochemistry 267, 1640–1650.

Sakai, M., Atsuta, S., Kobayashi, M., 1989. *Pseudomonas fluorescens* isolated from the diseased rainbow trout, *Oncorhynchus mykiss*. Kitasato Archives of Experimental Medicine 62, 157–162.

Seol, B., Naglic, T., Madic, J., et al., 2002. In vitro antimicrobial susceptibility of 183 *Pseudomonas aeruginosa* strains isolated from dogs to selected antipseudomonal agents. Journal of Veterinary Medicine B: Infectious Diseases and Veterinary Public Health 49, 188–192.

Spilker, T., Coenye, T., Vandamme, P., et al., 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. Journal of Clinical Microbiology 42, 2074–2079.

Sugimura, M., Maseda, H., Hanaki, H., et al., 2008. Macrolide Antibiotic-Mediated Down Regulation of MexAB-OprM Efflux Pump Expression in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 52 (11), 4141–4144.

Supaprom, C., Wang, D., Leelayuwat, C., et al., 2007. Development of real-time PCR assays and evaluation of their potential use for rapid detection of *Burkholderia pseudomallei* in clinical blood specimens. Journal of Clinical Microbiology 45, 2894–2901.

Swain, P., Behura, A., Dash, S., et al., 2007. Serum antibody response of Indian major carp, *Labeo rohita* to three species of pathogenic bacteria; Aeromonas hydrophila, Edwardsiella tarda and *Pseudomonas fluorescens*. Veterinary Immunology and Immunopathology 117, 137–141.

Tanpiboonsak, S., Paemanee, A., Bunyarataphan, S., et al., 2004. PCR-RFLP based differentiation of Burkholderia mallei and *Burkholderia pseudomallei*. Molecular and Cellular Probes 18, 97–101.

Teresa Tejedor, M., Martín, J.L., Navia, M., et al., 2003. Mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from canine infections. Veterinary Microbiology 94 (4), 295–301.

Thibault, F.M., Valade, E., Vidal, D.R., 2004. Identification and discrimination of *Burkholderia pseudomallei, B. mallei,* and *B. thailandensis* by real-time PCR targeting type III secretion system genes. Journal of Clinical Microbiology 42, 5871–5874.

Tomaso, H., Scholz, H.C., Al Dahouk, S., et al., 2006. Development of a 5′-nuclease real-time PCR assay targeting fliP for the rapid identification of *Burkholderia mallei* in clinical samples. Clinical Chemistry 52, 307–310.

Traub, W.H., Leonhard, B., Bauer, D., 1998. Antibiotic susceptibility of *Stenotrophomonas (Xanthomonas) maltophilia:* comparative (NCCLS criteria) evaluation of antimicrobial drugs with the agar dilution and the agar disk diffusion (Bauer–Kirby) tests. Chemotherapy 44, 164–173.

Tunney, M.M., Ramage, G., Field, T.R., et al., 2004. Rapid colorimetric assay for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 48, 1879–1881.

Tyler, S.D., Strathdee, C.A., Rozee, K.R., et al., 1995. Oligonucleotide primers designed to differentiate pathogenic pseudomonads on the basis of the sequencing of genes coding for 16S-23S rRNA internal transcribed spacers. Clinical and Diagnostic Laboratory Immunology 2, 448–453.

Ulett, G.C., Currie, B.J., Clair, T.W., et al., 2001. *Burkholderia pseudomallei* virulence: definition, stability and association with clonality. Microbes and Infection 3, 621–631.

Ulrich, R.L., DeShazer, D., 2004. Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*. Infection and Immunity 72, 1150–1154.

Ulrich, M.P., Norwood, D.A., Christensen, D.R., 2006. Using real-time PCR to specifically detect *Burkholderia mallei*. Journal of Medical Microbiology 55, 551–559.

Ulrich, R.L., Deshazer, D., Hines, H.B., 2004. Quorum sensing: a transcriptional regulatory system involved in the pathogenicity of *Burkholderia mallei*. Infection and Immunity 72, 6589–6596.

Van Couwenbergh, C., Cohen, S., 1994. Analysis of epidemic and endemic isolates of *Xanthomonas maltophilia* by contour-clamped homogeneous electric field gel electrophoresis. Infection Control and Hospital Epidemiology 15, 691–696.

Versalovic, J. (Ed.), 2011. American Society for Microbiology: Manual of Clinical Microbiology, tenth ed, Volume 1, ASM Press, Washington DC, pp. 692–713.

Walsh, A.L., Smith, M.D., Wuthiekanun, V., et al., 1994. Immunofluorescence microscopy for the rapid diagnosis of melioidosis. Journal of Clinical Pathology 47, 377–379.

Waters, V., Ratjen, F., 2008. Combination antimicrobial susceptibility testing for acute exacerbations in chronic infection of *Pseudomonas aeruginosa* in cystic fibrosis. Cochrane Database Systematic Reviews:CD006961.

Wattiau, P., Van Hessche, M., Neubauer, H., et al., 2007. Identification of *Burkholderia pseudomallei* and related bacteria by multiple-locus sequence typing-derived PCR and real-time PCR. Journal of Clinical Microbiology 45, 1045–1048.

Westbrock-Wadman, S., Sherman, D.R., Hickey, M.J., et al., 1999. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrobial Agents and Chemotherapy 43, 2975–2983.

Whitby, P.W., Carter, K.B., Burns, J.L., et al., 2000. Identification and detection of *Stenotrophomonas maltophilia* by rRNA-directed PCR.

Journal of Clinical Microbiology 38, 4305–4309.

Wolska, K., Szweda, P., 2008. A comparative evaluation of PCR ribotyping and ERIC PCR for determining the diversity of clinical *Pseudomonas aeruginosa* isolates. Polish Journal of Microbiology 57, 157–163.

Yao, J.D., Louie, M., Louie, L., et al., 1995. Comparison of E test and agar dilution for antimicrobial susceptibility testing of *Stenotrophomonas (Xanthomonas) maltophilia*. Journal of Clinical Microbiology 33, 1428–1430.

FURTHER READING

Larsen, J.C., Johnson, N.H., 2009. Pathogenesis of *Burkholderia pseudomallei* and *Burkholderia mallei*. Military Medicine 174 (6), 647–651.

Chapter 19

Aeromonas, *Plesiomonas* and *Vibrio* species

Genus Characteristics

The genus *Aeromonas* is a member of the family *Aeromonadaceae*, in the class *Proteobacteria*. The genus has undergone a number of nomenclatural revisions in recent years and there are now 30 recognized species in the genus *Aeromonas*. Both *A. hydrophila* and *A. salmonicida*, two of the main pathogens of veterinary interest in the genus, have five subspecies. *Plesiomonas shigelloides* is the only member of the genus. There are numerous members of the genus *Vibrio*, which is in the family *Vibronaaceae*, including many recently described marine vibrios. Members of the *Aeromonas*, *Plesiomonas* and *Vibrio* genera are Gram-negative rods (0.5–0.8 × 3.0–4.0 µm) which are either straight or curved. They are facultative anaerobes, catalase-positive and most are motile by polar flagella. *Aeromonas salmonicida* and other psychrophilic aeromonads are non-motile. All ferment glucose with acid production and a few *Aeromonas* spp. also produce gas. Many of the *Vibrio* spp. require sodium chloride for growth. *Plesiomonas* lacks exoenzymes whereas *Vibrio* and *Aeromonas* species produce diastase, lipase, DNase and various proteinases. Most of the species in the three genera will grow on common laboratory media at 35–37°C, although many of the saprophytic *Vibrio* and *Aeromonas* species have an optimum temperature for growth lower than 35°C.

Natural Habitat

Many of the species in the three genera are free-living saprophytes although some are associated with reptiles, fish and animals.

Aeromonas spp. are widespread in freshwater, sewage and soil. Their numbers rise with the amount of organic matter present. *Aeromonas hydrophila* is part of the normal flora of freshwater fish and is commonly present in fish ponds and tanks. Animals can be faecal carriers of *Aeromonas* spp.

Plesiomonas shigelloides is present in freshwater but its distribution is limited by its 8°C minimum temperature for growth and lack of halophilism. It has been isolated from a wide host range that includes freshwater fish, shellfish, oysters, toads, snakes, monkeys, dogs, cats, goats, pigs, cattle and poultry.

Vibrio spp. can be present in both fresh- and seawater as well as in the alimentary tracts of animals and man.

Pathogenesis and Pathogenicity

Aeromonas hydrophila is an opportunistic pathogen causing disease in fish and reptiles with rare reports of infections in mammals. The organism is important in fish suffering from stress or weakened by the presence of other diseases. As it is ubiquitous in aquatic environments, its importance as a secondary invader is not surprising when husbandry conditions in aquaculture are suboptimal. *Aeromonas hydrophila* produces a range of putative virulence factors including adhesins, exoenzymes, haemolysins, enterotoxins and an acyltransferase toxin secreted by a type III secretion system (Yu et al. 2004, Li et al. 2011). *Aeromonas hydrophila* can occasionally cause infections in humans that range from wound infections to septicaemia to self-limiting diarrhoea in children, as well as food poisoning. There are apparent differences between environmental and human clinical isolates but environmental isolates possess a wide range of virulence factors also and further studies are required to clarify their roles (Aguilera-Arreola et al. 2005). *Aeromonas salmonicida* is an obligate parasite of salmonid fish, causing furunculosis. As with *A. hydrophila* infections, acute or chronic stress is important and may trigger inapparent infections to become clinical. Virulence

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Table 19.1 Diseases and species affected by the principal pathogens in the genera *Aeromonas, Plesiomonas* and *Vibrio*

Genus and species	Animal species affected	Disease
Aeromonas hydrophila	Frogs	'Red-leg disease'
	Reptiles	Necrotic stomatitis in snakes, septicaemia
	Eels, cyprinids, pike	Skin lesions, haemorrhagic septicaemia
	Pike, grass carp	Swim bladder inflammation
	Mammals (rare infections): Dogs Cattle Turkeys Pigs Humans	 Neonatal septicaemia Mastitis Septicaemia Diarrhoea Food poisoning, wound infections; septicaemia in immunocompromised individuals
A. salmonicida subsp. *salmonicida*	Salmonids	Furunculosis
	Goldfish (carp)	'Ulcer disease'
Plesiomonas shigelloides	Humans	Gastroenteritis
	Animals and birds	Pathogenicity uncertain. It is isolated from clinical specimens
	Fish	Opportunistic infections
Vibrio parahaemolyticus	Humans	Food poisoning, associated with seafoods
Vibrio metschnikovii	Chickens and other birds	Enteric disease
Vibrio (*Listonella*) *anguillarum*	Salt-water eels and other fish	Skin necrosis, generalized disease with high mortality
Marine vibrios	Fish	Some members of this group cause significant disease with mortalities of up to 100%

factors of *A. salmonicida* include its outer protein coat, the S-layer, which functions as an adhesin, Type I and Type IV pili and a Type III secretion system (Dacanay et al. 2006, 2010).

Plesiomonas shigelloides has been reported as a cause of gastroenteritis in man with cases occurring mainly in tropical and subtropical regions. Virulent strains have been found to produce heat-stable and heat-labile enterotoxins. Its role in animal disease is uncertain but it can be isolated from diagnostic specimens.

At least five *Vibrio* species are human pathogens including *V. cholerae*, the cholera bacillus, and *V. parahaemolyticus* which causes food poisoning. Only *V. metschnikovii* is associated with disease in domestic animals. It causes a cholera-like disease in chickens and other birds but its geographical distribution is very limited. There are a large number of marine vibrios, including *V. anguillarum (Listonella anguillarum)* which cause infections in many species of fish. The pathogenicity of many of the newly identified species of marine vibrios was reported by Austin et al. (2005).

A summary of the diseases of veterinary importance caused by *Aeromonas*, *Plesiomonas* and *Vibrio* species is given in Table 19.1. Some of the principal virulence factors which have been identified in these pathogens are listed in Table 19.2.

Laboratory Diagnosis

Specimens

Specimens include swabs and scrapings, affected tissue, faeces and mastitic milk. As *A. hydrophila* is associated with fish and their environment, great care must be taken with specimens from freshwater fish to ensure the validity of cultural findings.

Table 19.2 Selected virulence factors which have been identified in strains of *Aeromonas hydrophila*, *A. salmonicida* and *V. anguillarum*

Pathogen	Gene(s) encoding virulence attribute	Virulence factor
Aeromonas hydrophila	*aer/hem, hly*A	Haemolysins
	*asp*A, *ahp*	Serine proteases
	gcat	Glycerophospholipid cholesterol acyltransferase
	alt	Heat-labile enterotoxin
	ast	Heat-stable enterotoxin
	*laf*A1 and A2	Lateral flagella
	*asc*V and *asc*U	Type III secretion system
	*aex*T	ADP-ribosylating toxin
A. salmonicida	*asc*V and *asc*U	Type III secretion system
	*aex*T	ADP-ribosylating toxin
	*vap*A	VapA, the major protein constituent of the S-layer
	fim	Type I pilus
	tap, flp, msh	Type IV pili
Vibrio (*Listonella*) *anguillarum*	65kbp plasmid	Iron uptake system
	*vah*1 to 5	Haemolysins

Direct microscopy

Many of the species are Gram-negative, straight rods without characteristic morphology, although some of the *Vibrio* species are distinctively curved and the findings from Gram-stained or DCF-smears from specimens may suggest the genus. A fluorescent antibody test has been developed for *A. salmonicida*.

Isolation

All cultures are incubated aerobically:

- *Aeromonas hydrophila*: blood agar and MacConkey agar, at 37°C for 24 hours.

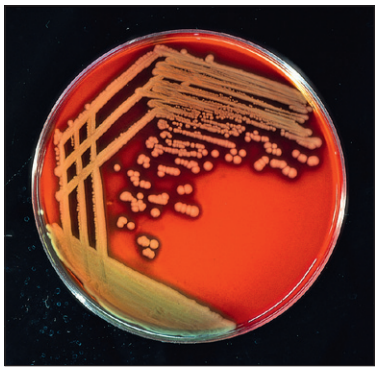

Figure 19.1 *Aeromonas hydrophila* on sheep blood agar, usually markedly haemolytic with large colonies after 48 hours. A putrid odour is characteristic of recent isolates.

- *Aeromonas salmonicida:* blood agar, furunculosis agar (BD Diagnostics) at 25°C for 48 hours.
- *Vibrio anguillarum*: blood agar composed of nutrient agar base with 2.0% NaCl at 20°C for 48 hours.
- *Plesiomonas shigelloides*, *V. metschnikovii* and *V. parahaemolyticus*: nutrient agar or blood agar at 37°C for 24–48 hours.

Selective media

- *Aeromonas hydrophila*: blood agar with 10 mg/litre ampicillin. CIN agar, which is used for the isolation of *Yersinia* spp., may also be useful (Janda & Abbott 2010).
- *Vibrio parahaemolyticus* will grow on TCBS agar for *V. cholerae* and other enteric vibrios and, being halophilic, also grows on mannitol salt agar containing 7.5% NaCl, normally used for the isolation of pathogenic staphylococci.

Identification

Colonial morphology

- *Aeromonas hydrophila*: colonies are large (2–3 mm), flat, greyish and surrounded by a large zone of beta-haemolysis (Fig. 19.1). As some other species of *Aeromonas* are haemolytic, particularly strains of *A caviae*, haemolytic activity may not be as useful as previously thought as a means of differentiating *A. hydrophila* (Abbott et al. 2003). Newly isolated strains have a pungent, foul odour. It grows well on MacConkey agar (Fig. 19.2) often with pale colonies (non-lactose-fermenting), but a minority of strains yield lactose-fermenting colonies. In addition, most *Aeromonas* strains produce tan to buff-coloured colonies on Trypticase soy agar (Abbott et al. 2003).
- *Aeromonas salmonicida*: forms small colonies on blood agar that produce haemolysis after 48 hours.

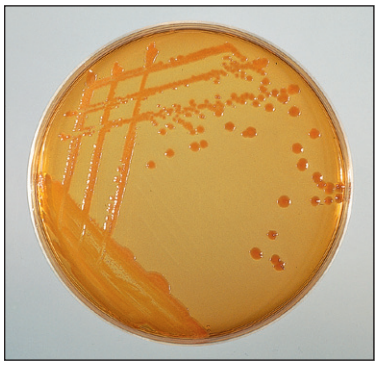

Figure 19.2 *Aeromonas hydrophila* on MacConkey agar. The ability to ferment lactose is variable.

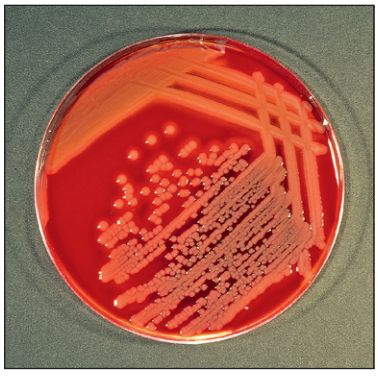

Figure 19.3 *Vibrio metschnikovii* on sheep blood agar showing large, glistening, haemolytic colonies.

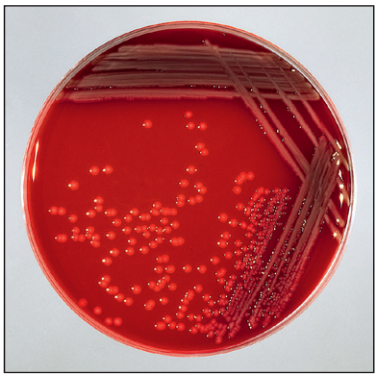

Figure 19.4 *Vibrio parahaemolyticus* on sheep blood agar after 24 hours' incubation at 37°C.

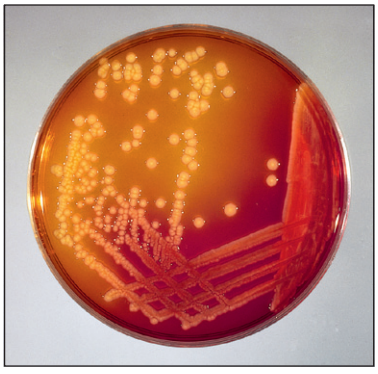

Figure 19.5 *Vibrio parahaemolyticus* on mannitol salt agar demonstrating its innate halophilic character and ability to ferment mannitol.

Brown pigment develops on Furunculosis agar (BD Diagnostics) and often also on nutrient agar.

- *Plesiomonas shigelloides* is non-haemolytic on blood agar and the colonies resemble those of the *Enterobacteriaceae*. It is a non-lactose fermenter on enteric media.
- *Vibrio metschnikovii*: smooth, transparent colonies that are 2–4 mm in diameter at 48 hours. It is haemolytic on blood agar (Fig. 19.3) and grows poorly on MacConkey agar.
- *Vibrio anguillarum*: small, smooth colonies within 48 hours. It is haemolytic on blood agar.
- *Vibrio parahaemolyticus*: moderate-sized (about 2 mm diameter) colonies in 24 hours, non-haemolytic on sheep blood agar (Fig. 19.4). It forms greenish colonies on TCBS agar and ferments mannitol to cause an acid reaction (yellow) on mannitol salt agar (Fig. 19.5).

Microscopic appearance

The *Aeromonas* and *Plesiomonas* species are medium-sized, straight, Gram-negative rods although *A. salmonicida* tends to be coccoid, in pairs, chains or clusters. The *Vibrio* species are Gram-negative rods, curved (Fig. 19.6) to a greater or lesser extent (Fig. 19.7).

Biochemical and other characteristics

A presumptive diagnosis of *Aeromonas* species is initially based on a positive oxidase reaction, growth on MacConkey and fermentation of carbohydrates (Janda & Abbott 2010). *Aeromonas hydrophila* produces acid and gas from glucose, but not all *Aeromonas* spp. are able to produce gas. Classical *A. salmonicida* strains are non-motile and five subspecies have been described. These subspecies vary in biochemical and other characteristics, none of which can be used to definitively identify each subspecies (Martínez-Murcia et al. 2005). *Aeromonas salmonicida* subsp. *salmonicida* can be identified presumptively based on the production of the brown pigment formed on Furunculosis Agar (BD Diagnostics), lack of motility, no gas production from glucose, a negative result in the indole test and positive reactions in the oxidase and catalase tests.

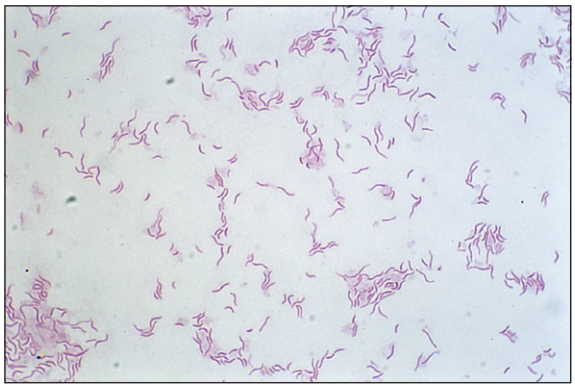

Figure 19.6 *Vibrio metschnikovii* in a Gram-stained smear from culture showing strongly curved Gram-negative rods. (×1000)

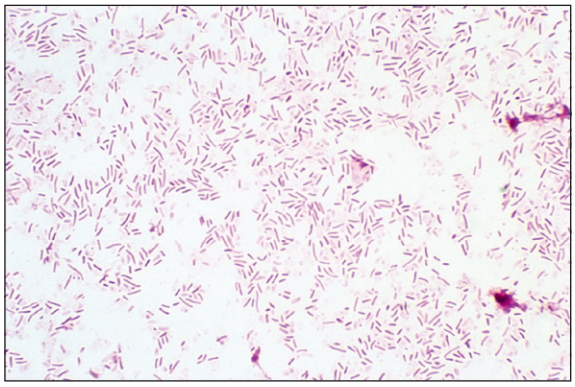

Figure 19.7 *Vibrio parahaemolyticus* in a Gram-stained smear from culture showing the Gram-negative straight rods.

Plesiomonas shigelloides does not produce exoenzymes and is therefore DNase-negative and fails to attack gelatin. It ferments glucose, inositol, maltose and trehalose but is otherwise not very reactive in 'sugars'. *Aeromonas hydrophila* is resistant to the vibriostat O/129 (Oxoid) although some sensitive Japanese strains have been isolated (Abbott et al. 2003). *Plesiomonas shigelloides* and most *Vibrio* species are sensitive to O/129, one of the exceptions being *V. parahaemolyticus*.

Vibrio cholerae and *V. mimicus* have only a slight requirement for Na^+ (NaCl), but most of the halophilic *Vibrio* spp. require the supplementation of biochemical tests with 1% NaCl. *Vibrio parahaemolyticus* belongs to the lysine-decarboxylase-positive, arginine-dihydrolase-negative group of *Vibrio* spp. It is distinguished from other members of the group by negative reactions for sucrose, salicin and cellobiose fermentation but a positive reaction for arabinose. *Vibrio metschnikovii* is the only clinically significant *Vibrio* sp. that is oxidase- and nitrate-reductase-negative.

Biochemical and other characteristics for identification of *A. hydrophila*, *A. salmonicida* subsp. *salmonicida*, *P. shigelloides*, *V. parahaemolyticus* and *V. metschnikovii* are given in Table 19.3.

Vibrio anguillarum is very sensitive to the vibriostat O/129 (Oxoid) and requires a high salt concentration for growth, the optimum being between 1.5 and 3.5% NaCl. Several biotypes and serotypes can be distinguished in this species (Pedersen et al. 1999). In addition, Tiainen et al. (1997) found considerable differences between strains of *V. anguillarum* isolated from fish and those isolated from other sources, the most significant being a negative reaction in the lysine decarboxylase test and a positive Voges–Proskauer test for fish-associated strains.

Antimicrobial susceptibility testing

Methods for antimicrobial susceptibility testing of *Aeromonas* spp. and *Pleisomonas shigelloides*, as well as associated interpretive criteria, are given in CLSI (2010a). Guidelines for methods of testing for fish pathogens have been published (CLSI 2006a, 2006b). Accepted breakpoints are available currently only for *A. salmonicida* (CLSI 2010b); data for other fish pathogens and antimicrobial compounds are the subject of ongoing investigation (Uhland & Higgins 2006).

Antimicrobial Resistance

Data on human isolates of *Aeromonas* spp. are available and show that inducible β-lactamases effective against a wide range of β-lactam antibiotics are the principal resistance mechanism in these organisms (Janda & Abbott, 2010). Although accepted breakpoints are not yet available, data using breakpoints accepted for bacteria isolated from animals suggest that antimicrobial resistance in fish pathogens is increasing (Schmidt et al. 2000, Akinbowale et al. 2006). Data on susceptibility to a wide range of antimicrobial agents in clinical and environmental isolates of *Plesiomonas shigelloides* were reported by Stock & Wiedemann (2001). These authors showed that *P. shigelloides* is resistant to β-lactam antibiotics, due to the production of β-lactamases. Isolates showed resistance to most macrolides, lincosamides, streptogramins and glycopeptides.

Strain Typing

Many of the organisms in the genus *Aeromonas* are difficult to type using phenotypic characteristics only. Thus several molecular methods have been investigated, including restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA genes, 16S rRNA gene sequencing, DNA-DNA hybridization, 16S-23S intergenic spacer region-RFLP, PCR amplification and sequencing of the *gyrB* and *rpoD* genes, use of random amplification of

Table 19.3 Characteristics of *Aeromonas, Plesiomonas* and *Vibrio* species

Characteristics	*A. hydrophila*	*A. salmonicida* subsp. *salmonicida*	*P. shigelloides*	*V. parahaemolyticus*	*V. metschnikovii*
Beta-haemolysis (BA)	+	(+)	–	–	–
Motility	+	–	+	+	+
Growth with 6.5% NaCl	+	–	–	+	(+)
Exoenzymes produced	+	+	–	+	–
DNase	+	(+)	–	+	(+)
Gelatin	+	+	–	+	(+)
Oxidase	+	+	+	+	–
Catalase	+	+	+	+	+
Sensitive to O/129 (150 µg)*	–	–	+	(–)	+
Indole production	+	–	+	+	(–)
Nitrate reduction	+	–	+	+	–
Urease	–	–	–	(–)	–
Aesculin hydrolysis	(+)	(+)	–	–	(+)
Lysine decarboxylase	(+)	v	+	+	(–)
Ornithine decarboxylase	–	–	+	+	–
Arginine dehydrolase	+	+	+	–	(+)
Glucose (gas)	+	(+)	–	–	–
Inositol	–	–	+	–	(–)
Arabinose	(+)	+	–	+	–
Mannitol	+	+	–	+	+
Sucrose	+	–	–	–	+
Lactose	v	–	v	–	v
Growth on MacConkey agar	+	NA	+	–	–

*=2,4-diamino-6,7-diisopropylpteridine phosphate (O/129, Oxoid), a vibriostat.

+ = positive reaction, (+) = most strains positive, v = variable reactions, (–) = most strains negative, – = negative reaction, BA = blood agar, NA = data not available

polymorphic DNA PCR markers and use of enterobacterial repetitive intergenic consensus PCR markers (Aguilera-Arreola et al. 2005, Martínez-Murcia et al. 2005, Ormen et al. 2005). Pulsed-field gel electrophoresis following digestion with XbaI, SpeI and SwaI endonucleases is reported as useful as is amplified fragment length polymorphism analysis (Janda & Abbott, 2010).

Vibrio anguillarum can be typed using serological methods but newer methods including ribotyping and plasmid profiling are now employed.

Molecular Diagnosis

Although there are many studies on the use of molecular methods for typing of *Aeromonas* and *Vibrio* species, there are relatively few reports on the use of molecular techniques for the detection of these organisms from clinical veterinary specimens. Warsen et al. (2004) described the development of a DNA microarray based on 16S ribosomal DNA polymorphisms for the detection of 15 fish pathogens including *Aeromonas* and *Vibrio* species.

REFERENCES

Abbott, S.L., Cheung, W.K.W., Janda, J.M., 2003. The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. Journal of Clinical Microbiology 41 (6), 2348–2357.

Aguilera-Arreola, M.G., Hernández-Rodríguez, C., Zúñiga, G., et al., 2005. *Aeromonas hydrophila* clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. FEMS Microbiology Letters 242, 2, 231–240.

Akinbowale, O.L., Peng, H., Barton, M.D., 2006. Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. Journal of Applied Microbiology 100 (5), 1103–1113.

Austin, B., Austin, D., Sutherland, R., et al., 2005. Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia nauplii*. Environmental Microbiology 7 (9), 1488–1495.

Clinical and Laboratory Standards Institute (CLSI), 2006a. Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals, Approved Guideline. CLSI document M42-A (ISBN 1-56238-611-5). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2006b. Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals, Approved Guideline. CLSI document M49-A (ISBN 1-56238-612-3). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2010a. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline, second ed. CLSI document M45-MA2 (ISBN 1-56238-732-4). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (CLSI), 2010b. Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals, First Informational Supplement. CLSI document M42/M49-S1 (ISBN 1-56238-727-8). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Dacanay, A., Knickle, L., Solanky, K.S., et al., 2006. Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. Microbiology (Reading) 152 (6), 1847–1856.

Dacanay, A., Boyd, J.M., Fast, M.D., et al., 2010. *Aeromonas salmonicida* type I pilus system contributes to host colonization but not invasion. Diseases of Aquatic Organisms 88 (3), 199–206.

Janda, J.M., Abbott, S.L., 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews 23 (1), 35–73.

Li, J., Ni, X.D., Liu, Y.J., et al., 2011. Detection of three virulence genes alt, ahp and aerA in *Aeromonas hydrophila* and their relationship with actual virulence to zebrafish. Journal of Applied Microbiology 110 (3), 823–830.

Martínez-Murcia, A.J., Soler, L., Saavedra, M.J., et al., 2005. Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. Microbiology 8 (4), 259–269.

Ormen, O., Granum, P.E., Lassen, J., et al., 2005. Lack of agreement between biochemical and genetic identification of *Aeromonas* spp. APMIS 113 (3), 203–207.

Pedersen, K., Kühn, I., Seppänen, J., et al., 1999. Clonality of *Vibrio anguillarum* strains isolated from fish from the Scandinavian countries, Sweden, Finland and Denmark. Journal of Applied Microbiology 86 (2), 337–347.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., et al., 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Applied and Environmental Microbiology 66 (11), 4908–4915.

Stock, I., Wiedemann, B., 2001. Natural antimicrobial susceptibilities of *Plesiomonas shigelloides* strains. Journal of Antimicrobial Chemotherapy 48 (6), 803–811.

Tiainen, T., Pedersen, K., Larsen, J.L., 1997. *Vibrio anguillarum* serogroup O3 and *V. anguillarum*-like serogroup O3 cross-reactive species – comparison and characterization. Journal of Applied Microbiology 82 (2), 211–218.

Uhland, F.C., Higgins, R., 2006. Evaluation of the susceptibility of *Aeromonas salmonicida* to oxytetracycline and tetracycline using antimicrobial disk diffusion and dilution susceptibility tests. Aquaculture 257 (1/4), 111–117.

Warsen, A.E., Krug, M.J., LaFrentz, S., et al., 2004. Simultaneous discrimination between 15 fish pathogens by using 16S ribosomal DNA PCR and DNA microarrays. Applied and Environmental Microbiology 70 (7), 4216–4221.

Yu, H.B., Rao, P.S.S., Lee, H.C., et al., 2004. A type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. Infection and Immunity 72 (3), 1248–1256.

Chapter 20

Actinobacillus species

Genus Characteristics

The genus *Actinobacillus* is within the *Pasteurellaceae* family, along with genera such as *Pasteurella*, *Haemophilus*, *Histophilus* and *Mannheimia*. The genus consists of many species that are commensals, some are pathogens of animals and more rarely humans. The *Actinobacillus* species are facultatively anaerobic fastidious Gram-negative, medium-sized rods (0.3–0.5 × 0.6–1.4 μm) that can produce coccal forms on routine solid media but longer forms in serum or sugar broths. Organisms may exhibit bipolar staining. Distinguishing features of the genus *Actinobacillus* from other Gram-negative rods can be found in Table 20.1. They are non-motile, non-spore-forming, non-acid-fast, indole-negative, usually oxidase-positive, reduce nitrates and produce beta-galactosidase. *Actinobacillus* species ferment carbohydrates within 24 hours without the production of gas. Most species grow on MacConkey agar as tiny lactose-fermenting colonies and produce urease. The reaction in the catalase test is variable. *Actinobacillus* species have a limited viability on solid media (seven to 10 days) and most have complex nutritional requirements. Growth is usually improved by a 5 to 10% CO_2 atmosphere.

Natural Habitat

The natural habitat for actinobacilli is primarily the mucous membranes of the upper respiratory tract and oral cavity of their hosts. They do not survive well in the environment. Some species are commensals while others are responsible for several distinct diseases of animals. The geographical distribution of the various *Actinobacillus* species is typically worldwide.

Pathogenesis and Pathogenicity

Actinobacillus species are responsible for several distinct diseases of animals (Rycroft & Garside 2000). The major pathogenic *Actinobacillus* species in veterinary medicine are presented in Table 20.2. Actinobacilli are usually transmitted via the aerosol route or by close contact. The organisms can also gain entry through breaks in the skin. *Actinobacillus pleuropneumoniae, A. suis, A. equuli* and *A. lignieresii* are the most significant veterinary pathogens. *Actinobacillus pleuropneumoniae* is the aetiological agent of porcine pleuropneumonia, a highly contagious and often fatal disease. The organism can also colonize the upper respiratory tract of healthy pigs; no other natural host has been described to date. The disease can be peracute, acute or chronic. The peracute and acute forms are characterized by a necrotizing, fibrinohaemorrhagic pneumonia with pleurisy. Haemorrhage and severe congestion are seen in the lungs with serosanguinous exudate in the pulmonary cavity. This form has high morbidity and mortality. The chronic form of the disease can be seen in animals that survive infection, characteristic lung lesions include focal necrotic abscesses with layers of fibrous tissue that result in scarring of the lung. A review of the pathogenesis of *A. pleuropneumoniae* has been published by Bosse et al. (2002). Virulence factors such as adhesins, iron-acquisition factors, capsule, lipopolysaccharide (LPS), RTX (Repeat in Toxins) cytotoxins are all important with regard to colonization, avoidance of host clearance mechanisms and damage of host tissues. A summary of the principal virulence factors of *A. pleuropneumoniae* can be found in Table 20.3.

Actinobacillus suis colonizes the upper respiratory tract and vagina of healthy pigs. It is an opportunistic pathogen that is more common in high-health status (or start-up)

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Table 20.1 Presumptive identification of *Actinobacillus* and differentiation from similar Gram-negative genera of significance in veterinary medicine

Test[a]	*Actinobacillus* species	*Pasteurella* species	*Mannheimia* species	*Haemophilus* species	*Aeromonas* species	*Vibrio* species
Glucose fermented	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Growth with 6% NaCl	–	–	–	–	–	+
Motility	–	–	–	–	+[b]	+
Urease	+	–[c]	–	v	–	–
Indole	–	v	–	v	v	v
Factor requirement (X or V)	–[d]	–	–	+	–	–

[a]+ = greater than or equal to 90% positive, – = less than or equal to 10% positive, v = variable, 11 to 89% positive

[b]= except *A. salmonicida* which is non-motile

[c]= except *P. dagmatis*, *P. pneumotropica*, *P. aerogenes* which are urease +

[d]= except *A. pleuropneumoniae* biotype 1 which needs V factor

Table 20.2 Main diseases caused by the major pathogenic *Actinobacillus* species in veterinary medicine

Species	Host(s)	Typical Diseases
A. arthritidis	Horse	Arthritis and septicaemia
A. capsulatus	Rabbit	Arthritis and septicaemia
A. equuli subsp. *equuli*	Horse	Septicaemia (sleepy foal disease), purulent arthritis (joint ill) and suppurative multifocal nephritis
	Pig	Septicaemia
A. equuli subsp. *haemolyticus*	Horse	Metritis, abortion, pneumonia and meningitis
A. lignieresii	Ruminants (mainly cattle)	Actinobacillosis, a granulomatous infection also referred to as wooden (timber) tongue
A. pleuropneumoniae	Pig	Fibrino-necrotic pleuropneumonia
A. rossii	Pig	Abortion, metritis
A. seminis	Sheep	Epididymitis, orchitis, infertility
A. suis	Piglet	Septicaemia and arthritis
	Pig	Pleuropneumonia, meningitis, abortion, myocarditis (mulberry heart disease), metritis, enteritis and cutaneous lesions

herds. Disease is sporadic and is characterized by an acute septicaemia in piglets that may be accompanied by neurological signs and arthritis. In grower-finisher pigs, the disease can resemble the pleuropneumonia associated with *A. pleuropneumoniae* infection. Meningitis, abortion, myocarditis (mulberry heart disease), metritis and skin lesions (resembling erysipelas) have been reported in adult pigs. Virulence factors similar to those documented for *A. pleuropneumoniae* have been reported including transferring-binding proteins (TbpA and TbpB), urease, capsule, LPS and RTX toxins (except ApxIV). However, *A. suis* shares only 50% DNA-DNA homology with *A.*

Table 20.3 Main virulence factors of *Actinobacillus pleuropneumoniae*

Virulence determinants	Functions
Apx toxins	RTX toxins
ApxI	Haemolytic and cytotoxic (strong), impairment of phagocytic function of macrophages and PMNs
ApxII	Haemolytic and cytotoxic (weak to moderate), impairment of phagocytic function of macrophages and PMNs
ApxIII	Cytotoxic only (strong), impairment of phagocytic function of macrophages and PMNs
ApxIV	Haemolytic (weak)*
Fimbriae of type 4	Mediate adherence to host cells
Proteases	May contribute to the pathogenesis of infection via cleavage of host proteins
Transferrin-binding proteins (TbpA and TbpB)	Iron uptake: High-affinity binding of porcine transferrin
FhuA	Iron uptake: Outer membrane receptor for ferric hydroxamate siderophores
HgbA	Iron uptake: A 104-kDa haemoglobin-binding protein
TonB-ExbB-ExbD	Three proteins transducing energy from the cytoplasmic membrane to the outer membrane receptor for iron transport into the periplasm
ABC-transport systems	Involved in uptake of iron across the cytoplasmic membrane
FhuBCD	Specific for ferric hydroxamate
afuABC	Periplasmic-binding-protein-dependent iron transport system, likely for unchelated Fe^{3+} across the cytoplasmic membrane
cbiKLMQO	High-affinity nickel uptake system
Capsule and/or LPS	Serum resistance
LPS	Activation of the alternative complement cascade, activation of alveolar and intravascular macrophages and production of pro-inflammatory cytokines
DnaK and Trigger factor	Stress response proteins essential for survival

*no data on its cytotoxic activity

pleuropneumoniae and distinct virulence factors presumably exist.

Actinobacillus equuli subsp. *equuli* is the agent of the sleepy foal disease, a frequently fatal septicaemia of neonatal foals. Infection of the foal is thought to occur via the upper respiratory tract or the umbilicus soon after birth. The chronic form of the disease is characterized by purulent arthritis (joint ill) and suppurative multifocal nephritis. *Actinobacillus equuli* subsp. *equuli* is also considered an opportunistic pathogen of pigs capable of causing septicaemia. *Actinobacillus equuli* subsp. *haemolyticus* is an opportunistic pathogen of the horse and can cause various infections such as metritis, abortion, pneumonia and meningitis. Little is known about the virulence factors of *A. equuli*. Haemolytic isolates seem to produce a RTX toxin, the AqxA protein, encoded by the *aqxA* gene of the *aqxCABD* operon.

Actinobacillus lignieresii is the cause of actinobacillosis, wooden (timber) tongue, in cattle and less commonly in sheep. It is a sporadic, insidious, granulomatous infection. The organism appears to be a commensal of the upper respiratory tract of ruminants, causing disease after inoculation into mucous membranes during abrasion by rough feed. The lesions, numerous small abscesses, are usually limited to the soft tissues of the jaw, throat and tongue. Ulcers filled with pus can be seen on the tongue. The granulomatous lesions can also involve the skin and underlying tissues of the head, neck and limbs. Spread of infection by the lymphatics can occur occasionally to affect the lungs and other internal organs. The pyogranulomatous lesions formed by *A. lignieresii* resemble those of *Actinomyces bovis* (actinomycosis). Small, greyish-white granules (about 1 mm in diameter) are present in exudates from lesions of *A. lignieresii*. If these granules are

Table 20.4 Differentiation of *Actinobacillus lignieresii* and *Actinomyces bovis* infections in cattle

Characteristics	*Actinobacillus lignieresii*	*Actinomyces bovis*
Specific disease	Bovine actinobacillosis or wonden (timber) tongue	Bovine actinomycosis or lumpy jaw
Granulomatous abscesses	Jaw, head, neck and limbs	Jaw region
Granules in exudates	Greyish-white, about 1 mm	Yellow 'sulphur' granules about 1 to 3 mm
Club colonies	+	+
Spread via lymphatics	+	–
Bone affected (osteomyelitis)	Uncommon	Common
Gram-stain reaction	Gram-negative rods	Gram-positive branching filaments or diphtheroidal forms
Atmospheric requirements	Growth in air (facultative anaerobe)	Anaerobic (H_2 + CO_2)

crushed on a slide and stained, club colonies are seen consisting of club-like processes of calcium phosphate with the Gram-negative rods of *A. lignieresii* in the centre. Clinically, it can be difficult to distinguish a granulomatous lesion of *A. lignieresii* in the soft tissues of the jaw area from lumpy jaw (*A. bovis*). Table 20.4 summarizes the differential features of the two conditions. Virulence factors of *A. lignieresii* are still unknown.

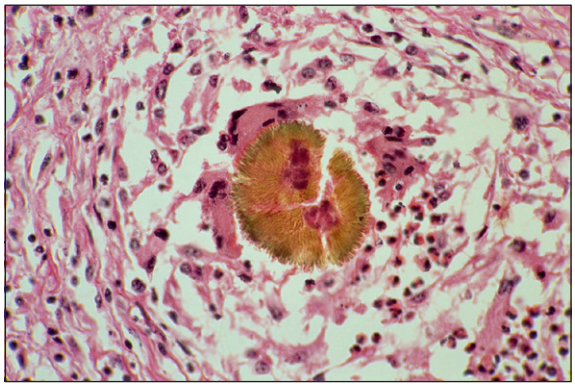

Figure 20.1 Club colonies (yellow) in a histopathological section from a case of bovine actinobacillus (*Actinobacillus lignieresii*) (H&E stain, ×400).

Laboratory Diagnosis

Specimens

Specimens should include pus, exudates from lesions, tissue biopsies, pneumonic lung samples and biopsies of granulomatous material. Serum should be submitted for serological testing to detect healthy carrier animals, to diagnose subclinical infections or for the control of porcine pleuropneumonia. Tonsils from clinically healthy carrier animals may also be tested using an immunomagnetic separation technique (Gagne et al. 1998) or PCR.

Direct microscopy

Direct microscopy is only worthwhile in *A. lignieresii* infections where exudates are available. Pus or exudates are washed with distilled water in a Petri dish to reveal the small greyish-white granules. A few of these granules are placed in a drop of 10% KOH on a microscope slide and crushed gently with a coverslip. The club-shaped structures that surround the bacterial colonies should be seen under the low-power objective. Stained histopathological sections (Fig. 20.1) can also demonstrate the characteristic club colonies. If a Gram-stained smear is made from the crushed granules, the presence of medium-sized Gram-negative rods confirms actinobacillosis rather than actinomycosis.

Isolation

Most of the actinobacilli can be cultured on blood agar (sheep or ox) and some will grow on MacConkey agar as tiny lactose-fermenting colonies. However, most strains of *A. pleuropneumoniae* require factor V that can be provided by chocolate agar or a staphylococcal streak on blood agar. The growth of all the actinobacilli, particularly *A. pleuropneumoniae*, is improved by 5–10% CO_2; a candle-jar is satisfactory for this. Most actinobacilli will grow on triple-sugar iron (TSI) slants with a typical orange to yellowish colour of the medium without gas. The inoculated plates or slants are incubated at 35°C for 24–72 hours.

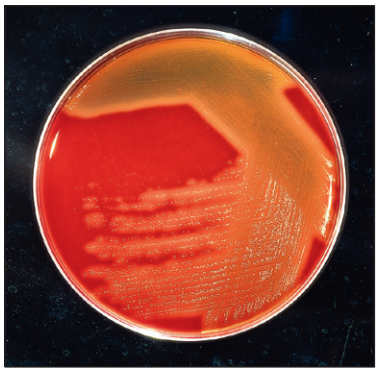

Figure 20.2 *Actinobacillus pleuropneumoniae* biotype 2 on sheep blood agar. The small colonies are surrounded by a narrow zone of beta-haemolysis.

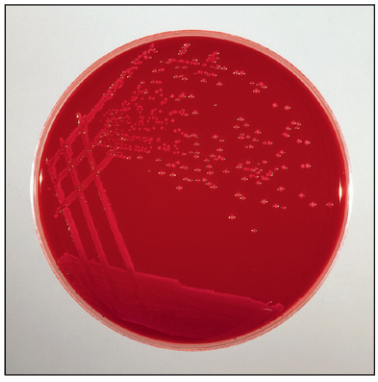

Figure 20.3 *Actinobacillus lignieresii* on sheep blood agar. On primary isolation the colonies are non-haemolytic, shiny and slightly sticky but this property is lost upon subculture.

Identification

Colonial appearance

The colonies of *A. pleuropneumoniae* are small (1 mm) colonies surrounded by a zone of beta-haemolysis, which somewhat resemble those of a beta-haemolytic *Streptococcus* (Fig. 20.2). Two distinct colony forms are possible, one waxy and the other a soft glistening type. No growth occurs on MacConkey agar. Two biotypes of *A. pleuropneumoniae* are recognised. Biotype 1 is NAD dependent (*S. aureus streak* needed on blood agar and biotype 1 satellites around the *S. aureus streak*). Biotype 2 is NAD-independent. All strains of *A. suis* are haemolytic with colonies similar to those of *A. lignieresii* but more sticky. The organism grows well on MacConkey agar and is a lactose-fermenter. *Actinobacillus lignieresii* produces small, glistening colonies which develop in 24 hours (Fig. 20.3). They are usually slightly sticky (viscid) on primary isolation but lose this characteristic on subculture. The colonies are non-haemolytic and develop to about 2 mm in diameter in 48 hours. The organism grows well on MacConkey agar, the colonies are

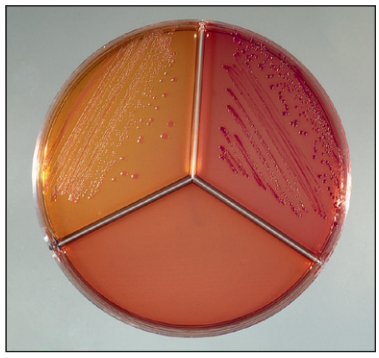

Figure 20.4 *Actinobacillus lignieresii* (left) and *A. equuli* (right) on MacConkey agar. *Actinobacillus pleuropneumoniae* (bottom) is unable to grow on this medium. *Actinobacillus equuli* ferments lactose but *A. lignieresii* gives a late reaction.

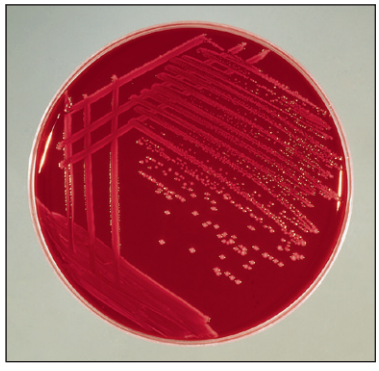

Figure 20.5 *Actinobacillus equuli* on sheep blood agar. Colonies are viscid and remain so on subculture.

at first pale but become pinkish as *A. lignieresii* is a late lactose-fermenter (Fig. 20.4). The colonies of *A. equuli* subsp. *haemolyticus* are haemolytic and sticky with this feature remaining on subculture, while those of *A. equuli* subsp. *equuli* are nonhaemolytic (Fig. 20.5). Both, *A. lignieresii* and *A. equuli*, are usually lactose-fermenters on MacConkey agar but growth on this medium can be variable (Fig. 20.4). The cells of *A. capsulatus* are capsulated and very sticky colonies are produced on blood agar. It grows well on MacConkey agar, appearing as a lactose-fermenter. *Actinobacillus seminis* colonies are small, round, pinpoint, greyish-white, nonhaemolytic and appear after 24–48 hours on blood agar. Usually no growth occurs on MacConkey agar.

Microscopic appearance

All actinobacilli are Gram-negative rods or coccobacilli (Fig. 20.6).

Biochemical reactions

Identification of significant veterinary species of *Actinobacillus* is possible using phenotypic characterization

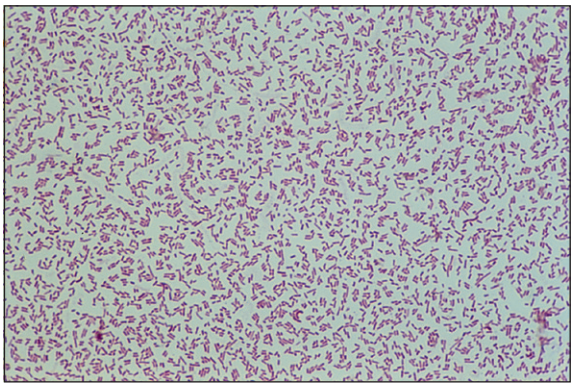

Figure 20.6 A Gram-stained smear showing the medium-sized Gram-negative rods of *A. lignieresii*, representative of the genus. (×1000)

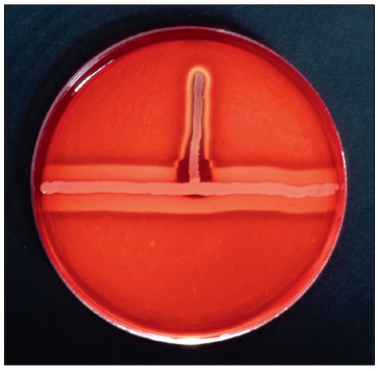

Figure 20.7 CAMP test with *A. pleuropneumoniae* biotype 2 and *Staphylococcus aureus* (horizontal streak) showing enhancement of the haemolytic effect of the staphylococcal beta-haemolysin.

combined with information on host of isolation (Christensen & Bisgaard 2004). The differential characteristics of the actinobacilli are shown in Table 20.5 (Murray et al. 2003). *Actinobacillus pleuropneumoniae* can be differentiated by its haemolytic effect from many other species of actinobacilli. The haemolysis caused by the beta-haemolysin of *Staphylococcus aureus* is enhanced by the presence of *A. pleuropneumoniae* and *A. equuli* subsp. *haemolyticus* in a CAMP test (Fig. 20.7). *Actinobacillis pleuropneumonia* biotype 1 is usually separated from *A. suis* by its NAD dependency. Lack of hydrolysis of aesculin, failure to ferment arabinose and trehalose as well as an ability to produce acid from mannitol are all additional characteristics differentiating these two species. *Actinobacillus equuli* isolates from horses are now separated into two subspecies. Haemolytic variants of *A. equuli* are classified as *A. equuli* subsp. *haemolyticus*, whereas the non-haemolytic ones are named *A. equuli* subsp. *equuli* (Christensen et al. 2002). The arabinose-positive strains of *A. equuli* have been shown to belong to *A. equuli* subsp. *equuli*.

Other tests

Actinobacillus pleuropneumoniae can be directly detected in lung tissues with techniques such as immunofluoresence, ring precipitation, latex agglutination, coagglutination, ELISA, counter-immunoelectrophoresis and fluorescent or immunoperoxidase antibody test (Dubreuil et al. 2000). In addition, an immunomagnetic separation technique for the selective recovery of particular serotypes of *A. pleuropneumoniae* from tonsils has been developed and this technique was shown to have a higher sensitivity than conventional culture (Gagne et al. 1998).

Antimicrobial Susceptibility Testing

Quality control standards for the in vitro antimicrobial susceptibility testing of *A. pleuropneumoniae* were developed in a multi-laboratory study according to procedures established by the Clinical and Laboratory Standards Institute (CLSI 2002, document M31-A2) for broth microdilution and disk diffusion methods (McDermott et al. 2001). The medium recommended for the broth microdilution test, the veterinary fastidious medium (VFM), is made of cation-adjusted Mueller–Hinton broth supplemented with 2% lysed horse blood, 2% yeast extract, and 2% supplement C (BD Diagnostics). The medium recommended for the disk diffusion test is the chocolate Mueller–Hinton agar. *Actinobacillus pleuropneumoniae* ATCC 27090 is the recommended quality control organism. Plates are incubated at 35°C in an atmosphere of 5% to 7% CO_2 for 20 to 24 hours before measuring the zones of inhibition or determining minimum inhibitory concentrations (MICs). For other veterinary actinobacilli, the currently recommended techniques for antimicrobial agent disk and dilution susceptibility testing as well as interpretive criteria for veterinary use can also be found in document M31-A2 (CLSI 2002). For most actinobacilli, there are no CLSI specific data for breakpoints and critical disk zones. The CLSI have published a laboratory guideline for antimicrobial susceptibility testing of infrequently encountered or fastidious bacteria not covered in previous CLSI publications. Various organisms can be found in these guidelines including *Actinobacillus* (Jorgensen & Hindler 2007).

Antimicrobial resistance

Many phenotypic studies on antimicrobial resistance (AMR) in *A. pleuropneumoniae* have been published over the last two decades. Most report that *A. pleuropneumoniae* is generally susceptible in vitro to antimicrobial agents such as colistin, enrofloxacin, sulphonamides, trimethoprim-sulfamethoxazole, tilmicosin, tiamulin, erythromycin, gentamicin and most beta-lactams (Aarestrup & Jensen 1999, Yoshimura et al. 2002). However, isolates are reported to be less susceptible to streptomycin, kanamycin, spiramycin, spectinomycin and lincomycin. Antimicrobial resistance to penicillins, chloramphenicol,

Table 20.5 Differential characteristics of *Actinobacillus* species of veterinary significance

Test[a]	*A. arthritidis*	*A. capsulatus*	*A. equuli* subsp. *equuli*	*A. equuli* subsp. *haemolyticus*	*A. lignieresii*	*A. pleuropneumoniae* (biotype 1)	*A. seminis*	*A. suis*
B-haemolysis	–	–	–	+	–	+	–	+
CAMP	–	–	–	+	–	+	–	–
Aesculin hydrolysis	–	*d*	-	–	–	–	*v*	+
Growth on MacConkey	+	+	*v*	*v*	*v*	–	–	+
NAD (factor V) dependence	–	–	–	–	–	+	–	–
ONPG	+	+	+	+	+	+	*v*	*v*
Urease	+	+	+	+	+	+	–	+
Acid from:								
Arabinose	*v*	–	*v*	–	–	–	*v*	+
Mannitol	+	+	+	–	+	+	*v*	–
Tréhalose	–	+	+	+	–	–	–	+

[a]+ = greater than or equal to 90% positive, – = less than or equal to 10% positive, v = variable, 11 to 89% positive, d = delayed

tylosin, and tetracycline has also been observed (Nadeau et al. 1988, Aarestrup & Jensen 1999). In the study by Yoshimura et al. (2002) ceftiofur and the fluoroquinolones danofloxacin and enrofloxacin were shown to be the most active compounds.

Other actinobacilli

Susceptibility studies are available for only a few other *Actinobacillus* species. Porcine isolates of *A. suis* are reported to be susceptible to most antibiotics used in veterinary medicine. However, resistance to some antibiotics has been recorded for particular isolates (Nelson et al. 1996, Daignault et al. 1999, Smith & Ross 2002).

Strain Typing

There are two biotypes (biovars) and 15 serotypes (serovars) of *A. pleuropneumoniae* described to date. Biotype 1 strains are dependent on nicotinamide adenine dinucleotide (NAD), while biotype 2 strains are NAD independent. Based on capsular polysaccharide (CPS) and lipopolysaccharide (LPS) antigens, serotypes 1 to 12, 15 and K2:O7 of *A. pleuropneumoniae* belong to biotype 1, while two other serotypes (serotypes 13 and 14) have been assigned to biotype 2. Serotypes 1 and 5 have been differentiated into 1a, 1b, and 5a, 5b respectively. Serotypes 1, 5, and 7 are the serotypes most commonly found in North America, while serotype 2 is predominant in many European countries (Jacques 2004). Serotyping of *A. pleuropneumoniae* is usually carried out at national or international reference laboratories and is recommended for confirmation of the bacterial identification of an isolate. The serotyping of *A. pleuropneumoniae* is complicated by cross-reactions amongst strains of different serotypes. Strong cross-reactions can be observed between serotypes 1, 9 and 11; 3, 6 and 8; 4 and 7 (Mittal et al. 1992). Various traditional serotyping methods such as slide agglutination, tube agglutination, ring precipitation, coagglutination, immunodiffusion and indirect haemagglutination have been used either alone or in combination for strain typing of *A. pleuropneumoniae* (Mittal et al. 1992).

Molecular methods have been investigated for differentiating serotypes of *A. pleuropneumoniae*. Because *apx* genes are associated with specific serotypes of *A. pleuropneumoniae*, a PCR identification and typing system based on the presence or absence of *apx* genes of the three toxins ApxI, ApxII and ApxIII in single serotypes was developed by Frey (2003). Molecular typing of the *omlA* gene by PCR and PCR-REA (Gram et al. 2000), restriction fragment length polymorphism (RFLP) analysis (Rychlik et al. 1994, Cho and Chae, 2003), ribotyping, sequence analysis of ribosomal intergenic regions, and pulsed-field gel electrophoresis (PFGE) (Fussing et al. 1998a, 1998b) have all been developed as alternatives to serotyping for *A. pleuropneumoniae*.

Isolates of *A. seminis* have been typed by PCR ribotyping, repetitive extragenic palindromic element (REP)-based PCR, and enterobacterial repetitive intergenic consensus (ERIC)-based PCR (Appuhamy et al. 1998).

Serology

Serology is considered the most powerful tool for diagnosis of subclinical cases of *A. pleuropneumoniae*. Various assays have been developed for the detection of antibodies against the toxins or somatic and/or capsular antigens. A dual-plate complement fixation (CF) assay and 3 commercially available enzyme-linked immunosorbent assays have been compared (Opriessnig et al, 2013). The techniques most commonly employed are ELISAs based on O-chain LPS as antigens (Dubreuil et al. 2000).

Molecular Diagnosis

Many PCR protocols have been designed for the specific detection of *A. pleuropneumoniae* including a commercially available PCR (Adiavet APP, Adiagene, Saint-Brieuc, France). Primers have been designed to hybridize to regions within the *omlA* gene, a *dsbE*-like gene and the *apxIVA* gene. Eight PCR assays were evaluated for their abilities to detect *A. pleuropneumoniae* in tonsils from subclinically infected swine by Fittipaldi et al. (2003). Sensitivities of direct PCRs were reported as variable (10^9 to 10^2 CFU/g of tonsil) while those of post-culture PCR were found to be similar (10^2 CFU/g of tonsil).

PCR tests are also available for the specific detection of other actinobacilli. A PCR method was developed for the rapid identification of *A. seminis* strains (Appuhamy et al. 1998). A repeat in toxin (RTX)-PCR can be used for identification of *A. suis*, *A. equuli* subsp. *haemolyticus*, and *A. equuli* subsp. *equuli* (Christensen & Bisgaard 2004). Alternatively, identification can be performed by 16S rRNA sequencing and homology searches in public databases. However, it is reported that neither the species *A. lignieresii* and *A. pleuropneumoniae* nor *A. equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticis* and *A. suis* can be separated based on 16S rRNA sequence results.

REFERENCES

Aarestrup, F.M., Jensen, N.E., 1999. Susceptibility testing of *Actinobacillus pleuropneumoniae* in Denmark. Evaluation of three different media of MIC-determinations and tablet diffusion tests. Veterinary Microbiology 64, 299–305.

Appuhamy, S., Coote, J.G., Low, J.C., 1998. PCR methods for rapid identification and characterization of *Actinobacillus seminis* strains. Journal

of Clinical Microbiology 36, 814–817.

Bosse, J.T., Janson, H., Sheehan, B.J., et al., 2002. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes and Infection 4, 225–235.

Cho, W.S., Chae, C., 2003. Differentiation of twelve *Actinobacillus pleuropneumoniae* serotypes by outer membrane lipoprotein gene-based restriction fragment length polymorphism. Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health 50, 90–94.

Christensen, H., Bisgaard, M., 2004. Revised definition of *Actinobacillus* sensu stricto isolated from animals. A review with special emphasis on diagnosis. Veterinary Microbiology 99, 13–30.

Christensen, H., Bisgaard, M., Olsen, J.E., 2002. Reclassification of equine isolates previously reported as *Actinobacillus equuli*, variants of *A. equuli*, *Actinobacillus suis* or Bisgaard taxon 11 and proposal of *A. equuli* subsp. *equuli* subsp. nov. and *A. equuli* subsp. *haemolyticus* subsp. nov. International Journal of Systematic Evolutionary Microbiology 52, 1569–1576.

Clinical and Laboratory Standards Institute (CLSI), 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, Approved Standard, second ed, CLSI M31-MA2. Clinical and Laboratory Standards Institute, Wayne, PA.

Daignault, D., Chouinard, L., Moller, K., et al., 1999. Isolation of *Actinobacillus suis* from a cat's lung. Canadian Veterinary Journal 40, 52–53.

Dubreuil, J.D., Jacques, M., Mittal, K.R., et al., 2000. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. Animal Health Research Reviews 1, 73–93.

Fittipaldi, N., Broes, A., Harel, J., et al., 2003. Evaluation and field validation of PCR tests for detection of *Actinobacillus pleuropneumoniae* in subclinically infected pigs. Journal of Clinical Microbiology 41, 5085–5093.

Frey, J., 2003. Detection, identification, and subtyping of *Actinobacillus pleuropneumoniae*. Methods in Molecular Biology 216, 87–95.

Fussing, V., Barfod, K., Nielsen, R., et al., 1998a. Evaluation and application of ribotyping for epidemiological studies of *Actinobacillus pleuropneumoniae* in Denmark. Veterinary Microbiology 62, 145–162.

Fussing, V., Paster, B.J., Dewhirst, F.E., et al., 1998b. Differentiation of *Actinobacillus pleuropneumoniae* strains by sequence analysis of 16S rDNA and ribosomal intergenic regions, and development of a species specific oligonucleotide for in situ detection. Systematic Applied Microbiology 21, 408–418.

Gagne, A., Lacouture, S., Broes, A., et al., 1998. Development of an immunomagnetic method for selective isolation of *Actinobacillus pleuropneumoniae* serotype 1 from tonsils. Journal of Clinical Microbiology 36, 251–254.

Gram, T., Ahrens, P., Andreasen, M., et al., 2000. An *Actinobacillus pleuropneumoniae* PCR typing system based on the apx and omlA genes – evaluation of isolates from lungs and tonsils of pigs. Veterinary Microbiology 75, 43–57.

Jacques, M., 2004. Surface polysaccharides and iron-uptake systems of *Actinobacillus pleuropneumoniae*. Canadian Journal of Veterinary Research 68, 81–85.

Jorgensen, J.H., Hindler, J.F., 2007. New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. Clinical Infectious Diseases 44, 280–286.

McDermott, P.F., Barry, A.L., Jones, R.N., et al., 2001. Standardization of broth microdilution and disk diffusion susceptibility tests for *Actinobacillus pleuropneumoniae* and *Haemophilus somnus*: quality control standards for ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole. Clinical Infectious Diseases 39, 4283–4287.

Mittal, K.R., Higgins, R., Lariviere, S., et al., 1992. Serological characterization of *Actinobacillus pleuropneumoniae* strains isolated from pigs in Quebec. Veterinary Microbiology 32, 135–148.

Murray, P.R., Baron, E.J., American Society for Microbiology, 2003. Manual of Clinical Microbiology, eighth ed. Washington DC, ASM Press.

Nadeau, M., Lariviere, S., Higgins, R., et al., 1988. Minimal inhibitory concentrations of antimicrobial agents against *Actinobacillus pleuropneumoniae*. Canadian Journal of Veterinary Research 52, 315–318.

Nelson, K.M., Darien, B.J., Konkle, D.M., et al., 1996. *Actinobacillus suis* septicaemia in two foals. Veterinary Record 138, 39–40.

Opriessnig, T., Hemann, M., Johnson, J.K., et al., 2013. Evaluation of diagnostic assays for the serological detection of *Actinobacillus pleuropneumoniae* on samples of known or unknown exposure. Journal of Veterinary Diagnostic Investigation 25, 61–71.

Rychlik, I., Bartos, M., Sestak, K., 1994. Use of DNA fingerprinting for accurate typing of *Actinobacillus pleuropneumoniae*. Veterinary Medicine (Praha) 39, 167–174.

Rycroft, A.N., Garside, L.H., 2000. *Actinobacillus* species and their role in animal disease. Veterinary Journal 159, 18–36.

Smith, M.A., Ross, M.W., 2002. Postoperative infection with *Actinobacillus* species in horses: 10 cases (1995–2000). Journal of the American Veterinary Medical Association 221, 1306–1310.

Yoshimura, H., Takagi, M., Ishimura, M., et al., 2002. Comparative in vitro activity of 16 antimicrobial agents against *Actinobacillus pleuropneumoniae*. Veterinary Research Communications 26, 11–19.

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Chapter 21

Pasteurella, Mannheimia, Bibersteinia and *Avibacterium* species

Genus Characteristics

The genera *Pasteurella* and *Mannheimia* belong to the family *Pasteurellaceae* along with other genera such as *Haemophilus* and *Actinobacillus*. *Pasteurella* and *Mannheimia* species are small (0.3–1.0 µm in width and 1.0–2.0 µm in length), facultatively anaerobic, Gram-negative coccobacilli or rods. They are non-motile, non-sporing, fermentative, generally oxidase-positive (except some *P. dagmatis*), catalase-positive (except for *P. caballi*) and non-haemolytic (except for *M. haemolytica*). Although unenriched media support their growth, they are nutritionally fastidious and grow best on media supplemented with serum or blood or on chocolate agar. Most of them cannot grow on media used for the *Enterobacteriaceae*, except for *M. haemolytica* which produces small, pinpoint colonies on MacConkey agar.

Classification of organisms in the genus *Pasteurella* is revised regularly and the taxonomy of the genus is complex. *Pasteurella* sensu stricto contains *P. multocida*, *P. canis*, *P. stomatis*, *P. dagmatis*, and *P.* species B. *Pasteurella caballi* and *P. pneumotropica* are both species of uncertain taxonomy, while *P. avium*, *P. gallinarum*, *P. volantium* and *P.* species A have all been reclassified under the genus *Avibacterium* (Blackall et al. 2005). *Haemophilus paragallinarum* has been reclassified as *Avibacterium paragallinarum* while *P. trehalosi* has been reclassified into the genus *Bibersteinia* (Blackall et al. 2007). *M. haemolytica*, formerly classified as *P. haemolytica* A serotypes (biogroup 1), is the main species of veterinary significance within the *Mannheimia* genus, followed by *M. granulomatis* and *M. varigena*.

Natural Habitat

Pasteurella, *Mannheimia*, *Bibersteinia* and *Avibacterium* species are worldwide in distribution with a wide spectrum of hosts. They have a predilection for the oral and respiratory tracts of animals and/or humans. Most are commensals on the mucous membranes of the upper respiratory and/or intestinal tracts of animals, humans and birds. The carrier rate for different species varies greatly. Many of them are opportunistic pathogens and cause disease only under certain conditions. Interestingly, *P. multocida* can survive in water for a year as well as in organic material for long periods (Bredy & Botzler 1989).

Pathogenesis and Pathogenicity

The principal hosts and diseases associated with *Pasteurella*, *Mannheimia*, *Bibersteinia* and *Avibacterium* species are listed in Tables 21.1 and 21.2. Isolates of the genera *Pasteurella* and *Mannheimia* cause a wide variety of diseases of great economic importance, mainly in poultry, pigs, cattle and rabbits. The review by Kehrenberg et al. (2001) provides a short summary of the infections caused by *Pasteurella* and *Mannheimia* isolates in food-producing animals. Infections caused by *Pasteurella* and *Mannheimia* species may be endogenous or exogenous. The portal of entry is usually via the respiratory tract and virulence is enhanced by animal-to-animal transmission, as occurs in pneumonic pasteurellosis. Subcutaneous infections are usually the result of animal bites. Various stresses, including concurrent viral infections, predispose to infection, as occurs in 'shipping fever'.

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Table 21.1 Main diseases caused by the major pathogenic *Pasteurella* species in veterinary medicine

Species	Host(s)	Typical Diseases
Pasteurella multocida		
Type A	Cattle	Part of the shipping fever complex and the enzootic pneumonia complex in calves (pneumonias), occasional but severe mastitis
	Sheep	Pleuropneumonia, mastitis
	Pigs	Pneumonia (often secondary), atrophic rhinitis (toxigenic serotype A) (with or without *Bordetella bronchiseptica*)
	Rabbits	One cause of snuffles (respiratory disease), pleuropneumonia, abscesses, otitis media, conjunctivitis, and genital infections
	Poultry	Fowl cholera (primary infection)
	Many domestic and wild animals	Pneumonia, bite wound contamination, and other infections in stressed animals
Type B	Cattle, water buffalo, bison, yak and other ruminants	Epizootic haemorrhagic septicaemia (nasopharynx of carrier animals, Southeast Asia and other countries) (B:2; B:2,5)
Type D	Pigs	Atrophic rhinitis (toxigenic serotype D) (with or without *Bordetella bronchiseptica*)
	Pigs and less commonly other domestic animals	Pneumonia (usually secondary)
	Poultry	Fowl cholera
Type E	Cattle and water buffalo	Epizootic haemorrhagic septicaemia (primary infection, Africa only) (E:2; E:2,5)
Type F	Poultry	Fowl cholera
	Rabbit	Fibrinopurulent pleuropneumonia or diffuse haemorrhagic pneumonia
P. canis	Dogs	Commensal in the oral cavity of dogs, bite-wound contamination (animals and humans), cellulitis, septicaemia, osteomyelitis
	Cattle and sheep	Pneumonia
P. dagmatis	Dogs and cats	Commensal in dogs and cats (oral and intestinal cavity), bite-wound contamination (animals and humans)
P. stomatis	Dogs and cats	Commensal in dogs and cats, bite-wound contamination (animals and humans), bronchitis
P. species B	Dogs and cats	Commensal in dogs and cats, bite-wound contamination (animals and humans)
P. caballi	Horses	Respiratory infections (pneumonia)
P. pneumotropica	Laboratory animals	Pneumonia (opportunistic pathogen)

The main virulence factors of *P. multocida* and *M. haemolytica* are described in Table 21.3. Endotoxins (lipopolysaccharides, LPS) are particularly important in the septicaemic diseases such as fowl cholera and bovine haemorrhagic septicaemia. *Pasteurella multocida* serotyes A and D can produce a cytotoxic protein named *P. multocida* toxin (PMT), which stimulates cellular cytoskeletal rearrangements and growth of fibroblasts. Interestingly, avirulent PMT-positive strains and virulent PMT-negative strains have both been reported (Rimler & Brogden 1986). However, PMT plays a role in atrophic rhinitis (mild to severe destruction of porcine nasal turbinate bones), while co-infection with toxigenic strains of *Bordetella bronchiseptica* promotes colonization by *P. multocida*. Iron

Table 21.2 Main diseases caused by the major pathogenic *Mannheimia, Bibersteinia* and *Avibacterium species* in veterinary medicine

Species	Host(s)	Typical Diseases
Mannheimia haemolytica	Cattle	Part of the shipping fever complex, pneumonia (primary or secondary)
	Sheep	Enzootic pneumonia, septicaemia in lambs, gangrenous mastitis (blue bag)
M. granulomatis	Cattle	Fibrogranulomatous disease, panniculitis
	Deer and hares	Bronchopneumonia and conjunctivitis
M. varisena	Pigs	Pneumonia, septicaemia, enteritis
	Cattle	Pneumonia, septicaemia, mastitis
Bibersteinia trehalosi	Sheep	Septicaemia in lambs
	Ruminants	Pulmonary diseases
Avibacterium avium	Chickens	Commensal
	Calves	Sinusitis and pneumonia
A. gallinarum	Birds	Possible low-grade infections (sinusitis, conjunctivitis, tracheitis)
A. paragallinarum	Chickens	Respiratory tract infections (avian coryza), conjunctivitis, oedema, possible diarrhoea
A. species A	Birds	Commensal, possible sinusitis and conjunctivitis

acquisition genes have been described in *P. multocida* (Bosch et al. 2002) which facilitate the removal of iron from the host and promote bacterial growth. The polysaccharide capsule of *P. multocida* offers protection against the host immune system by reducing macrophage uptake and diminishing susceptibility to the bactericidal activity of complement. Capsular adherence properties have also been described (Pruimboom et al. 1996). The composition of the capsule can vary from one serotype to another. Many fimbrial subunits and filamentous haemagglutinins have been described and are thought to play a role in adhesion to eucaryotic host cells (Al-Haddawi et al. 2000, May et al. 2001).

M. haemolytica serotype A1 produces a heat-labile leukotoxin, a member of the RTX (Repeats in ToXins) toxin family, with cytotoxic cell specificity for bovine leukocytes (Shewen & Wilkie 1982, Highlander 2001). This toxin acts by forming pores in leukocytes resulting in cell lysis. This virulence factor is considered of major importance in *M. haemolytica* A1 infections. *Mannheimia haemolytica* also secretes a sialoglycoprotease (metallo-endopeptidase) which cleaves the sialoglycoproteins on the surface of epithelial cells, macrophages and leukocytes (Abdullah et al. 1992). Therefore, this enzyme may have a role in adhesion and colonization of the respiratory tract by *M. haemolytica.* In addition, its ability to cleave bovine IgG1 may reduce the effectiveness of the immune response (Mellors and Lo 1995). The LPS of *M. haemolytica* has endotoxic activity (stimulates release of pro-inflammmatory cytokines, induces microvascular necrosis and thrombosis). The capsule has been reported to prevent phagocytosis, mask the cell surface, promote resistance to complement and aid adherence to host cells. Other virulence factors include fimbriae which facilitate adherence and a siderophore which promotes iron acquisition. Neuraminidase is involved in the removal of sialic acid residues from host glycoproteins, thus promoting adherence, while superoxide dismutase (metallo-enzyme) plays a role in the detoxification of free radicals.

Laboratory Diagnosis

Specimens

The specimens required depend on the animal species and on the disease syndrome. Portions of lung should be taken from the edge of pneumonic lesions. In cases of septicaemia, pieces of liver, spleen, kidney and lymph nodes can be submitted. Specimens from live animals might include pus, exudates, nasal swabs, bronchial lavages and mastitic milk. Transport media should be used since many of these bacteria have low viability outside the host.

Direct microscopy

The small, Gram-negative rods or coccobacilli are not always readily discernible in Gram-stained smears from affected tissues. In septicaemic conditions, such as fowl cholera, distinctive bipolar-staining *Pasteurella multocida* can be seen in Giemsa or Leishman-stained smears (Fig. 21.1).

Isolation

The routine medium for the isolation of *Pasteurella*, *Mannheimia*, *Bibersteinia* and *Avibacterium* species is ox or sheep blood agar. However, for *Avibacterium* species, a blood agar plate with 1.6 to 25 µg/mL of NADH or 20 to 100 µg/mL of NAD is recommended since some strains require V factor. NAD can also be provided by the use of a *Staphylococcus epidermidis* streak on a blood agar plate. In addition, some strains grow better under CO_2. Clinical materials should be inoculated onto both blood and MacConkey

Table 21.3 Main virulence factors of *Pasteurella multocida* and *Mannheimia haemolytica*

Bacteria	Virulence determinants	Functions
P. multocida		
	PMT toxin (serotypes A and D)	Cytotoxic protein that stimulates cell cytoskeleton rearrangements
	DnaK, DnaJ, DjlA, HscA, HscB, HtrA, GrpE, HslV, HslU, GroES, GroEL	Heat shock proteins
	Capsular polysaccharide	Prevent phagocytosis, resistance to complement, adherence
	SodA, SodC, Tpx, HktE, TsaA, ThdF	Detoxification
	Iron uptake system genes tonB, exbD and exbB HemB, HemE, HemH, HemL, HemU, Fur, RsgA1, RsgA2	Iron acquisition
	SurA, SurE	Stationary phase survival
	Filamentous hemagglutinins (PfhB1 and PfhB2), surface fibrils (Hsf_1 and Hfs_2), and fimbrial subunits (PtfA, FimA, Flp_1, Flp_2)	Adhesion to host cells, chemotaxis
M. haemolytica		
	LPS	Endotoxic activity, stimulates release of pro-inflammmatory cytokines, microvascular necrosis and thrombosis
	Leukotoxin (pore forming cytolysin)	Cytotoxic to ruminant leukocytes
	Capsule	Prevent phagocytosis, mask cell surface, resistance to complement, adherence
	Fimbriae	Adherence
	Siderophore	Iron acquisition
	Neuraminidase	Removal of sialic acid residues from host glycoproteins, thus promoting adherence
	Metallo-endopeptidase (sialoglycoprotease)	Cleavage of sialoglycoprotein on the surface of epithelial cells, macrophages or leukocytes
	Superoxide dismutase (metallo-enzyme)	Detoxification of free radicals

agars. Selective medium containing clindamycin (2 µg/ml) should be used for the isolation of *P. multocida* from porcine nasal swabs. The plates are incubated aerobically at 35°C for 24–48 hours. Intraperitoneal inoculation of mice has been used in the past to recover *P. multocida* from clinical specimens that contain large numbers of other, contaminating bacteria.

Identification

Colonial morphology

The colonies of all species are usually evident after 24 hours. They are of moderate size, round and greyish. Type A strains of *P. multocida* often produce relatively large, mucoid colonies due to their large capsules of hyaluronic acid (Fig. 21.2). *Pasteurella multocida* has a characteristic 'sweetish' odour, is non-haemolytic, does not grow on MacConkey agar and is a good indole producer. The colonies of *P. caballi* are smooth, round and non-haemolytic, while those of *P. pneumotropica* are non-haemolytic (Fig. 21.3) and somewhat similar to those of *P. multocida* (Figs 21.4 and 21.5). *Mannheimia haemolytica* is beta-haemolytic (Fig. 21.6) and usually tolerates the bile salts in MacConkey agar to grow as pinpoint red colonies (Fig. 21.7). It has no odour and does not produce indole. *Mannheimia granulomatis* is also haemolytic on blood agar. *Bibersteinia*

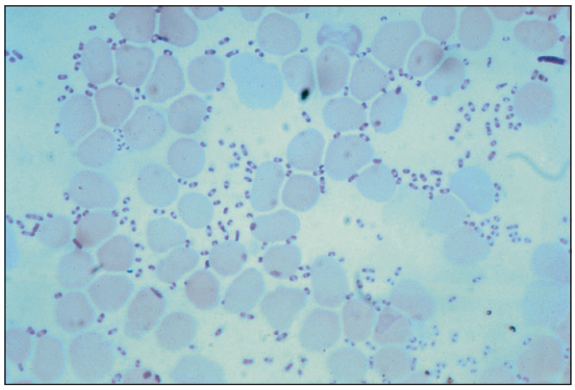

Figure 21.1 *Pasteurella multocida* in a bovine blood smear from a case of haemorrhagic septicaemia showing the characteristic bipolar staining. (Leishman stain, ×1000)

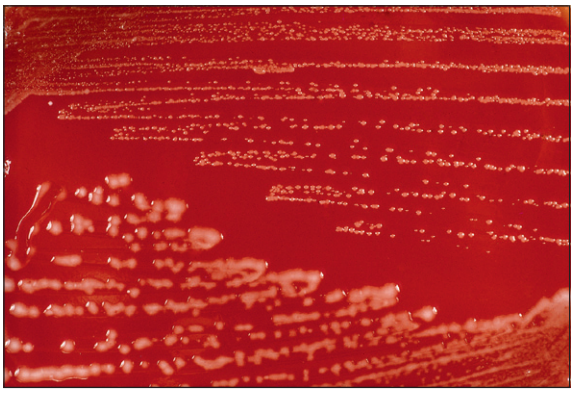

Figure 21.2 Comparison of the colonial types of *P. multocida*. The non-mucoid strain (top) was isolated from a dog, while the mucoid colonies (bottom) are those of a virulent type A strain from a pig.

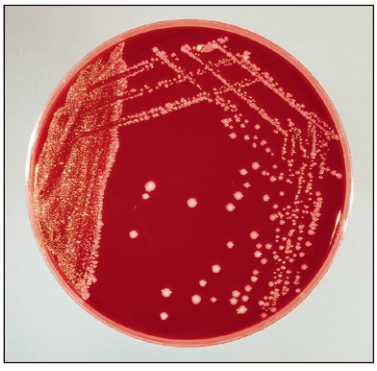

Figure 21.3 *Pasteurella pneumotropica* on sheep blood agar.

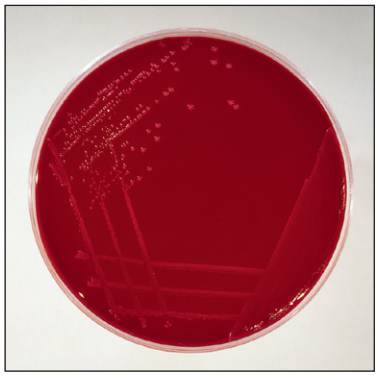

Figure 21.4 *Pasteurella multocida* on sheep blood agar. The colonies are non-haemolytic and have a characteristic sweetish odour.

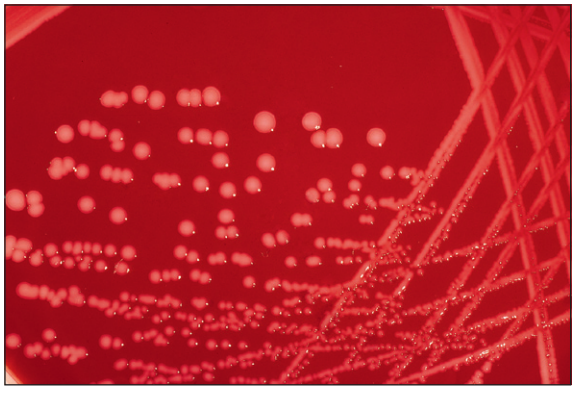

Figure 21.5 A close-up of the colonies of *P. multocida* shown in Figure 21.4.

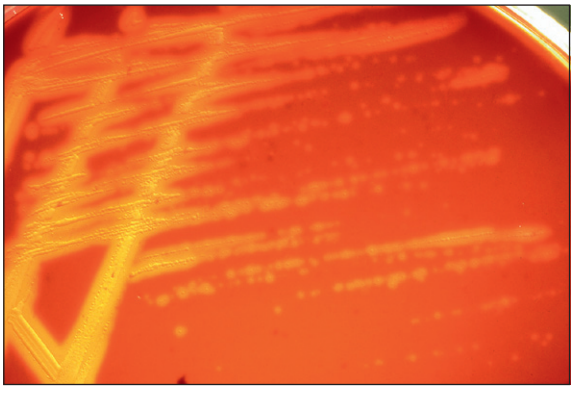

Figure 21.6 *Mannheimia haemolytica* on sheep blood agar, isolated from the pneumonic lung of a lamb, showing small colonies surrounded by a narrow zone of beta-haemolysis.

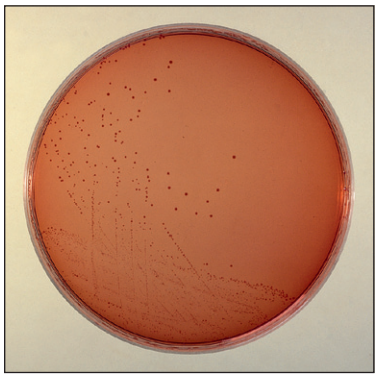

Figure 21.7 *Mannheimia haemolytica* on MacConkey agar: the small, red, pinpoint colonies indicate a tolerance of the bile salts in the medium.

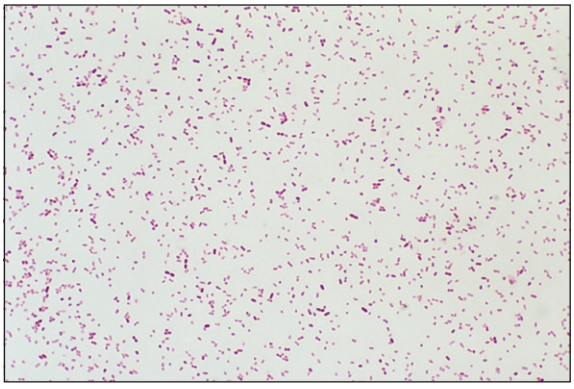

Figure 21.8 Gram-stained smear from a culture of *P. multocida*. Small Gram-negative rods with a tendency towards coccobacillary forms. (×1000)

trehalosi colonies are round, greyish and often have a double zone of haemolysis.

Microscopic appearance

Smears from colonies reveal small, Gram-negative rods or coccobacilli (Fig. 21.8). *Pasteurella* and *Mannheimia* species are non-motile and this can be confirmed in a wet mount or in motility medium.

Biochemical reactions

Identification may present some difficulties due to similar biochemical characteristics among species of *Pasteurella, Actinobacillus, Biberteinia* and *Avibacterium*. Characteristic colonies of *Pasteurella* and *Mannheimia* species yield small, Gram-negative rods or coccobacilli that are oxidase-positive (the enterobacteria are oxidase-negative) and catalase-positive (except for *P. caballi*). Suspect colonies are usually inoculated into TSI slopes. The usual reaction is a yellowish slant and butt with no gas or H_2S production. A suspect *Pasteurella* species isolate can then be inoculated into differential media to determine the identifying characteristics as listed in Table 21.4. This table also gives the reactions of the most important species of the genus *Pasteurella, Actinobacillus, Biberteinia* and *Avibacterium*. The API 20NE and the API ZYM tests can usually (95% of the time) identify *P. multocida* correctly (Vera Lizarazo et al. 2008). Confident identification may require molecular methods.

P. multocida has three subspecies that are differentiated by minor differences in their fermentation of carbohydrates (Table 21.5). *Pasteurella multocida* subsp. *multocida* strains cause significant disease in domestic animals. *Pasteurella multocida* subsp. *septica* strains can be recovered from various sources including dogs, cats, birds and man. *Pasteurella multocida* subsp. *gallicida* strains are recovered from birds and may occasionally cause fowl cholera. The identification of a particular subspecies may be of use in epidemiological studies rather than for routine diagnostic purposes.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing can be carried out using classical techniques such as disc diffusion, agar dilution and broth microdilution assays. The broth microdilution and the agar dilution methods are both used to determine the minimal inhibitory concentration (MIC) range and values for MIC50 and MIC90. Resistance of an isolate to an antimicrobial agent is determined by use of the breakpoint value when available. Most of the breakpoint values as well as the detailed methodology can be found in the Clinical and Laboratory Standards Institute document M31-A3 (CLSI 2008; CLSI formerly known as the National Committee for Clinical Laboratory Standards or NCCLS). The CLSI recommends the broth microdilution for *P. multocida* and the disc diffusion for *M. haemolytica*. For the broth microdilution assay, the cation-adjusted Mueller–Hinton broth can be used with incubation in a 35°C incubator. Some strains may require 24 hours' incubation instead of the18 hours' incubation which is usually recommended. For the disc diffusion test, a Mueller–Hinton agar with 5% sheep blood should be used and incubated in a 35°C incubator for 18 to 24 hours. *Staphylococcus aureus* ATCC 25923 is recommended for quality control testing, while *M. haemolytica* ATCC 33396 can be used as a quality control reference strain for both ceftiofur and tulathromycin (CLSI 2008; M31-A3). A pairwise comparison of disc diffusion with agar dilution results for seven antimicrobials revealed that the disc diffusion method is reliable for epidemiological studies such as surveillance programmes when the prevalence of resistance is low. However, it needs to be interpreted with caution in instances where levels of resistance are high (Catry et al. 2007).

Table 21.4 Presumptive identification of *Pasteurella, Mannheimia, Avibacterium* and *Bibersteinia* species of most significance in veterinary medicine

Test	*P. multocida*	*P. canis*	*P. dagmatis*	*P. caballi*	*M. haemolytica*	*B. trehalosi*	*A. paragallinarum*
Beta-haemolysis	–	–	–	–	+	+	–
NAD	–	–	–	–	–	–	+
Growth on MacConkey	–	–	–	–	+	weak	–
Indole	+	v	+	–	–	–	–
Urease	–	–	+	–	–	–	–
Ornithine decarboxylase	+	+	–	v	–	NA	NA
Acid from:							
Glucose	+	+	+ with gas	+ with gas	+	+	+
Lactose	–	–	–	+	+	NA	–
Sucrose	+	NA	–	NA	+	NA	NA
Maltose	–	–	+	+	+	+	v
Mannitol	+	–	–	+	–	+	+
Trehalose	v	v	+	–	–	+	NA

+ = greater than or equal to 90% positive, – = less than or equal to 10% positive, v = variable, 11 to 89% positive, NA = not available

Table 21.5 Differentiation of the *Pasteurella multocida* subspecies

Test	Subspecies *multocida*	Subspecies *septica*	Subspecies *gallicida*
Fermentation of:			
Trehalose	v	+	–
D-xylose	v	+	+
L-arabinose	–	–	v
Sorbitol	+	–	+
Dulcitol	–	–	+

+ = greater than or equal to 90% positive, –, less than or equal to 10% positive, v = variable, 11 to 89% positive

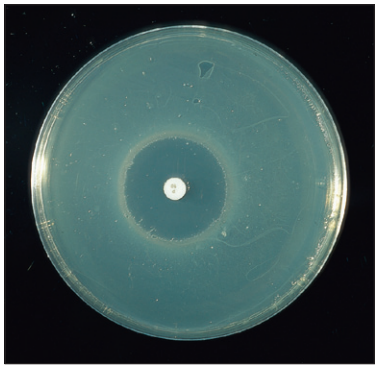

Figure 21.9 *Pasteurella multocida* on Isosensitest agar showing susceptibility to penicillin (uncommon for Gram-negative bacteria).

Antimicrobial resistance

Antimicrobial agents represent a powerful tool for the control of *Pasteurella* and *Mannheimia* infections. However, increasing rates of antimicrobial resistance may reduce the efficacy of the antimicrobial agents used to control these infections. Most *Pasteurella* species are susceptible to beta-lactam antibiotics such as penicillin (Fig. 21.9) and also to macrolides, tetracyclines and fluoroquinolones. They are usually resistant to amikacin and clindamycin. Moderate activity is observed with some aminoglycosides (Weber et al. 1984). However, routine antimicrobial susceptibility testing should be carried out on strains of *P. multocida* and *M. haemolytica* as plasmid-based resistance to sulphonamides and some commonly used antibiotics

has been widely encountered (Catry et al. 2005, Lizarazo et al. 2006). Beta-lactamase-positive strains of *P. multocida* have been reported for several years (Lion et al. 1999). The genetic basis of resistance of *Pasteurella* and *Mannheimia* isolates to beta-lactam antibiotics, tetracyclines, aminoglycosides, sulfonamides, and chloramphenicol has been reviewed (Kehrenberg et al. 2001).

Pasteurella multocida is the most commonly cultured bacterium from infected cat bite wounds and isolates from cats from the United States were reported to be highly susceptible to benzylpenicillin, amoxicillin-clavulanate, cefazolin, and azithromycin. These results indicate that these antimicrobials are still reliable choices for preventing and treating *P. multocida* bite-wound infections (Freshwater 2008).

In *B. trehalosi*, formerly *P. trehalosi*, floR-mediated resistance to chloramphenicol and florfenicol was associated with a plasmid. Intesrestingly, it also carried genes for resistance to sulphonamides and chloramphenicol *(catA3)* (Kehrenberg et al. 2006).

Strain Typing

Serotyping of *P. multocida* or *M. haemolytica* is usually performed in reference laboratories. Five types or serogroups of *P. multocida*, designated A, B, D, E and F, have been identified based on differences in capsular substances (polysaccharides). Sixteen somatic serotypes (lipopolysaccharides) have also been determined and given numbers (serotypes 1 to 16). A serotype is identified by its serogroup followed by its somatic type, for example the cause of bovine haemorrhagic septicaemia is B:6 in Southeast Asia and E:6 in Africa. *Mannheimia haemolytica* can be serotyped using the indirect haemagglutination test (Katsuda et al. 2008). Historically, *M. haemolytica* was organized into 17 serotypes based on capsular antigens with 13 A serotypes and 4 T serotypes. This classification has been revisited and *M. haemolytica* now encompasses the serotypes A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17; while serotype A11 was re-named *M. glucosida*, and serotypes T3, T4, T10, and T15 have been reassigned to *B. trehalosi*.

Novel typing methods rely on molecular techniques such as 16S rRNA gene sequencing (Boerlin et al. 2000), amplified fragment length polymorphism (Blehert et al. 2008), repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, single primer PCR assays (Shivachandra et al. 2008), random amplification polymorphic DNA (RAPD-PCR) (Katsuda et al. 2003), and pulsed field gel electrophoresis (PFGE) (Villard et al. 2008). A multiplex capsular typing system has been proposed for *P. multocida* (Townsend et al. 2001) which can be helpful in the distinguishing between closely related serogroups A and F. Diagnostic and selected typing systems for investigating disease caused by *P. multocida* are well described in the review by Dziva et al. (2008). Successive subculture of strains had no apparent effect on *B. trehalosi* strains whereas considerable variation was detected following subculture of *M. haemolytica* isolates as revealed by capsular and phenotypic typing and PFGE-based methods in a study by Villard et al. (2008).

Molecular Diagnosis

Many molecular diagnostic methods are now available to identify isolates of *Pasteurella* and *Mannheimia* including 16S rRNA sequencing and various PCR assays. Kuhnert et al. (2000) published a phylogenetic analysis of the three subspecies of *P. multocida* based on their 16S rRNA (rrs) gene sequence. A serotype-specific polymerase chain reaction (PCR) assay has been described for detection and identification of *P. multocida* serotype 1 (Rocke et al. 2002). A multiplex PCR assay has been designed to simplify investigations of porcine atrophic rhinitis and bronchopneumonia (Register & DeJong 2006). This assay can simultaneously detect three different targets: one common to all *P. multocida* strains (*kmt1* gene), one found only in toxigenic *P. multocida* strains (*toxA* gene), and one common to *B. bronchiseptica* strains (*flaA* gene). In situ hybridization has been utilized for the detection of the *P. multocida* toxin (PMT) gene in tissue sections of pneumonic lung from pigs (Ahn et al. 2008). The rapid and accurate identification of *Pasteurella* and *Mannheimia* isolates based on sequencing of the target sodA(int) gene, encoding the manganese-dependent superoxide dismutase has been reported (Gautier et al. 2005). A multiplex PCR assay has been described capable of identifying *M. haemolytica*, *M. glucosida* and *M. ruminalis* (Alexander et al. 2008). Chen et al. (1998a, 1998b) have published a PCR protocol for *A. paragallinarum*, which is rapid and specific.

REFERENCES

Abdullah, K.M., Udoh, E.A., Shewen, P.E., et al., 1992. A neutral glycoprotease of *Pasteurella haemolytica* A1 specifically cleaves O-sialoglycoproteins. Infection and Immunity 60, 56–62.

Ahn, K.K., Lee, Y.H., Ha, Y., et al., 2008. Detection by in-situ hybridization of *Pasteurella multocida* toxin (toxA) gene in the lungs of naturally infected pigs. Journal of Comparative Pathology 139, 51–53.

Alexander, T.W., Cook, S.R., Yanke, L.J., et al., 2008. A multiplex polymerase chain reaction assay for the identification of *Mannheimia haemolytica, Mannheimia glucosida* and *Mannheimia ruminalis*.

Veterinary Microbiology 130, 165–175.

Al-Haddawi, M.H., Jasni, S., Zamri-Saad, M., et al., 2000. In vitro study of *Pasteurella multocida* adhesion to trachea, lung and aorta of rabbits. Veterinary Journal 159, 274–281.

Blackall, P.J., Christensen, H., Beckenham, T., et al., 2005. Reclassification of *Pasteurella gallinarum, (Haemophilus) paragallinarum, Pasteurella avium* and P*asteurella volantium as Avibacterium gallinarum gen. nov., comb. nov., Avibacterium paragallinarum comb. nov., Avibacterium avium comb. nov.* and *Avibacterium volantium comb. nov*. International Journal of Systematic and Evolutionary Microbiology 55, 353–362.

Blackall, P.J., Bojesen, A.M., Christensen, H., et al., 2007. Reclassification of *(Pasteurella) trehalosi* as *Bibersteinia trehalosi gen. nov., comb. nov*. International Journal of Systematic and Evolutionary Microbiology 57, 666–674.

Blehert, D.S., Jefferson, K.L., Heisey, D.M., et al., 2008. Using amplified fragment length polymorphism analysis to differentiate isolates of *Pasteurella multocida* serotype 1. J Wildl Dis 44, 209–225.

Boerlin, P., Siegrist, H.H., Burnens, A.P., et al., 2000. Molecular identification and epidemiological tracing of *Pasteurella multocida* meningitis in a baby. Journal of Clinical Microbiology 38, 1235–1237.

Bosch, M., Garrido, M.E., Llagostera, M., et al., 2002. Characterization of the *Pasteurella multocida hgbA* gene encoding a hemoglobin-binding protein. Infection & Immunity 70 (11), 5955–5964.

Bredy, J.P., Botzler, R.G., 1989. The effects of six environmental variables on *Pasteurella multocida* populations in water. Journal of Wildlife Diseases 25, 232–239.

Catry, B., Haesebrouck, F., Vliegher, S.D., et al., 2005. Variability in acquired resistance of *Pasteurella* and *Mannheimia* isolates from the nasopharynx of calves, with particular reference to different herd types. Microbial Drug Resistance 11, 387–394.

Catry, B., Dewulf, J., de Kruif, A., et al., 2007. Accuracy of susceptibility testing of *Pasteurella multocida* and *Mannheimia haemolytica*. Microbial Drug Resistance 13, 204–211.

Chen, X., Chen, Q., Zhang, P., et al., 1998a. Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. Avian Pathology 27, 296–300.

Chen, X., Song, C., Gong, Y., et al., 1998b. Further studies on the use of a polymerase chain reaction test for the diagnosis of infectious coryza. Avian Pathology 27, 618–624.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard, third ed. CLSI document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

Dziva, F., Muhairwa, A.P., Bisgaard, M., et al., 2008. Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*. Veterinary Microbiology 128, 1–22.

Freshwater, A., 2008. Why your housecat's trite little bite could cause you quite a fright: a study of domestic felines on the occurrence and antibiotic susceptibility of *Pasteurella multocida*. Zoonoses Public Health 55, 507–513.

Gautier, A.L., Dubois, D., Escande, F., et al., 2005. Rapid and accurate identification of human isolates of *Pasteurella* and related species by sequencing the sodA gene. Journal of Clinical Microbiology 43, 2307–2314.

Highlander, S.K., 2001. Molecular genetic analysis of virulence in *Mannheimia (pasteurella) haemolytica*. Frontiers in Bioscience 6, D1128–D1150.

Katsuda, K., Kohmoto, M., Kawashima, K., et al., 2003. Molecular typing of *Mannheimia (Pasteurella) haemolytica* serotype A1 isolates from cattle in Japan. Epidemiology & Infection 131, 939–946.

Katsuda, K., Kamiyama, M., Kohmoto, M., et al., 2008. Serotyping of *Mannheimia haemolytica* isolates from bovine pneumonia: 1987–2006. Veterinary Journal 178, 146–148.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J.L., et al., 2001. Antimicrobial resistance in *Pasteurella* and *Mannheimia:* epidemiology and genetic basis. Veterinary Research 32, 323–339.

Kehrenberg, C., Meunier, D., Targant, H., et al., 2006. Plasmid-mediated florfenicol resistance in *Pasteurella trehalosi*. Journal of Antimicrobial Chemotherapy 58, 13–17.

Kuhnert, P., Boerlin, P., Emler, S., et al., 2000. Phylogenetic analysis of *Pasteurella multocida* subspecies and molecular identification of feline *P. multocida* subsp. *septica* by 16S rRNA gene sequencing. International Journal of Medical Microbiology 290, 599–604.

Lion, C., Lozniewski, A., Rosner, V., et al., 1999. Lung abscess due to beta-lactamase-producing *Pasteurella multocida*. Clinical Infectious Diseases 29, 1345–1346.

Lizarazo, Y.A., Ferri, E.F., de la Fuente, A.J., et al., 2006. Evaluation of changes in antimicrobial susceptibility patterns of *Pasteurella multocida* subsp *multocida* isolates from pigs in Spain in 1987–1988 and 2003–2004. American Journal of Veterinary Research 67, 663–668.

May, B.J., Zhang, Q., Li, L.L., et al., 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proceedings of the National Academy of Sciences USA 98, 3460–3465.

Mellors, A., Lo, R.Y., 1995. O-sialoglycoprotease from *Pasteurella haemolytica*. Methods in Enzymology 248, 728–740.

Pruimboom, I.M., Rimler, R.B., Ackermann, M.R., et al., 1996. Capsular hyaluronic acid-mediated adhesion of *Pasteurella multocida* to turkey air sac macrophages. Avian Diseases 40, 887–893.

Register, K.B., DeJong, K.D., 2006. Analytical verification of a multiplex PCR for identification of *Bordetella bronchiseptica* and *Pasteurella multocida* from swine. Veterinary Microbiology 117, 201–210.

Rimler, R.B., Brogden, K.A., 1986. *Pasteurella multocida* isolated from rabbits and swine: serologic types and toxin production. American Journal of Veterinary Research 47, 730–737.

Rocke, T.E., Smith, S.R., Miyamoto, A., et al., 2002. A serotype-specific polymerase chain reaction for

identification of *Pasteurella multocida* serotype 1. Avian Diseases 46, 370–377.

Shewen, P.E., Wilkie, B.N.. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect Immun 35 (1), 91–94.

Shivachandra, S.B., Kumar, A.A., Chaudhuri, P., 2008. Molecular characterization of avian strains of *Pasteurella multocida* serogroup-A:1 based on amplification of repetitive regions by PCR. Comparative Immunology, Microbiology & Infectious Diseases 31, 47–62.

Townsend, K.M., Boyce, J.D., Chung, J.Y., et al., 2001. Genetic organization of *Pasteurella multocida* cap Loci and development of a multiplex capsular PCR typing system. Journal of Clinical Microbiology 39, 924–929.

Vera Lizarazo, Y.A., Rodriguez Ferri, E.F., Gutierrez Martin, C.B., 2008. Evaluation of different API systems for identification of porcine *Pasteurella multocida* isolates. Research in Veterinary Science 85, 453–456.

Villard, L., Gauthier, D., Maurin, F., et al., 2008. Serotypes A1 and A2 of *Mannheimia haemolytica* are susceptible to genotypic, capsular and phenotypic variations in contrast to T3 and T4 serotypes of *Bibersteinia (Pasteurella) trehalosi*. FEMS Microbiology Letters 280, 42–49.

Weber, D.J., Wolfson, J.S., Swartz, M.N., et al., 1984. *Pasteurella multocida* infections. Report of 34 cases and review of the literature. Medicine (Baltimore) 63, 133–154.

Chapter 22

Francisella tularensis

Genus Characteristics

Fransicella species are tiny, pleomorphic, non-motile, Gram-negative coccobacilli. The genus *Francisella* can be distinguished from other genera of small Gram-negative coccobacilli using the features outlined in Table 22.1. The genus *Francisella* is within the *Francisellaceae* family and consists of *F. tularensis, F. noatunensis, F. hispaniensis, F. halioticida* and *F. philomiragia*. *Francisella* species are strict aerobes that grow best on blood agar supplemented with cystine or chocolate agar. They are oxidase-negative (except *F. philomiragia*), weakly catalase-positive and non-spore forming. They also have a limited range of carbohydrates fermentation (acid without gas) and a unique fatty acid composition.

Francisella tularensis is a facultative intracellular pathogen and the causative agent of tularaemia. *Francisella noatunensis, F. halioticida* and *F. philomiragia* are rarely isolated opportunistic pathogens of fish, closely linked to water. *F. tularensis* consists of four subspecies: *F. tularensis* subsp. *tularensis* (formerly Jellison type A or subspecies *nearctica*), *F. tularensis* subsp. *holarctica* (formerly Jellison type B or subspecies *palaearctica*), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*. The subspecies differ in host specificity, biochemical activity and geographical distribution.

Natural Habitat

Francisella tularensis is widely distributed in nature. However, it occurs mainly in the northern hemisphere and most frequently in Scandinavia, North America, Japan and Russia. It has also been reported from Turkey, Yugoslavia, Spain, Kosovo and Switzerland indicating a wider distribution (Ellis et al. 2002). *Francisella tularensis* subsp. *tularensis* (type A) is the most virulent strain and is found in North America, while *F. tularensis* subsp. *holarctica* (type B) is less virulent and is found in North America, Europe and Asia. Three biovars of *F. tularensis* subspecies *holarctica* have been suggested; biovar I (erythromycin sensitive), biovar II (erythromycin resistant) and biovar japonica (Kudelina & Olsufiev 1980, Olsufjev and Meshcheryakova 1982). *Francisella tularensis* subsp. *mediasiatica* has only been isolated from Central Asia and is considered to be of low virulence. Strains of the subspecies *novicida* cause severe disease in inbred mice, similar to *F. tularensis* subsp. *tularensis* human isolates, but are not pathogenic for immunocompetent humans (Baker et al. 1985).

Francisella tularensis has been isolated from about 250 wildlife species that can potentially transmit tularaemia to humans. Wild animals are thought to be reservoirs of infection, especially rabbits and hares but also beavers, muskrats, squirrels, woodchucks, opossums, skunks, deer, voles, foxes, rats and other rodents (Ellis et al. 2002, Zhang et al. 2006). The bacterium is most frequently transmitted by any one of a large range of biting arthropods including flies (*Chrysops* and *Tabanus*), mites, mosquitoes (*Culicidae*), lice and ticks (*Ixodes*, *Dermacentor* and *Amblyomma*). These arthropod vectors probably play a role in the mechanical transmission of the disease both between wild animals and from animals to humans. It is also thought that the tick may serve as a reservoir of infection (Foley & Nieto 2010). Rural populations and especially individuals who spend time in endemic areas such as hunters, agricultural and forestry workers have a higher risk of contracting tularaemia (Ellis et al. 2002). *Francisella tularensis* is quite resistant in the environment and can survive for months in soil, water, on carcasses and meat. It is susceptible to common disinfectants (70% ethanol, sodium hypochlorite, glutaraldehyde) and autoclaving.

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Table 22.1 Presumptive identification of *Francisella tularensis* and differentiation from similar Gram-negative genera of significance in veterinary medicine

Test[a]	*Francisella tularensis*	*Brucella* species	*Acinetobacter* species	*Bordetella bronchiseptica*	*Bartonella* species
Oxidase	–	+	–	+	–
Urea hydrolysis	–	+	v	+	–
Gram stain	Tiny ccb	Tiny ccb	Broad ccb	Thin rod	Thin rod
Cysteine enhancement	+	–	–	–	–
Motility	–	–	–	+	–[b]

[a]+ = greater than or equal to 90% positive, – = less than or equal to 10% positive, v = variable, 11 to 89% positive; ccb, coccobacilli;
[b]except *Bartonella bacilliformis*

Pathogenesis and Pathogenicity

Francisella tularensis is the aetiological agent of tularaemia, a serious and occasionally fatal disease of humans and animals. It has been designated by the Centers for Disease Control and Prevention (CDC) as a category A select agent because of its low infective dose (less than 10 colony-forming units), its transmission via aerosol, and its ability to produce severe morbidity and high mortality.

Francisella tularensis can infect a wide range of species of wild and domestic mammals, birds, fish, reptiles and amphibians. Tularaemia is acquired by direct contact with infected animals, through contaminated water or food, or from vectors such as biting insects or ticks. Airborne transmission also occurs, especially during processing of agricultural products. The disease is often epidemic, both in humans and in animals. Clinical manifestations depend on the type of reservoir involved and the means of transmission. An appreciable number of infections in farm animals can occur in some regions. Tularaemia is rarely seen in dogs which are considered relatively resistant. However, cats are more susceptible and there are reports of transmission from cats to humans. In animals, tularaemia has a large spectrum of clinical signs ranging from no signs of illness to death. Characteristic signs include depression, anorexia, fever, vomiting, diarrhoea, lymphadenomegaly, ulcers and haemorrhage. Human tularaemia can occur in several forms (ulceroglandular, glandular, oropharyngeal or gastrointestinal, oculoglandular, pneumonic and typhoidal), largely depending on the route of entry of the organism into the body. The most common form is the ulceroglandular disease that occurs as a consequence of an infected arthropod vector bite (Ohara et al. 1998, Ellis et al. 2002). In some cases, infection occurs via cuts or abrasions, typically in hunters after the handling of contaminated meat. An ulcer will then form at the site of infection and flu-like symptoms follow after an incubation period of three to six days.

In both human and animal cases of tularaemia, *F. tularensis* can be highly invasive. After infection, the organisms are disseminated from the inoculation site to the lymphatic system via the regional lymph nodes. These lymph nodes usually become enlarged and *F. tularensis* may then be disseminated to other tissues such as the lungs, spleen, liver, kidneys, intestine, central nervous system, and skeletal muscles. Granuloma formation may occur at these sites. The characteristic gross lesions seen in rabbits and other wild animals are small necrotic, granulomatous foci in the spleen, liver and lymph nodes.

Although *F. tularensis* is one of the most infectious bacterial pathogens, its virulence mechanisms (Table 22.2) are only beginning to be understood (Meibom & Charbit 2010). The organism resides within host macrophages in vivo. The capsule of *Francisella*, although essential for serum resistance, is not required for survival following phagocytosis by leukocytes (Sandstrom et al. 1988). Phase variation of the lipopolysaccharide (LPS) of *F. tularensis* has been reported and the different forms appear to affect both antigenicity (due to variations in the O antigen) and the nitric oxide (NO) response of macrophages (due to variation in the lipid A moiety). Phase variation of lipid A also affects the ability of the organism to grow intracellularly. One phase (reduced NO induction) results in bacterial growth, while another phase (increased NO production) suppresses growth, thus modulating the innate immune response (Cowley et al. 1996). In addition, the LPS of *F. tularensis* does not show the properties of a classical endotoxin. For example, it fails to induce interleukin-1 from mononuclear cells and poorly induces tumour necrosis factor. A *Francisella* pathogenicity island (FPI of 30 Kb) has been discovered (Nano et al. 2004) and several virulence-associated proteins identified (Santic et al. 2005, Brotcke et al. 2006, Lenco et al. 2007), in addition to a putative type IV secretion system, encoded by the FPI.

Table 22.2 Main virulence factors of pathogenic *Francisella tularensis*

Virulence determinants	Functions
AcpA protein	Acid phosphatase function, capable of inhibiting the respiratory burst
Capsule	Essential for serum resistance
LPS	Phase variation of LPS appears to affect antigenicity and the nitric oxide response of macrophages
Francisella pathogenicity island (FPI of 30 Kb)	Evasion of lysosomal fusion within macrophages
PdpA and PdpD	Required for intracellular replication in macrophages and for virulence in mice
iglABCD operon	Transcribed in response to iron limitation
IglC and IglA	Necessary for full virulence of *F. tularensis*
IglA and IglB	Thought to be involved in protein secretion
IglC	Required for growth in macrophages
mglAB operon	Likely a global transcriptional regulator which regulates the expression of a range of proteins in response to nutritional stress

To survive and evade phagosome–lysosome fusion, intracellular pathogens have evolved different mechanisms such as escaping from the phagosome into the cytoplasm after degradation of the phagosomal membrane or adapting to the acidic environment within the phagolysosomes. However, the more common strategy is to modulate phagosome biogenesis to faciliate intracellular replication. *Francisella tularensis* has a specific mechanism of intracellular development with a unique method of modulating phagosome biogenesis. Studies have shown that *F. tularensis* enters human macrophages by a novel process of engulfment within asymmetric, spacious pseudopod loops, a process that differs from all previously described uptake mechanisms (Clemens et al. 2005). The *Francisella*-containing phagosomes have limited maturation in the endocytic pathway and do not fuse with lysosomes (Clemens et al. 2004). Within hours the organism disrupts the phagosome and escapes into the cytoplasm where it replicates. Escape from the cell follows cell disruption due to pyroptosis, a form of apoptosis induced by the organism (Foley & Nieto 2010).

Laboratory Diagnosis

Frabcisella tularensis is highly infectious for humans. The greatest risk of laboratory-acquired infection is by aerosol inhalation while working with infected specimens or cultures. Clinical specimens should be handled by trained personnel under biosafety level 2 conditions and all work should be carried out in a biological safety cabinet. Specimens and cultures should be transferred to a biosafety level 3 laboratory as soon as *F. tularensis* is suspected (Murray et al. 2003). A modified live vaccine is available for laboratory personnel handling the organism.

Specimens

Clinical specimens (ulcer or wound swabs, fresh tissues or aspirates of affected lymph nodes) can be placed in Amies agar with charcoal, a commercial transport system specific for anaerobic and aerobic pathogens. The organism will remain viable for seven days at ambient room temperature when placed in Amies medium (Johansson et al. 2000a). Freezing of tissues such as spleen, lung, kidney, liver, and lymph nodes is also recommended. Representative portions should be selected and frozen at −30°C or −70°C for possible future reference. Blood samples for serology should be obtained.

Direct microscopy

Francisella tularensis cells are very small and because they stain faintly by direct Gram staining, they do not show up well in Gram-stained smears from specimens. Presumptive identification of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* can be achieved with direct fluorescent-antibody staining of smears from lesions using a labelled hyperimmune rabbit polyclonal antibody prepared by immunization with *F. tularensis* subsp. *tularensis*. Immunohistochemistry (IHC) has been used on formalin-fixed tissues to detect *F. tularensis* (DeBey et al. 2002). It is considered a valuable tool for establishing a rapid aetiological diagnosis under circumstances where fresh tissues may not be available for isolation and identification of the organism.

Isolation

Francisella tularensis is a fastidious organism which requires supplements such as cysteine, cystine, thiosulfate or IsoVitaleX (sulfhydryl compounds) to grow on laboratory medium. It will grow well on cysteine heart blood agar (supplemented with 9% chocolatized sheep blood), chocolate agar, buffered charcoal yeast extract agar, Thayer–Martin agar and in broth (thioglycolate, tryptic soy and

Mueller–Hinton) supplemented with 1% to 2% IsoVitaleX (Murray et al. 2003). However, *F. tularensis* will not grow on standard agar such as blood, trypticase soy and brain-heart infusion, except for subspecies *novicida*, which is cysteine independent. Material from specimens are usually plated on blood agar with and without cystine/cysteine or on chocolate agar. MacConkey agar plates can be inoculated in parallel in order to detect any Gram-negative pathogens or contaminants. The inoculated plates are incubated at 35°C to 37°C aerobically for 10 to 14 days. *Francisella tularensis* is indifferent to the presence or absence of carbon dioxide (CO_2).

Identification

Colonial morphology

Francisella tularensis grows slowly, requiring two to four days for maximum colony size. After 48 hours, on cysteine heart blood agar, the colonies are about 2 mm, smooth, pearl-white to ivory in colour, with a green tint and a prominent opalescent sheen (production of H_2S). The agar medium can have a green-yellow discoloration. On chocolate agar, colonies are 2–4 mm, grey, smooth, moist with no agar discoloration. On enriched blood agar, after 48 hours, the colonies are minute and dew-drop in appearance (Fig. 22.1). On further incubation the colonies tend to coalesce and a greenish discolouration is evident around them.

Microscopic appearance

Gram-stained smears from colonies reveal tiny (0.2 × 0.2–0.7 µm), poorly stained, pleomorphic, single (rarely in chains) Gram-negative coccobacilli.

Biochemical characteristics

Francisella tularensis is oxidase-negative and weakly catalase-positive. Biochemical characterization is difficult with only a few key differences between subspecies (Table 22.3).

Other tests

A positive test result with one of the immunological techniques such as immunofluorescence on smears from cultures, IHC on tissues, or slide agglutination test on cultures provides a presumptive diagnosis of tularaemia. The slide agglutination test will presumptively identify a suspected colony by mixing polyclonal rabbit antiserum against *F. tularensis* with safranin-stained cells (Murray et al. 2003). A capture enzyme-linked immunosorbent assay (cELISA) based on monoclonal antibodies specific for lipopolysaccharide (LPS) of *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* has been recently described (Grunow et al. 2000).

Serology

Testing of serum or plasma from citrated or heparinized blood, can be carried out using the tube agglutination (TA) or microagglutination (MA) tests. Both are standard serological methods for determining the presence of antibodies to *Franscisella tularensis* in cases of tularaemia. A commercial MA test (Difco Laboratories, Michigan, USA)

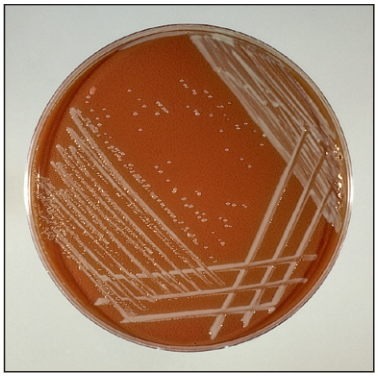

Figure 22.1 *Francisella tularensis* on enriched blood agar (ATCC 6223, avirulent type strain).

Table 22.3 Presumptive differentiation of *Francisella* subspecies

Characteristics	*F. tularensis* subspecies			
	Tularensis	Holarctica	Mediaasiatica	Novicida
Requires cystine/cysteine	+	+	+	–
Acid from:				
Glucose	+	+	–	+
Sucrose	–	–	+	+
Glycerol	+	–	+	+

+ = greater than or equal to 90% positive; – = less than or equal to 10% positive

containing a phenolized-inactivated suspension of *F. tularensis*, can be used to detect serum agglutinins. Haemagglutination, ELISA and indirect fluorescent antibody (IFA) staining methods (Magnarelli et al. 2007) are also available. In humans, a TA titre of >1 : 160 or a MA titre of >1 : 128 can be interpreted as presumptively positive (Murray et al. 2003). Microagglutination is the most commonly used test for detection of tularaemia antibodies in small animals. Titres tend to be lower than those observed in humans. Titres such as 1 : 140 to 1 : 160 are indicative of recent infections in dogs while lower serum antibody titres have been seen in infected cats. In general, a fourfold titre difference between acute and convalescent serum is confirmatory of infection.

Antimicrobial Susceptibility Testing

Due to safety concerns, *Francisella tularensis* antimicrobial susceptibility testing is not routinely performed in veterinary microbiology laboratories. Testing methods such as broth dilution, agar dilution (Baker et al. 1985), disc diffusion (Scheel et al. 1993) and E test (Biodisk, Solna, Sweden) (Ikaheimo et al. 2000) have been documented. Mueller–Hinton medium supplemented with 2% IsoVitaleX or cysteine heart blood agar supplemented with either 9% chocolatized sheep blood or 2% haemoglobin are suitable media for use with these methods. For the E test, the inoculum is adjusted to the density of a McFarland 0.5 turbidity standard and this results in confluent growth. The MICs are read after incubation overnight or after two nights at 35 °C in a 5% CO_2 atmosphere. Methods and interpretive standards for broth microdilution testing of *F. tularensis* are available in CLSI document M45-A2 (CLSI 2010). The drug of choice for the treatment of tularaemia in the past has been streptomycin, with tetracycline and chloramphenicol being used as alternatives (Murray et al. 2003). Gentamicin and quinolones have both been suggested as acceptable alternatives to streptomycin (Ikaheimo et al. 2000).

Antimicrobial Resistance

Little is known about drug resistance of isolates of *F. tularensis*. In a study by Ikaheimo et al. (2000), all strains tested were susceptible to streptomycin, tetracycline, chloramphenicol, tobramycin, gentamicin, ciprofloxacin, levofloxacin, grepafloxacin, and trovafloxacin. In contrast, all the strains were resistant to beta-lactams and azithromycin. *Francisella tularensis* produces a beta-lactamase. Erythromycin resistance of *F. tularensis* subsp. *holarctica* strains has been documented in certain geographical regions of northern Europe (Olsufjev & Meshcheryakova 1982, Georgi et al. 2012). A study of North American isolates did not reveal any significant resistance to seven antimicrobials (Urich & Petersen, 2008).

Strain Typing

The four subspecies of *F. tularensis* display a very close phylogenetic relationship. This has constrained the development of generally applicable typing methods. To overcome this problem, a variety of methods have been evaluated for the discrimination of subspecies and strains (Johansson et al. 2004). Sequencing of 16S rRNA can be used to determine genus, species and subspecies (Forsman et al. 1994). Many PCR methods have also been developed capable of typing strains (de la Puente-Redondo et al. 2000, Higgins et al. 2000, Johansson et al. 2000b, Tomaso et al. 2007). In the study by de la Puente-Redondo et al. (2000), three PCR methods were evaluated for epidemiological typing of *F. tularensis*: repetitive extragenic palindromic element PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and random amplified polymorphic DNA (RAPD) assay. On the basis of the combination of REP, ERIC, and RAPD fingerprints, *F. tularensis* strains were divided into 17 genetic groups (designated A to Q), and one *F. novicida* strain was classified in group R. Pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) analysis also appear to be promising tools for the epidemiological investigation of infections caused by different subspecies of *F. tularensis* (Garcia Del Blanco et al. 2002). The most discriminatory method seems to be multilocus variable tandem repeat analysis (MLVA) which targets highly variable regions of the *F. tularensis* genome (Johansson et al. 2004).

Molecular Diagnosis

A number of PCR assays have been developed for detection of *F. tularensis* (Forsman et al. 1994, Grunow et al. 2000). Use of a TaqMan 5′ nuclease assay (5NA) directed against the *F. tularensis* outer membrane protein (*Fop*) gene and a polymerase chain reaction-enzyme immunoassay (PCR-EIA) directed against the *tul 4* gene were investigated for detection of this organism in experimentally infected mice and in field-collected tick vectors (Higgins et al. 2000). These assays were genus-specific and a specially formulated filter paper (FTA) provided inexpensive and rapid template preparation for the ticks, mouse tissues and DNA from clinical samples.

Many specific and highly sensitive real-time PCR protocols for the rapid and specific identification of *F. tularensis* subspecies *tularensis* have been developed (Bystrom et al. 2005, Kugeler et al. 2006, Tomaso et al. 2007). The development of real-time PCR assays using minor groove binding probes for simultaneous detection of the *F. tularensis* 23 KDa gene, the *Bacillus anthracis pag* and *cap* genes, as well as the *Yersinia pestis pla* gene has provided a rapid tool for the simultaneous detection and identification of these three category A bacterial species (Skottman et al. 2007) which are listed as biological threats by the Centers for Disease Control and Prevention (CDC).

REFERENCES

Baker, C.N., Hollis, D.G., Thornsberry, C., 1985. Antimicrobial susceptibility testing of *Francisella tularensis* with a modified Mueller–Hinton broth. Journal of Clinical Microbiology 22, 212–215.

Brotcke, A., Weiss, D.S., Kim, C.C., et al., 2006. Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infection and Immunity 74, 6642–6655.

Bystrom, M., Bocher, S., Magnusson, A., et al., 2005. Tularaemia in Denmark: identification of a *Francisella tularensis* subsp. *holarctica* strain by real-time PCR and high-resolution typing by multiple-locus variable-number tandem repeat analysis. Journal of Clinical Microbiology 43, 5355–5358.

Clemens, D.L., Lee, B.Y., Horwitz, M.A., 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infection and Immunity 72, 3204–3217.

Clemens, D.L., Lee, B.Y., Horwitz, M.A., 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infection and Immunity 73, 5892–5902.

Clinical and Laboratory Standards Institute (CLSI), 2010. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, second ed. Approved Guideline M45-MA2. Clinical and Laboratory Standards Institute, Wayne, PA.

Cowley, S.C., Myltseva, S.V., Nano, F.E., 1996. Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Molecular Microbiology 20, 867–874.

DeBey, B.M., Andrews, G.A., Chard-Bergstrom, C., et al., 2002. Immunohistochemical demonstration of *Francisella tularensis* in lesions of cats with tularaemia. Journal of Veterinary Diagnostic Investigation 14, 162–164.

de la Puente-Redondo, V.A., del Blanco, N.G., Gutierrez-Martin, C.B., et al., 2000. Comparison of different PCR approaches for typing of *Francisella tularensis* strains. Journal of Clinical Microbiology 38, 1016–1022.

Ellis, J., Oyston, P.C., Green, M., et al., 2002. Tularaemia. Clinical Microbiology Reviews 15, 631–646.

Foley, J.E., Nieto, N.C., 2010. Tularaemia. Veterinary Microbiology 140 (3–4), 332–338.

Forsman, M., Sandstrom, G., Sjostedt, A., 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. International Journal of Systematic Bacteriology 44, 38–46.

Garcia Del Blanco, N., Dobson, M.E., Vela, A.I., et al., 2002. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. Journal of Clinical Microbiology 40, 2964–2972.

Georgi, E., Schacht, E., Scholz, H.C., et al., 2012. Standardized broth microdilution antimicrobial susceptibility testing of *Francisella tularensis* subsp. *holarctica* strains from Europe and rare *Francisella* species. Journal of Antimicrobial Chemotherapy 4 July 2012 (Epub ahead of print).

Grunow, R., Splettstoesser, W., McDonald, S., et al., 2000. Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clinical and Diagnostic Laboratory Immunology 7, 86–90.

Higgins, J.A., Hubalek, Z., Halouzka, J., et al., 2000. Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. American Journal of Tropical Medicine and Hygiene 62, 310–318.

Ikaheimo, I., Syrjala, H., Karhukorpi, J., et al., 2000. In vitro antibiotic susceptibility of *Francisella tularensis* isolated from humans and animals. Journal of Antimicrobial Chemotherapy 46, 287–290.

Johansson, A., Berglund, L., Eriksson, U., et al., 2000a. Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularaemia. Journal of Clinical Microbiology 38, 22–26.

Johansson, A., Ibrahim, A., Goransson, I., et al., 2000b. Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. Journal of Clinical Microbiology 38, 4180–4185.

Johansson, A., Forsman, M., Sjostedt, A., 2004. The development of tools for diagnosis of tularaemia and typing of *Francisella tularensis*. APMIS 112, 898–907.

Kudelina, R.I., Olsufiev, N.G., 1980. Sensitivity to macrolide antibiotics and lincomycin in Francisella tularensis holarctica. Journal of Hygiene, Epidemiology, Microbiology & Immunology 24, 84–91.

Kugeler, K.J., Pappert, R., Zhou, Y., et al., 2006. Real-time PCR for *Francisella tularensis* types A and B. Emerging Infectious Diseases 12, 1799–1801.

Lenco, J., Hubalek, M., Larsson, P., et al., 2007. Proteomics analysis of the *Francisella tularensis* LVS response to iron restriction: induction of the F. tularensis pathogenicity island proteins IglABC. FEMS Microbiology Letters 269, 11–21.

Magnarelli, L., Levy, S., Koski, R., 2007. Detection of antibodies to *Francisella tularensis* in cats. Research in Veterinary Science 82, 22–26.

Meibom, K.L., Charbit, A., 2010. The unraveling panoply of *Francisella tularensis* virulence attributes. Current Opinion in Microbiology 13 (1), 11–17.

Murray, P.R., Baron, E.J., American Society for Microbiology, 2003. Manual of Clinical Microbiology,

eighth ed, ASM Press, Washington DC.

Nano, F.E., Zhang, N., Cowley, S.C., et al., 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. Journal of Bacteriology 186, 6430–6436.

Ohara, Y., Sato, T., Homma, M.., 1998. Arthropod-borne tularaemia in Japan: clinical analysis of 1,374 cases observed between 1924 and 1996. Journal of Medical Entomology 35, 471–473.

Olsufjev, N.G., Meshcheryakova, I.S., 1982. Infraspecific taxonomy of tularaemia agent *Francisella tularensis* McCoy et Chapin. Journal of Hygiene, Epidemiology, Microbiology & Immunology 26, 291–299.

Sandstrom, G., Lofgren, S., Tarnvik, A., 1988. A capsule-deficient mutant of Francisella tularensis LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infection and Immunity 56, 1194–1202.

Santic, M., Molmeret, M., Klose, K.E., et al., 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiology 7, 969–979.

Scheel, O., Hoel, T., Sandvik, T., et al., 1993. Susceptibility pattern of Scandinavian *Francisella tularensis* isolates with regard to oral and parenteral antimicrobial agents. APMIS 101, 33–36.

Skottman, T., Piiparinen, H., Hyytiainen, H., et al., 2007. Simultaneous real-time PCR detection of *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis*. European Journal of Clinical Microbiology & Infectious Diseases 26 (3), 207–211.

Tomaso, H., Scholz, H.C., Neubauer, H., et al., 2007. Real-time PCR using hybridization probes for the rapid and specific identification of *Francisella tularensis* subspecies tularensis. Molecular and Cellular Probes 21, 12–16.

Urich, S.K., Petersen, J.M., 2008. In vitro susceptibility of isolates of *Francisella tularensis* types A and B from North America. Antimicrobial Agents and Chemotherapy 52 (6), 2276–2278.

Zhang, F., Liu, W., Chu, M.C., et al., 2006. *Francisella tularensis* in rodents, China. Emerging Infectious Diseases 12, 994–996.

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Chapter 23

Brucella species

Genus Characteristics

Brucella species are small Gram-negative rods (0.5–0.7 × 0.6–1.5 μm) that often appear coccobacillary. The brucellae were previously divided into six species based principally on host preference, pathogenicity and differences in biochemical properties. However, DNA–DNA hybridization studies revealed that the species were remarkably homogeneous and thus it was proposed that all species should be regarded as biovars of a single species, *Brucella melitensis*. This proposal has not gained general acceptance and many workers still adhere to the multiple species nomenclature which describes six nomenspecies: *Brucella abortus, B. melitensis, B. suis, B. neotomae, B. ovis* and *B. canis*. The first three of these are subdivided into biovars based on cultural and serological properties. Furthermore, with the recent isolation of *Brucella* species from marine mammals, two new species, *B. ceti* and *B. pinnipedialis,* have been recognized (Foster et al. 2007). Two further species, *B. microti* and *B. inopinata* have also been described. The *Brucella* genome is composed of two circular chromosomes, of approximately 2.1 and 1.2 Mbp, with the exception of *Brucella suis* biovar 3, which has a single chromosome. Full genomic sequences of *B. abortus*, *B. melitensis* and *B suis* have been published (Halling et al. 2005). The brucellae are non-motile, non-spore-forming and partially acid-fast in that they are not decolourized by 0.5% acetic acid used in the modified Ziehl–Neelsen (MZN) stain. The carbol fuchsin is retained and the brucellae appear as red-staining coccobacilli. The brucellae are aerobic and capnophilic (carboxyphilic), catalase-positive, oxidase-positive (except *B. ovis* and *B. neotomae*), urease-positive (except *B. ovis*) and will not grow on MacConkey agar. *B. ovis* and *B. abortus* biotype 2 require media enriched with blood or serum; the growth of the other brucellae is enhanced on enriched media but they will grow on nutrient agar.

Natural Habitat

Brucella species are obligate parasites and each species has a preferred natural host which serves as a reservoir of infection. Brucellae have a predilection for ungulate placentas, foetal fluids and testes of bulls, rams, boars and dogs. *Brucella abortus* is excreted in bovine milk and can remain viable in milk, water and damp soil for up to four months.

Pathogenesis and Pathogenicity

Table 23.1 lists the *Brucella* species and indicates the main hosts, diseases and geographical distribution. The principal means of transmission is by direct contact with infective excretors. The route of infection is often by ingestion but venereal transmission may occur and is the main route for *B. ovis.* Less commonly, infection may occur in utero, via conjunctival mucous membranes and by inhalation. *Brucella* species occur in both smooth and rough forms, the rough forms being those that lack O-polysaccharide on the cell surface and which belong to the species *B. ovis* and *B. canis.* Smooth forms of brucellae can infect, persist and replicate within the macrophages of the host. Soon after infection, before opsonization, the organisms enter the cells following interaction with cell surface microdomains known as lipid rafts (Watarai et al. 2002, Atluri et al. 2011). Once internalized, the organisms reside within the acidified phagosome which they then subvert in ways which are not yet fully understood. Acidification induces expression of the *virB* operon which in turn controls the expression of a type IV secretion system and its effectors. The outcome includes neutralization of the pH,

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Table 23.1 Diseases and principal hosts of the *Brucella* species

Species	Host(s)	Diseases	Geographical distribution
B. abortus	CATTLE*	Abortion and orchitis	Foci of infection can persist in wildlife such as bison and elk in the USA Biovars: 1. Worldwide (common) 2. Worldwide (not common) 3. India, Egypt, East Africa 4. Britain and Germany Other biotypes are infrequently isolated
	Sheep, goats, camels and pigs	Sporadic abortion	
	Horses	Associated with bursitis (poll evil and fistulous withers)	
	Humans	Undulant fever	
B. melitensis	GOATS	Abortion	Many sheep- and goat-raising regions except New Zealand, Australia and North America
	Sheep		
	Cattle, Camels	Occasional abortion and excretion in milk	
	Humans	Malta fever	
B. suis	PIGS, including wild boar	Abortion, orchitis, arthritis, spondylitis and herd infertility	Some biovars may persist in wildlife. Biovars: 1. Worldwide 2. Western and Central Europe, also infects hares 3. USA, Argentina and Singapore 4. Arctic Circle (Canada, Alaska and Siberia), also infects reindeer and caribou 5. Isolated from rodents in the former USSR
	Cattle (biovar 1)	Excretion in milk	
	Humans	Undulant fever (not biovar 2)	
B. canis	DOGS	Abortion, epididymitis, discospondylitis and permanent infertility in males	Worldwide but not common
	Humans	Undulant fever	
B. neotomae	DESERT WOOD RAT (*Neotoma lepida*)	Non-pathogenic for the wood rat and has not been recovered from any other animal species	USA (Utah)
B. ceti	CETACEANS	Little evidence of pathology, possible abortion, may cause secondary infections	Northern hemisphere, serological evidence of infection in southern hemisphere
B. pinnipedialis	PINNIPEDS	Little evidence of pathology, may cause secondary infections	Northern hemisphere, serological evidence of infection in southern hemisphere
B. microti	VOLES, isolated from foxes	Systemic infection and mortality in voles	Isolated in central Europe
B. inopinata	Human – Isolated from a mammary implant	Unknown	Unknown

*Natural host given in capital letters

Table 23.2 Virulence factors of *Brucella* species important for intracellular replication

Virulence factor	Function
Lipopolysaccharide O-chain	Entry to macrophage through lipid rafts; role in prevention of fusion of *Brucella*-containing vacuoles with lysosomes
Cyclic β-1,2-glucan	Role in inhibition of fusion of the *Brucella*-containing vacuoles with lysosomes
Vir B type IV secretion system	Late maturation of the *Brucella*-containing vacuole and fusion with the endoplasmic reticulum to form the 'brucellosome'
Siderophores	Iron acquisition

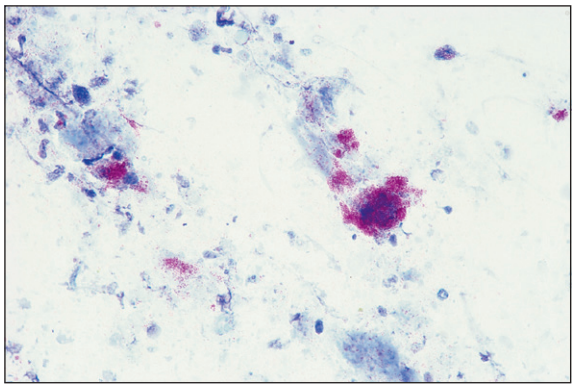

Figure 23.1 *Brucella abortus* in a MZN-stained smear of a cotyledon from a case of bovine abortion. The small, red (MZN-positive) coccobacilli characteristically occur in clumps reflecting their intracellular growth.

prevention of lysosomal fusion and multiplication of the organism within the 'brucellosome' (Celli 2006). The brucellae are transported to the regional lymph nodes, pass to the thoracic duct and then via the bloodstream to parenchymatous organs and other tissues such as joints. Localization occurs in the reproductive organs and associated glands. Table 23.2 lists major virulence factors of *Brucella* species which are important for intracellular replication.

Brucellosis is essentially a disease of the sexually mature animal, the predilection sites being the reproductive tracts of males and females, especially the pregnant uterus. Allantoic factors stimulate the growth of most brucellae. These factors include erythritol, possibly steroid hormones and other substances. Erythritol is present in the placenta and male genital tract of cattle, sheep, goats and pigs but not humans. Erythritol does not stimulate the growth of *B. ovis* and inhibits *B. abortus* strain 19, the attenuated vaccinal strain. In addition, acquisition of iron is essential to the brucellae in the acute replicative stage. A pyogranulomatous reaction occurs in affected placentae and abortion occurs from mid-gestation onwards. Apparently normal, but infected, calves can be born but the infection is of limited duration in these animals. Females usually only abort once, after which a degree of immunity develops. However, the animals remain infected and large numbers of brucellae can be excreted in foetal fluids at subsequent parturitions. Permanent infertility may occur in male dogs infected with *B. canis*.

Humans can be infected by all the *Brucella* species, except *B. ovis* and the non-pathogenic *B. neotomae*, leading to the development of undulant fever or Malta fever. The manifestations are an undulating pyrexia, malaise, fatigue, night sweats, muscle and joint pains, but not abortion. Osteomyelitis is the most common complication. Humans can also develop a hypersensitivity to the antigens of both virulent *B. abortus* and the vaccinal strain 19.

Laboratory Diagnosis

Extreme care must be exercised when working with brucellae as humans are highly susceptible to brucellosis and laboratory infections are not uncommon.

Specimens

In abortion cases a full range of specimens should be collected and submitted for a differential diagnosis. A whole foetus should be sent, if feasible, or foetal stomach contents, any foetal lesions, cotyledons, uterine discharges, colostrum, paired serum samples and sections of cotyledon and foetal lesions in 10% formalin for histopathology. Semen and tissue from epididymides or testes from males could be examined.

Direct examination

Smears are made from specimens and stained by the modified Ziehl–Neelsen (MZN) stain (Appendix 1). In smears containing cells, brucellae appear as small, red-staining, coccobacilli in clumps (Fig. 23.1) because of their intracellular growth.

Isolation

The brucellae grow well on 5–10% blood agar but other than foetal abomasal contents and colostrum, the specimens are likely to contain many contaminant bacteria and fungi, so selective media are required. The selective media contain a nutritive blood agar base with 5% sterile

sero-negative equine or bovine serum and an antibiotic supplement. The most widely used selective medium is Farrell's medium which is prepared by the addition of six antibiotics to a basal medium (Anon, 2012a). However, some of the antibiotics in Farrell's medium may have inhibitory effects on the growth of *B melitensis* and thus the sensitivity for *B. melitensis* isolation increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin medium (Anon. 2012a). The antibiotic supplement used in selective media for *B. ovis* usually differs from that for *B. abortus*. Terzolo et al. (1991) have suggested that Skirrow agar is a satisfactory medium for both the *Campylobacter fetus* subspecies and for *Brucella* species, including the most fastidious ones such as *B. abortus* biotype 2, *B. canis* and *B. ovis*. Formulae for suitable selective media are given in Appendix 2.

Liquid specimens can be inoculated straight onto plates. Scrapings from cotyledons can be used. Tissue samples should be homogenized and aliquots used for culture. Milk or colostrum can be centrifuged at 2000 x *g* for 20 minutes and loopfuls of both cream and sediment used to inoculate the plates. A loopful of cream from a positive *Brucella* milk ring test often yields *B. abortus.* The inoculated plates are incubated at 37°C under 5–10% CO_2 for up to 15 days.

Identification

Colonial morphology

After three to five days' incubation on selective serum agar, pinpoint, smooth, glistening, bluish, translucent colonies appear. As they age the colonies become opaque and about 2–3 mm in diameter (Fig. 23.2). Strains of *B. abortus, B. suis, B. melitensis* and *B. neotomae* are usually in the smooth form when first isolated. Colonies of rough morphology occur in each of these species on subculture. *Brucella ovis* and *B. canis* are always in the rough form. The rough colonies are dull, yellowish, opaque and when touched with an inoculation loop are found to be friable. Brucellae are non-haemolytic on blood agar (Fig. 23.3).

Microscopic appearance

MZN-stained smears from suspect colonies show small red-staining (MZN-positive) coccobacilli. Some laboratories use a fluorescent antibody test that identifies the organism to the generic level.

Biochemical tests

For routine identification, a few biochemical tests, together with colonial morphology and staining properties, will presumptively identify the isolate as a *Brucella* sp. In summary, the brucellae are non-motile, catalase-positive, oxidase-positive (except *B. ovis* and *B. neotomae*), give rapid urease activity (except *B. ovis* and some strains of *B. melitensis*), reduce nitrate and are indole-negative. Using known positive antisera a rapid slide agglutination test can be used to identify *B. abortus*.

Animal inoculation

Guinea pig inoculation is not commonly used but it is the most sensitive test for isolating pathogenic brucellae. Guinea pigs are inoculated intramuscularly with 0.5–1.0 mL of suspect tissue homogenate. Two animals per sample are usually used and euthanised at three and six weeks after inoculation. Serum is taken for serology and the spleen, together with any abnormal tissue, is collected for bacteriological examination.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Susceptibility testing using broth microdilution methods is carried out by specialized reference laboratories. The E-test method has also been used and interpretive standards for slow-growing bacteria (CLSI 2009, Document

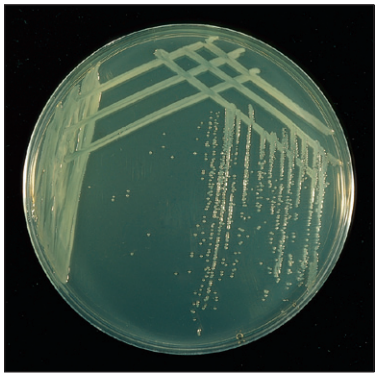

Figure 23.2 *Brucella abortus* on selective serum agar after four days at 37°C.

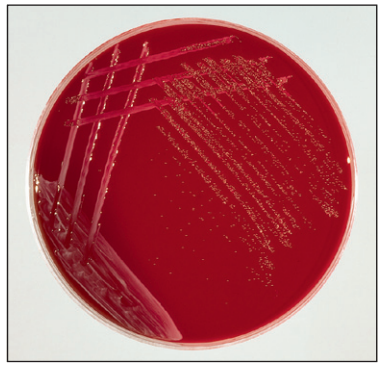

Figure 23.3 *Brucella abortus* on sheep blood agar: small, glistening, non-haemolytic colonies becoming opaque with age.

M100-S19) employed because interpretative tables for *Brucella* species are not available (Maves et al. 2011). There is limited information available on the antimicrobial susceptibility of *Brucella* species, most of which pertains to human isolates. Treatment of brucellosis in farm animals is not usually carried out.

Strain Typing

Brucella biotyping

This is usually carried out in reference laboratories and the tests involved definitively identify the species and biotype (Table 23.3). Full descriptions of the tests for biotyping brucellae are given by Alton et al. (1975) and Corbel & Morgan (1975).

CO_2 requirement for primary isolation: this test must be carried out immediately after isolation as the brucellae can quickly adapt to growth without CO_2. Duplicate, non-selective medium plates are streaked and incubated aerobically and under CO_2.

- *H_2S production*: the brucellae are not strong producers of H_2S so the sensitive lead-acetate-strip method is used. The *Brucella* isolate is grown on a trypticase soy (tryptose) agar slant with the test strip suspended over the slant. The strip is examined daily for four days and replaced each day. Blackening of the strip indicates a positive test.
- *Urease activity*: a Christensen urea slope is inoculated with a loopful of a culture. The test is incubated at room temperature and examined at half-hour intervals. All species are positive except *B. ovis* and some strains of *B. melitensis. Brucella suis, B. canis* and *B. neotomae* usually show urease activity (slope turning pink) within half an hour and *B. abortus* biotypes within two hours. The test is regarded as negative if there is no reaction after 24 hours.
- *Growth in the presence of dyes*: the conventional test is carried out by incorporating the dyes thionin (blue) or basic fuchsin (red), separately in trypticase soy agar at a concentration of 20 µg/mL (1:50,000) or 40 µg/mL (1:25,000). The medium is prepared by heating a 0.1% solution of either dye in a boiling water bath for 20 minutes and then adding it to the required amount of autoclaved agar. The dye is mixed with the agar and poured into Petri dishes. A sterile swab is used to inoculate the media containing the dye with a suspension of the test strain and a reference strain as a control. Six cultures, including the control, may be tested per agar plate. The inoculated plates are incubated at 37°C under 5–10% CO_2 for 3–4 days and then examined for growth. An example of the growth patterns on dye plates is illustrated (Fig. 23.4).
- *Agglutination with monospecific sera: B. abortus, B. melitensis* and *B. suis* possess two important surface antigens named A and M, which are present on the lipopolysaccharide–protein complex. The basis of the test is that the biotypes of the three species have the two antigens in different amounts. Only the permanently rough species, *B. ovis* and *B. canis,* will agglutinate with the R, anti-rough, monospecific serum. A dense suspension of the test *Brucella* is prepared in 0.5% phenolsaline and heated at 60°C for one hour. A drop of the suspension is added to a drop of each monospecific antiserum and mixed. Agglutination should occur within one minute. Control cultures of *B. abortus* biotype 1, *B. melitensis* biotype 1, *B. ovis* and *B. canis* are recommended to monitor the test.
- *Phage typing*: a number of bacteriophages are available to aid in the identification of the brucellae. The reactions of the Tbilisi (Tb) phage are given in Table 23.3. All biotypes of *B. abortus* will be lysed at the routine test dilution (RTD) and both *B. abortus* and *B. suis* biotypes will lyse at a phage concentration of 10^4 x RTD.
- *Oxidative metabolic rates* on selected substrates are conducted in reference laboratories. These rather involved tests need only be carried out if the results of the above tests are equivocal.

Differentiation of *B. abortus* biotype 1 and strain 19 (S19)

The vaccinal strain 19, derived from *B. abortus* biotype 1, gives similar reactions in the biotyping tests to CO_2-independent biotype 1. Strain 19 is inhibited by erythritol and is not commonly isolated from clinical specimens but recovery of strain 19 from diagnostic samples has been recorded. Table 23.4 gives the tests to distinguish strain 19 from *B. abortus* biotype 1 isolates.

Molecular Diagnosis and Typing

Numerous PCR-based assays for the detection of *Brucella* species have been published since the late 1980s (reviewed by Bricker 2002, Cutler et al. 2005). Diagnostic PCR has been applied to blood, milk, tissues and semen, with genus-specific assays using unique genetic loci that are highly conserved in *Brucella* species, e.g. genes such as *bcsp31* or *IS711*. Although difficulties may arise such as problems with co-purification of PCR inhibitors with the DNA of the organism or from interference with excessive host DNA, once these problems are resolved, sensitivity can be better than that achieved by culture.

Molecular tests to differentiate between *Brucella* species and/or biovars must target loci that are variable between species. These are uncommon in brucellae as the genus is extremely homogeneous but a number of assays capable of discriminating between species have been developed. One of the most successful of these is the AMOS-PCR,

Table 23.3 Differential characteristics of the species and biotypes in the genus *Brucella*

					Growth in the presence of dyes		Agglutination using monospecific sera			Lysis by phage Tbilisi		
Species	Biotype	CO_2 required	H_2S production	Urease activity (hours)	Thionin 20 μg/ml	Basic fuchsin 20 μg/ml	A	M	R	RTD	$10^4 \times$ RTD	Main host(s)
B. abortus	1	(+)	+	1–2+	–	+	+	–	–	+	+	Cattle
	2	(+)	+	1–2+	–	–	+	–	–	+	+	Cattle
	3	(+)	+	1–2+	+	+	+	–	–	+	+	Cattle
	4	(+)	+	1–2+	–	(+)	–	+	–	+	+	Cattle
	5	–	–	1–2+	+*	+	–	+	–	+	+	Cattle
	6	–	(+)	1–2+	+*	+	+	–	–	+	+	Cattle
	7	(–)	+	1–2+	+*	+	–	+	–	+	+	Cattle
Strain	19	–	+	1–2+	–	+	+	–	–	+	+	Vaccine
B. melitensis	1	–	–	v	+*	+	–	+	–	–	–	Goats, sheep
	2	–	–	v	+*	+	+	–	–	–	–	Goats, sheep
	3	–	–	v	+*	+	+	+	–	–	–	Goats, sheep
B. suis	1	–	+	0–0.5+	+	(–)	+	–	–	–	+	Pigs
	2	–	–	0–0.5+	+*	–	+	–	–	–	+	Pigs, hares
	3	–	–	0–0.5+	+	+	+	–	–	–	+	Pigs
	4	–	–	0–0.5+	+	(–)	+	+	–	–	+	Reindeer, caribou
	5	–	–	0–0.5+	+	–	–	+	–	–	+	Rodents
B.ovis		+	–	–	+*	(–)	–	–	+	–	–	Sheep
B. canis		–	–	0–0.5+	+	–	–	–	+	–	–	Dogs
B. neotomae		–	+	0–0.5+	–	–	+	–	–	–	+	Desert wood rat

+ = positve, (+) = most strains positive, (–) = most strains negative, – = negative, v = variable reactions, * = inhibited by 40 μg/ml thionin

A = *B. abortus* anigen, M = *B. melitensis* antigen, R = rough, RTD = routine test dilution

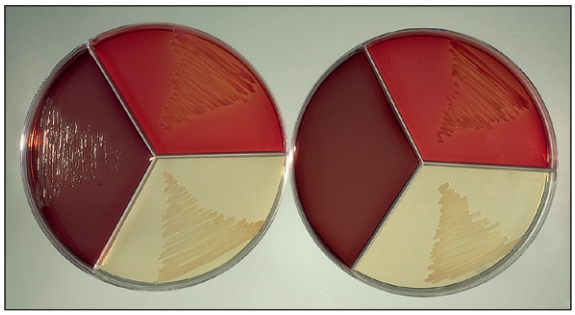

Figure 23.4 Growth of *B. abortus* biotype 3 (left plate) and biotype 1 (right plate) on serum agar containing the dyes thionin (left) and basic fuchsin (right). A control segment, without dye, is included for each biotype. Biotype 3 is able to grow in the presence of both dyes at a 20 µg/mL concentration but biotype 1 will only tolerate basic fuchsin (20 µg/mL).

Table 23.4 Differentiation of *Brucella abortus* biotype 1 and strain 19

	Brucella abortus	**Strain 19**
Growth on media containing:		
Thionin blue 2 µg/ml	+	–
Erythritol 1 µg/ml	+	–
Penicillin (5 unit disc)	R	S

which is particularly useful as it can differentiate between vaccinated animals and those infected with field strains (Bricker & Halling 1994, Ewalt & Bricker 2000). This technique is so named because it can differentiate between *B. abortus* biovars 1, 2 and 4, *B. melitensis, B. ovis* and *B. suis* biovar 1. A multiplex PCR (Bruce-ladder) which can identify and differentiate all currently described *Brucella* species has been reported (López-Goñi et al. 2011, Anon. 2012a).

In addition to the traditional methods for strain typing of *Brucella* isolates, many different molecular methods have been developed. A MLST method was reported by Whatmore et al. (2007) and hundreds of *Brucella* strains have now been typed. MLVA methods are also available and provide high levels of discrimination between strains when required for epidemiological investigation. Other typing methods which have been successfully used for *Brucella* species include outer membrane protein typing (Cloeckaert et al. 1995, 1996; Vizcaíno et al. 1997, 2004), IS 711 typing (Cloeckaert et al. 2000) and amplified fragment length polymorphism (Whatmore et al. 2005). Although these techniques do not always permit resolution of *Brucella* strains into the same groups as phenotypic identification schemes, they can be useful for epidemiological tracing purposes, particularly when more than one typing technique is employed (Cutler et al. 2005).

Immunological Tests for Detecting Antibodies to *Brucella abortus*

A wide range of immunological tests have been developed, in conjunction with state-controlled National Eradication Schemes, for bovine brucellosis. Some of the most commonly used serological tests are summarized in Table 23.5. The techniques can be obtained by reference to the texts listed in the Further Reading. Although the standard tube agglutination test has been widely used in many countries for control of bovine brucellosis, it is susceptible to false-positive reactions by cross-reacting antibody and therefore, its discontinuation is recommended by the OIE (Anon. 2012a).

In cattle naturally infected with *B. abortus* there is a subsequent rise in both IgM and IgG antibodies. However, the IgM titre declines and later the predominant immunoglobulin present is IgG. In some cases of chronic brucellosis the animal can have high levels of IgG1 which agglutinate poorly and can also mask the normally efficient agglutinating properties of any IgM antibodies that may be present.

In adults, vaccinated with strain 19, IgM antibody reaches peak values at about 13 days post-inoculation and the highest IgG values are reached at 28–42 days. Unlike natural infections, the IgM antibody titres remain high after vaccination.

Shared antigens occur between the brucellae and other Gram-negative bacteria such as *Francisella, Campylobacter, Salmonella, Pasteurella* and *Yersinia enterocolitica* (especially serotype O9) involving the somatic antigens. In Europe, the pig is the species most frequently infected with *Y. enterocolitica* and this can cause cross-reactions, particularly in the agglutination test. Cross-reactions have also been recorded in cattle, dogs and other species.

Immunological Tests for Detecting Antibodies to *B. melitensis, B. ovis, B. suis* and *B. canis*

The following tests are described in detail in the OIE Manual of Diagnostic Tests and Vaccine for Terrestrial Animals (Anon. 2012a, 2012b).

- *Brucella melitensis*: serological tests such as the complement fixation test, indirect ELISA, agar-gel immunodiffusion (AGID) and the Rose Bengal plate test and buffered antigen plate agglutination test (BPAT) are available. The CFT and the BPAT are the prescribed tests for international trade. Indirect and competitive ELISA tests have been developed and are considered sensitive and specific; however, further

Table 23.5 Summary of the serological tests for bovine brucellosis

Serological test	Principal immunoglobulin class identified	Remarks
Brucella milk ring test	IgM, IgG1, IgA	Conducted on bulk milk from a herd. If positive, sera are taken from individual cows in the herd and subjected to one or more of the other tests
Milk ELISA*	IgG	Extremely sensitive test
Plate agglutination tests: Rose-Bengal plate test* and the Card test	IgG1, IgM	Useful screening tests. Antigens buffered to pH 3.65–4.0 and this allows IgG1 to cause agglutination
Brucella serum agglutination test (SAT) (tube agglutination)	IgM, IgG2, (IgG1)	Widely used test but often IgG1 antibodies fail to agglutinate, so false negatives may occur. Not suitable for international trade
Brucella complement fixation test (CFT)*	IgG1, IgM	Specific but cumbersome to perform and more modern assays such as ELISA or FPA now give comparable or better results
Enzyme-linked immunosorbent assay (ELISA)*	Has capacity to detect all immunoglobulins. OIE approved version tests for IgG	Reliable, easily automated. Both indirect and competitive ELISAs are described
Fluoresence polarization assay (FPA)*	Has capacity to detect all immunoglobulins	Extremely rapid test with results available within minutes. The FPA can be performed in glass tubes or a 96-well plate format
Supplementary tests		
Coombs antiglobulin test	IgM, IgG2, IgG1	Very sensitive test, will detect 'incomplete antibodies' that do not react in the *Brucella* SAT
Heat inactivation	IgG	These are designed to differentiate the 'non-specific' reactions by destroying IgM, the antibody most commonly produced by adult vaccination
Rivanol precipitation	IgG	
Mercaptoethanol treatment	IgG	

*Prescribed tests for international trade

work on standardization of reagents is required before they could be considered as prescribed tests for trade. Milk ring tests are unreliable with sheep milk.

- *Brucella ovis*: the complement fixation test and the PBAT are the OIE-prescibed tests for trade. The AGID is in widespread use also. However, the indirect ELISA is thought to be both more sensitive and specific than either the CFT or AGID.
- *Brucella suis*: the tube agglutination test has been used on a herd basis but is not reliable for individual animals because of low titres to *B. suis* and non-specific reactions. Indeed, all serological tests for *B. suis* infections give more satisfactory results on a herd rather than individual basis. The indirect and the competitive ELISA are the prescribed tests for the purposes of international trade with the BPAT, Rose Bengal test (RBT) and fluoresence polymerization assays (FPA) as alternative tests.
- *Brucella canis*: the serological tests include a commercially available rapid slide agglutination test, a mercaptoethanol tube agglutination test (TAT), complement fixation test and an agar-gel immunodiffusion (AGID) test. The tests are subject to the occasional false-positive reaction. The slide agglutination test should only be used as a screening test and a definite diagnosis must be based on the results of a TAT or AGID test together with the culture of uterine discharges. A FPA has been described also (Nielsen et al. 2004).

REFERENCES

Alton, G.G., Jones, L.M., Pietz, D.E., 1975. Laboratory techniques in brucellosis, second ed. Monograph Ser. No. 55. World Health Organization, Geneva.

Atluri, V.L., Xavier, M.N., de Jong, M.F., et al., 2011. Interactions of the human pathogenic *Brucella* species with their hosts. Annual Review of Microbiology 65, 523–541.

Anon., 2012a. Manual of Diagnostic Tests and Vaccine for Terrestrial Animals 2009, Bovine Brucellosis. Available at: http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/ (accessed 14 January 2013).

Anon., 2012b. Manual of Diagnostic Tests and Vaccine for Terrestrial Animals 2012, Caprine and Ovine Brucellosis. Available at: http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/ (accessed 14 January 2013).

Bricker, B.J., 2002. PCR as a diagnostic tool for brucellosis. Veterinary Microbiology 90 (1–4), 435–446.

Bricker, B.J., Halling, S.M., 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis,* and *Brucella suis* bv. 1 by PCR. Journal of Clinical Microbiology 32 (11), 2660–2666.

Celli, J., 2006. Surviving inside a macrophage: the many ways of *Brucella*. Research in Microbiology 157, 93–98.

Clinical and Laboratory Standards Institute (CLSI), 2009. Performance standards for antimicrobial susceptibility testing, vol 29, 19th International Supplement. M100-S19-MIC. Clinical and Laboratory Standards Institute, Wayne, PA.

Cloeckaert, A., Verger, J.M., Grayon, M., et al., 1995. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. Microbiology 141, 2111–2121.

Cloeckaert, A., Verger, J.M., Grayon, M., et al., 1996. Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of *Brucella ovis*: Evidence for antigenic shift, compared with other *Brucella* species, due to a deletion in the gene. Infection and Immunity 64 (6), 2047–2055.

Cloeckaert, A., Grayon, M., Grepinet, O., 2000. An IS711 element downstream of the bp26 gene is a specific marker of *Brucella* spp. isolated from marine mammals. Clinical and Diagnostic Laboratory Immunology 7 (5), 835–839.

Corbel, M.J., Morgan, W.J.B., 1975. Proposal for minimal standards for descriptions of new species and biotypes of the genus *Brucella*. International Journal of Systematic Bacteriology 25, 83–89.

Cutler, S.J., Whatmore, A.M., Commander, N.J., 2005. Brucellosis – new aspects of an old disease. Journal of Applied Microbiology 98 (6), 1270–1281.

Ewalt, D.R., Bricker, B.J., 2000. Validation of the abbreviated Brucella AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strain isolates and the vaccine strains, 19 and RB51. Journal of Clinical Microbiology 38 (8), 3085–3086.

Foster, G., Osterman, B.S., Godfroid, J., et al., 2007. *Brucella ceti sp. nov.* and *Brucella pinnipedialis sp. nov.* for *Brucella* strains with cetaceans and seals as their preferred hosts. International Journal of Systematic and Evolutionary Microbiology 57, 2688–2693.

Halling, S.M., Peterson-Burch, B.D., Bricker, B.J., et al., 2005. Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. Journal of Bacteriology, 187, 2715–2726.

López-Goñi, I., García-Yoldi, D., Marín, C.M., et al., 2011. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Veterinary Microbiology 154 (1–2), 152–155.

Maves, R.C., Castillo, R., Guillen, A., 2011. Antimicrobial susceptibility of *Brucella melitensis* isolates in Peru. Antimicrobial Agents and Chemotherapy 55 (3), 1279–1281.

Nielsen, K., Smith, P., Conde, S., et al., 2004. Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B. ovis, B. canis,* and *B. abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay. Journal of Immunoassay Immunochemistry 25 (2), 171–182.

Terzolo, H.R., Paolicchi, F.A., Moreira, A.R., et al., 1991. Skirrow agar for simultaneous isolation of *Brucella* and *Campylobacter* species. Veterinary Record 129, 531–532.

Vizcaíno, N., Verger, J.M., Grayon, M., et al., 1997. DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology 143 (9), 2913–2921.

Vizcaíno, N., Caro-Hernández, P., Cloeckaert, A., et al., 2004. DNA polymorphism in the omp25/omp31 family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. Microbes and Infection 6 (9), 821–834.

Watarai, M., Makino, S., Fujii, Y., et al., 2002. Modulation of *Brucella*-induced macropinocytosis by lipid rafts mediates intracellular replication. Cell Microbiology 4 (6), 341–355.

Whatmore, A.M., Murphy, T.J., Shankster, S., et al., 2005. Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. Journal of Clinical Microbiology 43 (2), 761–769.

Whatmore, A.M., Perrett, L.L., MacMillan, A.P., 2007. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiology 7, 34.

FURTHER READING

Hollett, R.B.. 2006. Canine brucellosis: outbreaks and compliance. Theriogenology 66, 575–587

Nicoletti, P.. 1990. Serological diagnosis of canine brucellosis. In: Carter, G.R., Cole, J.R. Jr (Eds.), Diagnostic Procedures in Veterinary Bacteriology and Mycology, fifth ed, Academic Press Inc., New York, pp. 102–104

United States Department of Agriculture, 1965. Manual Nos. 64A, B, C and D, Ames, Iowa, Agricultural Research Service, National Animal Disease Center, Diagnostic Reagents Division

Chapter 24

Campylobacter, *Arcobacter* and *Helicobacter* species

Genus Characteristics

The *Campylobacter* species are members of the family *Campylobacteraceae*, together with *Arcobacter* species and *Bacteroides ureolyticus*. They are thin, curved, Gram-negative motile rods. The cells are 0.2–0.5 µm in width and when daughter cells remain joined, S-shaped, seagull-shaped and sometimes long spiral forms may be seen (Fig. 24.1). They are motile by a single polar flagellum, oxidase-positive, catalase-variable and most are microaerophilic. They grow best on nutritious basal media supplemented with 5–10% blood under reduced oxygen tension. Members of the *Campylobacteraceae* do not utilize carbohydrates but obtain energy from amino acids or intermediates of the tricarboxylic acid cycle. Some species of *Campylobacter* are thermophilic, growing best at 42 °C, the most important pathogen in this category being *C. jejuni*. The most significant animal pathogens are *C. fetus* subsp. *fetus, C. fetus* subsp. *venerealis* and *C. jejuni*.

Members of the genus *Arcobacter* species are emerging animal and human pathogens (Snelling et al. 2006). They are described as aerotolerant *Campylobacter*-like organisms although recent phylogenetic analysis has cast doubt on whether the genus should be included in the *Campylobacteraceae* (Miller et al. 2007). *Arcobacter* species have been isolated from cases of abortion in animals and diarrhoea in humans.

Helicobacter pylori is associated with gastric ulcers in humans but there are many *Helicobacter* species which have been isolated from animals also. Non-*H. pylori* organisms are long and spiral-shaped and many of them are difficult to culture in vitro. Their role in disease production in animals is uncertain and their principal importance may be as zoonotic organisms.

Natural Habitat

The *Campylobacter* species are worldwide in distribution. Many species are commensals on the mucosa of the oral cavity and intestinal tract of animals and birds. *Campylobacter fetus* subsp. *venerealis* occurs in the prepuce of bulls and in the genital tract of cows in herds where bovine genital campylobacteriosis is, or has been, present. There are also some non-pathogenic species that are saprophytes in the environment. Arcobacters may be found in the faeces of many animal species and it has been suggested that poultry may act as reservoirs of this organism. However, genomic studies suggest that *A. butzleri* may be a free-living, water-borne organism (Miller et al. 2007). Helicobacters are found in the gastrointestinal tracts of many animals and birds.

Pathogenesis and Pathogenicity

Transmission of many of the *Campylobacter* species including *C. fetus* subsp. *fetus*, is by the faecal–oral route. *Campylobacter fetus* subsp. *venerealis* is transmitted by coitus and infection of the female genital tract may lead to metritis with resulting death and resorption of the embryo (infertility), or occasionally to abortion. The diseases associated with the *Campylobacter* and related species and/or their commensal status, are given in Table 24.1.

There are two subspecies of *C. jejuni*, subspecies *jejuni* and subspecies *doylei*. Subspecies *jejuni* is pathogenic whereas the role of subspecies *doylei* in animals and humans is unclear. Pathogenic isolates of *C. jejuni* show several virulence attributes. Motility, chemotaxis and temperature stress responses are important pathogenic factors as mutants lacking these characteristics are unable to

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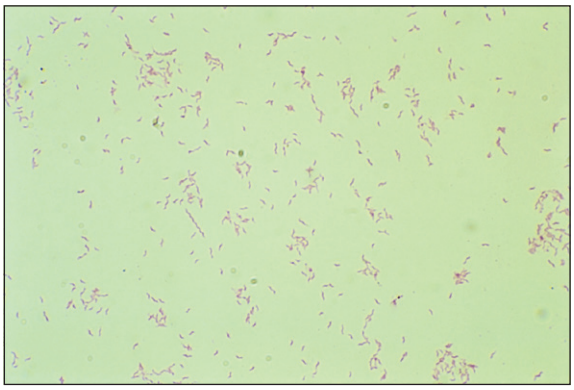

Figure 24.1 *Campylobacter fetus* subsp. *fetus* in a DCF-stained smear from culture showing the curved rods and 'seagull' forms characteristic of the genus (× 1000).

colonize the intestines of chickens (Konkel et al. 1998, Fields & Swerdlow 1999). Flagella play a role in adhesion in addition to motility. Other adhesins involved in both adherence and invasion have been identified (Konkel et al. 1997, Pei et al. 1998). Following adhesion, *C. jejuni* is internalized into the epithelial cells lining the intestine and may replicate once inside its membrane-bound compartment within the cell. Exocytosis can occur at the basolateral surface of the cell. Mechanisms of disease production are unclear although the organism is known to produce a cytolethal-distending toxin. Outer membrane constituents lipo-oligosaccharides and polysaccharides are involved in adhesion, endotoxicity and serum resistance. In addition, phase variation may be important in immune evasion.

The pathogenic mechanisms of *C. fetus* in the production of reproductive loss remain largely unknown.

Table 24.1 Pathogenic and non-pathogenic *Campylobacter* and *Arcobacter* species

Species	Principal host(s)	Disease and/or commensal status
C. fetus subsp. *venerealis*	Cattle	Bovine genital campylobacteriosis (epizootic bovine infertility): infertility, early embryonic death and occasional abortion Prepuce of asymptomatic bulls
C. fetus subsp. *fetus*	Sheep	Ovine genital campylobacteriosis: outbreaks of abortion
	Cattle	Occasional abortions
	Man	Occasional infections
	Cattle, sheep	Commensal in the intestinal tract
C. jejuni subsp. *jejuni*	Sheep	Outbreaks of abortion
	Dogs, cats, other animals	Associated with enteritis and diarrhoea
	Poultry	Role in avian vibrionic hepatitis is unclear
	Humans	Enterocolitis
	Many domestic and wild animals and birds	Commensal in the intestinal tract
C. coli	Pigs	Commensal in the intestinal tract
	Humans	Enterocolitis
C. helveticus	Dogs, cats	Present in intestinal tract
C. hyoilei	Pigs	Commensal in intestinal tract
C. hyointestinalis	Pigs	Present in gastrointestinal tract
C. lari	Dogs, birds, other animals	Commensal in intestinal tract
	Humans	May cause enteritis
C. jejuni subsp. *doylei*	Humans	Isolated from clinical specimens
C. mucosalis	Pigs	Commensal in intestinal tract
C. sputorum biovar Sputorum	Cattle, sheep	Present in genital tract
	Humans	Isolated from faeces and gingivae

Table 24.1 Pathogenic and non-pathogenic *Campylobacter* and *Arcobacter* species—cont'd

Species	Principal host(s)	Disease and/or commensal status
C. sputorum biovar Faecalis	Sheep, cattle	Commensal in intestinal and genital tracts
	Cattle	Isolated from cases of bovine digital dermatitis, pathogenicity uncertain
C. upsaliensis	Dogs, humans	Isolated from diarrhoeic and normal individuals
Arcobacter butzleri	Humans	May cause diarrhoea
	Cattle, pigs	Implicated in abortion
	Pigs, cattle, horses	Associated with enteritis
A. cryaerophilus	Many species	Present in intestinal tract
	Sheep, horses, cattle	Isolated from normal and aborted foetuses
	Cattle	Rare cases of mastitis
A. skirrowii	Cattle	Present in prepuce
	Cattle, sheep, pigs	Isolated from aborted foetuses
Helicobacter species	Many animals species	Isolated from the gastrointestinal tract
	Pigs, cats, dogs	Associated with gastritis
	Sheep	The organism '*Flexispira rappini*' associated with abortion is now thought to comprise several *Helicobacter* species

Genomic studies suggest that this species possesses many of the same genes encoding virulence attributes as *C. jejuni*, including genes encoding outer membrane proteins, flagella, regulatory and secretion systems (Moolhuijzen et al. 2009). In addition, a genomic island encoding the components of a type IV secretion system has been identified in *C. fetus* subspecies *venerealis* only (Gorkiewicz et al. 2010). Extensive studies have been conducted on the surface layer (S-layer) proteins which this species produces. The S-layer, which is a paracrystalline protein structure external to the bacterial outer membrane, confers resistance to complement-mediated killing in non-immune serum. In addition, eight antigenic variants of the protein can be expressed by the bacterium and this may delay the host immune response (Grogono-Thomas et al. 2003).

Some of the virulence attributes of *C. jejuni* and *C. fetus* are given in Table 24.2.

Virulence mechanisms of *Arcobacter* and *Helicobacter* species in animals are currently unclear.

Laboratory Diagnosis

Specimens

Table 24.3 summarizes the specimens required from the various clinical conditions for the diagnosis of *Campylobacter* species. Transport medium should be used for the collection of specimens for the isolation of *C. fetus*. A study by Monke et al. (2002) suggests that Weybridge transport enrichment medium in combination with Skirrow agar and culture within four hours of sampling gives enhanced sensitivity of isolation. See Appendix 2 for composition of transport and culture media.

Direct microscopy

Both subspecies of *Campylobacter fetus* can be demonstrated in foetal abomasal contents using the DCF stain (dilute carbol fuchsin for four minutes) while they tend to stain poorly in Gram-stained smears. Fluorescent antibody staining of smears from foetal abomasal contents, cervical mucus and preputial washings is the most reliable staining method, especially when small numbers of the bacterium are present.

Campylobacter jejuni can be seen in wet mounts of faeces by phase contrast or darkfield microscopy. The typical darting motility of corkscrew-like organisms is suggestive of *Campylobacter* species. Characteristic slender, curved rods can be demonstrated in DCF-stained smears, or by phase contrast of ovine foetal abomasal contents and in bile from chickens with hepatitis.

Other *Campylobacter* species can be demonstrated in ways similar to those described above and detailed descriptions of these techniques can be found in the OIE Manual (Anon. 2008).

Table 24.2 Selected virulence attributes of *Campylobacter* species

Pathogen	Virulence factor	Role in the host
Campylobacter jejuni	Heat shock proteins	Survival in the environment. DnaJ shown to be important for colonization in vivo
	Flagella	Corkscrew motility for penetration of mucus; colonization and invasion
	Outer membrane adhesion proteins	Adhesion
	Lipo-oligosaccharide and lipopolysaccharide	Serum resistance, endotoxic, adhesion
	Superoxide dismutase and catalase	Survival within intestinal cells
	Cytolethal-distending toxin	Cell death
Campylobacter fetus	S-layer	Resistance to complement-mediated killing. Delayed host immune response due to antigenic variation
	Genes encoding several other potential virulence factors identified but role in animals not proven	

Table 24.3 Suitable specimens for detection of *Campylobacter* species

Clinical entity	Specimens
Bovine infertility (*C. fetus* subsp. *venerealis*)	Vaginal or cervicovaginal mucus from females collected on a herd basis (10–20 animals). Specimens can be collected by swabbing, suction or by washing the vaginal cavity; a sterile speculum should be used to ensure collection of good-quality samples. Samples should always be placed in transport medium Preputial mucus or smegma can be obtained by scraping, suction or preputial washing. Semen samples are suitable also. Transport medium is recommended
Bovine and ovine abortion (*C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus* and *C. jejuni*)	Foetal abomasal contents preferred although the placenta, liver and lungs of the foetus may be submitted. Further samples from dam and foetus to exclude other infectious agents
Diarrhoea (*C. jejuni* and others)	Rectal faeces. If swabs are used or if culture of faecal samples will be delayed, samples should be stored in transport media such as Amies, Cary–Blair or Stuart media

Isolation procedures

Culture media and isolation procedures for *C. fetus, C. jejuni* and other *Campylobacter* species are presented in detail in the OIE Manual (Anon. 2008).

1. *Campylobacter fetus* (both subspecies): cervical mucus and preputial washings can be passed through a 0.65 µm membrane filter to reduce contamination. Foetal abomasal contents and filtrates are inoculated onto a nutritious base (*Brucella,* Columbia or brain heart infusion agars) supplemented with 5–10% blood. The medium can be made selective by the addition of polymyxin B sulphate (2 units/mL), novobiocin (2 µg/mL) and cycloheximide (20 µg/mL). As cycloheximide has toxic effects and is likely to become unavailable in future years, it can be replaced by 10 µg/mL of amphotericin B (Aspinall et al. 1993). A number of selective media for the isolation of *C. fetus* have been described including Skirrow agar and Clark's selective medium (Anon. 2008). Non-selective blood agar should be inoculated simultaneously for the possible isolation of other pathogens. The plates are incubated at 37°C for four to six days in a microaerophilic atmosphere containing 6% O_2, 10% CO_2, and 85% N_2. These atmospheric conditions must be adhered to strictly and can be attained by one of the following methods (the first two methods are preferable):
   - Filling an anaerobic/CO_2 jar with the specified ready-mixed gas in a cylinder obtained from a commercial gas supplier.
   - Using special gas-generating envelopes such as CampyPak™ II (BBL) or BR56/BR60 (Oxoid) with a palladium catalyst in an anaerobic/CO_2 jar.

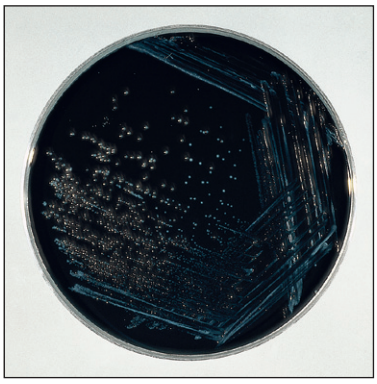

Figure 24.2 *Campylobacter jejuni* on charcoal-cefoperazone-deoxycholate agar.

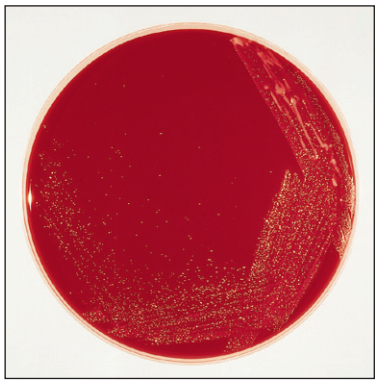

Figure 24.3 *Campylobacter fetus* subsp. *fetus* on sheep blood agar demonstrating the small colonies characteristic of both subtypes *fetus* and *venerealis*.

- An anaerobic gas-generating envelope in the jar without a catalyst.
- Incubating a plate inoculated with a facultative anaerobe together with the plate for isolation of *Campylobacter* species. Both plates are placed in an airtight plastic bag.

2. *Campylobacter jejuni*: rectal swabs or faeces are inoculated onto one of several selective media, which can be obtained commercially, such as charcoal-cefoperazole-deoxycholate agar (Oxoid) (Fig. 24.2) or Blaser's Campy-BAP medium. This latter medium is Brucella agar with 5% sheep blood, vancomycin (10 µg/mL), polymyxin B sulphate (2–5 units/mL), trimethoprim lactate (5 g/mL), cephalothin (15 µg/mL) and amphotericin B (2 µg/mL). The plates should be incubated under the atmosphere described above for two to three days at 42°C as 37°C is suboptimal for this bacterium. Procedures for the isolation of thermophilic *Campylobacter* spp. from food are laid down by the International Organisation for Standardisation in ISO 10272 (ISO 2006).
3. Other *Campylobacter* species: those of faecal origin can be isolated in a similar manner to *C. jejuni* and those from the reproductive tract in the same manner as *C. fetus*.

Arcobacter species can be isolated on commercially available isolation media such as cefoperazone, amphotericin B and teicoplanin agar (CAT) for most *Arcobacter* species and charcoal cefoperazone, deoxycholate agar (CCDA) specifically developed for *A. butzleri* (Oxoid). Isolation may require an enrichment step at 25°C (Lehner et al. 2005).

Helicobacter species require enriched media and are microaerophilic; Skirrow's medium is suitable for some species. However, members of this genus are frequently difficult to culture and detection by molecular methods is frequently employed.

Figure 24.4 *Campylobacter jejuni* on sheep blood agar. The colonies have a spreading, watery appearance on slightly moist plates.

Identification

Colonial morphology

Both subspecies of *C. fetus* have small (1 mm), round, slightly raised, smooth, translucent colonies said to have a 'dew-drop' appearance (Fig. 24.3) or may be slightly grey-pink in colour. The colonies of *C. jejuni* are usually flat, greyish in appearance on charcoal-based media, larger than those of *C. fetus* and can be spreading and watery on moist plates (Fig. 24.4). Other *Campylobacter* species vary somewhat in colonial appearance. *Campylobacter coli* produces a pink-tan pigment.

Microscopic appearance

1. DCF-stained smears from colonies show small, curved or seagull-shaped rods.
2. Wet mounts under phase contrast or darkfield microscopy reveal the characteristic curved forms with darting motility.

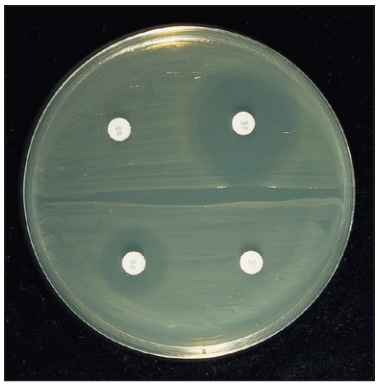

Figure 24.5 Susceptibility or resistance to 30 µg discs of nalidixic acid (NA) or cephalothin (KF) as an aid to the identification of *Campylobacter* species. *Campylobacter jejuni*, susceptible to nalidixic acid (right), is shown above and *C. fetus* subsp. *venerealis*, susceptible to cephalothin (left), is shown below.

3. Fluorescent antibody-stained smears can be used to identify *C. fetus,* but the technique does not distinguish between the subtypes, so this must be done by biochemical tests (Table 24.4).

Biochemical and other tests

Susceptibility or resistance to nalidixic acid or cephalothin (Fig. 24.5), hydrogen sulphide production, nitrate reduction, growth at 25 °C or 45 °C and the catalase reaction are some of the criteria on which a definitive identification of the *Campylobacter* species is based (Table 24.4). Susceptibility to nalidixic acid was considered a most useful test in the past but has become more difficult to interpret due to increasing numbers of nalidixic-acid-resistant *C. jejuni* strains and the occurrence of nalidixic-acid-sensitive *C. lari* strains (Anon. 2008). Presumptive differentiation of *C. fetus* subspecies is based on growth in a basic blood medium containing glycine (15 mL of medium and 1.65 mL of a 10% aqueous solution of glycine). Control strains of *C. fetus* ssp. *fetus* and *C. fetus* ssp. *venerealis* must be included in each test. Lack of growth is suggestive of *C. fetus* ssp. *venerealis* strains.

Campylobacter jejuni is the only species that hydrolyses sodium hippurate. For this test a large loopful of a 24–48 hour culture of *C. jejuni* is emulsified in 0.4 mL of a 1% aqueous solution of sodium hippurate and incubated at 37 °C for two hours. Then 0.2 mL of a ninhydrin solution is added to the tubes at 37 °C. A positive reaction is given by a deep purple colour developing after 10 minutes. The ninhydrin solution is prepared by adding 3.5 g of ninhydrin to 100 mL of a 1 : 1 mixture of acetone and butanol. Commercial disks for testing hippurate hydrolysis are available.

Serology

1. A vaginal mucus agglutination test is useful for the detection of infected herds, and it is ideally carried out approximately two months post-infection. It is unreliable for the identification of individual infected animals. An ELISA test may be used to detect antibodies in vaginal mucus also. Both of these tests are described in detail in the OIE Manual of Diagnostic Tests (Anon. 2008).
2. As *C. jejuni* is present in the intestines of many normal animals, its isolation from faeces may not necessarily be significant. Infection is associated with the production of mucosal and systemic antibody but no validated serological tests are available.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Agar dilution or E-test methods are often employed for antimicrobial susceptibility testing of isolates of *Campylobacter* spp. However, agar dilution methods are time-consuming and the E test method is expensive and beyond the means of many laboratories. Luber et al. (2003) compared broth microdilution to both of these methods and found good agreement between the results of the three methods. Standard methods and interpretive criteria for testing of *C. jejuni* and *C. coli* using broth microdilution or disk diffusion are now available (CLSI 2010).

Substantial information is available on antimicrobial resistance patterns of intestinal isolates of *Camplylobacter* species, including *C. jejuni* and *C. coli*. Increasing resistance to the fluoroquinolone and macrolide antibiotics in particular is a cause of concern worldwide, owing to their importance as therapeutic agents in human medicine (Moore et al. 2006). Unfortunately, in spite of changes in the use of fluoroquinolones in animals, including halting its use in poultry production in many countries, fluoroquinolone resistance persists in poultry isolates of *C. jejuni* (Snelling et al. 2006). This may be due in part to enhanced fitness of fluoroquinolone-resistant isolates (Luo et al. 2005). High levels of resistance to tetracyclines and increasing levels of erythromycin resistance are reported (Gibreel & Taylor 2006). Isolates of *C. coli* from pigs before and after slaughter also exhibited substantial levels of resistance to these two antimicrobial agents (Abley et al. 2012). Resistance to fluoroquinolones is frequently mediated by a mutation in the *gyrA* gene (Griggs et al. 2005) and resistance to macrolide antibiotics is due to ribosomal mutation (Gibreel & Taylor 2006). In addition, efflux pumps may be important in resistance to both of these classes of antimicrobial agent (Gibreel & Taylor 2006).

There are few recent publications on antimicrobial susceptibility testing of animal isolates of *C. fetus* subspecies.

Table 24.4 Differentiation of the principal *Campylobacter* and *Arcobacter* species

Species	Growth at		Catalase	Hippurate hydrolysis	Nitrate reduction	H_2S production			Susceptibility to	
	25°C	42°C				TSI (trace amounts)	Growth in 1% glycine	Growth in 3.5% NaCl	Nalidixic acid	Cephalothin
C. fetus subsp. *venerealis*	+	–	+	–	+	–	–	–	v	S
C. fetus subsp. *fetus*	+	v	+	–	+	–	+	–	R	S
C. jejuni subsp. *jejuni*	–	+	+	+	+	–	+	–	S	R
C. coli	–	+	+	–	+	v	+	–	S	R
C. helveticus	–	+	–	–	+	–	v	–	S	S
C. hyointestinalis	v	+	+	–	+	+	+	–	R	v
C. lari	–	+	+	–	+	–	+	–	v	R
C. jejuni subsp. *doylei*	–	–	v	+	–	–	v	–	S	S
C. mucosalis	–	+	–	–	–	+	v	–	R	S
C. sputorum	–	+	v	–	+	+	+	v	v	S
C. upsaliensis	—	+	–	–	+	–	+	––	–	v
Arcobacter butzleri	+	v	v	–	+	–	–	–	v	R
A. cryaerophilus	+	–	+	–	+	–	–	–	S	R
A. skirrowii	+	v	+	–	+	–	–	+	S	R

v = variable reaction, R = resistant, S = susceptible, TSI = triple sugar iron agar

However, Hänel et al. (2011) reported reduced susceptibility of *C. fetus* ssp. *venerealis* isolates to lincomycin and spectinomycin.

Strain Typing

Previously serotyping was used for differentiation of *Campylobacter* strains but molecular typing is now more commonly employed. Pulsed field gel electrophoresis (PFGE)-based methods are widely used for subtyping, especially in investigation of human campylobacteriosis but also in veterinary medicine. PulseNet International is a system of interconnected laboratory networks through which PFGE patterns of isolates of foodborne pathogens, including *Campylobacter* species generated using standard protocols, can be transmitted around the world (Gerner-Smidt et al. 2006). This enables rapid identification and investigation of outbreaks of disease. PFGE patterns are also used for the investigation of abortions in sheep due to *C. jejuni* (Mannering et al. 2006, Sahin et al. 2008) and to investigate the molecular epidemiology of *C. fetus* subsp. *fetus* strains (van Bergen et al. 2006).

MLST is widely employed for characterization of isolates of *C. coli* and *C. jejuni* and data on many isolates are available on the MLST database (http://pubmlst.org/campylobacter/ accessed 21 December 2012). This typing method has been applied to *C. fetus* isolates also and demonstrated the homogeneous nature of this species compared to other campylobacters (van Bergen et al. 2006).

PCR-based methods can be successfully employed for differentiation of the subspecies of *C. fetus*. However, Willoughby et al. (2005) reported that depending on the clones present in a particular area, PCR procedures developed in one geographical region may not be useful in another.

Molecular Diagnosis

Because of its importance in public health, significant research on the molecular detection of thermophilic campylobacters in food and environmental samples has been conducted. Many of the methods are PCR-based, including 'real-time' PCR (Bohaychuk et al. 2005, Oliveira et al. 2005, Nam et al. 2005). However, methods for the direct detection of *Campylobacter* species in clinical specimens from animals are being developed. A PCR assay for the direct detection of *C. fetus* subsp. *venerealis* has been reported by McMillen et al. (2006).

REFERENCES

Abley, M.J., Wittum, T.E., Funk, J.A., et al., 2012. Antimicrobial susceptibility, pulsed-field gel electrophoresis, and multi-locus sequence typing of *Campylobacter coli* in swine before, during, and after the slaughter process. Foodborne Pathogens and Disease. 9 (6), 506–512.

Anon, 2008. Manual of Diagnostic Tests and Vaccine for Terrestrial Animals 2009: Bovine genital campylobacteriosis. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.05_BGC.pdf accessed 17 December 2012.

Aspinall, G.O., McDonald, A.G., Pang, H., et al., 1993. An antigenic polysaccharide from *Campylobacter coli* serotype O:30. Structure of a teichoic acid-like antigenic polysaccharide associated with the lipopolysaccharide. Journal of Biological Chemistry 268 (24), 18321–18329.

Bohaychuk, V.M., Gensler, G.E., King, R.K., et al., 2005. Evaluation of detection methods for screening meat and poultry products for the presence of foodborne pathogens. Journal of Food Protection 68 (12), 2637–2647.

Clinical and Laboratory Standards Institute (CLSI), 2010. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, Approved Guideline, second ed, CLSI document M45-A2 (ISBN 1-56238-732-734). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Fields, P.I., Swerdlow, M.D., 1999. *Campylobacter jejuni*. Clinics in Laboratory Medicine 19, 489–504.

Gerner-Smidt, P., Hise, K., Kincaid, J., et al., 2006. PulseNet USA: a five-year update. Foodborne Pathogens and Disease 3 (1), 9–19.

Gibreel, A., Taylor, D.E., 2006. Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. Journal of Antimicrobial Chemotherapy 58 (2), 243–255.

Gorkiewicz, G., Kienesberger, S., Schober, C., et al., 2010. A genomic island defines subspecies-specific virulence features of the host-adapted pathogen *Campylobacter fetus* subsp. *venerealis*. Journal of Bacteriology 192 (2), 502–517.

Griggs, D.J., Johnson, M.M., Frost, J.A., et al., 2005. Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp. isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. Antimicrobial Agents and Chemotherapy 49 (2), 699–707.

Grogono-Thomas, R., Blaser, M.J., Ahmadi, M., et al., 2003. Role of S-layer protein antigenic diversity in the immune responses of sheep experimentally challenged with *Campylobacter fetus* subsp. *fetus*. Infection and Immunity 71 (1), 147–154.

Hänel, I., Hotzel, H., Müller, W., et al., 2011. Antimicrobial susceptibility testing of German *Campylobacter fetus* subsp. *venerealis* isolates by agar disk diffusion method. Berliner und Munchener Tierarztliche Wochenschrift 124 (5–6), 198–202.

ISO 10272-10271, 2006. Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp. – Part 1: Detection method. International Organisation for Standardisation, Geneva, Switzerland.

Konkel, M.E., Garvis, S.G., Tipton, S.L., et al., 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Molecular Microbiology 24, 953–963.

Konkel, M.E., Kim, B.J., Klena, J.D., et al., 1998. Characterization of the thermal stress response of *Campylobacter jejuni*. Infect Immun 66, 3666–3672.

Lehner, A., Tasara, T., Stephan, R., 2005. Relevant aspects of *Arcobacter* spp. as potential foodborne pathogen. International Journal of Food Microbiology 102 (2), 127–135.

Luber, P., Bartelt, E., Genschow, E., et al., 2003. Comparison of broth microdilution, E Test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. Journal of Clinical Microbiology 41 (3), 1062–1068.

Luo, N., Pereira, S., Sahin, O., et al., 2005. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proceedings of the National Academy of Sciences USA 102 (3), 541–546.

McMillen, L., Fordyce, G., Doogan, V.J., et al., 2006. Comparison of culture and a novel 5′ Taq nuclease assay for direct detection of *Campylobacter fetus* subsp. *venerealis* in clinical specimens from cattle. Journal of Clinical Microbiology 44 (3), 938–945.

Mannering, S.A., West, D.M., Fenwick, S.G., et al., 2006. Pulsed-field gel electrophoresis of *Campylobacter jejuni* sheep abortion isolates. Veterinary Microbiology 115 (1–3), 237–242.

Miller, W.G., Parker, C.T., Rubenfield, M., et al., 2007. The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. PLOS ONE 2 (12), e1358.

Monke, H.J., Love, B.C., Wittum, T.E., et al., 2002. Effect of transport enrichment medium, transport time, and growth medium on the detection of *Campylobacter fetus* subsp. *venerealis*. Journal of Veterinary Diagnostic Investigation 14 (1), 35–39.

Moolhuijzen, P.M.; Lew-Tabor, A.E.; Wlodek, B.M., et al., 2009. Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiology 9, 86.

Moore, J.E., Barton, M.D., Blair, I.S., et al., 2006. The epidemiology of antibiotic resistance in *Campylobacter*. Microbes and Infection 8 (7), 1955–1966.

Nam, H.M., Srinivasan, V., Murinda, S.E., et al., 2005. Detection of *Campylobacter jejuni* in dairy farm environmental samples using SYBR Green real-time polymerase chain reaction. Foodborne Pathogens and Disease 2 (2), 160–168.

Oliveira, T.C., Barbut, S., Griffiths, M.W., 2005. Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. International Journal of Food Microbiology 104 (1), 105–111.

Pei, Z., Burucoa, C., Grignon, B., et al., 1998. Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infection and Immunity 66, 938–943.

Sahin, O., Plummer, P.J., Jordan, D.M., et al., 2008. Emergence of a tetracycline-resistant *Campylobacter jejuni* clone associated with outbreaks of ovine abortion in the United States. Journal of Clinical Microbiology 46, 1663–1671.

Snelling, W.J., Matsuda, M., Moore, J.E., et al., 2006. Under the microscope: *Arcobacter*. Letters in Applied Microbiology 42, 7–14.

van Bergen, M.A., van der Graaf-van Bloois, L., Visser, I.J., et al., 2006. Molecular epidemiology of *Campylobacter fetus* subsp. *fetus* on bovine artificial insemination stations using pulsed field gel electrophoresis. Veterinary Microbiology 112 (1), 65–71.

Willoughby, K., Nettleton, P.F., Quirie, M., et al., 2005. A multiplex polymerase chain reaction to detect and differentiate *Campylobacter fetus* subspecies *fetus* and *Campylobacter fetus* species *venerealis*: use on UK isolates of C. fetus and other *Campylobacter* spp. Journal of Applied Microbiology 99 (4), 758–766.

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Chapter 25

Lawsonia intracellularis

Genus Characteristics

Lawsonia intracellularis, a slender, curved Gram-negative rod, is classified in the delta subdivision of the *Proteobacteria*. The full genome has been sequenced and is available on the EMBL-EBI website (www.ebi.ac.uk/embl/Contact/collaboation.html accessed 21 December 2012). It is most closely related to *Bilophila wadsworthia* and the sulphate-reducing *Desulphovibrio* species. *Lawsonia intracellularis* is an obligate intracellular pathogen and causes disease primarily in pigs. However, it is increasingly recognized as a cause of diarrhoea in foals and disease has been recorded in a number of other species including hamsters, deer and ostriches. Strains isolated from different species appear to be closely related (Cooper et al. 1997). The organism can be grown only in enterocyte cell lines and is microaerophilic.

Natural Habitat

Lawsonia intracellularis grows intracellularly in enterocytes, particularly those of the terminal ileum, in pigs and other species. It can survive in the environment for periods of approximately two weeks.

Pathogenesis and Pathogenicity

The characteristic lesion associated with infection is a proliferative enteropathy but clinical disease can range from an acute haemorrhagic syndrome to a subclinical condition associated with poor production. In pigs, all ages are susceptible to infection but under farm conditions, infection appears to occur approximately six weeks after weaning, with shedding of the organism persisting for between two and six weeks (Stege et al. 2004). Interaction with other microorganisms of the intestinal flora appears to be essential for the development of clinical disease as neither infection nor colonization results from the exposure of gnotobiotic pigs to pure cultures of *L. intracellularis*. In addition, recent studies indicate that active proliferation and differentiation of crypt cells, as occurs at weaning, as well as infection and multiplication of *Lawsonia* organisms, is necessary for the production of the characteristic proliferative lesions (McOrist et al. 2006).

The pathogenesis of proliferative enteropathy has been reviewed by Smith & Lawson (2001). The principal cells infected are the epithelial cells lining the intestinal crypts. Exact mechanisms involved in colonization and invasion of enterocytes have not been identified although a surface antigen apparently involved in attachment to and entry into epithelial cells of the intestine has been documented (McCluskey et al. 2002). The cell membrane remains intact as the organism enters the cells but the organisms do not remain in the vacuole formed but multiply free in the cytoplasm and are located principally in the apical area of the cell. Following infection, the cells begin to proliferate and migrate to populate the epithelium. In more severe disease, the organism may be found in tissues other than the epithelium, being found within macrophages in the lamina propria, submucosa and tonsils and in intestinal capillaries and lymphatics (Love & Love 1979, Jensen et al. 2000). It is unclear whether differences in disease manifestation reflect differences in virulence of bacterial strains or alterations in the host immune response. It is suggested that the organism may be able to modulate the immune response as the inflammatory response is notably limited, particularly in the early stages of disease (Rowland & Rowntree 1972).

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Laboratory Diagnosis

Lawsonia intracellularis can be grown only in enterocyte cell lines and few laboratories worldwide have the expertise required. Thus, isolation of the organism from clinical specimens is not generally available as a method of diagnosis. Vannucci et al. (2012) recently described an alternative method for culture of *Lawsonia* organisms using an Original Space Bag (Storage Packs, San Diego, CA). This method allows culture without the need for a tri-gas incubator and thus may be feasible for a greater number of laboratories than the original methods described.

In the past, presumptive diagnosis of infection with *L. intracellularis* was based on history, clinical signs and the demonstration of typical gross and histopathological lesions following postmortem examination. Silver staining of histopathological sections has been used to reveal numerous curved intracellular organisms in the epithelial lining of the ileum, but the sensitivity of this technique was poor. Immunohistochemical techniques were developed and are still used as sensitive and specific diagnostic methods (Jensen et al. 2010).

In recent years considerable progress has been achieved in developing diagnostic tests suitable for use in the live animal, although the most accurate results still require the demonstration of the organism in intestinal tissues collected from animals postmortem. Screening of herds for infection can be carried out using either serological tests or by detection of the organism in faecal samples. The serological tests used include the indirect fluorescent antibody test, an immunoperoxidase monolayer assay and ELISA techniques; some of these tests are available commercially. Detection of the organism in faecal samples can be done using PCR, including 'real-time' PCR (Lindecrona et al. 2002). Multiplex PCR techniques for the detection of *Salmonella* species, *Brachyspira hyodysenteriae* and *L. intracellularis* (Elder et al. 1997, Suh & Song 2005) as well as *Brachyspira hyodysenteriae*. *Brachyspira piloscoli* and *L. intracellularis* (La et al. 2006) have been described. Jacobson et al. (2004) evaluated serology, PCR techniques and postmortem examination for diagnosis of *L. intracellularis* infection and concluded that serology or repeated faecal sampling for PCR, or both, should be used to determine the presence of the microorganism in a herd. *Lawsonia intracellularis* can also be demonstrated in infected faeces using techniques such as immunomagnetic separation and ATP bioluminescence (Watarai et al. 2005) but such techniques are not widely available in laboratories.

Antimicrobial Resistance

Methods for the detection of antimicrobial resistance in *L. intracellularis* have been developed (McOrist et al. 1995) but are seldom employed. Thus, there is little information available on antimicrobial resistance patterns. Nevertheless, Wattanaphansak et al. (2008) evaluated antimicrobial susceptibility of 10 North American and European isolates and found tiamulin and valnemulin to be most effective. Yeh et al. (2011) tested two *Lawsonia* isolates from Korea and reported tylosin and tilmicosin to be the most effective agents.

REFERENCES

Cooper, D.M., Swanson, D.L., Barns, S.M., et al., 1997. Comparison of the 16S ribosomal DNA sequences from the intracellular agents of proliferative enteritis in a hamster, deer, and ostrich with the sequence of a porcine isolate of *Lawsonia intracellularis*. International Journal of Systematic Bacteriology 47 (3), 635–639.

Elder, R.O., Duhamel, G.E., Mathiesen, M.R., et al., 1997. Multiplex polymerase chain reaction for simultaneous detection of *Lawsonia intracellularis, Serpulina hyodysenteriae*, and salmonellae in porcine intestinal specimens. Journal of Veterinary Diagnostic Investigation 9, 281–286.

Jacobson, M., Aspan, A., Königsson, M.H., et al., 2004. Routine diagnostics of *Lawsonia intracellularis* performed by PCR, serological and postmortem examination, with special emphasis on sample preparation methods for PCR. Veterinary Microbiology 102 (3–4), 189–201.

Jensen, T.K., Møller, K., Lindecrona, R., et al., 2000. Detection of *Lawsonia intracellularis* in the tonsils of pigs with proliferative enteropathy. Research in Veterinary Science 68 (1), 23–26.

Jensen, T.K., Boesen, H.T., Vigre, H., et al., 2010. Detection of *Lawsonia intracellularis* in formalin-fixed porcine intestinal tissue samples: comparison of immunofluorescence and in-situ hybridization, and evaluation of the effects of controlled autolysis. Journal of Comparative Pathology 142 (1), 1–8.

La, T., Collins, A.M., Phillips, N.D., et al., 2006. Development of a multiplex-PCR for rapid detection of the enteric pathogens *Lawsonia intracellularis, Brachyspira hyodysenteriae,* and *Brachyspira pilosicoli* in porcine faeces. Letters in Applied Microbiology 42 (3), 284–288.

Lindecrona, R.H., Jensen, T.K., Andersen, P.H., et al., 2002. Application of a 5′ nuclease assay for detection of *Lawsonia intracellularis* in fecal samples from pigs. Journal of Clinical Microbiology 40 (3), 984–987.

Love, D.N., Love, R.J., 1979. Pathology of proliferative haemorrhagic enteropathy in pigs. Veterinary Pathology 16, 41–48.

McCluskey, J., Hannigan, J., Harris, J.D., et al., 2002. LsaA, an antigen

involved in cell attachment and invasion, is expressed by *Lawsonia intracellularis* during infection in vitro and in vivo. Infection and Immunity 70 (6), 2899–2907.

McOrist, S., Gebhart, C.J., Boid, R., et al., 1995. Characterization of *Lawsonia intracellularis gen. nov., sp. nov.*, the obligately intracellular bacterium of porcine proliferative enteropathy. International Journal of Systematic Bacteriology 45, 820–825.

McOrist, S., Gebhart, C.J., Bosworth, B.T., 2006. Evaluation of porcine ileum models of enterocyte infection by *Lawsonia intracellularis*. Canadian Journal of Veterinary Research 70, 155–159.

Rowland, A.C., Rowntree, P.G.M., et al., 1972. A haemorrhagic bowel syndrome associated with intestinal adenomatosis in the pig. Veterinary Record 91, 235–241.

Smith, D.G., Lawson, G.H., 2001. *Lawsonia intracellularis*: getting inside the pathogenesis of proliferative enteropathy. Veterinary Microbiology 82 (4), 331–345.

Stege, H., Jensen, T.K., Møller, K., et al., 2004. Infection dynamics of *Lawsonia intracellularis* in pig herds. Veterinary Microbiology 104, 197–206.

Suh, D.K., Song, J.C., 2005. Simultaneous detection of *Lawsonia intracellularis, Brachyspira hyodysenteriae* and *Salmonella* spp. in swine intestinal specimens by multiplex polymerase chain reaction. Journal of Veterinary Science 6, 231–237.

Vannucci, F.A., Wattanaphansak, S., Gebhart, C.J., 2012. An alternative method for cultivation of *Lawsonia intracellularis*. Journal of Clinical Microbiology 50 (3), 1070–1072.

Watarai, M., Yamato, Y., Murakata, K., 2005. Detection of *Lawsonia intracellularis* using immunomagnetic beads and ATP bioluminescence. Journal of Veterinary Medical Science 67 (4), 449–451.

Wattanaphansak, S., Singer, R.S., Gebhart, C.J., 2008. In vitro antimicrobial activity against 10 North American and European *Lawsonia intracellularis* isolates. Veterinary Microbiology 134, 305–310.

Yeh, J.Y., Lee, J.H., Yeh, H.R., et al., 2011. Antimicrobial susceptibility testing of two *Lawsonia intracellularis* isolates associated with proliferative hemorrhagic enteropathy and porcine intestinal adenomatosis in South Korea. Antimicrobial Agents and Chemotherapy 55 (9), 4451–4453.

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Chapter 26

Haemophilus and *Histophilus* species

Genus Characteristics

There have been a number of recent name changes to members of the *Haemophilus* genus. The species *Histophilus somni* now encompasses the organisms formerly known as *Haemophilus somnus, Histophilus ovis* and *Histophilus agni*. *Haemophilus paragallinarum* has been reassigned to the genus *Avibacterium* (see Chapter 21). *Haemophilus piscium* strains are included in the species *Aeromonas salmonicida* (see Chapter 19). *Haemophilus* and *Histophilus* species are small Gram-negative rods, less than 1 μm wide by 1–3 μm, but can be coccobacillary or produce short filaments. Capsules can be produced by *H. influenzae* and *H. parasuis*. Traditionally *Haemophilus* species had to have, by definition, an absolute requirement for one or both of the growth factors: X (haemin) that is heat-stable and V (nicotinamide adenine dinucleotide; NAD) that is heat-labile. However, *Histophilus somni* is able to grow in the absence of these factors although growth is enhanced by their presence. *Haemophilus* and *Histophilus* species are motile, facultative anaerobes, produce acid from glucose, reduce nitrates, and are variable in the oxidase and catalase tests. They are nutritionally fastidious, will not grow on MacConkey agar and grow best on chocolate agar (supplying the X and V factors) under 5–10% CO_2 at 37°C.

Natural Habitat

Haemophilus and *Histophilus* species are commensals of the mucous membranes of humans and animals, most commonly of the upper respiratory and lower genital tracts. *Haemophilus parasuis* inhabits the nasopharynx of normal pigs, *Histophilus somni* is present in the respiratory and genital tracts of healthy cattle and sheep and *Haemophilus haemoglobinophilus* is a commensal of the genital tract of dogs.

Pathogenesis

The most significant pathogens of these species are *Haemophilus parasuis* which causes Glasser's disease in pigs and *Histophilus somni* which casuses a variety of conditions in domestic ruminants (Table 26.1). Even within the pathogenic species, there is a high level of strain variability; some strains appear to be avirulent whereas others are consistently isolated from pathological specimens and possess a range of virulence factors. Traditionally *Haemophilus* species are classified by serotyping. Unfortunately no clear relationship exists between serotype and pathogenicity although some serotypes such as *H. parasuis*, serotype 3, are isolated only from mucosae and never from systemic sites (Oliveira et al. 2003). Genetic characterization of these organisms has identified many putative virulence genes but in most cases the role of the virulence attributes has not been conclusively demonstrated in vivo. *Histophilus somni* is resistant to the lethal effects of phagocytes and serum, in part through the production of immunoglobulin-binding proteins which bind the Fc portion of bovine IgG. It can adhere to epithelium and is toxic to endothelial cells to which it also adheres. Immunoglobulin-binding proteins may mediate adherence to host cells and in addition, these proteins have two direct repeat regions which contain Fic (filamentation-induced by c-AMP) domains. These motifs mediate cytotoxicity through induction of cytoskeletal collapse (Worby et al. 2009). Infections of lungs, body cavities and joints are serofibrinous and/or suppurative. Thrombotic vasculitis leading to encephalitis and meningitis as well as

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Table 26.1 Diseases of *Histophilus somni* and *Haemophilus* species

Organism	Host(s)	Disease or significance
Histophilus somni	Cattle	Infectious thromboembolic meningoencephalitis (TEME): septicaemia with infarcts in cerebellum
		Respiratory disease: pneumonia and pleurisy, often in mixed infections with other agents
		Genital infections such as endometritis and abortion. The organism can occur in semen and genital tract of bulls
	Sheep	Commensal of genital tract of sheep and causes epididymitis and orchitis in rams. May also cause pneumonia, mastitis, polyarthritis, meningtitis and septicaemia
Haemophilus parasuis	Pigs	Primary agent of Glasser's disease: polyserositis and meningitis in young pigs
		Arthritis and pneumonia in older pigs
		Secondary invader in swine influenza or in enzootic pneumonia (*Mycoplasma hyopneumoniae*)
H. influenzae	Humans	Variety of diseases ranging from respiratory infections to meningitis
H. parainfluenzae	Humans	Normal flora of upper respiratory tract and implicated in urethritis, endocarditis and occasionally in other conditions
H. haemoglobinophilus	Dogs	Commensal of lower genital tract and sometimes causes cystitis and neonatal infections. Isolated from balanoposthitis and vaginitis but the role of the bacterium in these conditions is uncertain
H. paracuniculus	Rabbits	Significance unknown. Isolated from the intestine of rabbits with mucoid enteritis
'H. influenzaemurium'	Mice	Respiratory infections and conjunctivitis

haemorrhagic necrotizing processes are caused by *H. somni*. Young or previously unexposed animals are most susceptible to *H. somni* and *Haemophilus* infections with stress factors contributing to the development of signs of disease.

Virulence attributes of *Haemophilus parasuis* are not well defined. In common with *H. somni* it produces lipo-oligosaccharide and a number of outer membrane proteins. Table 26.1 summarizes the diseases or significance of *H. somni* and *Haemophilus* species associated with animals, while virulence factors are summarized in Table 26.2.

Laboratory Diagnosis

Specimens

Haemophilus species are fragile and the specimens should be protected from drying and cultured as soon as possible (within 24 hours) after collection. Refrigeration and transport media may not be particularly beneficial and deep freezing, below −60°C, is the only reliable method for the preservation of these bacteria. The type of specimen required will depend on the disease or lesions present. Results of isolation require careful interpretation because of the prevalence of commensal, apparently non-pathogenic, strains on mucosal sites. Isolation of these organisms from mucosal sites is not necessarily proof of their involvement in the clinical signs observed and further tests for pathogenicity may be required. Studies are ongoing to establish definitive virulence markers in the major animal pathogens, *Histophilus somni* and *Haemophilus parasuis*. Isolation from systemic sites is diagnostically significant.

Direct microscopy

Demonstration of these small Gram-negative rods in tissues is often difficult and specific fluorescent antibody staining is a more sensitive method.

Isolation

X or V factors must be supplied for all the *Haemophilus* species but not for *Histophilus somni*. The X factor (haemin) is heat-stable and present in adequate amounts in 5%

Table 26.2 Virulence factors of *Histophilus somni*

Virulence factor	Function
Lipo-oligosaccharide (LOS)	Lipid A component has endotoxic activity Induces apoptosis of pulmonary and brain vascular endothelial cells Phase variation of LOS helps in evasion of host immune response Role in colonization of respiratory tract
Sialylation of LOS	Sialic acid is non-immunogenic and confers resistance to antibody binding
Immunoglobulin-binding proteins	Bind Fc portion of immunoglobulins Fic (filamentation-induced by c-AMP) domains responsible for cytotoxicity Filamentous haemagglutinin-like domain mediates adhesion to host cells
Inhibition of the oxidative burst	Probable role in intracellular survival within phagocytes
Outer membrane proteins	Exact roles remain unclear
Biofilm	Evasion of immune response
Iron binding and utilization proteins	Iron acquisition

blood agar. V factor is present in red cells and is susceptible to NADases present in most bloods. In chocolate agar the V factor is released from the red cells, the NADases are destroyed, and the heat-stable X factor is still present. *Staphylococcus aureus* grown as a streak across a blood agar plate will provide the V factor and V-factor-requiring haemophili will grow as satellite colonies near the streak. Commercial media, with supplements, are available for *Haemophilus* species but chocolate agar is the most satisfactory medium for the haemophili isolated from animals. Selective media have been designed for *H. somni* but their performance has not been consistently successful. The chances of isolating *H. somni* from contaminated specimens is increased if the specimens are first incubated in an infusion broth.

The growth of many of the *Haemophilus* species is enhanced by 10% CO_2. As this atmosphere is not inhibitory for any of them, CO_2 should be used for routine isolation. Inoculated chocolate agar plates are incubated under 10% CO_2 at 35–37°C for three to four days, although some growth may be seen after 24 hours.

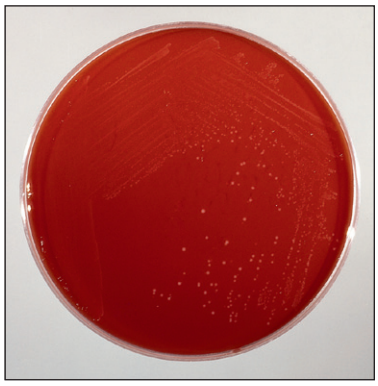

Figure 26.1 Typical dew-drop type colonies of many *Haemophilus* species on chocolate agar.

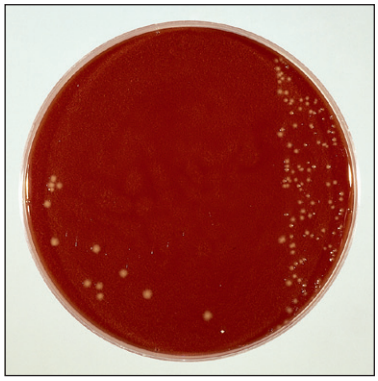

Figure 26.2 *Histophilus somni* on chocolate agar showing the characteristic yellowish tinge of the colonies.

Identification

Colonial morphology

Small dewdrop-like colonies may appear after 24–48 hours' incubation (Fig 26.1). None of the species is consistently haemolytic. A few strains of *H. somni* may show a frank clearing around the colonies especially on Columbia-base sheep blood agar. *Histophilus somni* colonies may appear yellowish (Fig 26.2) especially in a loopful of growth or on a confluent lawn.

Microscopic appearance

Haemophili are small Gram-negative rods that can be coccobacillary in form (Fig 26.3). More rarely short filaments occur.

Biochemical reactions

Some differential biochemical reactions for the *Haemophilus* species isolated from animals are given in Table 26.3. In non-specialist laboratories a presumptive

Table 26.3 Differential characteristics for *Histophilus somni* and *Haemophilus* species isolated from animals

	Requirement for factors		Catalase	Oxidase	CO_2 enhances growth	Haemolysis	Indole	Urease	Glucose (acid)	Nitrate reduction	Sucrose	Lactose	D-Xylose	Mannitol	Ornithine decarboxylase	Arginine dihydrolase
	X	V														
Histophilus somni	–	–	–	+	+	v	v	–	+	+	–	v	+	v	+	–
Haemophilus parasuis	–	+	+	–	v	–	–	–	+	+	+	v	–	–	–	–
H. haemoglobinophilus	+	–	+	+	–	–	+	–	+	+	+	–	+	+	–	–
H. paracuniculus	–	+	+	+	+	–	+	+	+	+	+	–	–	–	+	+
'H. influenzaemurium'	+	–	+	–	–	–	–	–	+	+	+	–	–	–	na	na

+ = over 90% strains positive, – = less than 90% strains positive, v = variable, na = data not available

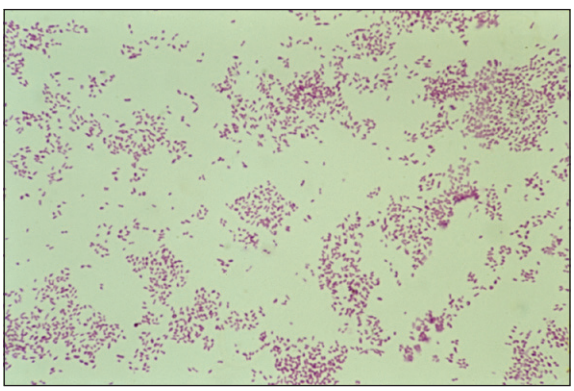

Figure 26.3 Microscopic appearance of *Haemophilus* species: small Gram-positive rods, often coccobacillary.

identification of the fastidious *Haemophilus* species is based on the host species, clinical signs and lesions, colonial and microscopic characteristics, X and V factor requirements, oxidase and catalase reactions and whether or not CO_2 enhances growth. *Histophilus somni* is rather variable in biochemical activities and the most reliable reactions are oxidase-positive, catalase-negative and CO_2 atmosphere giving a considerable enhancement of growth. If the indole test is positive, this is useful diagnostically.

- *Indole test*: the substrate is 0.1% L-tryptophan in 0.05 M phosphate buffer at pH 6.8. This is inoculated with a loopful of the suspect *Haemophilus*/*Histophilus* species and incubated for four hours. The test is shaken with 0.5 mL of Kovac's reagent. A red colour in the upper alcohol phase indicates the presence of indole.
- *Urease test*. The test medium contains:
  - 0.1 g KH_2PO_4
  - 0.1 g K_2HPO_4
  - 0.5 g NaCl
  - 0.5 mL of 0.2% phenol red
  - 100 mL distilled water

The 0.2% phenol red is prepared by dissolving 0.2 g phenol red crystals in 8.0 ml 1M NaOH and 92.0 mL distilled water. The test medium pH is adjusted to 7.0 with 5M NaOH. This is autoclaved and then 10.4 mL of a 20% filter-sterilized urea solution is added. The medium is inoculated with the test species and incubated for four hours at 37°C. A red colour indicates urease activity.

- *Ornithine and arginine tests*. Commercial Moller's medium can be used but should be inoculated with a heavy loopful of the suspect culture. A purple colour that develops after four to 24 hours' incubation indicates a positive reaction.
- *Carbohydrate fermentation*. A phenol red broth (BD Difco) containing 1% of the sugar and filter-sterilized X and V factors (10 mg/L of each) is used. Additions should be made for some of the species; 1% serum in the medium for *H. parasuis,* a drop of defibrinated blood/mL for *H. somni*. *Haemophilus paracuniculus* is tested in bromocresol purple broth as it may not grow in the phenol red broth.

Figure 26.4 Satellitism, typical of *Haemophilus* species, around a V factor-producing bacterium such as *Staphylococcus aureus*.

Tests for X and V factor requirements

- *V factor*: the need for the V factor can be demonstrated by satellitism around a V factor-producing bacterium such as *Staphylococcus aureus* (Fig 26.4). The test is carried out on tryptose agar that does not contain either the X or V factor.
- *Disc Method for X and V factors*: three commercial discs impregnated with V factor, X factor and XV factors, respectively, are placed on a lawn of the test bacterium on a tryptose agar plate. Colonies will cluster around the disc(s) supplying the required growth factor(s). However, the results of this test are invalidated if:
  - there is a carry-over of, particularly, the X factor from a previous richer medium
  - a contaminant colony is present on the plate, this may act as a feeder-organism
  - the test medium contains traces of X or V factors.
- *Porphyrin test*: this is the most satisfactory method for testing the requirement for the X factor. A loopful of growth from a young culture is suspended in 0.5 mL of a 2 mM solution of delta-aminolevulinic acid (ALA) hydrochloride (Sigma) and 0.8 mM $MgSO_4$ in 0.1 M phosphate buffer at pH 6.9. It is incubated for at least four hours at 37°C and exposed to a Wood's UV lamp in a dark room. A red fluorescence indicates that porphyrin is present and the X factor is not required. The test is based on X factor-independent strains being able to convert ALA, a porphyrin precursor, to porphyrin (an intermediate in the haemin biosynthetic pathway). Haemin-dependent strains do not have the appropriate enzymes. Filter paper discs impregnated with ALA are available commercially.

For further information on biochemical or X and V factor requirement tests Killian and Biberstein (1984) should be consulted.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Haemophilus species and *Histophilus somni* can be tested for antimicrobial susceptibility using disc diffusion or broth microdilution methods. Specific breakpoints for antimicrobials used in a veterinary setting are available only for *H. somni* in bovine respiratory disease (CLSI 2008). Where studies have been carried out, results suggest that most *H. somni* isolates remain broadly susceptible to many antimicrobials used for treatment of infections in animals. Lamm et al. (2012) reported that *H. somni* isolated from feedlot cattle with respiratory disease were mostly susceptible to tilmicosin, enrofloxacin and ceftiofur despite treatment with these agents. Isolates were, however, resistant to tetracycline. Aarestrup et al. (2004) reported that *H. parasuis* and *H. somni* isolates from Denmark were susceptible to most agents commonly used in pig and cattle production there. However, recent reports from China suggest that high levels of resistance to fluoroquinolones and potentiated sulphonamides are present in *H. parasuis* isolates in that country (Zhou et al. 2010, Xu et al. 2011).

Molecular Diagnosis and Strain Typing

Owing to the fastidious nature of *Haemophilus* species and *Histophilus somni*, cultural methods may not be the most suitable for their detection, in particular where specimens are of poor quality or are contaminated. Tegtmeier et al. (2000) reported that a PCR procedure based on amplification of the 16S rDNA gene was superior to culture, immunohistochemical and in situ hybridization methods for the detection of *H. somni* in cases of pneumonia in cattle. Likewise, Turni and Blackall (2007) suggested that PCR-based methods may be the most useful for detection of *H. parasuis* in certain instances. Saunders et al. (2007) developed a multiplex PCR-based method for detection of *H. somni*, *Actinobacillus seminis* and *Brucella ovis* in ram semen.

Serotyping is commonly used in some laboratories for typing of *H. parasuis* and the 15 capsular serovars of this species have been broadly classified into highly virulent, moderately virulent and avirulent groups with serovars 1, 5, 10, 12, 13 and 14 being most virulent. However, there is no complete correlation between serotype and pathogenicity. Molecular methods are also used for strain characterization. Enzymic digestion followed by pulsed field gel electrophoresis is used for both *H. somni* (D'Amours et al. 2011) and *H. parasuis* (Xu et al. 2011) and may be useful in epidemiological investigations. Differentiation of bovine and ovine strains of *H. somni* may be carried out using restriction enzyme analysis of the *rpoB* gene (Tanaka et al. 2005).

REFERENCES

Aarestrup, F.M., Seyfarth, A.M., Angen Ø, 2004. Antimicrobial susceptibility of *Haemophilus parasuis* and *Histophilus somni* from pigs and cattle in Denmark. Veterinary Microbiology 101, 143–146.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard, third ed. CLSI document M31-MA3. Clinical and Laboratory Standards Institute, Wayne, PA.

D'Amours, G.H., Ward, T.I., Mulvey, M.R., et al., 2011. Genetic diversity and tetracycline resistance genes of *Histophilus somni*. Veterinary Microbiology 150 (3–4), 362–372.

Killian, M., Biberstein, E.L., 1984. Haemophilus. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol 1, Williams and Wilkins Co., Baltimore, USA, pp. 558–596.

Lamm, C.G., Love, B.C., Krehbiel, C.R., et al., 2012. Comparison of antemortem antimicrobial treatment regimens to antimicrobial susceptibility patterns of postmortem lung isolates from feedlot cattle with bronchopneumonia. Journal of Veterinary Diagnostic Investigation March 24 (2), 277–282.

Oliveira, S., Blackall, P.J., Pijoan, C., 2003. Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. American Journal of Veterinary Research 64 (4), 435–442.

Saunders, V.F., Reddacliff, L.A., Berg, T., et al., 2007. Multiplex PCR for the detection of *Brucella ovis*, *Actinobacillus seminis* and *Histophilus somni* in ram semen. Australian Veterinary Journal 85 (1–2), 72–77.

Tanaka, A., Hoshinoo, K., Hoshino, T., et al., 2005. Differentiation between bovine and ovine strains of *Histophilus somni* based on the sequences of 16S rDNA and rpoB gene. Journal of Veterinary Medical Science 67 (3), 255–262.

Tegtmeier, C., Angen, O., Ahrens, P., 2000. Comparison of bacterial cultivation, PCR, in situ hybridization and immunohistochemistry as tools for diagnosis of *Haemophilus somnus* pneumonia in cattle. Veterinary Microbiology 76 (4), 385–394.

Turni, C., Blackall, P.J., 2007. Comparison of sampling sites and detection methods for *Haemophilus parasuis*. Australian Veterinary Journal 85 (5), 177–184.

Worby, C.A., Mattoo, S., Kruger, R.P., et al., 2009. The *fic* domain: regulation of cell signaling by adenylylation. Molecular Cell 34, 93–103.

Xu, C., Zhang, J., Zhao, Z., et al., 2011. Antimicrobial susceptibility and PFGE genotyping of *Haemophilus parasuis* isolates from pigs in South China (2008–2010). Journal of Veterinary Medical Science 73 (8), 1061–1065.

Zhou, X., Xu, X., Zhao, Y., et al., 2010. Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates. Veterinary Microbiology 141 (1–2), 168–173.

Chapter 27

Taylorella species

Taylorella equigenitalis and the recently described *Taylorella asinigenitalis* (Jang et al. 2001), are Gram-negative rods about 0.8 × 5.0 µm in size. Both species are facultative anaerobes, non-motile, oxidase-positive, catalase-positive, phosphatase-positive and produce no acid from carbohydrates. *Taylorella* species are fastidious and slow-growing bacteria; optimal growth is obtained on chocolate agar with a rich base (Eugon or Columbia agar) at 37°C under 5–10% CO_2, with *T. asinigenitalis* growing more slowly than *T. equigenitalis*. *Taylorella* species do not grow on MacConkey agar.

Natural Habitat

Taylorella equigenitalis is the causal agent of contagious equine metritis (CEM). It resides exclusively in the equine genital tract. Both sexes can remain carriers indefinitely but stallions do not develop signs of disease. The disease is highly contagious. The geographical distribution is limited, but from Europe the disease has spread to Japan, Australia and the USA. *Taylorella asinigenitalis* has been isolated from the genital tract of male donkeys and from mares bred by natural service to infected donkeys but its pathogenicity is unclear.

Pathogenesis and Pathogenicity

Transmission of *T. equigenitalis* is essentially venereal, but mares can also be infected by attendants and via veterinary instruments. The organisms can be isolated from neonatal and virgin animals. A purulent metritis develops within a few days of infection and the mare often has a copious mucopurulent uterine discharge. The infectious process is limited to the mucous membranes of the uterus, cervix and vagina. There is erosion and degenerative change in the endometrium. After endometrial repair is complete, within a few weeks, the organism may still be present in the clitoral sinuses and fossa, remaining there for long periods. No clinical signs occur in the stallion but *T. equigenitalis* can be found on the surface of the penis, in preputial smegma and in the urethral fossa. The infection in mares causes a temporary infertility and occasionally abortion within the first 60 days of pregnancy. Little is known of the virulence mechanisms of *T. equigenitalis* although studies attempting to characterize pathogenic properties such as adherence and invasion have been described (Bleumink-Pluym et al. 1996, Lapan et al. 1991). The genomes of both *Taylorella* species have been sequenced recently and these studies identified a number of putative virulence genes including genes encoding Type II, III, IV and VI Secretion Systems, haemagglutinins and iron acquisition systems (Hauser et al. 2012, Hébert et al. 2012). However, the role of these genes in vivo remains to be confirmed. In addition, use of PCR-based techniques for the detection of the organism indicates that carriage of the organism may be more widespread than cultural techniques would suggest and genotyping procedures have demonstrated the existence of several different strains of *T. equigenitalis.* These findings suggest that differences in pathogenicity exist between strains. Application of molecular techniques such as microarray analysis should increase our understanding of the relationship between infection with *T. equigenitalis* and the pathogenesis of contagious equine metritis in the future.

Laboratory Diagnosis

Specimens

In many countries contagious equine metritis is controlled by a government body or by a thoroughbred breeders'

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association. These bodies lay down the method of sample taking, the type of samples to be taken and the culture media to be used, when examining asymptomatic stallions and mares for the carrier state of the disease. Many of the recommendations are based on the Code of Practice of the Horserace Betting Levy Board in the UK (Horserace Betting Levy Board 2012). Requirements may be amended periodically. Often only approved laboratories are licensed to process and culture the specimens. Details of required sampling and cultural techniques are also available from the OIE (2012).

In general, acceptable samples are swabs or biopsies from:

- Mares: cervix, uterus, clitoral fossa and clitoral sinuses
- Stallions: urethra, urethral fossa and diverticulum, prepuce and pre-ejaculatory fluid.

In some cases it is specified that a stallion serves two maiden mares and these are sampled instead of the stallion himself. The specimens are collected using sterile swabs and these are placed into Amies transport medium with charcoal. They must reach the laboratory, under refrigeration, within 48 hours of collection.

Direct microscopy

Gram-stained smears are only of use on uterine exudates from a mare with clinical disease. *Taylorella equigenitalis* can appear as Gram-negative rods, coccobacilli or short filaments.

Isolation

The medium routinely used is chocolate agar with a highly nutritive base such as Eugon or Columbia agar base and preferably equine blood. The inoculated plates are incubated at 37°C under 10% CO_2. Growth may occur at 48 hours but negative plates should be examined daily for up to seven days before discarding them. Selective media are usually required to suppress contaminating bacteria. If streptomycin is used as one of the selective agents, two plates should be inoculated in parallel, with and without streptomycin, as many strains of *T. equigenitalis* are susceptible to this antibiotic. Examples of this type of medium are:

- Plate 1: Eugon chocolate agar with 10% horse blood and 200 µg/mL streptomycin.
- Plate 2: Eugon chocolate agar with 10% horse blood, 5 µg/mL amphotericin B and 1 µg/mL crystal violet.

Timoney et al. (1982) suggested a selective medium that proved very effective in controlling the bacterial and fungal flora in material on swabs taken from the external genitalia of mares and stallions. As the medium does not contain streptomycin, it is suitable for the isolation of both streptomycin-sensitive and streptomycin-resistant strains of *T. equigenitalis*. The formula for the CEM selective medium (Timoney et al. 1982) is given in Appendix 2.

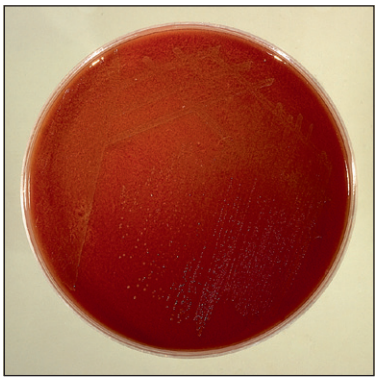

Figure 27.1 *Taylorella equigenitalis* on chocolate agar after three days at 37°C.

Identification

Colonial morphology

After 48 hours' incubation the colonies are under 1 mm in diameter, shiny, smooth and yellowish-grey. They may attain a size of 2 mm on further incubation (Fig. 27.1).

Microscopic appearance

Gram-negative pleomorphic coccobacilli are seen in smears from the colonies.

Biochemical reactions

Colonies with the correct macroscopic and microscopic appearance which are catalase-positive and oxidase-positive are subcultured onto Eugon chocolate agar without antibiotics and subjected to further tests:

- Inability to grow in air
- Agglutination with *T.-equigenitalis*-specific antiserum in a slide test. Weak spontaneous agglutination may sometimes occur in the saline control
- Phosphatase activity: 0.5 mL of *p*-nitrophenyl phosphate solution (1 mg/mL) is added to a suspension of the suspect colonies in 0.5 mL of Tris buffer (pH 8.0). The mixture is incubated at 37°C for up to two hours. A yellow colour indicates a positive result
- It is generally unreactive in other biochemical tests.

Serology

Complement fixing antibodies are consistently detectable from the third to seventh weeks post-infection in mares. However, this is rather late for the test to be useful

diagnostically and the CFT titres do not correlate sufficiently well with the carrier state to be reliable. Demonstration of CFT antibodies may be useful, in retrospect, to confirm a past infection.

Antimicrobial Susceptibility

Conventional antibiotic susceptibility tests are difficult with this slow-growing, fastidious bacterium. Agar dilution methods for the determination of minimum inhibitory concentrations can be used (Ensink et al. 1993). Topical treatment of the clitoral fossa in mares and external genitalia of stallions with disinfectant, together with systemic antimicrobial treatment may be required for the elimination of the carrier state (OIE 2012). Apart from some strains which are resistant to streptomycin, most *T. equigenitalis* isolates remain susceptible to most commonly used antimicrobial agents (Erdman et al. 2011).

Strain Typing

Isolates can be identified using a commercially available latex agglutination test. Monoclonal antibodies for use in immunofluoresence tests are available also. No serological differences between isolates examined in differenct parts of the world have been observed.

Molecular identification of isolates can be carried out using 16S rDNA sequencing (Bleumink-Pluym et al. 1993). Several different methods for genotyping of *Taylorella* isolates are described and have been reviewed by Matsuda and Moore (2003). To date, digestion with restriction enzymes followed by pulsed-field gel electrophoresis (PFGE) or variations of PFGE such as field inversion gel electrophoresis or crossed-field gel electrophoresis appear most useful and have identified up to 28 different strains (Matsuda & Moore 2003). Aalsburg and Erdman (2011) demonstrated the usefulness of these techniques in epidemiological investigations; in a recent USA outbreak, all isolates were shown to be related but clearly distinguishable from strains isolated in previous outbreaks.

Molecular Diagnosis

Several authors have reported the successful use of PCR-based methods for the detection of *Taylorella equigenitalis* in clinical specimens (Bleumink-Pluym et al. 1994, Chanter et al. 1998, Anzai et al. 1999, Moore et al. 2001). A real-time PCR for use on genital swabs, which discriminates between the two *Taylorella* species, has been developed by Wakeley et al. (2006). Duquesne et al. (2007) have described a PCR technique which can be used directly on clinical specimens without the need for preliminary DNA extraction.

REFERENCES

Aalsburg, A.M., Erdman, M.M., 2011. Pulsed-field gel electrophoresis genotyping of *Taylorella equigenitalis* isolates collected in the United States from 1978 to 2010. Journal of Clinical Microbiology 9 (3), 829–833.

Anzai, T., Eguchi, M., Sekizaki, T., et al., 1999. Development of a PCR test for rapid diagnosis of contagious equine metritis. Journal of Veterinary Medical Science 61 (12), 1287–1292.

Bleumink-Pluym, N.M., van Dijk, L., van Vliet, A.H., et al., 1993. Phylogenetic position of *Taylorella equigenitalis* determined by analysis of amplified 16S ribosomal DNA sequences. International Journal of Systematic Bacteriology 43 (3), 618–621.

Bleumink-Pluym, N.M., Werdler, M.E., Houwers, D.J., et al., 1994. Development and evaluation of PCR test for detection of *Taylorella equigenitalis*. Journal of Clinical Microbiology 32 (4), 893–896.

Bleumink-Pluym, N.M., ter Laak, E.A., Houwers, D.J., et al., 1996. Differences between *Taylorella equigenitalis* strains in their invasion of and replication in cultured cells. Clinical and Diagnostic Laboratory Immunology 3 (1), 47–50.

Chanter, N., Vigano, F., Collin, N.C., et al., 1998. Use of a PCR assay for *Taylorella equigenitalis* applied to samples from the United Kingdom. Veterinary Record 143 (8), 225–227.

Duquesne, F., Pronost, S., Laugier, C., et al., 2007. Identification of *Taylorella equigenitalis* responsible for contagious equine metritis in equine genital swabs by direct polyermase chain reaction. Research in Veterinary Science 82, 47–49.

Ensink, J.M., van Klingeren, B., Houwers, D.J., et al., 1993. In-vitro susceptibility to antimicrobial drugs of bacterial isolates from horses in The Netherlands. Equine Veterinary Journal 25 (4), 309–313.

Erdman, M.M., Creekmore, L.H., Fox, P.E., et al., 2011. Diagnostic and epidemiologic analysis of the 2008–2010 investigation of a multi-year outbreak of contagious equine metritis in the United States. Preventive Veterinary Medicine 101 (3–4), 219–228.

Hauser, H., Richter, D.C., van Tonder, A., et al., 2012. Comparative genomic analyses of the Taylorellae. Veterinary Microbiology 159 (1–2), 195–203.

Hébert, L., Moumen, B., Pons, N., et al., 2012. Genomic characterization of the *Taylorella* genus. PLOS ONE 7 (1), e29953.

Horserace Betting Levy Board, 2012. Code of Practice on Contagious Equine Metritis, Horserace Betting Levy Board, London, UK. Available at: http://codes.hblb.org.uk/

index.php/page/19 (accessed 21 December 2012).

Jang, S.S., Donahue, J.M., Arata, A.B., et al., 2001. *Taylorella asinigenitalis* sp. nov., a bacterium isolated from the genital tract of male donkeys (Equus asinus). International Journal of Systematic and Evolutionary Microbiology 51 (3), 971–976.

Lapan, G., Awad-Masalmeh, M., Hartig, A., et al., 1991. *Taylorella equigenitalis*: cell wall proteins, gene fingerprints, plasmids, adhesion and toxicity. Zentralblatt fur Veterinarmedizin 38 (8), 589–598.

Matsuda, M., Moore, J.E., 2003. Recent advances in molecular epidemiology and detection of *Taylorella equigenitalis* associated with contagious equine metritis (CEM). Veterinary Microbiology 97 (1–2), 111–122.

Moore, J.E., Buckley, T.C., Millar, B.C., et al., 2001. Molecular surveillance of the incidence of *Taylorella equigenitalis* and *Pseudomonas aeruginosa* from horses in Ireland by sequence-specific PCR. Equine Veterinary Journal 33, 319–322.

OIE, 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.5.2. Contagious equine metritis. Available online at: http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/ (accessed 21 December 2012).

Timoney, P.J., Shin, S.J., Jacobson, R.H., 1982. Improved selective medium for isolation of the contagious metritis organism. Veterinary Record 111, 107–108.

Wakeley, P.R., Errington, J., Hannon, S., et al., 2006. Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from T. asinigenitalis. Veterinary Microbiology 118, 247–254.

Chapter 28

Bordetella species

Genus Characteristics

Bordetella species are small (0.2–0.5 µm in diameter and 0.5–2.0 µm in length), Gram-negative rods which tend to be coccobacillary. They belong to the *Alcaligenaceae* family. The genus *Bordetella* accomodates currently eight species including the cause of pertussis or whooping cough in humans, *B. pertussis*. Three *Bordetella* species can be considered contagious respiratory pathogens in animals: *B. bronchiseptica* (porcine atrophic rhinitis; canine infectious tracheobronchitis, synonyms: kennel cough, canine cough, canine croup), *B. avium* (turkey coryza) and *B. parapertussis* (pneumonia in lambs).

Bordetella species are strict aerobes (except *B. petrii*) and do not ferment carbohydrates (asaccharolytic) but derive energy from the oxidation of amino and carboxylic acids. All are catalase-positive and oxidase-positive. *Bordetella bronchiseptica* and *B. avium* will grow on MacConkey agar. *Bordetella avium* and *B. bronchiseptica* are motile by peritrichous flagella, while *B. pertussis* and *B. parapertussis* are non-motile.

Natural Habitat

Bordetella species are contagious, obligatory parasites of the upper respiratory tract of healthy and diseased humans, animals and birds. Their distribution is worldwide. *Bordetella bronchiseptica* can be present as part of the flora of the upper respiratory tract of pigs, dogs, cats, rabbits, guinea-pigs, rats, horses and possibly other animals. The carrier rates for dogs, pigs and rabbits are reported to be high. *Bordetella avium* inhabits the respiratory tract of infected poultry, principally turkeys. *Bordetella pertussis* and *B. parapertussis* are human pathogens causing whooping cough and a mild form of whooping cough, respectively. *Bordetella parapertussis* has also been associated with a non-progressive pneumonia in lambs.

Mammalian infections are mainly transmitted by aerosol via expired droplets. Infected animals represent the main sources of infection. In turkeys, indirect spread can occur via water and litter. The environmental survival capacity of *Bordetella* species is short and they are easily killed by UV radiation, pH, temperature and common disinfectants. However, *B. avium* and *B. bronchiseptica* are more resistant to various physical and chemical conditions than *B. pertussis* and *B. parapertussis*.

Pathogenicity and Pathogenesis

The bordetellae are associated with contagious respiratory diseases with high morbidities and low mortalities. Typical clinical signs include coughing with or without dyspnea, ocular or nasal discharge and weight loss. The severity of the disease increases with concomitant infection with other pathogenic respiratory agents. Table 28.1 summarizes the main diseases and hosts of *Bordetella* species. *Bordetella bronchiseptica* is the aetiological agent of infectious tracheobronchitis (kennel cough) in dogs and atrophic rhinitis in pigs. Respiratory infections associated with *B. bronchiseptica* have also been described in rabbits, cats, horses, rats, guinea pigs and less commonly in wildlife. Zoonotic diseases such as wound infections, bacteraemia and respiratory infections caused by *B. bronchiseptica* have been reported in the human literature but seem to be uncommon. *Bordetella avium* is the aetiological agent of turkey coryza or bordetellosis, a respiratory disease responsible for substantial economic losses in the poultry industry, especially in turkey production. This bacterium can also infect various species of fowl and songbirds (Raffel et al. 2002). *Boredetella hinzii* is generally regarded

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Table 28.1 Main diseases caused by the major pathogenic *Bordetella* species

Species	Host(s)	Diseases
Bordetella avium	Turkeys and less commonly other birds	Turkey coryza: rhinotracheitis and sinusitis in young poults. Morbidity high but mortality low
Bordetella bronchiseptica	Pigs	Atrophic rhinitis (with or without *Pasteurella multocida*) Bronchopneumonia seen in young pigs
	Dogs	Canine infectious tracheobronchitis (kennel cough) with or without concurrent respiratory viruses. Secondary invader in canine distemper
	Rabbits	Snuffles-like syndrome with upper respiratory tract infection, bronchopneumonia or septicaemia
	Guinea pigs and rats	Similar to the disease in rabbits
	Horses, cats	Respiratory infections
	Humans	Occasionally isolated from wounds and body fluids (presumed to be zoonotic infections)
Bordetella parapertussis	Humans	Mild form of whooping cough
	Lambs	Pneumonia
Bordetella pertussis	Humans	Classical form of whooping cough
	Chimpanzees	Rare cases of whooping-cough-like disease in captive animals

as non-pathogenic but some strains may be capable of causing disease in turkeys (Register & Kunkle 2009). *Bordetella parapertussis* is the cause of a nonprogressive form of pneumonia in lambs and a mild form of whooping cough in humans. Ovine and human strains of *B. parapertussis* are distinct with different host specificities.

Bordetella species virulence factors are summarized in Table 28.2. Virulence factors such as fimbriae (FIM), filamentous haemagglutinin (FHA), pertactin (PTN) and products of the type III secretion system promote adherence and colonization in *Bordetella* species. Virulence factors such as dermonecrotic toxin (DNT), osteotoxin, adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), pertussis toxin (PTX), type III secretion proteins and lipopolysaccharide (LPS) alter host tissues and promote lesion formation. Mattoo et al. (2001) reviewed the mechanisms of *Bordetella* pathogenesis.

Virulent strains of *Bordetella* species attach firmly to ciliated respiratory epithelium via attachment factors (FHA, FIM or PTN) and this is followed by rapid proliferation, ciliary paralysis and an inflammatory response. *Bordetella* species also have LPS which likely plays a role in colonization of the respiratory tract (Spears et al. 2000). Certain species of *Bordetella* produce an extracellular enzyme, adenylate cyclase haemolysin (ACT), which is a member of the RTX (Repeat in Toxins) family and has antiphagocytic activity. Dermonecrotic toxin (DNT) is a thermolabile intracellular polypeptide primarily responsible for nasal turbinate atrophy via alteration of osteoblast differentiation in *B. bronchiseptica* infections of pigs. It also produces necrotic lesions if injected intradermally. The effect on the turbinate bones is most serious in young pigs under three weeks of age when osteogenesis is most active. Atrophic rhinitis in pigs is transient and self-limiting when caused by *B. bronchiseptica* alone but the bacterium aids the establishment of the piliated and toxigenic strains of *Pasteurella multocida* and the combined infection causes more serious and permanent lesions (Fig. 28.1). *Bordetella avium* produces an osteotoxin which also alters osteoblast functions but without any dermonecrotic activity.

Bordetella species infections depress the respiratory clearance mechanisms facilitating invasion by other organisms. Both *B. bronchiseptica* and *B. parapertussis* have a type III secretion system (TTSS) which contributes to bacterial survival in the lower respiratory tract of the host (Pilione & Harvill 2006). The type III secretion products seem to be involved in cytotoxicity, apoptosis and inactivation of NF-κB (transcription factor) of eukaryotic cells. It is thought that persistent colonization by *B. bronchiseptica* may rely on the ability of the bacteria to differentially modulate both macrophage and dendritic cell functions leading to a modified adaptive immune response and subsequent bacterial colonization (Siciliano et al. 2006).

Bordetella species have genetic regulatory systems which contribute to pathogenesis of disease through modulation of virulence factors. *Bordetella* species utilize the *BvgAS* (*Bordetella* virulence gene) two-component signal

Table 28.2 Main virulence factors of pathogenic *Bordetella* species in veterinary medicine

Genes	Virulence determinants	Functions
*cya*ABCDE operon	Adenylate cyclase-haemolysin (ACT)	Inhibits phagocytic cell functions
dnt gene	Dermonecrotic toxin (DNT)	Inhibits osteoblast differentiation and produces skin lesions
*fim*ABCD (in FHA operon)	Fimbriae (FIM)	Attachment and colonization in the respiratory tract
*fha*B, *fha*C (in FHA operon)	Filamentous haemagglutinin (FHA)	FHA is a large (>200 kDa), rod-shaped protein that is both surface-associated and secreted. FHA mediates bacterial adherence to epithelial cells and macrophages in vitro and is required for tracheal colonization in vivo
locus *wlb*	LPS	Colonization of the respiratory tract, endotoxin activities
metC gene	Osteotoxin	A beta-cystathionase that inhibits osteoblast differentiation
ptx and *ptl* genes	Pertussis toxin (PTX)	An A-B bacterial toxin with ADP-ribosylating activity which disrupts different eukaryotic cellular functions, secreted only by *B. pertussis*
prn gene	Pertactin (PTN)	An outer membrane protein adhesin (autotransporter family) involved in eukaryotic cell binding (via a RGD sequence, proline- and leucine-rich repeats)
NA	Tracheal cytotoxin (TCT)	A secreted muramyl dipeptide which stimulates Il-1 production that results in nitric oxide accumulation which causes damage to ciliated tracheal epithelial cells
bscN locus	Type III secretion system (TTSS)	Products of this secretion system are required for the induction of necrosis in infected mammalian cells
bvgAS locus	Sensory transduction system	Locus that encodes a transacting transcriptional regulator and a membrane-spanning sensor protein triggered by environmental signals

NA = not available

transduction system to sense the environment and regulate gene expression with at least three phases: a virulent *Bvg*+ phase, a non-virulent *Bvg*- phase, and an intermediate *Bvg*i phase. Genes expressed in the *Bvg*+ phase encode virulence factors including adhesins (FHA and FIM) and toxins such as ACT. In the *Bvg*i phase, FHA and FIM continue to be expressed, however ACT expression is significantly downregulated. *Bordetella bronchiseptica* can form biofilms in vitro, the generation of biofilm is maximal in the *Bvg*i phase.

Laboratory Diagnosis

Specimens

Specimens may include nasal or tracheal swabs, fluid from transtracheal apirates, endotracheal or bronchoalveolar lavages and pneumonic lung tissue. If nasal swabs are to be taken from animals where the nasal orifice is small, such as in young pigs, dogs and laboratory animals, the narrow gauge, flexible swabs designed for human infants (such as Mini-Tip Culturette swabs, Marion Scientific, USA) should be used. Nasal swabs are viewed as inferior specimens for culture since they contain large numbers of members of the normal flora which can result in overgrowth during isolation. Aspirates of lavage fluids are more likely to be representative of disease-causing organisms. Specimens should be either plated directly or placed in a suitable transport medium for bordetellae such as Amies medium. For *B. pertussis* or *B. parapertussis*, the Regan–Lowe medium or Amies medium with charcoal should be used.

Direct microscopy examination

Since bordetellae are small Gram-negative coccobacilli, direct Gram-stained smears from specimens are not usually very helpful. A fluorescent antibody technique could be useful for *B. bronchiseptica* and *B. avium*. An indirect fluorescent antibody staining technique using monoclonal antibody has been developed to detect *B. avium* in tracheal sections of turkeys (Saif & Barnes 2003).

Figure 28.1 Pig with early signs of atrophic rhinitis. Note the lacrimation and the shortened, deeply wrinkled snout.

Isolation

Bordetella species have simple growth requirements except for *B. pertussis* which can be easily inhibited by substances such as fatty acids in the medium. The routine media for *Bordetella* species are sheep blood and MacConkey agars. *Bordetella avium* and *B. bronchiseptica* grow well on both media as well as on Centrimide agar. The plates are incubated aerobically at 35°C for 24–48 hours. If isolations are to be attempted from specimens containing a large number of bacterial contaminants, such as nasal swabs, a selective medium is required: to prevent overgrowth and to maintain the alkaline to neutral conditions for the *Bordetella* species. Even a few fermentative bacteria on a medium containing carbohydrates can produce sufficient acid to inhibit *Bordetella* species. Selective media for *B. bronchiseptica* are usually based on its characteristic property of resistance to nitrofuran resistance. Several selective media have been described including MacConkey agar with 1% glucose and 20 µg/mL furaltadone and blood agar with 2 µg/mL clindamycin and 4 µg/mL neomycin. However, Smith and Baskerville (SB) medium (Smith & Baskerville 1979) gives a high isolation rate and is also an indicator medium (Appendix 2). This medium was designed for the isolation of *B. bronchiseptica* from pigs. If it is used for the isolation of strains from dogs or rabbits, then gentamicin should be omitted as some of these isolates have been reported as being susceptible to gentamicin. *Bordetella avium* will grow well on SB medium, with or without the antibiotic supplement. The inoculated SB medium is incubated aerobically at 35°C for 48 hours.

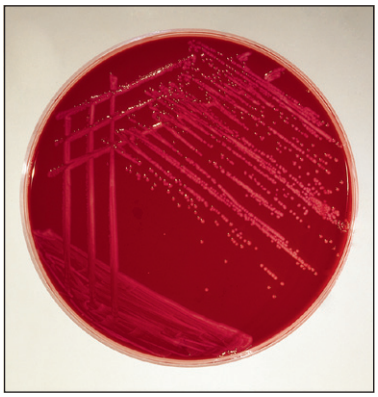

Figure 28.2 *Bordetella bronchiseptica* on sheep blood agar.

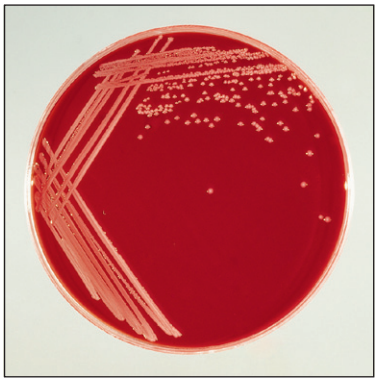

Figure 28.3 *Bordetella avium* on sheep blood agar.

Identification

Colonial morphology

On sheep, bovine or horse blood agar *B. bronchiseptica* forms very small (0.5–1 mm), convex, smooth colonies with an entire edge after 24 hours at 35°C (Fig. 28.2). Some strains may be beta-haemolytic. The colonies of *B. avium* are similar (Fig. 28.3) but are non-haemolytic. Phase modulation occurs in both species and this is thought to be due to loss of a capsule-like structure on subculture. The virulent, encapsulated phase I colonies are convex and shiny, those of phase II are larger, circular and convex with a smooth surface and the avirulent phase III colonies are large, flat and granular with an irregular edge.

The colonies on MacConkey agar are small, pale with a pinkish hue and amber discolouration of the underlying medium. Both *B. avium* (Fig. 28.4) and *B. bronchiseptica* (Fig. 28.5) have colonies of similar appearance on MacConkey agar. *Bordetella avium* colonies may have raised brown-tinged centres.

Smith–Baskerville (SB) medium contains the pH indicator bromothymol blue and the agar is green at pH 6.8.

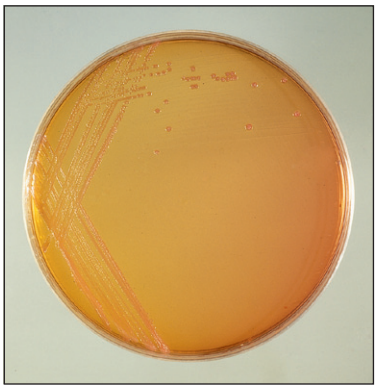

Figure 28.4 *Bordetella avium* on MacConkey agar.

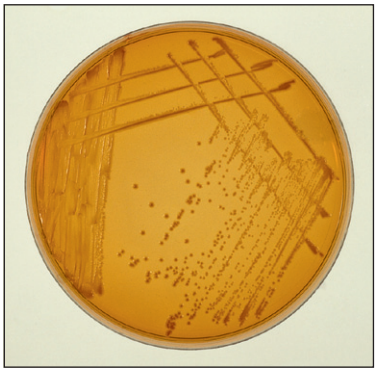

Figure 28.5 *Bordetella bronchiseptica* on MacConkey agar showing the pale, slightly tan colonies.

After 24 hours' incubation the colonies of *B. avium* and *B. bronchiseptica* are small (0.5 mm diameter or less) and blue with a lighter blue (alkaline) reaction in the medium around them. After 48 hours' incubation, the colonies are 1.0–2.0 mm diameter, blue or blue with a green centre and the surrounding medium is blue. The colonies of non-fermentative contaminants such as *Alcaligenes, Pseudomonas* and *Flavobacterium* species, at 24 and 48 hours' incubation, tend to be larger and more greenish than those of the *Bordetella* species. Any fermentative contaminants give an acid reaction with the colonies and surrounding medium appearing yellow (Fig. 28.6).

Microscopic appearance

Bordetella species are small Gram-negative coccobacilli.

Biochemical and other tests

Bordetella bronchiseptica is positive to the oxidase, catalase, citrate, urease and nitrate tests. It is motile and does not ferment carbohydrates. *Bordetella avium* and *Alcaligenes faecalis* have similar reactions to those of *B. bronchiseptica* but are urease-negative and nitrate-negative. *Alcaligenes faecalis*

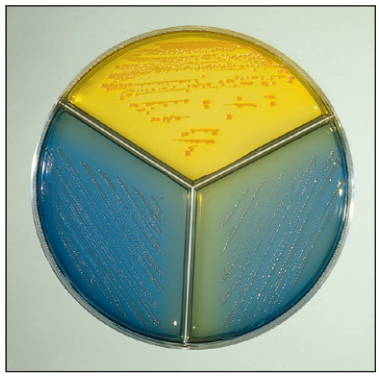

Figure 28.6 Smith–Baskerville medium with *B. bronchiseptica* (left), a lactose and/or glucose-fermenting bacterium (top) and *B. avium* (right). The uninoculated medium, with bromothymol blue as the pH indicator, is green.

is present in soil, water and faeces and as a result it can occasionally be present as a contaminant in clinical specimens. It has many properties in common with *B. avium* from which it must be distinguished using specific biochemical tests or media (Table 28.3).

Both automated and miniaturized commercial identification systems such as API 20NE, API ZYM and API 50CH are available for glucose-non-fermenting bacteria (see Chapter 30) including *B. bronchiseptica, B. avium* and *A. faecalis*. The colonies can also be identified by slide agglutination tests. A monoclonal antibody-based latex bead agglutination test for the detection of *B. avium* was developed as a rapid method to distinguish *B. avium* from closely related *Bordetella* species. This latex bead agglutination test may be useful as an aid in the identification of *B. avium* when used in conjunction with other criteria (Suresh & Arp 1993). *Bordetella hinzii* can exhibit a biochemical profile identical to that of *B. avium* with the following three commercial identification systems: API 20E, API 20 NE, and VITEK GNI+ card. However, their cellular fatty acid profiles are different. Definitive identification can also be achieved by 16S rRNA gene sequence analysis (Kattar et al. 2000). Phenotypic features useful in distinguishing *B. hinzii* from *B. avium* are production of alkali from malonate and resistance to several antimicrobial agents.

Haemagglutination test

Bordetella bronchiseptica possesses a haemagglutinin and will haemagglutinate washed sheep erythrocytes. A young 24-hour culture should be used as older colonies tend to lose their haemagglutinating ability. Two colonies of a suspected *B. bronchiseptica* culture are suspended in a drop of physiological saline on a slide. An equal volume of a 3% suspension of washed sheep red cells is added and mixed. To check for autoagglutination, controls should include a suspension of colonies without erythrocytes and

Table 28.3 Presumptive differentiation of *Bordetella* and *Alcaligenes* species

Characteristic	*Bordetella avium*	*Bordetella bronchiseptica*	*Alcaligenes faecalis*
Beta-haemolysis (blood agar)	–	(+/–)	–
Growth on MacConkey agar	+ (delayed)	+ (delayed)	+
Growth on SS agar	+	+	+
Nitrate reduction	–	+	–
Nitrite reduction	–	–	+
Urease	–	+ (<4h)	–
Sole source of carbon for growth:			
Citrate	+ (weak)	+	+
Malonate	–	–	+
Glycolate	–	–	+
Assimilation of adipate	+	d	–
Assimilation of caprate	–	d	+
Colonial morphology on SB medium	Small, blue	Small, blue	Larger, greenish
Motility	+	+	+
Oxidase	+	+	+
Tetrazolium reduction	+	+	–

+ = activity or growth present, – = not present, +/– = most strains positive, d = delayed, SB medium = Smith-Baskerville medium, SS agar = salmonella-shigella agar

a suspension of erythrocytes alone. *B. bronchiseptica* will haemagglutinate the red cells in one to two minutes. *Bordetella avium* agglutinates guinea pig erythrocytes.

Determination of pathogenicity

The dermonecrotic toxins of *B. bronchiseptica* and *B. avium* are thought to be important virulence factors. There appears to be no cross-reactivity between the two toxins. They are intracellular, heat-labile toxins and the dermonecrotizing toxin of *B. bronchiseptica* is lethal if inoculated intraperitoneally into mice and produces skin necrosis when injected intradermally into guinea pigs. Fatal infections can also be produced in guinea pigs by injection of young, intact cells intraperitoneally.

Serology

Tube agglutination, microagglutination (MAT), indirect immunofluorescence, dot-immunobinding assay and ELISA procedures have been developed for *B. avium* and *B. bronchiseptica*. Serology is used to measure antibody levels in turkeys to detect natural infection with *B. avium*; ELISA kits (KPL, Maryland, USA) are commercially available (Barbour et al. 1991, Tsai & Saif 1991, Raffel et al. 2002).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) for *Bordetella* species can be obtained by broth microdilution and agar dilution according to the recommendations of the Clinical and Laboratory Standards Institute (formerly known as the NCCLS) document M31-A3 (CLSI 2008). The E-test (AB Biodisk, Sweden) method has also been performed where MICs of antimicrobial agents are determined based on the diffusion of a pre-defined antibiotic gradient from a plastic strip. Comparisons with the E-test demonstrated an overall agreement (+/– 1 $\log^2$ dilution) with the agar dilution method of 79.4% and an agreement within +/– 2 $\log^2$ dilutions of 96.2% (Speakman et al. 1997). Standardized Kirby–Bauer disc diffusion antimicrobial susceptibility testing can also be performed (Rougier et al. 2006).

Antimicrobial resistance

A study of *B. bronchiseptica* isolates from respiratory tract infections of pigs revealed the lowest MICs 90 were those

of tetracycline and enrofloxacin, whereas the highest MICs 90 were those of tilmicosin, cephalothin and streptomycin (Kadlec et al. 2004). Burton et al. (1996) reported that ceftiofur was not active against *B. bronchiseptica* as measured by MIC. Canine isolates of *B. bronchiseptica* were reported as susceptible to tetracycline, doxycycline, enrofloxacin, and amoxycillin/clavulanic acid. The majority were also susceptible to ampicillin, trimethoprim, and sulfadiazine (Speakman et al. 2000). Another study reported that the majority of feline isolates of *B. bronchiseptica* tested by the agar dilution method were resistant to trimethoprim and ampicillin but susceptible to tetracycline, doxycycline and enrofloxacin (Speakman et al. 1997). A study in pet rabbits reported gentamicin was slightly more effective for *B. bronchiseptica* than marbofloxacin, which exhibited the highest level of activity of all the fluoroquinolones tested (Rougier et al. 2006). Comparison of antimicrobial susceptibilities of *B. avium* and *B. bronchiseptica* isolates revealed that in both species, isolates were consistently resistant to penicillin and cefuroxime but susceptible to mezlocillin, piperacillin, gentamicin, amikacin, and cefoperazone (Mortensen et al. 1989). Resistance to penicillin, the cephalosporins and sulphonamides has been reported in canine and feline isolates (Schwarz et al. 2007). Bemis (1992) recommended the use of tetracyclines and fluoroquinolones for treatment of *B. bronchiseptica* infections in dogs and cats.

To date, little is known regarding the molecular basis of antimicrobial resistance in *B. bronchiseptica* and other *Bordetella* species. However, timethoprim, chloramphenicol and sulphonamide resistance genes and class 1 integrons were reported in *B. bronchiseptica* porcine isolates (Kadlec et al. 2005). Florfenicol resistance was apparently mediated by the *floR* gene in some isolates and by an efflux system in others (Kadlec et al. 2007) For *B. pertussis*, erythromycin treatment failures and in vitro resistance have been reported. This is likely due to a mutation of the erythromycin-binding site in the 23S rRNA gene of the 50S ribosomal subunit (Bartkus et al. 2003).

Strain Typing

Pulsed-field gel electrophoresis (PFGE) analysis has proven to be a valuable tool for tracking the transmission of *B. pertussis* during outbreaks. It has also been used for typing *B. parapertussis* and *B. bronchiseptica*, typically using XbaI as the restriction enzyme (Porter et al. 1996, Binns et al. 1998). Other genotyping methods for typing bordetellae include restriction enzyme analysis (Sacco et al. 2000a, 2000b), randomly amplified polymorphic DNA (Yuk et al. 1998), PCR amplification of a specific repetitive element (Moissenet et al. 1996) and ribotyping (Register et al. 1997). No correlation was found between the results of PCR-RFLP and PFGE when applied to the typing of feline isolates of *B. bronchiseptica* (Winstanley et al. 2001).

Molecular Diagnosis

At present, presumptive routine identification of *Bordetella* relies mainly on isolation and biochemical testing in veterinary diagnostic laboratories. However, because of complex phylogenetic links with *Alcaligenes* and *Achromobacter*, accurate and definitive genus-level identification is difficult using phenotypic characteristics only (Yabuuchi et al. 1998). Polymerase chain reaction (PCR) assays have been developed for discriminative detection of *Bordetella* species. A PCR assay for detection of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* uses the up-stream sequence of the flagellin gene as the target DNA region and is able to differentiate these species by utilizing two different sets of primers (Hozbor et al. 1999). A multiplex PCR assay has been developed as a reliable method for identifying *B. bronchiseptica* and both toxigenic and non-toxigenic *P. multocida*, simplifying investigations of swine progressive atrophic rhinitis and bronchopneumonia (Register & DeJong 2006). PCR is also reported to be a useful method for the rapid detection of *B. avium* (Savelkoul et al. 1993, Register & Yersin 2005).

REFERENCES

Barbour, E.K., Brinton, M.K., Torkelson, S.D., et al., 1991. An enzyme-linked immunosorbent assay for detection of *Bordetella avium* infection in turkey flocks: sensitivity, specificity, and reproducibility. Avian Diseases 35, 308–314.

Bartkus, J.M., Juni, B.A., Ehresmann, K., et al., 2003. Identification of a mutation associated with erythromycin resistance in *Bordetella pertussis:* implications for surveillance of antimicrobial resistance. Journal of Clinical Microbiology 41, 1167–1172.

Bemis, D.A., 1992. Bordetella and mycoplasma respiratory infections in dogs and cats. Veterinary Clinics of North America: Small Animal Practice 22, 1173–1186.

Binns, S.H., Speakman, A.J., Dawson, S., et al., 1998. The use of pulsed-field gel electrophoresis to examine the epidemiology of *Bordetella bronchiseptica* isolated from cats and other species. Epidemiology & Infection 120, 201–208.

Burton, P.J., Thornsberry, C., Cheung Yee, Y., et al., 1996. Interpretive criteria for antimicrobial susceptibility testing of ceftiofur against bacteria associated with swine respiratory disease. Journal of Veterinary Diagnostic Investigation 8, 464–468.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk

and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard, third ed. CLSI document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

Hozbor, D., Fouque, F., Guiso, N., 1999. Detection of *Bordetella bronchiseptica* by the polymerase chain reaction. Research in Microbiology 150, 333–341.

Kadlec, K., Kehrenberg, C., Wallmann, J., et al., 2004. Antimicrobial susceptibility of *Bordetella bronchiseptica* isolates from porcine respiratory tract infections. Antimicrobial Agents and Chemotherapy 48, 4903–4906.

Kadlec, K., Kehrenberg, C., Schwarz, S., 2005. Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*. Journal of Antimicrobial Chemotherapy 56, 485–490.

Kadlec, K., Kehrenberg, C., Schwarz, S., 2007. Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica:* identification of a novel chloramphenicol exporter. Journal of Antimicrobial Chemotherapy 59 (2), 191–196.

Kattar, M.M., Chavez, J.F., Limaye, A.P., et al., 2000. Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicemia. Journal of Clinical Microbiology 38, 789–794.

Mattoo, S., Foreman-Wykert, A.K., Cotter, P.A., et al., 2001. Mechanisms of *Bordetella* pathogenesis. Frontiers in Bioscience 6, e168–e186.

Moissenet, D., Valcin, M., Marchand, V., et al., 1996. Comparative DNA analysis of *Bordetella pertussis* clinical isolates by pulsed-field gel electrophoresis, randomly amplified polymorphism DNA, and ERIC polymerase chain reaction. FEMS Microbiology Letters 143, 127–132.

Mortensen, J.E., Brumbach, A., Shryock, T.R., 1989. Antimicrobial susceptibility of *Bordetella avium* and *Bordetella bronchiseptica* isolates. Antimicrobial Agents and Chemotherapy 33, 771–772.

Pilione, M.R., Harvill, E.T., 2006. The *Bordetella bronchiseptica* type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance. Infection and Immunity 74, 1043–1049.

Porter, J.F., Connor, K., Donachie, W., 1996. Differentiation between human and ovine isolates of *Bordetella parapertussis* using pulsed-field gel electrophoresis. FEMS Microbiology Letters 135, 131–135.

Raffel, T.R., Register, K.B., Marks, S.A., et al., 2002. Prevalence of *Bordetella avium* infection in selected wild and domesticated birds in the eastern USA. Journal of Wildlife Diseases 38, 40–46.

Register, K.B., Yersin, A.G., 2005. Analytical verification of a PCR assay for identification of *Bordetella avium*. Journal of Clinical Microbiology 43, 5567–5573.

Register, K.B., Boisvert, A., Ackermann, M.R., 1997. Use of ribotyping to distinguish *Bordetella bronchiseptica* isolates. International Journal of Systematic Bacteriology 47, 678–683.

Register, K.B., DeJong, K.D., 2006. Analytical verification of a multiplex PCR for identification of *Bordetella bronchiseptica* and *Pasteurella multocida* from swine. Veterinary Microbiology 117, 201–210.

Register, K.B., Kunkle, R.A., 2009. Strain-specific virulence of *Bordetella hinzii* in poultry. Avian Diseases 53 (1), 50–54.

Rougier, S., Galland, D., Boucher, S., et al., 2006. Epidemiology and susceptibility of pathogenic bacteria responsible for upper respiratory tract infections in pet rabbits. Veterinary Microbiology 115, 192–198.

Sacco, R.E., Register, K.B., Nordholm, G.E., 2000a. Restriction endonuclease analysis discriminates *Bordetella bronchiseptica* isolates. Journal of Clinical Microbiology 38, 4387–4393.

Sacco, R.E., Register, K.B., Nordholm, G.E., 2000b. Restriction enzyme analysis and ribotyping distinguish *Bordetella avium* and *Bordetella hinzii* isolates. Epidemiology & Infection 124, 83–90.

Saif, Y.M., Barnes, H.J., 2003. Diseases of Poultry, eleventh ed. Iowa State Press, Ames, Iowa, pp. 705–718.

Savelkoul, P.H., de Groot, L.E., Boersma, C., et al., 1993. Identification of *Bordetella avium* using the polymerase chain reaction. Microbial Pathogenesis 15, 207–215.

Schwarz, S., Alesík, E., Grobbel, M., et al., 2007. Antimicrobial susceptibility of *Pasteurella multocida* and *Bordetella bronchiseptica* from dogs and cats as determined in the BfT-GermVet monitoring program 2004–2006. Berliner Munchener Tierarztliche Wochenschrift 120 (9–10), 423–430.

Siciliano, N.A., Skinner, J.A., Yuk, M.H., 2006. *Bordetella bronchiseptica* modulates macrophage phenotype leading to the inhibition of CD4+ T cell proliferation and the initiation of a Th17 immune response. Journal of Immunology 177, 7131–7138.

Smith, I.M., Baskerville, A.J., 1979. A selective medium facilitating the isolation and recognition of *Bordetella bronchiseptica* in pigs. Research in Veterinary Science 27, 187–192.

Speakman, A.J., Binns, S.H., Dawson, S., et al., 1997. Antimicrobial susceptibility of *Bordetella bronchiseptica* isolates from cats and a comparison of the agar dilution and E-test methods. Veterinary Microbiology 54, 63–72.

Speakman, A.J., Dawson, S., Corkill, J.E., et al., 2000. Antibiotic susceptibility of canine *Bordetella bronchiseptica* isolates. Veterinary Microbiology 71, 193–200.

Spears, P.A., Temple, L.M., Orndorff, P.E., 2000. A role for lipopolysaccharide in turkey tracheal colonization by *Bordetella avium* as demonstrated in vivo and in vitro. Molecular Microbiology 36, 1425–1435.

Suresh, P., Arp, L.H., 1993. A monoclonal antibody-based latex bead agglutination test for the detection of *Bordetella avium*. Avian Diseases 37, 767–772.

Tsai, H.J., Saif, Y.M., 1991. Detection of antibodies against *Bordetella avium* in turkeys by avidin-biotin enhancement of the enzyme-linked immunosorbent assay and the

dot-immunobinding assay. Avian Diseases 35, 801–808.

Winstanley, C., Shina, A., Dawson, S., et al., 2001. Variation in *Bordetella bronchiseptica* flaA does not correlate with typing by macro-restriction analysis by pulsed-field gel electrophoresis. Journal of Medical Microbiology 50, 255–260.

Yabuuchi, E., Kawamura, Y., Kosako, Y., et al., 1998. Emendation of genus *Achromobacter* and *Achromobacter xylosoxidans* (Yabuuchi and Yano) and proposal of *Achromobacter ruhlandii* (Packer and Vishniac) comb. nov., Achromobacter piechaudii (Kiredjian et al.) comb. nov., and *Achromobacter xylosoxidans* subsp. *denitrificans* (Ruger and Tan) comb. nov. Microbiology and Immunology 42, 429–438.

Yuk, M.H., Heininger, U., Martinez de Tejada, G., et al., 1998. Human but not ovine isolates of *Bordetella parapertussis* are highly clonal as determined by PCR-based RAPD fingerprinting. Infection 26, 270–273.

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Chapter 29

Moraxella species

The genus *Moraxella* is a member of the family *Moraxellaceae*. *Moraxella catarrhalis* and *Branhamella catarrhalis* are homotypic synonyms and it has been suggested that the genus should be divided into two subgenera, *Moraxella* and *Branhamella*. The moraxellae are short, plump, Gram-negative rods (1.0–1.5 × 1.5–2.5 μm) that characteristically occur in pairs (particularly those of the subgenus *Branhamella*). Some strains approach a completely coccal shape. They are strict aerobes, oxidative, oxidase-positive, usually catalase-positive, non-motile and do not attack carbohydrates. Although they will grow on non-enriched media, their growth is enhanced by the addition of blood or serum and the optimal temperature for growth is 33–35°C. Most *M. phenylpyruvica* strains will grow on MacConkey agar, but *M. bovis* and *M. lacunata* are unable to do so. The principal animal pathogen in the group is *Moraxella bovis* but *M. ovis* and *M. equi* are also associated with disease in animals.

Natural Habitat

The moraxellae are commensals on the mucous membranes of man and other mammals. The reservoir of *M. bovis* is thought to be the conjunctiva or nasopharynx of asymptomatic cattle over two years of age. The *Moraxella* species are susceptible to desiccation and do not survive well away from the animal host. Transmission is by direct contact or via flying insects.

Pathogenesis and Pathogenicity

Moraxella bovis, the cause of infectious bovine keratoconjunctivitis, is the major animal pathogen in the genus. The pathogenicity of *M bovis* strains depends on the production of type IV pili and a cytotoxin, in addition to other putative virulence factors (Table 29.1). The cytotoxin is a haemolysin and belongs to the repeat-in-toxin family of toxins (Angelos et al. 2001). Strains which are not haemolytic or lack pili are apathogenic for cattle. In addition to the pili, which have a major role in attachment of the organism, filamentous-haemagglutinin-like proteins may be important in the infectivity of the organism (Kakuda et al. 2006). Other enzymes which may play a role in tissue destruction include fibrinolysins, proteases and phospholipases. Acquisition of iron through transferrin and lactoferrin-binding proteins may contribute to virulence also.

Predisposing environmental factors are implicated in infectious bovine keratoconjunctivitis. These include irritation to the eyes by ultra-violet light (in sunlight), dust, long vegetation and flies. The incidence of the disease is highest in the summer months. Concurrent infections by such agents as *Mycoplasma bovoculi, Listeria monocytogenes, Chlamydophila pecorum*, bovine herpesvirus 1, bovine adenoviruses and the nematode *Thelazia* species may complicate the disease. Young animals under two years of age are most commonly affected and in a typical outbreak of disease about 60% of the young cattle may be affected while only about 20% of the older animals are affected.

The early signs of infectious bovine keratoconjunctivitis are lacrimation, blepharospasm and conjunctivitis. Later an ulcer develops on the cornea. Corneal opacity and oedema surround the ulcer and in severe cases vascularization of the cornea occurs from the limbus to the ulcer. The corneal opacity then involves the entire cornea. In the healing stage, granulation tissue forms on the ulcer floor and a characteristic red cone of granulation tissue will project from the cornea (Fig. 29.1). The granulation tissue and the ulcer itself will eventually regress leaving a white corneal scar. The scar may or may not be permanent. Mild

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Table 29.1 Virulence factors of *Moraxella bovis*

Virulence factor	Encoding gene(s)	Comments
Type IV pili	Pilin gene depends on strain	The genes encoding pilin from the prototype strain of each of the seven serogroups of *M. bovis* have been characterized by Atwell et al. (1994)
Cytotoxin (MbxA)	*mbxA*	Member of the RTX family. Haemolytic, cytotoxic and leukotoxic properties (Angelos et al. 2001)
Phospholipase	*plb*	Displays phospholipase B activity (Farn et al. 2001)
Filamentous haemaglutinin-like protein	*flpA* and *flpB*	Genes located on a plasmid, recently described by Kakuda et al. (2006)
Transferrin and lactoferrin-binding proteins	*tbpA, tbpB, lbpA* and *lbpB*	Bind bovine transferrin and lactoferrin for use as a source of iron. Characterized by Yu et al. (2002)

Figure 29.1 Young steer with infectious keratoconjunctivitis showing the healing stage with the characteristic red cone of granulation tissue projecting from the cornea.

cases are similar, but ulcers resolve without vascularization occurring and most eyes become clinically normal in two to three weeks.

Table 29.2 summarizes the sites of isolation and diseases of the moraxellae.

Laboratory Diagnosis

Specimens

A swab of lacrimal secretions is taken from deep in the inner canthus of the eye. Ideally blood agar plates should be inoculated with the lacrimal secretions immediately after collection. If this is not possible, each swab should be placed in about 1–2 mL of sterile distilled water, to prevent desiccation, and the specimens taken to the laboratory within two hours of collection.

Direct microscopy

Gram-stained smears are of little practical use, but use of the fluorescent antibody technique on smears will demonstrate and identify *M. bovis* if sufficient cells are present.

Isolation

Lacrimal secretions should be inoculated as soon as possible after collection on blood agar and incubated at 35 °C for 48–72 hours. The inoculation of a MacConkey plate is useful to gauge the degree of contamination by other Gram-negative bacteria, *M. bovis* is unable to grow on MacConkey agar.

Identification

Colonial appearance

After 48 hours' incubation the colonies of *M. bovis* are flat, round, small (1 mm diameter), greyish-white and friable, surrounded by a narrow zone of complete haemolysis (Fig. 29.2). The appearance is not unlike that of a beta-haemolytic *Streptococcus*. New isolates are often piliated and corrode the agar, sinking into it. Colonial growth will autoagglutinate when suspended in saline. On subculture, colonial variation is common, pili are no longer formed and the colonies are butyrous and less likely to autoagglutinate. Some colonies can become non-haemolytic. Strains of *M. equi* are non-haemolytic even on primary isolation. *Moraxella lacunata* and *M. phenylpyruvica* are non-haemolytic on blood agar and some strains of *M. phenylpyruvica* will grow on MacConkey agar.

Microscopic appearance

Gram-stained smears from colonial growth reveal fat Gram-negative rods or cocci, characteristically in pairs (Fig. 29.3). Specific identification of *M. bovis* can be

Table 29.2 Summary of the diseases and sites of isolation of selected *Moraxella* species

Species	Host	Disease and/or site of isolation
Moraxella boevrei	Goats	Isolated from the upper respiratory tract of healthy goats
M. bovis	Cattle	Infectious bovine keratoconjunctivitis ('pink-eye' or 'New Forest disease')
M. bovoculi	Cattle	Associated with keratoconjunctivitis
M. canis	Dogs	Isolated from the oral flora of dogs and from dog bite wounds in humans
M. caprae	Goats	Isolated from the upper respiratory tract of healthy goats
M. cuniculi	Ruminants	Isolated in association with other organisms from infectious bovine keratoconjunctivitis. Isolated from cases of respiratory disease in ruminants, usually in association with other organisms
M. equi	Horses	Conjunctivitis
M. lacunata	Many animal species	Isolated from a number of pathological conditions in animals including goats, dogs, pigs and aborted equine foetuses. Pathogenicity unknown
	Humans	May cause conjunctivitis
M. ovis	Ruminants	Isolated from healthy animals and those with keratoconjunctivitis
M. phenylpyruvica	Many animal species	Isolated from a number of sites but pathogenicity uncertain

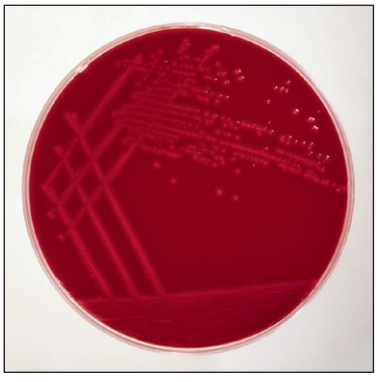

Figure 29.2 *Moraxella bovis* on sheep blood agar showing small haemolytic colonies. Some strains have a larger zone of beta-haemolysis.

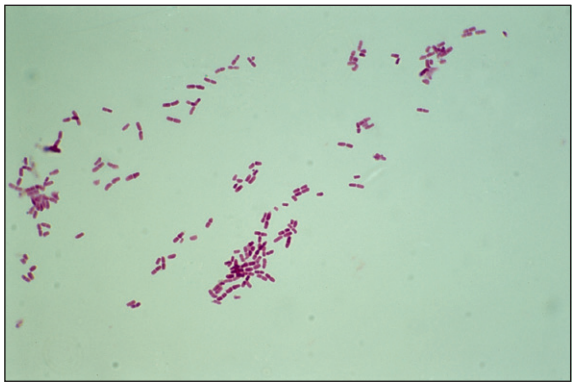

Figure 29.3 *Moraxella bovis* in a Gram-stained smear from a culture showing fat Gram-negative rods in pairs. Some strains occur as Gram-negative cocci in pairs.

obtained by a fluorescent antibody technique on smears from colonies.

Biochemical reactions

None of the moraxellae attack carbohydrates with the formation of acid. They are non-motile, indole-negative and all are sensitive to penicillin. *Moraxella bovis* will slowly pit a Loeffler serum slope and is relatively salt-tolerant, growing on media containing 5% NaCl. Litmus milk medium inoculated with *M. bovis* becomes alkaline (blue) and develops three zones: a deep blue upper layer, a soft blue curd in the centre and a coagulated, white (reduced) bottom layer. Table 29.3 gives the characteristics of the moraxellae isolated from animals. *Moraxella* species can also be differentiated by analysis of the cellular fatty acid profile.

Antimicrobial Susceptibility Testing and Antimicrobial Resistance

Disc diffusion and broth microdilution methods for antimicrobial susceptibility testing of *Moraxella bovis* isolates have been described by Webber et al. (1982). Specific interpretive criteria for *M. bovis* are not available but criteria for pathogens associated with bovine respiratory disease have been used (Angelos et al. 2011). Data on the

Table 29.3 Differentiation of the *Moraxella* species isolated from animals

Characteristic	*Moraxella boevrei*	*M. bovis*	*M. bovoculi*	*M. canis*	*M. caprae*	*M. cuniculi*	*M. equi*	*M. lacunata*	*M. ovis*	*M. phenylpyruvica*
Beta-haemolysis	+	+	(+)	+	+	–	–	–	(+)	–
Growth on MacConkey agar	–	–	–	–	–	–	–	–	–	(+)
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	(+)	+	+	+	+	+	+	+	+
Nitrate reduction	+	–	(+)	+	+	–	–	+	+	(+)
Phenylalanine deaminase	–	–	+	–	–	–	–	–	–	+
Gelatinase	+	(+)	–	–	–	–	+	+	–	–

+ = positive reaction, (+) = most strains positive, – = negative reaction

antimicrobial susceptibility of *M. bovis* from the USA (Shryock et al. 1998), Argentina (Zielinski et al. 2000) and Australia (McConnel et al. 2007) suggest that the organism remains susceptible to the majority of antimicrobials used in clinical practice.

Molecular Diagnosis and Strain Typing

PCR-based methods have been developed for the detection and differentiation of the moraxellae in animals. Shen et al. (2011) developed both conventional and 'real-time' multiplex PCR procedures for the identification and differentiation of *M. bovix*, *M. bovoculi* and *M. ovis* in either clinical samples or in culture.

Fimbriate isolates of *M. bovis* can be assigned to seven serogroups, A to G (Moore and Lepper 1991). Diversity of isolates has been investigated using randomly applied polymorphic DNA (RAPD) analysis (Conceição et al. 2004) and through characterization of lipopolysaccharides, outer membrane proteins and PCR-DNA fingerprinting (Prieto et al. 1999).

REFERENCES

Angelos, J.A., Hess, J.F., George, L.W., 2001. Cloning and characterization of a *Moraxella bovis* cytotoxin gene. American Journal of Veterinary Research 62 (8), 1222–1228.

Angelos, J.A., Ball, L.M., Byrne, B.A., 2011. Minimum inhibitory concentrations of selected antimicrobial agents for *Moraxella bovoculi* associated with infectious bovine keratoconjunctivitis. Journal of Veterinary Diagnostic Investigation 23 (3), 552–555.

Atwell, J.L., Tennent, J.M., Lepper, A.W., et al., 1994. Characterization of pilin genes from seven serologically defined prototype strains of *Moraxella bovis*. Journal of Bacteriology 176 (16), 4875–4882.

Conceição, F.R., Dellagostin, O.A., Paolichi, F., 2004. Molecular diversity of *Moraxella bovis* isolated from Brazil, Argentina and Uruguay over a period of three decades. Veterinary Journal 167 (1), 53–58.

Farn, J.L., Strugnell, R.A., Hoyne, P.A., et al., 2001. Molecular characterization of a secreted enzyme with phospholipase B activity from *Moraxella bovis*. Journal of Bacteriology 183 (22), 6717–6720.

Kakuda, T., Sarataphan, N., Tanaka, T., 2006. Filamentous-haemagglutinin-like protein genes encoded on a plasmid of *Moraxella bovis*. Veterinary Microbiology 118 (1–2), 141–147.

McConnel, C.S., Shum, L., House, J.K., 2007. Antimicrobial susceptibility of Australian bovine *Moraxella* isolates. Australian Veterinary Journal 85 (1–2), 70–71.

Moore, L.J., Lepper, A.W., 1991. A unified serotyping scheme for *Moraxella bovis*. Veterinary Microbiology 29 (1), 75–83.

Prieto, C.I., Aguilar, O.M., Yantorno, O.M., 1999. Analyses of lipopolysaccharides, outer membrane proteins and DNA fingerprints reveal intraspecies diversity in *Moraxella bovis* isolated in Argentina. Veterinary Microbiology 70 (3–4), 213–223.

Shen, H.G., Gould, S., Kinyon, J., 2011. Development and evaluation of a multiplex real-time PCR assay for the detection and differentiation of *Moraxella bovis, Moraxella bovoculi* and *Moraxella ovis* in pure culture isolates and lacrimal swabs collected from conventionally raised cattle. Journal of Applied Microbiology 111 (5), 1037–1043.

Shryock, T.R., White, D.W., Werner, C.S., 1998. Antimicrobial susceptibility of *Moraxella bovis*. Veterinary Microbiology 61 (4), 305–309.

Webber, J.J., Fales, W.H., Selby, L.A., 1982. Antimicrobial susceptibility of *Moraxella bovis* determined by agar disk diffusion and broth microdilution. Antimicrobial Agents and Chemotherapy 21 (4), 554–557.

Yu, R.H., Schryvers, A.B., 2002. Bacterial lactoferrin receptors: insights from characterizing the *Moraxella bovis* receptors. Biochemistry and Cell Biology 80 (1), 81–90.

Zielinski, G., Piscitelli, H., Perez-Monti, H., et al., 2000. Antibiotic sensitivity of an Argentine strain collection of *Moraxella bovis*. Veterinary Therapeutics 1 (3), 199–204.

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Chapter 30

Glucose non-fermenting, Gram-negative bacteria

The glucose non-fermenting, Gram-negative bacteria use glucose oxidatively or not at all. The genera *Pseudomonas*, *Bordetella* and *Moraxella*, which include significant veterinary pathogens, belong to this category and these are described in other chapters. Other genera belonging to the glucose non-fermenting bacteria which are of minor veterinary importance include *Alcaligenes*, *Acinetobacter*, *Achromobacter*, *Flavobacterium*, *Chryseobacterium*, *Neisseria*, and some other species. Some of these organisms, such as *Acinetobacter* species are opportunistic pathogens of immunocompromised patients in hospitals. They are frequently environmental organisms which are intrinsically resistant to many antimicrobial agents and may also acquire resistance to further agents. Strains of *Acinetobacter* species have now emerged in human medicine which are resistant to all commercially available antibiotics (Munoz-Price & Weinstein 2008). Pathogens belonging to this group of glucose non-fermenting, Gram-negative bacteria have also been documented as causing post-surgical wound infections in veterinary hospitals (Weese 2008). These bacteria are described in this chapter and their significance is summarized in Table 30.1.

Laboratory Diagnosis

Isolation

These glucose non-fermenting bacteria are fairly commonly isolated from clinical specimens incubated aerobically. Most grow at 37°C although this may be above their optimum temperature. Pigment is produced by some of these bacteria, including *Flavobacterium* species (Fig. 30.1).

Identification

A presumptive identification can be made on the basis of a few comparatively simple tests. A general comparison of the genera is given in Table 30.2 and some characteristics of the species commonly isolated from clinical materials are shown in Table 30.3. The tests are as follows:

- Gram-stained smears for morphology. The genera *Neisseria* (Fig. 30.2), *Branhamella*, *Acinetobacter* and *Moraxella* can all be Gram-negative diplococci. However, if the smears are made from the culture near the edge of a zone of inhibition around a penicillin disc the following morphology is usually seen:

	Cocci	Rods
Neisseria	+	
Branhamella	+	
Acinetobacter		+
Moraxella		+

- Motility is shown by *Pseudomonas* (polar flagella). *Bordetella* and *Alcaligenes* are peritrichous. A flagellar stain (West et al. 1977) can determine the flagellar arrangement.
- The O-F and other tests are carried out as described in the section 'Identification of bacterial pathogens' in Chapter 2.

In diagnostic laboratories identification is usually carried out with the aid of commercially available phenotypic

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Table 30.1 The significance of glucose non-fermenting Gram-negative bacteria

Genus and species	Host(s)	Significance
Alcaligenes species	Environment, many animal species	Saprophytes that are occasionally isolated from the intestinal tract of vertebrates
Achromobacter xylosoxidans	Environment	Nosocomial pathogen in humans. Occasional reports of opportunistic infections in animals
Acinetobacter spp.	Environment Animals and humans	Commonly found in soil, water, sewage, food and milk Part of the normal flora. Nosocomial pathogen in humans and potentially in animals also. Opportunistic pathogen in animals
Bergeriella denitrificans	Guinea pigs	Isolated from the nasopharynx
Bergeyella (*Weeksella*) *zoohelcum*	Dogs and cats Humans	Part of the normal flora of the oral cavity and paws. Not thought to be pathogenic for animals Infected animal bites and scratches
Elizabethkingia meningoseptica (*Chryseobacterium meningosepticum*)	Environment Humans	Commonly found in natural and hospital environments Meningitis and septicaemia in hospitalized infants. Pneumonia and meningitis in immunocompromised adults
Flavobacterium spp. *F. columnare* *F. psychrophilum*	Environment Humans (animals) Salmon Salmonid fish	Soil and water: widespread Nosocomial infections Columnaris disease Cold water disease
Neisseria meningitidis *N. gonorrhoeae* *N. animalis* *N. animaloris* (CDC group EF-4a) *N. canis* *N. elongata* subsp. *nitroreducens* *N. flavescens* and *N. sicca* *N. lactamica* *N. mucosa* *N. zoodegmatis* (CDC group EF-4b) *N. weaveri* (CDC group M5)	Humans Humans Guineapigs Dogs and cats Humans Cats and dogs Humans Dogs Dogs Dolphins Dogs and cats Humans Dogs	Meningococcal meningitis Gonorrhoea Isolated from the pharynx Commensal in oral cavity and nasopharynx. Occasional reports of localized infection Infection of dog and cat bites Isolated from the nasopharynx Isolated from clinical specimens, endocarditis Commensal in canine oropharynx Isolated from the conjunctival sac Respiratory tract of healthy dolphins Commensal in oral cavity and nasopharynx Infected dog bites, endocarditis Commensal in canine oropharynx

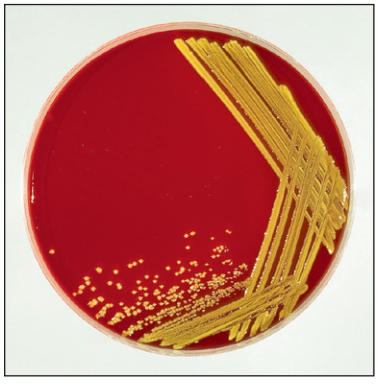

Figure 30.1 *Flavobacterium* species on sheep blood agar showing distinctive yellow pigmentation.

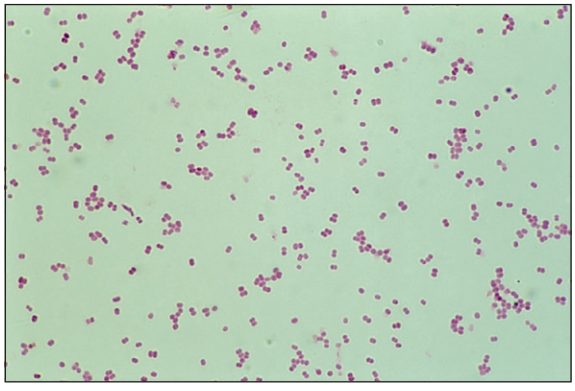

Figure 30.2 *Neisseria* species in a Gram-stained smear from a culture showing the typical fat cocci in pairs. (×1000)

Table 30.2 Comparison of the characteristics of Gram-negative, glucose non-fermenting bacteria

	Morphology	Acid from oxidation of glucose*	Haemolysis	Growth on MacConkey agar	Motility	Catalase	Oxidase	Pigment	Other characteristics
Pseudomonas	Medium-sized rods	+	+β (*P. aeruginosa*)	+	(+) polar	+	+	(+)	*P. aeruginosa* has grape-like odour and bluish pigment
Moraxella	Fat rods or cocci in pairs	–	(+)β (*M. bovis*)	(–)	–	(+)	+	–	Pathogenic *M. bovis* usually haemolytic
Bordetella	Coccobacilli	–	(+)β (*B. bronchi-septica*)	(+)	(+) peri-trichous	+	+	–	*B. bronchiseptica* urease + and *B. avium* urease –. Both citrate +
Alcaligenes	Rods or coccobacilli	–	–	+	+ peri-trichous	+	+	–	*A. faecalis* urease – and citrate +
Acinetobacter	Rods or diplococci	v	–	+	–	+	–	–	Blue colonies on EMB medium
Bergeyella	Rods	–	vα	–	–	+	+	–	Hydrolyse urease rapidly. Small, sticky colonies
Elizabethkingia	Rods	+	+α	+	–	+	+	–	
Flavobacterium	Rods or coccobacilli	(+)	+α	(+)	–	+	+	+ yellow	
Neisseria	Cocci in pairs	(+)	(–)	–	–	(+)	+	+ yellow	Growth enhanced by 10% carbon dioxide

*O-F test, + = oxidative, and – = unreactive. R = resistant and S = susceptible

+ = positive, (+) = most isolates positive, (–) = most isolates negative, – = negative, v = variable reactions

identification systems. These systems include API 20NE (bioMérieux, Marcy l'Etoile, France) and the automated systems VITEK GNI Plus or VITEK 2 ID-GNB (bioMérieux, Marcy l'Etoile, France).

Genotypic approaches are also used and include 16S rRNA gene sequence analysis.

A completely different method which can be used for the identification of microorganisms and which has recently been successfully applied to this group of glucose non-fermenting Gram-negative bacteria is the Fourier transform infrared spectroscopic technique (Bosch et al. 2008).

Table 30.3 Reactions of some Gram-negative, glucose non-fermenting bacteria

	Acid from glucose	Haemolysis (blood agar)	Growth on MacConkey agar	Motility	Catalase	Oxidase	Nitrate reduction	Urease	Indole	Gelatinase	Pigment
Alcaligenes faecalis	–	–	+	+	+	+	–	–	–	–	–
Achromobacter xylosoxidans subsp. *xylosoxidans*	v	–	+	+	+	+	+	–	–	–	–
A. xylosoxidans subsp. *denitrificans*	–	–	+	+	+	+	+	v	–	–	–
A. piechaudii	–	–	+	+	+	+	+	–	–	–	–
Acinetobacter calcoaceticus-baumanii complex	+	–	+	–	+	–	–	v	–	–	–
A. lwoffii	–	–	+	–	+	–	–	–	–	–	–
Bergeriella denitrificans	O	–	–	–	+	+	–	–	–	–	v (yellow)
Bergeyella (*Weeksella*) *zoohelcum*	–	v(α)	–	–	+	+	–	+	+	+	–
Chryseobacterium indologenes	O	v(α)	v	–	+	+	v	(–)	+	(+)	yellow
Elizabethkingia meningoseptica (*Chryseobacterium meningosepticum*)	O	v(α)	+	–	+	+	–	–	+	+	v (yellow)
Neisseria animaloris (CDC group EF-4a)	(O)	–	v	–	+	+	+	–	–	–	yellow/tan
N. canis	–	–	–	–	+	+	+	–	–	+	yellow
N. elongata subsp. *nitroreducens*	–	–	v	–	–	+	+	–	–	–	–
N. flavescens	–	–	–	–	+	+	–	–	–	–	yellow
N. sicca	O	v	–	–	+	+	–	–	–	–	v (yellow)
N. lactamica	O	–	–	–	+	+	–	–	–	–	yellow
N. mucosa	O	–	–	–	+	+	+	–	–	–	v (yellow)
N. zoodegmatis (CDC group EF-4b)	(O)	–	v	–	+	+	+	–	–	–	yellow/tan
N. weaveri (CDC group M5)	–	vβ	v	–	+	+	–	–	–	–	–

O = oxidative, – = unreactive. (O) = most strains oxidative

+ = positive reaction, (+) = most strains positive, (–) = most strains negative, – = negative reaction, v = variable reactions, β = beta-haemolysis, α = alpha haemolysis, () = most strains

REFERENCES

Bosch, A., Miñán, A., Vescina, C., et al., 2008. Fourier transform infrared spectroscopy for rapid identification of non-fermenting Gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. Journal of Clinical Microbiology 46, 2535–2546.

Munoz-Price, L.S., Weinstein, R.A., 2008. *Acinetobacter* infection. New England Journal of Medicine 358 (12), 1271–1281.

Weese, J.S., 2008. A review of multidrug resistant surgical site infections. Veterinary and Comparative Orthopaedics and Traumatology 21, 1–7.

West, M., Burdash, N.M., Freimuth, F., 1977. Simplified silver-plating for flagella. Journal of Clinical Microbiology 6, 414–419.

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Chapter 31

The spirochaetes

The order *Spirochaetales* includes the families *Spirochaetaceae, Brachyspiraceae* and *Leptospiraceae*. The genera of significance in animals and humans are *Leptospira* (*Leptospiraceae*), *Treponema* and *Borrelia* (*Spirochaetaceae*) and *Brachyspira* (*Brachyspiraceae*). The spirochaetes are slender, motile, flexuous, unicellular, helically coiled bacteria that range from 0.1–3.0 µm in width. The outer sheath, the outermost layer of a spirochaete cell, is a multilayered membrane that completely surrounds the periplasmic endoflagella and the helical protoplasmic cylinder. The cylinder consists of the nuclear material, cytoplasm, cytoplasmic membrane and the peptidoglycan portion of the cell wall. The periplasmic flagella are wrapped around the cylinder and are in the periplasmic space of these Gram-negative bacteria. One end of each flagellum is inserted near a pole of the protoplasmic cylinder and attached by plate-like structures called insertion discs. The distal end of each flagellum is not inserted and extends to the centre of the cell and may overlap the flagellum from the opposite end (Figs. 31.1 and 31.2). The periplasmic flagella facilitate the motility of the bacteria in viscid and non-viscid environments. Table 31.1 and Figure 31.3 summarize the main differences between the genera *Leptospira*, *Brachyspira* and *Borrelia*, the principal genera of importance in animals.

LEPTOSPIRA SPECIES

The genus *Leptospira* was previously divided into two species, *L. interrogans* (parasitic) and *L. biflexa* (saprophytic); however, this phenotypic classification has been replaced by a genotypic one. There are currently 17 recognized genospecies including *L. biflexa*, *L. borgpetersenii*, *L. interrogans* and *L. noguchii*. Some species contain both pathogenic and non-pathogenic strains. Only non-pathogenic strains of *L. biflexa* have been described to date. Using the previous classification methods in which leptospires were differentiated on the basis of antigenic composition and serological reactions, more than 250 serovars in 23 serogroups were defined (Yasuda et al. 1987, Perolat et al. 1998). Serogroups and serovars do not correspond with the current genospecies and strains within the same serogroup or serovar may be found in multiple species (Table 31.2). Further taxonomic revisions of this genus are likely. Molecular classification is not yet used in clinical microbiological laboratories and until DNA-based identification methods are widely available, these laboratories are likely to retain the serological classification of pathogenic leptospires.

Many serovars appear to have a certain animal species as a natural host, but animals and humans can be infected with a wide variety of serovars. The serovars causing disease in animals vary between countries and sometimes between regions in the same country. Usually the majority of infections in domestic animals, in a particular region, are caused by only a few serovars. Table 31.3 lists some of the serovars, and the genospecies and serogroups to which they belong, which have been commonly isolated from domestic and wild animals.

Natural Habitat

Leptospires are present in the tubules of mammalian kidneys and are excreted in urine, often for several months. Some serovars may persist in the genital tract of carrier animals. Characteristically, a reservoir or maintenance host shows minimal or no clinical signs. A protracted shedder state is usual in maintenance hosts and they are

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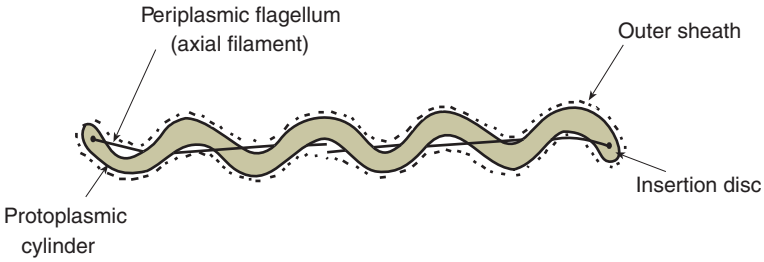

Figure 31.1 Diagrammatic representation of a spirochaete.

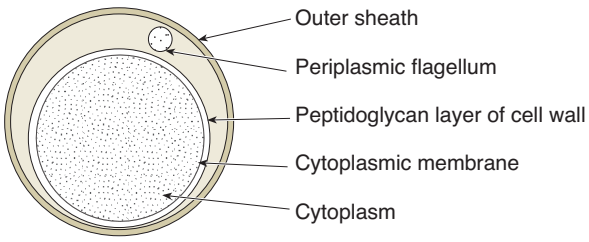

Figure 31.2 Section through a spirochaete demonstrating structural features.

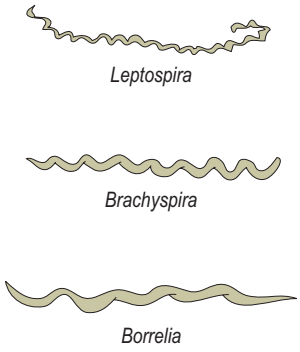

Figure 31.3 A comparison of the general morphology of *Leptospira, Brachyspira* and *Borrelia* (see also Table 31.1).

Table 31.1 Main differences between the genera *Leptospira, Brachyspira* and *Borrelia*

	Leptospira	***Brachyspira***	***Borrelia***
Morphology	Many spirals, tight and fine	6–14 regular spirals with 1 μm amplitude	4–8 loose spirals with 3 μm amplitude
Length	6–20 μm	5–20 μm	3–20 μm
Width	0.1–0.2 μm	0.1–0.5 μm	0.2–0.5 μm
Periplasmic flagella	2	6–10	15–20
Insertion discs	3–5	1	2
Ends	One or both ends hooked	Pointed	Tapers to a fine filament
Atmosphere	Aerobic	Microaerophilic or anaerobic	Microaerophilic
Arthropod vectors required	No	No	Yes
Direct microscopy:			
Dark-field	+	+	+
DCF stain	–	+	+
Giemsa	–	Poor	+
Silver impregnation	+	+	+
FA technique	+	+	+
Movement	Rotation around central axis, flexing and translational, undulatory movement	Rotation and majestic translational movement and stiff flexion	Frequent reversal of translational movement, corkscrew-like and lashing

the main source of environmental contamination. Transmission of infection can then occur to other animal species, termed incidental hosts. Incidental hosts species are less susceptible to infection than maintenance hosts but exhibit more severe disease and are inefficient transmitters of infection to other animals. Nevertheless, protacted shedding of leptospires in urine can occur in animals which have recovered from clinical disease. Significant reservoirs of animal and human leptospirosis are shown in Table 31.4. Indirect exposure depends on wet environmental conditions and a mild climate that favour the survival of leptospires. Streams and ponds can be a source of infection as can aerosols of urine in cowsheds and milk from infected cows. Leptospirosis occurs worldwide.

Table 31.2 Some leptospiral serovars found in multiple species*

Serovar	Species
Bataviae	*L. interrogans, L. santarosai*
Grippotyphosa	*L. interrogans, L. kirschneri*
Hardjo	*L. borgpetersenii, L. interrogans, L. meyeri*
Icterohaemorrhagiae	*L. interrogans, L. inadai*
Pomona	*L. interrogans, L. noguchii*
Pyrogenes	*L. interrogans, L. santarosai*
Szwaijizak	*L. interrogans, L. santarosai*

*From Levett (2001).

Pathogenesis

The leptospiral genome is made up of two chromosomes, a larger one of 4400 kb and a smaller one of 350 kb in size. The full genome sequences of two serovars of *Leptospira interrogans*, two strains of *Leptospira borgpetersenii* serovar Hardjo and two strains of *L. biflexa* serovar *Patoc* have been completed (Ren et al. 2003, Nascimento et al. 2004, Picardeau et al. 2008). Comparative analysis of these genomes provides insight into the physiology and pathogenesis of pathogenic leptospires. However, many of the identified genes have no known function as it appears that the pathogenic mechanisms of leptospires differ from those of most other bacterial pathogens and homologues of these genes have not been identified in other organisms.

Table 31.3 Selected leptospiral serovars and animals from which they are commonly isolated

Serogroup	Serovar	Occurrence in:						
		Cattle	Pigs	Dogs	Horses	Sheep	Rodents	Other wildlife
Australis	Australis	+						+
	Bratislava	+	+		+			
Autumnalis	Autumnalis						+	+
Ballum	Ballum						+	+
Bataviae	Bataviae						+	
Canicola	Canicola	+	+	+			+	+
Grippotyphosa	Grippotyphosa	+	+	+	+		+	+
Hebdomadis	Hebdomadis							+
	Szwaijizak	+						
Ictero-haemorrhagie	Ictero-haemorrhagiae	+	+	+	+		+	+
	Copenhageni	+		+			+	
Pomona	Pomona	+	+	+	+	+		+
Sejroe	Balcanica	+						
	Hardjo	+			+	+		
	Sejroe	+					+	+
Tarassovi	Tarassovi		+					

Table 31.4 Significant reservoirs of pathogenic leptospires in animals

Serovar	Main reservoir(s)	Alternative reservoir(s)
Autumnalis	Wildlife	
Ballum	Wildlife	
Bratislava	Pigs, horses, cattle	
Canicola	Dogs	Cattle, pigs, rodents
Grippotyphosa	Wildlife	Cattle, pigs
Hardjo	Cattle	Sheep, deer
Icterohaemorrhagiae	Rats	Dogs, cattle, pigs
Pomona	Pigs, cattle	Dogs, wildlife

Table 31.5 Virulence attributes of pathogenic leptospires

Virulence factor	Comment
Adhesion to renal epithelial cells	Adhesin not identified
Fibronectin-binding protein	Identified in virulent strains
Outer membrane proteins (Omps)	Mutation of associated gene results in attenuation
Several surface proteins, including the leptospiral immunoglobulin-like proteins	Bind host proteins and found only in pathogenic species but role in virulence unknown
Haemolysins	Sphingomyelinases identified in pathogenic species
Haem oxygenase	Utilization of haem as an iron source
Flagella	Mutation of *fliY* gene reduces tissue dissemination
Lipopolysaccharide	Although leptospiral LPS has reduced toxicity compared to the LPS of conventional Gram-negative pathogens, mutation and alteration of LPS biosynthesis reduces the ability of affected strains to colonize and cause disease

Leptospires gain entry through mucous membranes or damaged skin as a result of direct or indirect contact. Motility and chemotaxis are essential for penetration of host tissues at this stage of infection. At least 79 motility-associated genes have been identified in the *L. interrogans* genome, 42 of which are common to *Borrelia burgdorferi* and *Treponema pallidum* (Nascimento et al. 2004). Colonization of host tissues is essential in the early stages of infection and afimbrial adhesins of *Leptospira* serovars have been characterized. After epithelial penetration there is haematogenous spread with localization and proliferation in parenchymatous organs, particularly the liver, kidneys, spleen and sometimes meninges. This phase of infection is likely to be mediated by the secretion of enzymes capable of degrading host cell membranes (Ren et al. 2003). Genes encoding both haemolysins and proteases have been identified. Some of the putative virulence factors of leptospires are given in Table 31.5. The role of some putative virulence attributes in the pathogenesis of disease has been proven through the development of mutagenesis systems for leptospires but many aspects of molecular pathogenesis remain unknown. Penetration and multiplication in the foetus can occur in pregnant animals leading to foetal death and resorption, abortion or weak offspring. The foetus, if infection occurs in the third trimester, can produce specific antibodies and may overcome the infection. Antibody production in infected animals begins a few days after the onset of leptospiraemia aand leptospires are cleared from the circulation by about 10 days after infection. Leptospires are phagocytosed by macrophages in the presence of specific antibody but virulent leptospires can induce apoptosis of macrophages both in vivo and in vitro (Mérien et al. 1997, 1998). Leptospires tend to persist in sites such as renal tubules, eyes and uterus where antibody activity is minimal. In the kidneys the organisms localize in the lumen of the proximal convoluted tubules. The relative ease with which establishment and persistence of infection occurs in maintenance hosts may be explained in part by the down regulation of some outer membrane lipoproteins in vivo and the consequent lack of recognition of infection by the immune response of the host (Haake et al. 1998).

Leptospires damage vascular endothelium resulting in haemorrhages. Serovars in the serogroups Autumnalis, Grippotyphosa, Icterohaemorrhagiae and Pomona produce a haemolysin that is probably responsible for the haemoglobinuria (redwater) in young calves infected with these serovars. The pathogenesis of equine recurrent uveitis is thought to involve the production of antibodies against leptospiral antigens, probably membrane proteins, which cross-react with ocular tissues (Verma et al. 2005). Table 31.6 summarizes the syndromes of leptospirosis in domestic animals. Serological evidence indicates that a variety of leptospiral serovars can infect cats but disease appears to be uncommon in this animal.

Table 31.6 Leptospirosis in domestic animals

Host	Disease syndrome
Cattle	• Subclinical with or without leptospiruria • Milk-drop syndrome, with or without any other clinical signs (often Hardjo) • Abortion and neonatal mortality: abortion 'storms' (Pomona) and sporadic abortions (Hardjo) • Haemoglobinuria, jaundice and fever in calves, and less commonly, in young adults. Serovars commonly involved are Pomona, Grippotyphosa and Icterohaemorrhagiae. Occasionally, some animals show signs of meningitis
Pigs	• Subclinical, often with leptospiruria (especially Pomona) • Fever and focal non-suppurative mastitis and leptospiruria • Infertility, abortions and stillbirths (Canicola, Pomona, Icterohaemorrhagiae or Bratislava) • Fever, anorexia, jaundice, haemoglobinuria and high mortality in young pigs (often Icterohaemmorhagiae)
Dogs	• Subclinical with leptospiruria (often Canicola) • Acute haemorrhagic disease: high fever, vomiting, prostration and often early death (usually Icterohaemorrhagiae) • Acute renal failure (many serovars including Canicola, Icterohaemorrhagiae, Grippotyphosa and Bratislava) • Infertility, abortions and stillbirths (Bratislava)
Horses	• Recurrent iridocyclitis ('periodic ophthalmia' or 'moon blindness') which can result in blindness • Occasionally abortion with foetuses of six months to term • Rarely fever, anorexia, depression and icterus
Sheep	• Mainly subclinical infections with leptospiruria (serovars such as Hardjo) • Occasionally, acute leptopirosis with depression, dyspnoea, haemoglobinuria, anaemia and high mortality in lambs (often Pomona)

Laboratory Diagnosis

Diagnosis of infection with leptospires by demonstration of the organism using conventional methods is problematic. Specimens of body fluids can be examined by dark-field microscopy but this is an insensitive technique as approximately 10^4 organisms/mL must be present for one leptospire to be visible per field. Culture of blood or urine is a highly specialized technique, requires prolonged incubation times and is performed by few laboratories for veterinary specimens. Diagnosis in animals relies largely on serology although newer immunological and molecular techniques for demonstration of the presence of the organisms are described and may greatly assist with diagnosis in the future.

Specimens

- Whole blood (serum) for serological tests.
- Mid-stream urine for dark-field examination:
  - If the urine cannot be examined within 20 minutes, it should be neutralized with N/10 HCl or N/10 NaOH.
  - To preserve the morphology of the leptospires for several days, 20 mL of mid-stream urine should be added immediately to 1.5 mL of 10% formalin in a 30 mL bottle.
- Mid-stream urine for attempted culture should be diluted 1 : 10 with 1% bovine serum albumin (BSA) for transport to the laboratory. Alternatively, a few drops of the urine can be aseptically added to culture media on a 'beside-the-animal' basis.
- Whole blood (5 mL) from an animal in the leptospiraemic phase can be collected in 0.5 mL of 1.0% Na oxalate or 0.1 mL of 1.0% heparin for attempted culture of leptospires.
- Kidneys:
  - A small plug of kidney, taken from inside the organ with a sterile glass Pasteur pipette, can be macerated with a little distilled water and examined under dark-field microscopy.
  - Kidney tissue can be used for culture. Leptospires remain viable in unfrozen kidneys for several days after the death of the animal.
  - Kidney and/or liver sections in 10% formalin can be used for histopathology (Fig. 31.4).
- Foetal abomasal contents, cotyledons and uterine discharge for differential diagnosis in abortion cases.

Leptospires can disintegrate quickly in a urine sample, especially if it is acidic. By adding formalin, the leptospires will be killed but will retain their morphology for several days and can be examined by dark-field microscopy. In suspected leptospirosis in cattle and pigs, the infection should be investigated on a herd basis, with urine and

serum samples taken from clinically affected and in-contact animals. Leptospires may be excreted in urine intermittently and leptospiral antibody titres can vary considerably between individual animals. Figure 31.5 summarizes the specimens and laboratory tests for the diagnosis of leptospirosis.

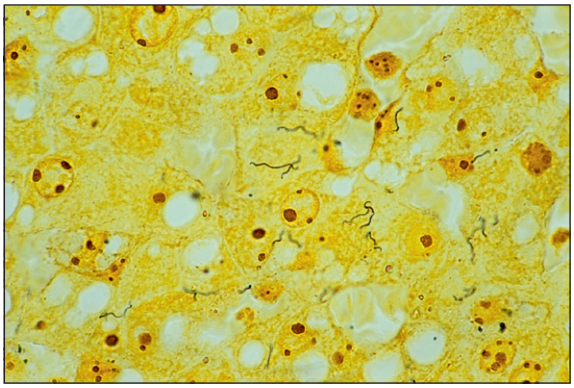

Figure 31.4 *Leptospira interrogans* serovar Icterohaemorrhagiae in a section of canine liver. (Leviditi stain, ×1000)

Direct microscopy

Leptospires average only 0.1–0.2 µm in width and cannot be resolved under the light microscope. They stain weakly, or not at all, with both aniline and Romanowsky stains. Leptospires can be demonstrated in urine, other body fluids and tissues by dark-field microscopy (Fig. 31.6) and fluorescent antibody (FA) technique (Fig. 31.7). Urine is centrifuged at 1500 × *g* for 30 minutes to concentrate the leptospires. Unclotted blood is centrifuged at low speed to sediment the red cells after which the plasma can be removed and centrifuged at 1500 × *g* for 30 minutes and a wet mount prepared from the sediment. Tissue specimens are ground and prepared as a 10% suspension in 1% bovine serum albumin (BSA, Fraction V powder). A drop of the preparation is placed on a microscope slide, under a cover slip, and examined by dark-field microscopy. The FA technique can be carried out on urinary deposits, tissue impression smears or frozen tissue sections. Silver impregnation or immunoperoxidase staining techniques can be used on tissue sections of kidney, liver or other tissues.

Under dark-field microscopy leptospires can just be seen under the low power if large numbers are present. Under high-dry or oil-immersion objectives they appear as silver, beaded (due to the helical coils), slender rods with a distinct hook at each end (Fig. 31.6). Leptospiral serovars are

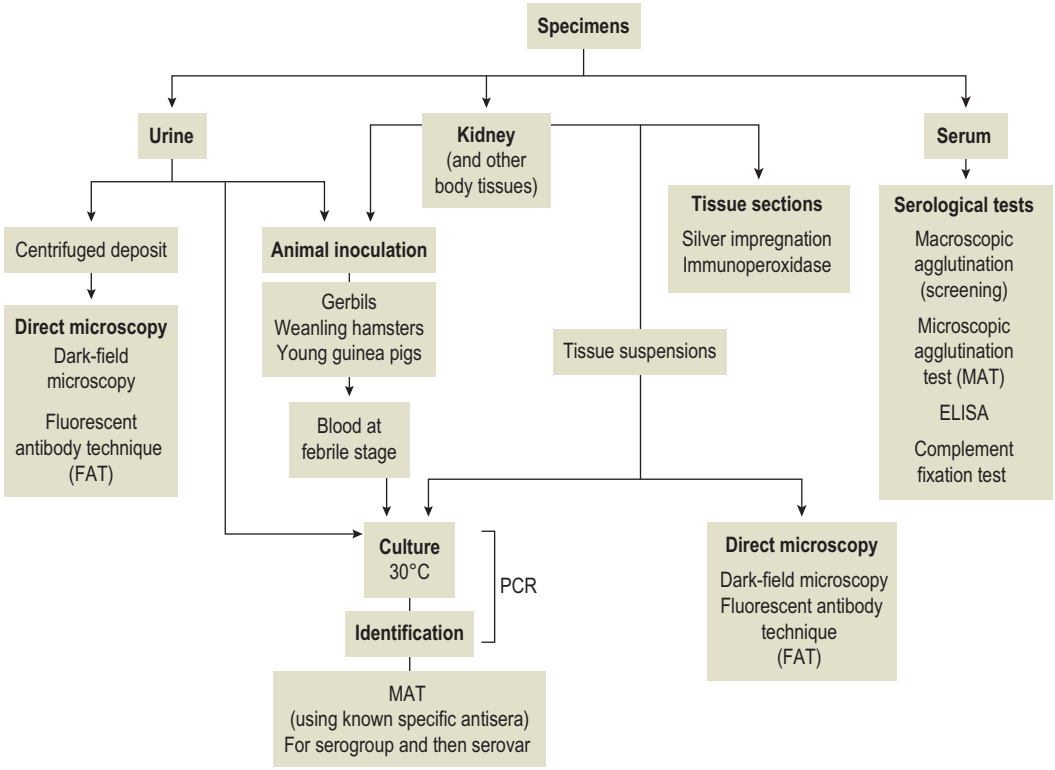

Figure 31.5 Summary of the laboratory procedures for the diagnosis of leptospirosis.

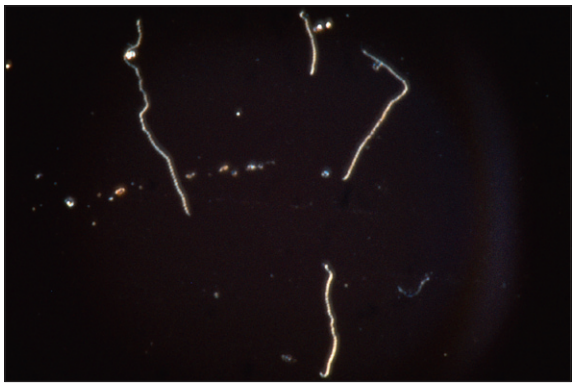

Figure 31.6 *Leptospira interrogans* serovar Canicola from a young, actively dividing culture. (Darkfield, ×1000)

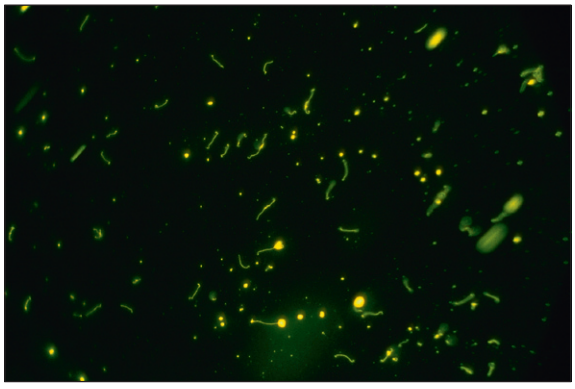

Figure 31.7 *Leptospira interrogans* serovar Hardjo in a bovine urinary deposit. (Direct FA technique, ×400)

obviously motile, if they are still viable, with flexuous movements and spinning around the long axis. The characteristic morphology can often be seen in FA or silver impregnation preparations.

Culture

The media used for the culture of leptospires include: Korthof and Stuart broths, Fletcher semisolid medium, EMJH (Ellinghausen, McCullough, Johnson and Harris) and Tween 80-albumin medium (OAC – oleic albumin complex). The latter are liquid but can be made semisolid by the addition of l.5 g agar/L of medium. Contaminated samples (or cultures) can be filtered through a 0.45 µm bacteriological filter and inoculated into a medium containing 5-fluorouracil at 200 µg/mL. The media can be inoculated with one to three drops of carefully taken urine within a few minutes of collection, or urine diluted 1 : 10 with 1% BSA, as soon as possible after collection.

A 10% tissue suspension in 1% BSA (one to two drops) or a few drops of oxalated or heparinized blood can also be inoculated into media. The cultures are incubated at 30 °C for up to 13 weeks. A drop of the culture is examined by dark-field microscopy once weekly. Serovar Hardjo is one of the slowest growing serovars and Bratislava is difficult to culture in laboratory media.

Antimicrobial susceptibility testing

There is currently no accepted standard methodology for assessing the in vitro activity of antimicrobial agents against *Leptospira* species. Murray and Hospenthal (2004) described a broth microdilution technique for susceptibility testing which gave results within five days and employed alamarBlue as a growth detector. This group have subsequently verified that the results obtained with laboratory-adapted strains hold true for virulent organisms, lethal for hamsters (Griffith et al. 2006). Ressner et al. (2008) tested human clinical isolates from several countries worldwide and found them to be susceptible to a wide range of antimicrobial agents although there was some regional variation and the highest MICs were detected for doxycycline and tetracycline, agents traditionally used for the treatment of leptospirosis in humans.

Animal inoculation

Weanling gerbils, hamsters and guinea pigs can be inoculated intraperitoneally with 0.5–1.0 mL of neutralized urine, unclotted blood or a 10% tissue suspension in EMJH or 1% BSA. Cardiac blood is taken aseptically when a temperature rise is detected or at five, eight, 10 and 14 days post-inoculation. Media are inoculated with two to three drops of the freshly collected blood. Animals surviving for 21 days post-inoculation can be killed and blood samples taken for serology.

Identification

Fluorescent antibody techniques or immunoperoxidase staining are usually carried out on the original specimens. Isolated leptospires are identified either by serological methods or by molecular methods in leptospiral reference laboratories. Panels of monoclonal antibodies can be used in the microscopic agglutination test and result in relatively rapid identification of isolates. Definitive culture typing is described in detail by Faine (1982). Molecular identification methods are described below.

Serology

Macroscopic and microscopic agglutination tests, and the ELISA technique are used for the detection of leptospiral antibodies in serum. The macroscopic agglutination test is a screening test and uses dead antigens but suffers from lack of specificity. The leptospiral microscopic agglutination test is the reference method for the serological diagnosis of leptospirosis and uses live leptospires as antigen.

It is serogroup-specific, but may indicate infection with a specific serovar where only one serovar from a serogroup is common in a particular geographical region and animal species. Details for carrying out the macroscopic and microscopic agglutination tests are given by Cole (1990). A titre of 1:100 is suspicious for infection in the MAT while titres of 1:200 or more are regarded as positive if accompanied by clinical signs consistent with leptospirosis. The titres are often 1:800 or more in an active infection. These interpretations apply particularly to infections in incidental hosts and where the background level of exposure in the particular animal population is low. Serological diagnosis of infection is much more difficult in maintenance hosts when titres may be low or absent and do not correlate with shedding of leptospires in urine. Many ELISAs have been developed for the diagnosis of leptospiral infection in animals and are usually based on detection of antibodies to the predominant serovars occurring in those countries in which they are used. Tests for the detection of both IgM and IgG are available and include rapid tests which can be performed 'in the field'.

Molecular diagnosis and typing

Molecular methods for the detection of leptospiral infection in animals are undergoing rapid development and promise to solve many of the difficulties associated with conventional diagnostic techniques. Several primer pairs for the detection of leptospires have been described, with the primers described by Mérien et al. (1992) and Gravekamp et al. (1993) having been widely used in clinical studies. More recently, PCR techniques which are specific for pathogenic leptospires (Branger et al. 2005, Palaniappan et al. 2005) and which are also quantitative (Levett et al. 2005, Mérien et al. 2005) have been described. These methods require large-scale validation.

Molecular typing methods for the identification of leptospires have been extensively investigated and include digestion of chromosomal DNA by restriction endonucleases, restriction fragment length polymorphism, ribotyping, pulsed field gel electrophoresis and PCR-based approaches (reviewed by Levett 2001). Multilocus sequence typing (MLST) has also been described for characterization of isolates and an online database is available (Ahmed et al. 2006).

BRACHYSPIRA AND *TREPONEMA* SPECIES

Members of these host-associated spirochaetal genera are found in the oral cavity, intestinal tract and genital region of animals and humans. The cells are wider, not as tightly coiled as the leptospires and can be stained by aniline dyes. The principal species of veterinary significance is *Brachyspira hyodysenteriae*, the cause of swine dysentery. Other organisms in this group may be associated with disease in animals: *Brachspira pilosicoli* (porcine intestinal spirochaetosis), *Brachyspira intermedia* (spirochaetal colitis in chickens and pigs), *B. alvinipulli* (diarrhoea and poor laying performance in poultry), *Treponema paraluiscuniculi* (vent disease of rabbits) and *Treponema* organisms associated with digital dermatitis of cattle and contagious ovine digital dermatitis. *Brachyspira innocens* and *B. murdochii*, present in the intestinal tract of pigs, are considered non-pathogenic. Brachyspires are anaerobic and can be grown on laboratory media, but *T. paraluiscuniculi* has not been cultured in vitro.

Normal Habitat

The reservoir of *B. hyodysenteriae* is the intestinal tract of pigs, wild rats and mice. Recovered, asymptomatic pigs can excrete brachyspires in faeces for three months or more. *Brachyspira pilosicoli* is found in the intestines of chickens, dogs, wild birds, rodents and non-human primates. *Brachyspira* species can persist in the environment for several weeks if protected from dessication and *B. hyodysenteriae* can survive for at least three days in slurry. Infection is by the faecal–oral route. The epidemiology of treponema infection associated with ruminant digital disease is unclear but it is thought that infection is introduced by carrier animals. *Treponema paraluiscuniculi* produces a benign venereal disease of rabbits and is present in lesions in the genitoperineal area of rabbits. It causes latent infection in mice, guinea pigs and hamsters. The treponemes can be found in the lymph nodes of these animals.

Pathogenesis

After *B. hyodysenteriae* enters a susceptible pig the organism invades goblet cells of the colonic mucosa, multiplies in the crypts of Lieberkuhn and causes necrosis and erosion of the mucosal cells and an outpouring of mucus. Haemolytic activity, demonstrated in vitro, correlates with pathogenicity and four haemolysins have been identified, while genes for a further three putative haemolysins have been reported (ter Huurne et al. 1994, Bellgard et al. 2009). The faecal material is watery and contains mucus, blood and necrotic debris. Only the large intestine is involved and colonic malabsorption occurs. Motility in mucus is an essential virulence factor; disruption of the genes encoding flagellar proteins resulted in mutants with abnormal motility which had significantly reduced ability to colonize and infect mice (Kennedy et al. 1997). Organisms in which the *nox* gene, which codes for NADH oxidase, was disrupted were 100-fold more susceptible to oxygen inactivation and were less virulent for pigs (Stanton et al. 1999). Dietary and immune factors are also important in the development of disease (Jacobson et al. 2004,

Table 31.7 Virulence factors of *Brachyspira hyodysenteriae*

Virulence factor	Pathogenic role
Flagella	Confer motility in intestinal mucus
Haemolysins	Damage to intestinal mucosa
NADH oxidase	Resistance to oxidative damage
Lipo-oligosaccharide	Endotoxic effects

Jonasson et al. 2004). Virulence factors of *B. hyodysenteriae* are summarized in Table 31.7.

Brachyspira pilosicoli causes transient diarrhoea usually lasting from seven to 10 days, with consequent production losses. Differences in the severity of clinical signs observed suggest that strains may vary in virulence. Diets containing poorly digestible non-starchy materials appear to enhance colonization with *B. pilosicoli* and increase the severity of the diarrhoea.

Laboratory Diagnosis

Specimens

Deep mucosal scrapings should be taken from a portion of the affected large intestine from a dead pig, or rectal swabs and faeces from several live affected pigs. The numbers of *B. hyodysenteriae* can sometimes be low in faeces and it may be difficult to visualize the organisms using direct microscopy. Sections of affected colon in 10% formalin should be taken for histopathology.

Direct microscopy

Various methods can be used to visualize *B. hyodysenteriae*:

- A portion of deep scrapings from the mucosa or faecal material is examined in a drop of water under dark-field microscopy.
- Fixed smears of mucosal scrapings or faeces are stained by dilute carbol fuchsin (DCF) for four to six minutes, or by Victoria blue 4-R (Appendix 1) (Fig. 31.8) or by a silver impregnation technique (Fig. 31.9).
- Histological sections of colon can be stained by a silver impregnation stain or Victoria blue 4-R.
- Fluorescent antibody technique can be carried out on mucosal scrapings or faeces (Fig. 31.10).

Three to five brachyspires per high-power field is considered significant. *Brachyspira hyodysenteriae* is 0.3–0.4 µm in width, 6–8 µm in length, is loosely coiled with two to four coils and tapered ends. It is motile by flexing movements under dark-field microscopy. Pigs normally have other non-pathogenic brachyspires such as *B. innocens* and

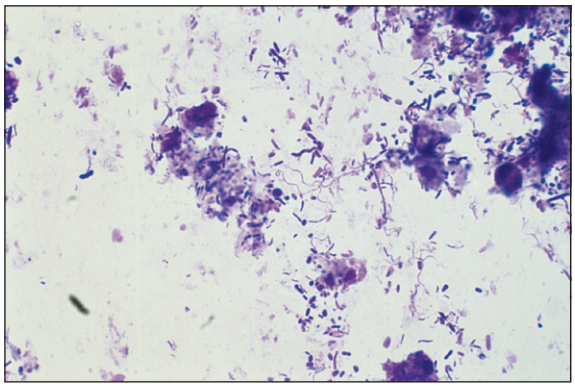

Figure 31.8 *Brachyspira hyodysenteriae*: characteristic, delicate, spiral forms in a faecal smear from a pig with swine dysentery. (Victoria blue stain, ×1000)

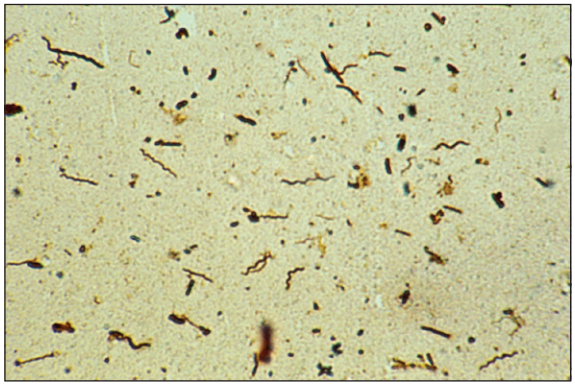

Figure 31.9 *Brachyspira hyodysenteriae* in porcine faeces. (Silver stain, ×1000)

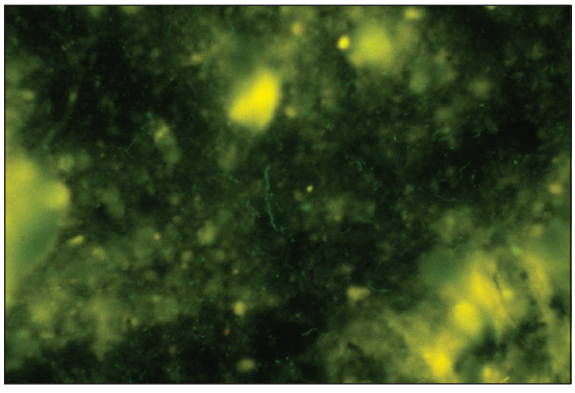

Figure 31.10 *Brachyspira hyodysenteriae* in a faecal smear from a pig with swine dysentery. (Direct FA technique, ×400)

smaller, tightly coiled spirochaetes in the intestinal tract (Fig. 31.11) that can complicate the interpretation of dark-field examination or stained smears.

Isolation

Brachyspira hyodysenteriae is anaerobic but oxygen-tolerant and growth is enhanced by CO_2. It grows well on freshly poured, or pre-reduced, trypticase soy agar with 5% blood and 400 µg/mL spectinomycin at 42 °C for two to six days under an atmosphere delivered by an $H_2 + CO_2$ envelope. Vancomycin and colistin are commonly used antimicrobials in selective media and a recently described selective medium incorporated flavomycin, rifampicin, spectinomycin and colistin in a blood agar modified medium (Råsbäck et al. 2005, Lugsomya et al. 2012). Increased growth has been obtained using Fastidious Anaerobe Agar (Lab M) with 5% sheep blood and also by the incorporation of 1% sodium RNA into media. Sodium RNA can be sterilized by filtration or by autoclaving. It is a growth enhancer and also increases the difference in haemolysis between *B. hyodysenteriae* and *B. innocens*. Colonic mucosal scrapings or faeces can be prepared as a 1 : 10 suspension in saline and clarified by centrifugation at slow speed. The supernatant can be passed, serially, through 0.8, 0.65 and 0.45 µm filters and this material is used to streak the culture medium. Cultures contaminated by other bacteria may be purified through the use of filtration as described by Jansson et al. (2011): a loopful of growth is suspended in broth, 150 µL of which is then pipetted onto a 0.45 µm filter paper on a fastidious anaerobe agar plate. Following 72 hours of incubation, the swarming growth of *B. hyodysenteriae* adjacent to the filter paper is subcultured.

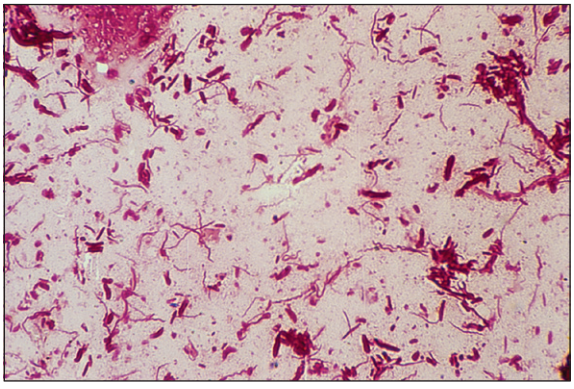

Figure 31.11 Spirochaetes in porcine faeces showing the characteristic morphology. (DCF stain, ×1000)

Identification

Colonial characteristics

After about 48 hours' incubation *B. hyodysenteriae* appears as small, translucent colonies with a zone of clear haemolysis. *Brachyspira pilosicoli* and *B. innocens* are weakly beta-haemolytic.

Microscopic appearance

DCF-stained smears or dark-field microscopy from the colony will reveal the typical helical brachyspire.

Biochemical characteristics

Brachyspira hyodysenteriae must be distinguished from other porcine brachyspires. Rapid and simple differentiation can be based on the combined results of haemolysis, the haemolysis intensification test, an indole spot test, hippurate hydrolysis and γ-galactosidase, γ-glucosidase and β-glucosidase activity (Table 31.8).

- Haemolysis: a distinctive characteristic of *B. hyodysenteriae* is the production of a strong beta-haemolysis that is best demonstrated on sheep blood agar. *Brachyspira pilosicoli* and *B. innocens* give a weak reaction.

Table 31.8 Laboratory differentiation of *Brachyspira* species isolated from pigs

Species	Laboratory tests						
	Haemolysis	Haemolysis intensification test	Indole spot test	Hippurate hydrolysis	∝-galactosidase activity	∝-glucosidase activity	β-glucosidase activity
B. hyodysenteriae	Strong	+	+*	–	+	+/–	+
B. pilosicoli	Weak	–	–	+/–	+/–	+/–	–
B. intermedia	Weak	–	+	–	–	+	+
B. innocens	Weak	–	–	–	+/–	+/–	+
B. murdochii	Weak	–	–	–	–	–	+

*Indole-negative strains have been reported

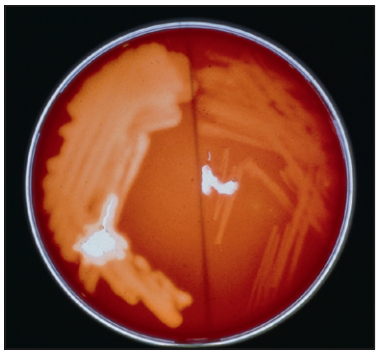

Figure 31.12 *Brachyspira hydrodysenteriae* (left) showing strong beta-haemolysis and the 'ring phenomenon'. *Brachyspira innocens* (right) gives a weaker haemolysis and no 'ring phenomenon'.

- Haemolysis intensification: this test can be carried out by cutting an agar block (0.5 cm^2) from a four-day culture and by incubating the plate for another four days. The intensification of haemolysis is recognized by a lighter zone, 1–2 mm wide, along the cut line. *Brachyspira hyodysenteriae* gives this reaction (ring phenomenon) but other species do not (Fig. 31.12).
- Indole spot test for anaerobes: the reagent is 1% *p*-dimethyl-aminocinamaldehyde in 10% conc. HCl. This is used to saturate a strip of filter paper in a Petri dish and growth from a culture of the test bacterium is smeared on the filter paper. *Brachyspira hyodysenteriae* gives a positive reaction indicated by a blue colour usually within one to three minutes. *Brachyspira pilosicoli* and *B. innocens* give a negative reaction and the filter paper remains pink.

Tests for *B. hyodysenteriae* antigens

- The fluorescent antibody test is useful as a screening test for identification of *B. hyodysenteriae* in specimens but both false-positive and false-negative results can occur (Lysons & Lemcke 1983).
- A slide agglutination test (Burrows & Lemcke 1981) and a microscopic agglutination test (Lysons 1991) using absorbed polyclonal serum, have been described. The slide test requires a considerable amount of pure culture and non-specific clumping in the saline control can occur. The use of monoclonal antibodies might increase the specificity of both tests.

Molecular diagnosis and typing

Techniques such as PCR can be used to confirm the identity of isolates and are sometimes used directly on clinical specimens. The most common PCR methods used are based on the *nox* or 23S rRNA genes for all porcine *Brachyspira* species, the 16s rRNA gene for *B. pilosicoli* and the *tly*A gene for *B. hyodysenteriae* (Park et al. 1995, Leser et al. 1997, Fellström et al. 2001, La et al. 2003, Råsbäck et al. 2006). Multiplex quantitative PCR procedures which detect several enteric pathogens of pigs simultaneously have also been developed, such as that described by Ståhl et al. (2011).

Typing of isolates is now commonly carried out using MLST procedures and there is now a MLST database of *B. hyodysenteriae* and other *Brachyspira* isolates. Other methods such as random amplified polymorphic DNA analysis and PFGE-based techniques may also be used for epidemiological investigations.

Antimicrobial susceptibility testing and antimicrobial resistance

At present there is no standardized method for susceptibility testing of *Brachyspira* species (Råsbäck et al. 2005). Broth or agar dilution methods using extended antimicrobial dilution series gave the best results in a ring trial with 10 participating laboratories from around Europe (Råsbäck et al. 2005). Internationally agreed interpretive criteria are not available although a recent publication by Pringle et al. (2012) proposes cut-off values for six antimicrobial agents. Susceptibility of isolates to tylosin and lincomycin has decreased in many countries but tiamulin and valnemulin, antimicrobials commonly used in the treatment of swine dysentery, appear to retain good activity against *B. hyodysenteriae* in most countries. However, there is evidence of significant resistance in some countries with consequent problems for effective treatment.

Tests for *B. hyodysenteriae* antibodies

Several serological procedures have been developed for the serodiagnosis of swine dysentery or for the detection of carrier animals. The ELISA appears to be the most useful and tests based on whole cell antigens and on outer membrane lipoprotein are available. Although both types of tests gave satisfactory results, the tests based on whole cell antigens resulted in better discrimination between infected and non-infected herds (Song et al. 2012).

BORRELIA SPECIES

The *Borrelia* species (Fig. 31.13) are spirochaetes highly adapted to arthropod transmission. The infections affecting animals and humans have blood-borne phases and disease may be localized or generalized. The animal pathogens are *B. anserina* (avian spirochaetosis) and *B. burgdorferi* sensu lato (Lyme disease). *Borrelia theileri* is associated with tick spirochaetosis of cattle and *B. coriaceae*

with epizootic bovine abortion in the USA but the exact pathogenic significance of these two species is uncertain.

Borrelia burgdorferi sensu lato comprises all strains that cause Lyme disease in animals and humans and can be divided into a number of genospecies, the most important relative to clinical disease being *B. burgdorferi* sensu stricto (in North America and Europe), *B. garinii* (Eurasia) and *B. afzelii* (Eurasia). The genome of *B. burgdorferi* sensu stricto comprises a linear chromosome and 21 plasmids (Fraser et al. 1997). Unlike other bacteria, some of these plasmids appear to be essential for growth, survival and virulence of the organism.

Natural Habitat

Ticks are the main reservoir of borreliae affecting animals. *Borrelia burgdorferi* sensu lato has a complex life cycle in which *Ixodes* ticks, small mammals and birds, and larger mammals play an essential part. Small rodents and birds constitute the main reservoir hosts for *Borrelia burgdorferi* sensu lato and ticks acquire infection when larval or nymphal stages feed on these infected hosts. Infection persists in the tick until adulthood but infection does not pass transovarially. Adult ticks feed preferentially on larger mammals such as deer and sheep and infect these species. Infection in humans and domestic animals can be acquired through the bite of a nymph or adult tick. As nymphs are smaller and thus more difficult to detect, they may play a more significant role in transmission of infection, particularly in humans as they may persist longer on the host before detection and removal. Transmission of infection requires the presence of the tick on the host for between 24 and 48 hours. Table 31.9 indicates the vectors and geographical distribution of the *Borrelia* species of veterinary significance.

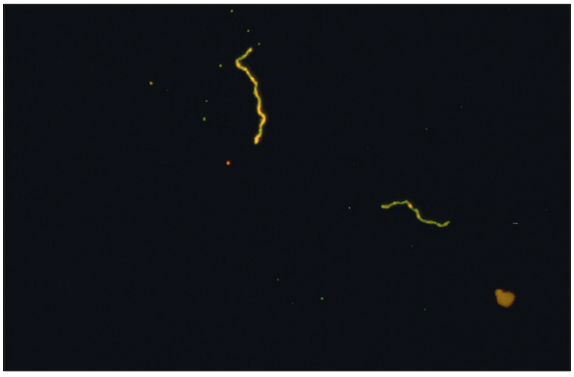

Figure 31.13 *Borrelia burgdorferi*. (Direct FA technique, ×1000)

Pathogenesis and Pathogenicity

Infection with *Borrelia burgdorferi* sensu lato in animals is much more common than disease and in endemic areas it can be difficult to determine whether clinical signs observed are attributable to infection with borreliae or to other disease. Clinical signs are most often observed in dogs. One of the first manifestations of Lyme disease in man is a skin rash around the site of the tick bite (Fig. 31.14). A rash has not been reported in dogs where the characteristic clinical feature is acute or intermittent lameness with swelling in one or more joints. Fever, lethargy and anorexia may accompany the lameness. In addition, a renal syndrome, CNS dysfunction and myocarditis have been associated with *B. burgdorferi* infection in dogs.

Table 31.9 Distribution and vectors of *Borrelia* species affecting domestic animals

Borrelia species	Arthropod vector	Reservoir	Distribution	Disease
B. anserina	*Argas persica* *A. miniatus* *A. reflexus*	Birds	All continents	Avian spirochaetosis
B. burgdorferi sensu lato	*Ixodes scapularis*	Rodents (white footed and deer mice)	East and Midwest USA	Lyme disease
	Ixodes pacificus	Rodents	Western USA	Lyme disease
	Ixodes ricinus	Rodents, birds	Europe	Lyme disease
	Ixodes persulcatus	Rodents	Eurasia	Lyme disease
B. theileri	*Rhipicephalus* species *Margaropus* species *Boophilus* species	Cattle, sheep, horses	South Africa, Australia, North America, Europe	Tick spirochaetosis
B. coriaceae	*Ornithodorus* species	Cattle, deer	Western USA	Associated with epizootic bovine abortion

Clinical signs may be dependent in part on the genospecies prevalent in a particular geographical region; lameness is the most frequently observed clinical sign in dogs in the USA where *B. burgdorferi* sensu stricto is prevalent, whereas neurological manifestations are more common in Europe and Japan where other genospecies predominate. Although *B. afzelii* is associated with skin disease in humans and *B. garinii* with neuroborreliosis, definitive proof of clinical disease associated with these species is lacking in dogs.

Outer surface proteins (Osps) are key to the transmission of infection between ticks and hosts. While in the midgut of the tick, *Borrelia burgdorferi* expresses mainly OspA. During feeding on a blood meal, changes in temperature and pH downregulate expression of OspA and upregulate the expression of OspC (Ohnishi et al. 2001). Once in the host, antibodies produced against OspC after about 10 days of infection will eliminate organisms expressing that protein and select for those not expressing OspC. The organism is able to change many of its surface antigens and this antigenic variation is one important mechanism enabling persistence in the host. Persistence may be facilitated also by the formation of spheroplasts or L-forms in hostile environments such as CSF. Non-viable remnants of the organism may remain bound to host cell membranes and stimulate an immune response; this is considered one of the possible explanations for chronic disease in which detection of the full genome of *B. burgdorferi* is not possible (Littman 2003). The organism is not thought to produce any known toxins, nor does its cell envelope contain any lipopolysaccharide. The lesions produced during infection with *B. burgdorferi* sensu lato are therefore considered to be due in large part to the inflammatory response induced by the organism in the host.

Table 31.10 summarizes the hosts and diseases caused by the borreliae of veterinary importance, while virulence attributes of *B. burgdorferi* sensu lato are presented in Table 31.11.

Figure 31.14 Typical skin rash of Lyme disease (*Borrelia burgdorferi*) in a human following a tick-bite (*Ixodes* species).

Laboratory Diagnosis

Borrelia anserina

- Demonstration of the borreliae (8–20 µm in length) in buffy coat, spleen and liver smears by dark-field

Table 31.10 Pathogenicity and hosts of *Borrelia* species affecting domestic animals

Borrelia species	Hosts	Disease
B. anserina	Chickens, ducks, geese, turkeys, pigeons, pheasants, canaries, and some wild birds	Avian spirochaetosis affects all ages but higher mortality in young birds. There is fever, depression and anorexia. Birds become cyanotic and produce a greenish diarrhoea. Later, paralysis and anaemia may develop. Mortality 10–99%. On postmortem the spleen is enlarged and mottled
B. burgdorferi sensu lato (Lyme disease)	Humans	Skin rash, stiffness of joints and neck, headache and lymphadenopathy. Sequelae if not treated include chronic arthritis, cardiac signs and chronic neurological symptoms
	Dogs	Sudden onset of lameness involving several joints. The affected joints are swollen and painful. The arthritis is characteristically migratory. Synovial fluid has a high neutrophil count. Some dogs can develop renal disorders. Neurological disturbance is a frequent clinical feature in Europe and Japan
	Cattle and horses	Arthritis, encephalitis, uveitis and laminitis
B. theileri	Cattle (sheep, horses)	Tick spirochaetosis: fever, weight loss, weakness and anaemia. There are one or more febrile attacks before recovery. The febrile attacks are of minor clinical significance in sheep and horses

Table 31.11 Virulence attributes of *Borrelia burgdorferi* sensu lato

Virulence attribute	Pathogenic role
Outer surface proteins (Osps) OspA/OspB OspC	Expressed in the mid-gut of unfed ticks, role in attachment and persistence in tick host. Production of this protein is up-regulated during blood meal, essential for the early stages of mammalian infection
Resistance-nodulation-division-type (RND-type) protein export system	Multidrug resistance, may also be important for survival in the mammalian host
Surface lipoproteins BptA (Borrelial persistence in ticks A) BRA64	Major inflammatory agonists Important for persistence in ticks May be required for transmission from tick to mammalian host
Host tissue-binding proteins	Dissemination and establishment of infection
VlsE (variable major protein-like sequence)	Antigenic variation and evasion of the immune response
CRASPs (complement regulator-acquiring surface proteins)	Evasion of complement-mediated lysis by binding of host serum proteins factor H and factor H-like proteins
Porin-like proteins P66	Adhesive properties
Flagella	Motility. Movement towards site of tick feeding in the mammalian host, evasion of phagocytosis
Induction of host matrix metalloproteases	Degradation of extracellular matrix proteins, facilitating invasion and dissemination

microscopy, Giemsa stain or fluorescent antibody (FA) technique.
- Culture in six- to 10-day-old fertile chicken or turkey eggs.
- Immunodiffusion tests can be used to demonstrate either antigen or antibody.

Borrelia theileri

Demonstration of the organism in blood during the febrile stage. It is a large (20–30 µm in length), loosely coiled spirochaete that can be visualized using dark-field microscopy or Giemsa-stained smears.

Borrelia burgdorferi sensu lato

Laboratory diagnosis is difficult in animals and relies mainly on a history of exposure to *Ixodes* ticks, together with compatible clinical signs and serological evidence of infection.

- Culture is difficult but the organism will grow in modified Kelley's medium under microaerophilic conditions (Barbour et al. 1983). The cultures are examined weekly by dark-field microscopy for growth. Improvements in detection of *B. burgdorferi* sensu lato in the early stages of human disease have been achieved through the culture of plasma and increasing the volume of material cultured (Aguero-Rosenfeld et al. 2005). Comparable improvements in diagnosis of canine disease are unlikely as the characteristic rash which accompanies the early stages of disease in humans is not reported in dogs and clinical signs are not usually observed until four to six weeks after exposure to infection.
- Indirect evidence of exposure is provided by positive serological titres. ELISA and IFA tests are the prinicipal methods used for antibody detection. An ELISA based on a recombinant C6 peptide antigen is now available. The C6 peptide is part of the invariable region of a variable surface antigen which is expressed in vivo during both early and late infection but not expressed in vitro. The ELISA can differentiate between naturally exposed and vaccinated animals and is reported to be both sensitive and specific. Western blotting is frequently used as a confirmatory test in cases of samples giving equivocal results and it is also possible to differentiate natural infection and vaccinal titres

using this technique; vaccinated animals express antibodies to OspA which are rarely detected in naturally infected dogs.

Molecular Diagnosis and Typing

Several molecular procedures have been developed to identify *B. burgdorferi* sensu lato: PCR in particular has proved useful (Wang 2002, Aguero-Rosenfeld et al. 2005). However, the numbers of organisms present in the tissues of affected animals are low and thus false-negative results may be a problem even with this sensitive procedure.

Typing of *B. burgdorferi* sensu lato is frequently carried out using multilocus sequence typing (MLST), sometimes in combination with sequencing of specific genes encoding particular proteins such as the Osps or the detection of single nucleotide polymorphisms (Margos et al. 2011). For example, analysis of North American isolates using MLST revealed three geographically separated subpopulations, one in the West transmitted by *Ixodes pacificus*, one in the Midwest and one in the Northeast (Margos et al. 2012). Techniques employing pulsed-field gel electrophoresis procedures may also be used for characterization of borreliae at both the genospecies and strain level (Lin et al. 2004).

Antimicrobial Susceptibility Testing and Resistance

Broth microdilution procedures are used for antimicrobial susceptibility testing of borreliae. Such procedures can be adapted for use with colorimetric growth detection methods (Hunfeld et al. 2000a, 2000b). However, owing to difficulties in culturing these organisms, such testing is carried out in specialized laboratories.

Borrelia burgdorferi sensu lato is generally susceptible to tetracyclines, amoxicillin, macrolides and cephalosporins. Fluoroquinolones are usually ineffective against these organisms. It is suggested that failure of antimicrobial therapy in vivo may be due to a lack of efficacy of antimicrobials against round body forms and biofilm-like colonies and this suggestion is supported by recent in vitro findings (Sapi et al. 2011).

REFERENCES

Aguero-Rosenfeld, M.E., Wang, G., Schwartz, I., et al., 2005. Diagnosis of lyme borreliosis. Clinical Microbiology Reviews 18 (3), 484–509.

Ahmed, N., Devi, S.M., Valverde Mde, L., et al., 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Annals of Clinical Microbiology and Antimicrobials 5, 28.

Barbour, A.G., Burgdorfer, W., Hayes, S.F., et al., 1983. Isolation of a cultivable spirochete from Ixodes ricinus ticks of Switzerland. Current Microbiology, 8, 123–126.

Bellgard, M.I., Wanchanthuek, P., La, T., et al., 2009. Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine. PLOS ONE 4 (3), e4641.

Branger, C., Blanchard, B., Fillonneau, C., et al., 2005. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene hap1 encoding the hemolysis-associated protein-1. FEMS Microbiology Letters 243 (2), 437–445.

Burrows, M.R., Lemcke, R.M., 1981. Identification of *Treponema hyodysenteriae* by a rapid slide agglutination test. Veterinary Record 108, 187–189.

Cole Jr., J.R., 1990. Spirochetes. In: Carter, G.R., Cole Jr., J.R. (Eds.), Diagnostic Procedures in Veterinary Bacteriology and Mycology, fifth ed. Academic Press Inc, New York, pp. 41–60.

Faine, S. (Ed.), 1982. Guidelines for the Control of Leptospirosis. WHO offset publication no 67. World Health Organization, Geneva.

Gravekamp, C., Van de Kemp, H., Franzen, M., et al., 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. Journal of General Microbiology 139 (8), 1691–1700.

Fellström, C., Zimmerman, U., Aspan, A., et al., 2001. The use of culture, pooled samples and PCR for identification of herds infected with *Brachyspira hyodysenteriae*. Animal Health Research Reviews 2 (1), 37–43.

Fraser, C.M., Casjens, S., Huang, W.M., et al., 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390 (6660), 580–586.

Griffith, M.E., Hospenthal, D.R., Murray, C.K., 2006. Antimicrobial therapy of leptospirosis. Current Opinion in Infectious Diseases 19 (6), 533–537.

Haake, D.A., Martinich, C., Summers, T.A., et al., 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infection and Immunity 66 (4), 1579–1587.

Hunfeld, K-P, Kraiczy, P., Wichelhaus, T.A., et al., 2000a. Colorimetric in vitro susceptibility testing of penicillins, cephalosporins, macrolides, streptogramins, tetracyclines, and aminoglycosides against *Borrelia burgdorferi* isolates. International Journal of Antimicrobial Agents 15, 11–17.

Hunfeld, K.-P., Kraiczy, P., Wichelhaus, T.A., et al., 2000b. New colorimetric microdilution method for in vitro susceptibility testing of *Borrelia burgdorferi* against antimicrobial substances. European Journal of Clinical Microbiology & Infectious Diseases 19, 27–32.

Jacobson, M., Fellström, M., Lindberg, C., et al., 2004. Experimental swine dysentery: comparison between infection models. Journal of Medical Microbiology 53, 273–280.

Jansson, D.S., Persson, M., Zimmerman, U., et al., 2011. Phenotypic and genetic diversity among intestinal spirochaetes (genus *Brachyspira*) in free-living wild mallards (Anas platyrhynchos) sampled in southern Sweden. Systematic and Applied Microbiology 34 (8), 566–575.

Jonasson, R., Johannisson, A., Jacobson, M., et al., 2004. Differences in lymphocyte subpopulations and cell counts before and after experimentally induced swine dysentery. Journal of Medical Microbiology 53, 267–272.

Kennedy, M.J. Rosey, E.L., Yancey, R.J., 1997. Characterization of flaA- and flaB-mutants of *Serpulina hyodysenteriae*: both flagellin subunits, FlaA and FlaB, are necessary for full motility and intestinal colonization. FEMS Microbiology Letters 153, 119–128.

La, T., Phillips, N.D., Hampson, D.J., 2003. Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. Journal of Clinical Microbiology 41 (7), 3372–3375.

Leser, T.D., Møller, K., Jensen, T.K., et al., 1997. Specific detection of *Serpulina hyodysenteriae* and potentially pathogenic weakly beta-haemolytic porcine intestinal spirochetes by polymerase chain reaction targeting 23S rDNA. Molecular and Cellular Probes 11 (5), 363–372.

Levett, P.N., 2001. Leptospirosis. Clinical Microbiology Reviews 14 (2), 296–326.

Levett, P.N., Morey, R.E., Galloway, R.L., et al., 2005. Detection of pathogenic leptospires by real-time quantitative PCR. Journal of Medical Microbiology 54 (Pt 1), 45–49.

Lin, T., Oliver Jr., J.H., Gao, L., 2004. Molecular characterization of *Borrelia* isolates from ticks and mammals from the southern United States. Journal of Parasitology 90 (6), 1298–1307.

Littman, M.P., 2003. Canine borreliosis. Veterinary Clinics of North America: Small Animal Practice 33 (4), 827–862.

Lugsomya, K., Tummaruk, P., Hampson, D.J., et al., 2012. Development of a modified selective medium to enhance the recovery rate of *Brachyspira hyodysenteriae* and other porcine intestinal spirochaetes from faeces. Letters in Applied Microbiology 54 (4), 330–335.

Lysons, R.J., 1991. Microscopic agglutination: a rapid test for identification of *Treponema hyodysenteriae*. Veterinary Record 129, 315–316.

Lysons, R.J., Lemcke, R.M., 1983. Swine dysentery: to isolate or to fluoresce. Veterinary Record 112, 203.

Margos, G., Vollmer, S.A., Ogden, N.H., et al., 2011. Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. Infection, Genetics and Evolution 20 11 (7), 1545–1563.

Margos, G., Tsao, J.I., Castillo-Ramírez, S., et al., 2012. Two Boundaries Separate *Borrelia burgdorferi* Populations in North America. Applied and Environmental Microbiology 78 (17), 6059–6067.

Mérien, F., Amouriaux, P., Perolat, P., et al., 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. Journal of Clinical Microbiology 30 (9), 2219–2224.

Mérien, F., Baranton, G., Perolat, P., 1997. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. Infection and Immunity 65 (2), 729–738.

Mérien, F., Truccolo, J., Rougier, Y., et al., 1998. In vivo apoptosis of hepatocytes in guinea pigs infected with *Leptospira interrogans* serovar *icterohaemorrhagiae*. FEMS Microbiology Letters 169 (1), 95–102.

Mérien, F., Portnoi, D., Bourhy, P., et al., 2005. A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. FEMS Microbiology Letters 249 (1), 139–147.

Murray, C.K., Hospenthal, D.R., 2004. Broth microdilution susceptibility testing for *Leptospira* spp. Antimicrobial Agents and Chemotherapy 48 (5), 1548–1552.

Nascimento, A.L.T.O., Ko, A.I., Martins, E.A.L., et al., 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. Journal of Bacteriology 186, 2164–2172.

Ohnishi, J., Piesman, J., de Silva, A.M., 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. Proceedings of the National Academy of Sciences USA 98 (2), 670–675.

Palaniappan, R.U., Chang, Y.F., Chang, C.F., et al., 2005. Evaluation of lig-based conventional and real time PCR for the detection of pathogenic leptospires. Molecular and Cellular Probes 19 (2), 111–117.

Park, N.Y., Chung, C.Y., McLaren, A.J., et al., 1995. Polymerase chain reaction for identification of human and porcine spirochaetes recovered from cases of intestinal spirochaetosis. FEMS Microbiology Letters 125 (2–3), 225–229.

Perolat, P., Chappel, R.J., Adler, B., et al., 1998. *Leptospira fainei sp. nov.*, isolated from pigs in Australia. International Journal of Systematic Bacteriology 48, 851–858.

Picardeau, M., Bulach, D.M., Bouchier, C., et al., 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLOS ONE 3 (2), e1607.

Pringle, M., Landén, A., Unnerstad, H.E., et al., 2012. Antimicrobial susceptibility of porcine *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* isolated in Sweden between 1990 and 2010. Acta Veterinaria Scandinavica 54 (1), 54.

Råsbäck, T., Fellström, C., Bergsjø, B., et al., 2005. Assessment of diagnostics and antimicrobial susceptibility testing of *Brachyspira*

species using a ring test. Veterinary Microbiology 109 (3–4), 229–243.

Råsbäck, T., Fellström, C., Gunnarsson, A., et al., 2006. Comparison of culture and biochemical tests with PCR for detection of *Brachyspira hyodysenteriae* and Brachyspira pilosicoli. Journal of Microbiological Methods 66 (2), 347–353.

Ren, S.X., Fu, G., Jiang, X.G., et al., 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 422, 888–893.

Ressner, R.A., Griffith, M.E., Beckius, M.L., et al., 2008. Antimicrobial susceptibilities of geographically diverse clinical human isolates of *Leptospira*. Antimicrobial Agents and Chemotherapy 52 (8), 2750–2754.

Sapi, E., Kaur, N., Anyanwu, S., et al., 2011. Evaluation of in-vitro antibiotic susceptibility of different morphological forms of *Borrelia burgdorferi*. Journal of Infection and Drug Resistance 4, 97–113.

Song, Y., Frey, B., Hampson, D.J., 2012. The use of ELISAs for monitoring exposure of pig herds to *Brachyspira hyodysenteriae*. BMC Veterinary Research 17 (8), 6.

Ståhl, M., Kokotovic, B., Hjulsager, C.K., et al., 2011. The use of quantitative PCR for identification and quantification of *Brachyspira pilosicoli*, *Lawsonia intracellularis* and *Escherichia coli* fimbrial types F4 and F18 in pig feces. Veterinary Microbiology 151 (3–4), 307–314.

Stanton, T.B., Rosey, E.L., Kennedy, M.J., et al., 1999. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete *Brachyspira (Serpulina) hyodysenteriae,* etiologic agent of swine dysentery. Applied and Environmental Microbiology 65 (11), 5028–5034.

ter Huurne, A.A.H.M., Muir, S., van Houten, M., et al., 1994. Characterization of three putative *Serpulina hyodysenteriae* haemolysins. Microbiological Pathogenesis 16, 269–282.

Verma, A., Artiushin, S., Matsunaga, J., et al., 2005. LruA and LruB, novel lipoproteins of pathogenic *Leptospira interrogans* associated with equine recurrent uveitis. Infection and Immunity 73 (11), 7259–7266.

Wang, G., 2002. Direct detection methods for Lyme Borrelia, including the use of quantitative assays. Vector-Borne and Zoonotic Diseases 2 (4), 223–231.

Yasuda, P.H., Steigerwalt, A.G., Sulzer, K.R., et al., 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. International Journal of Systematic Bacteriology 37 (4), 407–415.

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Chapter 32

Miscellaneous Gram-negative bacteria

These bacteria, not included in any other pathogenic groups, are not commonly isolated but may have importance in either animal or human disease, or as zoonotic infections. The habitats, host species and diseases caused by these species are given in Table 32.1.

BARTONELLA SPECIES

There are 25 species described in the genus *Bartonella*, present in a wide range of animals and man. The principal pathogen in the genus is *Bartonella henselae*, which has an animal reservoir, and is the causative agent of cat scratch disease in humans. Several other species are associated with disease in humans and are increasingly reported in association with disease in domestic animals. Bartonellae which have been isolated from domestic animals and their associated clinical conditions are summarized in Table 32.2.

Bartonella species are pleomorphic, Gram-negative slightly curved bacilli. They can be isolated only on blood-enriched media and growth may take three or four weeks to become apparent.

Natural Habitat

Bartonella species have been isolated from the blood of many domestic animals as indicated in Table 32.2. *Bartonella henselae* is carried by domestic and wild cats. Co-infection with a number of *Bartonella* species has been shown in cats and experimental infection with one species does not confer protection against heterologous challenge (Breitschwerdt 2008). In humans, as in cats, the prevalence of infection is low in northern latitudes and higher in warm and humid regions. Cats may be bacteraemic for weeks to months with kittens being most likely to show prolonged bacteraemia. Transmission of infection from cat to cat is via the cat flea (*Ctenocephalides felis*). Transmission to humans is usually through a scratch from a cat and less frequently from a cat bite. It is likely, although not definitively proven, that the method of transmission is through contamination of the scratch by infected flea faeces adhering to the claws of the cat.

Pathogenicity

The organism is intracellular and is found in red blood cells and vascular endothelium. It appears that *B. henselae* invades haemopoeitic progenitor cells in humans following infection and that this results in the presence of the organism in circulating red blood cells (Mändle et al. 2005).

Cat scratch disease in humans is manifested principally by regional lymphadenopathy in immunocompetent individuals. Approximately seven to 12 days following a cat scratch a papule and then a pustule develop at the scratch site. Lymphadenopathy involving usually only a single lymph node nearest the site of infection follows one to three weeks later and may persist for weeks or months. Headaches, weight loss, vomiting, sore throat and other non-specific signs may accompany the lymphadenopathy. Atypical disease occurs in a small number of affected humans and includes ocular and neurological manifestations. Disseminated disease has been described occasionally. In immunocompromised patients, a vasoproliferative response to infection occurs and bacillary angiomatosis may develop.

Although *Bartonella* species typically cause no disease in cats, cases of endocarditis, lymphadenitis and abscessation

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Table 32.1 Hosts, habitat and diseases of the miscellaneous Gram-negative bacterial pathogens

Organism	Hosts	Diseases
Bartonella species	*Bartonella* species have been isolated from many wild and domesticated animals	*B. henselae* • Cat scratch disease in humans • Hepatitis in dogs • Frequently asymptomatic in cats, may cause endocarditis, granulomatous inflammation in lymph nodes and other organs
Chromobacterium violaceum	Saprophyte of soil and water in subtropical and tropical regions	Occasional disease (abscessation, septicaemia) reported in humans, pigs, cattle, dogs
Capnocytophaga canimorsus	Commensal in the nasopharynx of dogs	Infection of dog bites in humans, septicaemia if immunocompromised
Ornithobacterium rhinotracheale	Chickens, turkeys	Respiratory disease
Riemerella anatipestifer	Ducklings	Septicaemia
Streptobacillus moniliformis	Commensal in the nasopharynx of wild and laboratory rats and cats	Rat-bite (Haverhill) fever in man Epizootics in laboratory mice characterized by swelling of the extremities, arthritis, conjunctivitis, lymphadenitis and acute septicaemia Laboratory rats may show signs of bronchopneumonia, otitis media and conjunctivitis Septicaemia or tenosynovitis following rat bites in turkeys Pneumonic lesions have been reported in calves

Table 32.2 *Bartonella* species detected in domestic animals, their vectors and associated diseases in humans and animals

Bartonella species	Primary animal reservoir	Vector	Disease in humans	Disease in animals
Bartonella henselae *B. clarridgeiae* *B. koehlerae*	Cats	Cat flea	Cat scratch disease	Endocarditis, hepatitis and other clinical syndromes in dogs and less frequently in cats (*B. koehlerae* not identified as a cause of disease to date)
B. vinsonii subsp. *berkoffii*	Coyotes, dogs	Possibly ticks	Endocarditis	Endocarditis, myocarditis, lymphadenitis and a variety of other clinical conditions in dogs
B. alsatica	Rabbit	Possibly fleas	Endocarditis	Disease not recorded
B. chomelii	Cattle	Unknown	Disease not recorded	Disease not recorded
B. bovis	Cattle	Unknown	Disease not recorded	Endocarditis in cattle

in liver and spleen have been reported (Breitschwerdt 2008). Endocarditis has been documented in dogs due to *B. vinsonii* subsp. *berkhoffii, B. clarridgeiae* and *B. quintana,* (Chomel et al. 2006) and also in cattle due to *B. bovis* (Maillard et al. 2007). *Bartonella henselae* causes hepatitis in dogs and was associated with chronic arthropathy and presumptive vasculitis in horses (Jones et al. 2008).

Laboratory Diagnosis

In tissues, *Bartonella* organisms stain well with silver stains such as the Warthin–Starry stain.

Bartonella species can be difficult to isolate but *B. henselae* can be isolated from the blood of bacteraemic cats using conventional blood culture systems, although blind

subculture onto solid media is required as the organism produces little or no CO_2 (Sander 2005). Solid media must be supplemented with 5–10% sheep or rabbit blood. Chocolate agar can be used also. Cell lysis by centrifugation or freezing of the blood sample prior to culture, improves recovery of the organism. Cultures are incubated at 35–37°C for at least four weeks. Isolation from tissue specimens from affected people is usually unsuccessful although isolation rates have been improved by the development of a liquid growth medium (Chenoweth et al. 2004). Colony morphology is variable on primary isolation and colonies are whitish-cream and may be small and smooth or rough and irregular. They are usually adherent and deeply embedded in the agar.

Identification cannot be performed using conventional biochemical tests although whole cell fatty acid analysis is useful for identification to genus level. Molecular, PCR-based tests are frequently used for detection in clinical specimens and for identification of isolates.

Diagnosis of disease is frequently dependent on serological tests with IFAT and ELISA being most widely used. Seroconversion in people and animals such as dogs can be demonstrated and is useful for diagnosis in conjunction with disease signs. Serology in cats is not useful for the diagnosis of disease as cats are frequently asymptomatic. In addition, serology cannot be used to predict bacteraemia as bacteraemic cats may be seronegative (Breitschwerdt 2008).

Antimicrobial susceptibility testing can be performed using agar dilution or E-test methods (Dörbecker et al. 2006).

STREPTOBACILLUS MONILIFORMIS

Streptobacillus moniliformis, the only species in the genus, is an extremely pleomorphic Gram-negative rod (0.1–0.7 to 1–5 µm) with rounded or pointed ends but can form unbranched filaments 10–150 µm long. The filaments bear knobby irregularities, hence the specific name, *moniliformis* (Latin) meaning in the form of a 'necklace' or 'string of beads'. The bacterium ferments a range of carbohydrates but is non-motile, catalase-negative, oxidase-negative and relatively unreactive in other biochemical tests. L-phase and transitional variants are readily formed. *Streptobacillus moniliformis* is fastidious and requires media enriched with blood, serum or ascitic fluid. It requires microaerophilic conditions for growth.

Natural Habitat and Pathogenicity

The reservoir is the nasopharynx of rats and possibly of small carnivores. Humans can acquire the disease rat-bite fever from the bite of a laboratory rat but it is most common in children. Historically the condition was associated with poverty and rat-infested accommodation but the increasing popularity of keeping rats as pets means that disease is now seen in the owners of such pets and in pet shop personnel (Elliott 2007). The organism has been demonstrated in the mouth of dogs, which had known contact with rats but the importance of dogs as a source of infection for humans is not known (Wouters et al. 2008). The incubation period is usually one to four days and clinical signs include fever, headache, rash on hands and feet, and later polyarthralgia in about two-thirds of patients. The bite wound usually heals well but the disease can develop into a chronic relapsing illness. Transmission in Haverhill fever (Haverhill, Massachusetts) is by the ingestion of contaminated milk or water to which rats have had access. The clinical signs in Haverhill fever are similar to those of rat-bite fever but gastrointestinal and respiratory signs are more common. '*Spirillum minus*' (Fig. 32.1) is another cause of rat-bite fever in man but this bacterium does not affect animals.

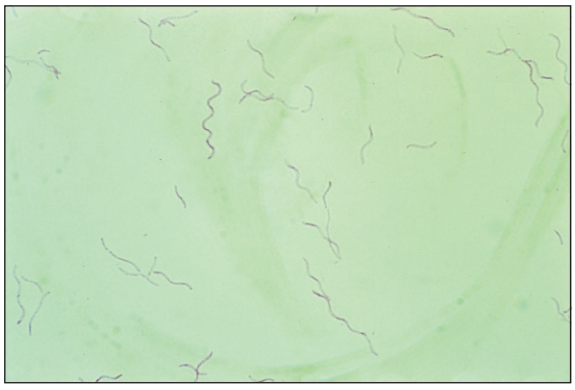

Figure 32.1 *Spirillum* species showing the spiral form of the bacterium. (DCF stain, ×1000)

Laboratory Diagnosis

Specimens

These include joint fluid, citrated blood (equal volume of blood and 2.5% sodium citrate), pus, exudates and tissue lesions.

Direct microscopy

Streptobacillus moniliformis may not stain strongly with the Gram stain so either DCF or Giemsa stains should be used. It is less pleomorphic in animal and human tissues than in smears from cultures. An immunofluorescence technique is also used in some laboratories.

Isolation

An enriched medium consisting of brain heart infusion base enriched with 15% blood, 20% horse or calf serum,

or 5% ascitic fluid is required. The L-forms are more fastidious and a clear medium is also preferable. Rogosa (1985) suggested a heart infusion base enriched with 20% horse serum and yeast extract. Thioglycollate and meat infusion broths, both with added serum, will support the growth of the bacterium. The sodium-polyanethol sulfonate (SPS) present in most blood culture media is inhibitory. Inoculated media are incubated in a humified l0% CO_2 atmosphere at 35–37°C for up to seven days.

Identification

Colonial morphology

Colonies on blood agar are non-haemolytic, round, greyish, smooth, glistening with a diameter of l–2 mm after two to three days. Spontaneous L-form microcolonies may occur adjacent to the bacterial-type colonies. These L-form colonies have a 'fried-egg' appearance and can be stained by the Dienes' stain (as for mycoplasmal microcolonies) and examined under a low-power objective of a microscope.

In thioglycollate and other semisolid or liquid media, colonies of *S. moniliformis* grow as characteristic fluff-balls near the bottom of the tube.

Microscopic appearance

Under the most favourable cultural conditions, *S. moniliformis* is rod-shaped and relatively uniform with an occasional short filament or knob-like irregularity (Fig. 32.2). In less favourable conditions or in older cultures the bacterium is highly pleomorphic and consists of filaments and bulbous swellings. The L-form microcolonies yield small Gram-negative coccal forms.

Biochemical reactions

Fermentation tests on sugars are best conducted in cystine tryptic agar (CTA) base with added serum or ascitic fluid. The alkaline phosphatase test is characteristically positive. Some of the other biochemical reactions are given in Table 32.3.

A typical fatty acid profile can be identified for this bacterium using gas-liquid chromatography.

PCR-based assays have been used successfully for the diagnosis and identification of *S. moniliformis* also (Elliott 2007).

CHROMOBACTERIUM VIOLACEUM

Chromobacterium violaceum is a Gram-negative rod (0.6–0.9 to l.5–3.5 µm), which is sometimes slightly curved with the cells occurring singly or in pairs. It often shows barred or bipolar staining in a Gram-stained smear. There is a characteristic type of flagellar arrangement with a short polar flagellum which stains poorly and has a relatively long wavelength and one to four longer lateral flagella that stain well and have a shorter wavelength. Most strains are facultative anaerobes but a few are aerobic. It is catalase-positive, oxidase-positive and grows on nutrient and MacConkey agars at 37°C but not at 4°C. Most strains of *C. violaceum* produce a non-diffusible violet pigment, known as violacein, which requires the presence of oxygen for its formation.

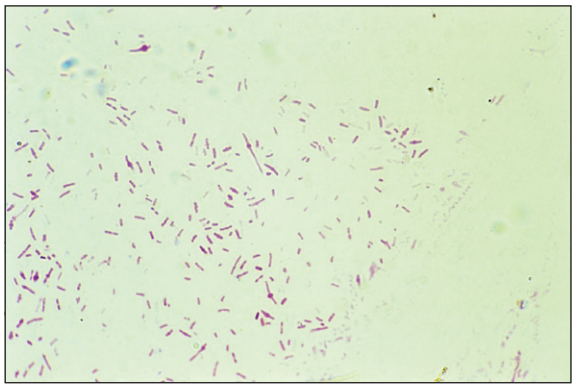

Figure 32.2 *Streptobacillus moniliformis*: a highly pleomorphic Gram-negative rod with filamentous forms bearing knobby irregularities. (Gram stain, ×1000)

Natural Habitat

Chromobacterium violaceum is a saprophyte in soil and water of subtropical and tropical regions, which occasionally causes disease in man and animals. Human cases have been reported from southeast Asia, southeast USA, South America and northern Australia. Outbreaks of disease have been recorded in buffaloes, cattle, pigs, dogs and gibbons. Comparatively few infections occur, despite the bacterium being widespread within the regions where it occurs.

Pathogenicity

In humans, *C. violaceum* can cause skin abscesses or an overwhelming septicaemia with pulmonary, liver and subcutaneous abscesses, occasionally resembling melioidosis (*Burkholderia pseudomallei*). Suppurative pneumonia and other infections occur in animals.

Laboratory Diagnosis

Isolation

Direct microscopy is not rewarding. Specimens such as pus or material from tissue lesions can be used to inoculate blood and MacConkey agars. Humidity may be important as the bacterium dies rapidly on over-dried plates or agar

Table 32.3 Summary of the biochemical reactions of Gram-negative organisms uncommonly isolated in veterinary diagnostic laboratories

	Streptobacillus moniliformis	*Chromobacterium violaceum*	*Capnophaga canimorsus*	*Ornithobacterium rhinotracheale*	*Riemerella anatipestifer*
Catalase	–	+	+	–	+
Oxidase	–	+	+	+	+
Growth on MacConkey agar	–	+	–	–	–
Citrate	–	v	–	–	–
Urease	–	v	–	+	v
Lysine decarboxylase	–	–	–	–	ND
Ornithine decarboxylase	–	–	–	–	ND
Arginine dehydrolase	v	+	v	+	+
Indole	–	–*	–	–	–[b]
Gelatin liquefaction	–	+	–	ND	+
Nitrate reduction	–	+	–	–	–
Motility	–	+[a]	–	–	–
Acid from:					
Galactose	+	–	+	ND	–
Glucose	+	+	+	–	–
Fructose	+	+	–	–	–
Lactose	+	–	+	+	–
Maltose	+	v	+	–	–
Mannitol	–	–	–	–	–
Raffinose	–	–	–	ND	–
Sucrose	v	v	–	–	–
Trehalose	–	+	–	–	–
Xylose	v	–	–	–	–
Pigment production	–	+	–	–	–

[a]Possesses pili and may show twitching motility

[b]Indole-positive and -negative variants are recognized

*Some pigmented strains are indole-positive

ND = data not available, + = more than 80% strains positive, v = 20–80% strains positive, – = less than 80% strains positive

slopes on prolonged storage. Inoculated plates are incubated aerobically at 35–37°C for 24–48 hours.

Identification

Colonial morphology

After 24 hours on blood agar the colonies may appear black, 1–2 mm in diameter, round, smooth, with a variable degree of partial haemolysis and smell of cyanide. Violacein production is enhanced by tryptophan and aerobic incubation at room temperature (22°C). Non-pigmented strains occur and these are occasionally isolated from both soil and clinical specimens. Growth occurs on MacConkey agar.

Microscopic appearance

Gram-stained smears show Gram-negative rods which may be slightly curved and tend to show barred or bipolar staining. The unique flagellar arrangement is seen best in preparations from young cultures grown on solid media. The lateral flagella are rare in broth cultures.

Biochemical reactions

Chromobacterium violaceum is included in the list of bacteria which can be identified by API 20E (bioMérieux) strips. The catalase test is positive but may be weak. If the violet pigment interferes with the oxidase reaction, the test should be performed on a culture grown anaerobically, as oxygen is required for pigment production. The sugar fermentation tests can be conducted in peptone water sugars. A biochemical profile of this bacterium is given in Table 32.3.

Rapid identification of the organism using a multiplex PCR assay has been reported (Scholz et al. 2006).

CAPNOCYTOPHAGA SPECIES

Capnocytophaga species belong to the order *Cytophagales*, and lack flagella but exhibit a characteristic gliding motility. They are Gram-negative, slender or filamentous rods, facultative anaerobes and capnophilic (carbon-dioxide-loving). All species ferment carbohydrates but never mannitol or xylose.

Natural Habitat and Pathogenicity

The *Capnocytophaga* species are commensals in the oral cavity of humans and although formerly associated with periodontal diseases, are now considered commensals of dental plaque (Jolivet-Gougeon et al. 2007). They cause a number of opportunistic infections and systemic disease, especially in immunocompromised humans.

Capnocytophaga canimorsus and *C. cynodegmi* are commensals in the nasopharynx, mouth and saliva of dogs and probably cats. Infections in man are associated with dog bites (occasionally cat bites) and vary from local sepsis to severe septicaemia, with endocarditis and meningitis in immunosuppressed, splenectomized or alcoholic patients.

Laboratory Diagnosis

Isolation

Specimens are inoculated on a heart-infusion agar with 5–10% blood and incubated under 5–10% CO_2 at 35–37°C for two to three days. Gliding motility is seen best on heart-infusion agar with 5% rabbit blood and the agar content increased to 2–3%.

Identification

Colonial morphology

The colonies of *C. canimorsus*, the principal species in the genus associated with dog bites, are 1–2 mm in diameter after 48 hours' incubation and are convex, smooth and circular.

Microscopic appearance

Capnocytophaga canimorsus organisms are long, thin Gram-negative rods (1–3 µm) with tapering ends. They can resemble the cells of *Fusobacterium* species in Gram-stained smears.

Biochemical characteristics

In tests for acid production from carbohydrates, positive results are more certain if relatively large inocula are used in small volumes of fluid. The broth base (3 mL) should contain 0.3 mL of filter-sterilized l0% carbohydrate solution and 0.1 mL sterile rabbit serum. The indole test is performed from 4 mL heart infusion broth containing 0.1 mL sterile rabbit serum. The culture is incubated for four days at 35°C, extracted with xylene and tested with Ehrlich reagent. Nitrate reduction is tested in 4 mL heart infusion broth containing 0.2% potassium nitrate and 0.1 mL sterile rabbit serum. Tubes should be incubated for four days.

The biochemical results for the *Capnocytophaga* species are summarized in Table 32.3. More recently, molecular techniques such as 16S rRNA gene sequencing, have proved useful in the identification of these organisms (Janda et al. 2006).

ORNITHOBACTERIUM RHINOTRACHEALE

The organism *O. rhinotracheale* was first characterized and named in the early 1990s although it had been isolated from clinical cases of respiratory disease in birds in the

1980s. It is a Gram-negative, highly pleomorphic rod-shaped organism and is closely related to another pathogen of birds, *Riemerella anatipestifer*. Respiratory disease associated with this pathogen has been reported worldwide although the severity of disease varies greatly depending on management factors and the presence of concurrent infections.

Natural Habitat and Pathogenicity

The organism has been isolated from many species of wild and domesticated birds. Both vertical and horizontal transmission of infection occur. Disease is reported most frequently in chickens and turkeys, with older birds showing more severe disease signs. Other respiratory pathogens such as *E. coli* or *Bordetella avium* are often isolated with *O. rhinotracheale* and contribute to the clinical lesions observed.

Laboratory Diagnosis

The organism can be readily isolated on 5–10% sheep blood agar, with optimal growth occurring at 37°C in an atmosphere supplemented with 5–10% CO_2. Colonies are small, grey and non-haemolytic. *Ornithobacterium rhinotracheale* does not grow on MacConkey agar. The organism can be identified using biochemical tests although results can be inconsistent. Cellular fatty acid composition is also used. Isolates are classified according to their serotype using agar gel precipitation; 18 serotypes are currently recognized. PCR-based typing has been reported by Thachil et al. (2007) and is suggested as a useful tool for characterization of clinical isolates and development of vaccines.

RIEMERELLA ANATIPESTIFER

Infection with *Riemerella anatipestifer* classically causes septicaemia in affected ducklings under six weeks of age. The organism can cause disease in ducks and turkeys and has been isolated from pigs, guillemots, a herring gull and a budgerigar (Hinz et al. 1998). It is a small Gram-negative rod and was previously known as *Pasteurella anatipestifer*.

Natural Habitat and Pathogenicity

Although mainly regarded as a pathogen of domestic ducks and geese, *R. anatipestifer* can cause significant losses in turkeys and has been isolated from a large number of wild and domestic bird species. Transmission of infection occurs via the respiratory tract and through skin wounds. The disease is known as 'new duck disease' and 'duck septicaemia' among other names and is precipitated by stress. Mortality may be up to 70%. Postmortem lesions include fibrinous exudate in the serosal cavities and the air sacs and meningitis may be present. Chronic localized skin lesions may occur.

Laboratory Diagnosis

The organism can be isolated on blood agar at 37°C. Optimal growth occurs in an atmosphere of 5–10% CO_2 following incubation for 48–72 hours. Colonies are small, transparent and glistening. There is no growth on MacConkey agar and most strains are non-haemolytic. As carbohydrates are not fermented in routine sugar fermentation tests, confirmation of identity is frequently performed using cellular fatty acid profiles. Biochemical properties can be used for identification (Table 32.3). Strains are classified using agar-gel precipitation or agglutination techniques and 21 serotypes are recognized. As more than one serotype may be present on a farm and serotypes may change over time, serotyping does not always provide useful information for epidemiological investigation. Molecular typing using PCR-based techniques or *Sma*I macrorestriction analysis may provide more useful information for such studies (Kiss et al. 2007).

REFERENCES

Breitschwerdt, E.B., 2008. Feline bartonellosis and cat scratch disease. Veterinary Immunology and Immunopathology 123, 167–171.

Chenoweth, M.R., Somerville, G.A., Krause, D.C., et al., 2004. Growth characteristics of *Bartonella henselae* in a novel liquid medium: primary isolation, growth-phase dependent phage induction, and metabolic studies. Applied and Environmental Microbiology 70, 656–663.

Chomel, B.B., Boulouis, H.J., Maruyama, S., et al., 2006. *Bartonella* spp. in pets and effect on human health. Emerging Infectious Diseases 12, 389–394.

Dörbecker, C., Sander, A., Oberle, K., et al., 2006. In vitro susceptibility of *Bartonella* species to 17 antimicrobial compounds: comparison of Etest and agar dilution. Journal of Antimicrobial Chemotherapy 58, 784–788.

Elliott, S.P., 2007. Rat bite fever and *Streptobacillus moniliformis*. Clinical Microbiology Reviews 20, 13–22.

Hinz, K.H., Ryll, M., Kohler, B., et al., 1998. Phenotypic characteristics of *Riemerella anatipestifer* and similar micro-organisms from various hosts. Avian Pathology 27, 33–42.

Janda, J.M., Graves, M.H., Lindquist, D., et al., 2006. Diagnosing *Capnocytophaga canimorsus* infections.

Emerging Infectious Diseases 12, 340–342.

Jolivet-Gougeon, A., Sixou, J.L., Tamanai-Shacoori, Z., et al., 2007. Antimicrobial treatment of *Capnocytophaga* infections. International Journal of Antimicrobial Agents 29, 367–373.

Jones, S.L., Maggi, R., Shuler, J., et al., 2008. Detection of *Bartonella henselae* in the blood of 2 adult horses. Journal of Veterinary Internal Medicine 22, 495–498.

Kiss, I., Kardos, G., Nagy, J., et al., 2007. DNA fingerprinting of *Riemerella anatipestifer* isolates from ducks. Veterinary Record 160, 26–28.

Maillard, R., Petit, E., Chomel, B., et al., 2007. Endocarditis in cattle caused by *Bartonella bovis*. Emerging Infectious Diseases 13, 1383–1385.

Mändle, T., Einsele, H., Schaller, M., et al., 2005. Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*. Blood 106, 1215–1222.

Rogosa, M., 1985. *Streptobacillus moniliformis* and *Spirillum minus*. In: Lennette, E.H., Balows, A., Hausler Jr., W.J., et al. (Eds.), Manual of Clinical Microbiology, fourth ed. American Society for Microbiology, Washington DC, pp. 400–406.

Sander, A., 2005. *Bartonella* and *Apfia*. In: Borriello, S.P., Murray, P.R., Funke, G. (Eds.), Topley and Wilson's Microbiology and microbial infections, Bacteriology Volume 2, tenth ed. Hodder Arnold, ASM Press, pp. 1885–1900.

Scholz, H.C., Witte, A., Tomaso, H., et al., 2006. Detection of *Chromobacterium violaceum* by multiplex PCR targeting the prgI, spaO, invG, and sipB genes. Systematic and Applied Microbiology 29, 45–48.

Thachil, A.J., Velayudhan, B.T., Lopes-Berkas, V.C., et al., 2007. Application of polymerase chain reaction fingerprinting to differentiate *Ornithobacterium rhinotracheale* isolates. Journal of Veterinary Diagnostic Investigation 19, 417–420.

Wouters, E.G., Ho, H.T., Lipman, L.J., et al., 2008. Dogs as vectors of *Streptobacillus moniliformis* infection? Veterinary Microbiology 128, 419–422.

Chapter 33

Chlamydiales

This large group of obligate intracellular bacteria is characterized by a unique, biphasic developmental cycle involving replication in cytoplasmic inclusion vacuoles within eukaryotic cells (Fig. 33.1). The order *Chlamydiales* consists of four families, *Chlamydiaceae, Parachlamydiaceae, Simkaniaceae* and *Waddliaceae*. A rapid expansion in the number of organisms within the order has occurred in recent years to include members from numerous animal species including invertebrates and environmental sources. Taxonomic changes have accompanied the expansion of the order (Everett et al. 1999). *Neochlamydia hartmannellae*, an amoebic endosymbiont, has been associated with ocular disease in cats (von Bomhard et al. 2003). *Waddlia chondrophila* and *Parachlamydia* species have been implicated in bovine abortion (Ruhl et al. 2009). From a veterinary and medical point of view, the most significant species are found in the family *Chlamydiaceae*. Currently nine species are recognized. There is on-going debate regarding the allocation of these species to a single genus (Stephens et al. 2009, Greub 2010a, 2010b, Kuo et al. 2011) or to two genera (Everett & Andersen 1999, Everett et al. 1999). Phylogenetic analyses (Voigt et al. 2012) support the recognition of *Chlamydophila* species as a distinct evolutionary branch (Table 33.1). Chlamydiae have been termed 'energy parasites' on account of their apparent inability to generate ATP and resulting dependence on the host cell to supply NTPs. Both plasmids and bacteriophages are found in chlamydiae.

The developmental cycle of chlamydiae involves morphologically distinct infectious and reproductive forms (Figs. 33.1 and 33.2). The infectious extracellular form, termed the elementary body (EB), is small (200–300 nm), metabolically inert and osmotically stable. The EB is surrounded by a conventional bacterial cytoplasmic membrane, a periplasmic space and a lipopolysaccharide-containing outer envelope. The periplasmic space does not contain a detectable peptidoglycan layer and the EB relies on disulphide cross-linked envelope proteins for osmotic stability (Hatch 1996). The EB enters a host cell by receptor-mediated endocytosis. Acidification of the endosome and subsequent phagolysosomal fusion is prevented by a mechanism that is not fully understood. A process of reorganization lasting several hours follows and results in the conversion of the EB into the reticulate body (RB). The RB is about 1 µm in diameter, metabolically active, osmotically fragile and divides by binary fission to produce more RBs. In cells infected with *C. trachomatis* and containing more than one inclusion, fusion of the inclusions is possible. At about 20 hours post infection the cycle becomes asynchronous with some RBs continuing to divide while others condense and mature to form EBs. In general the cycle continues till about 30–72 hours after infection when the host cell lyses, releasing several hundred EBs, RBs and intermediate forms. Under stressful conditions such as nutrient deprivation, particularly shortages of the amino acids tryptophan and cysteine, certain immunological responses such as the presence of gamma interferon or exposure to antibiotics such as penicillin, chlamydial replication may become suspended resulting in a state of persistent infection and the appearance of large, aberrant RBs. Delayed infections of this nature appear to be important in the development of immunopathology, associated with diseases such as trachoma and pelvic inflammatory disease in humans.

Natural Habitat

Elementary bodies are relatively resistant, remaining viable in the environment for several days if conditions are suitable. The gastrointestinal tract appears to be the

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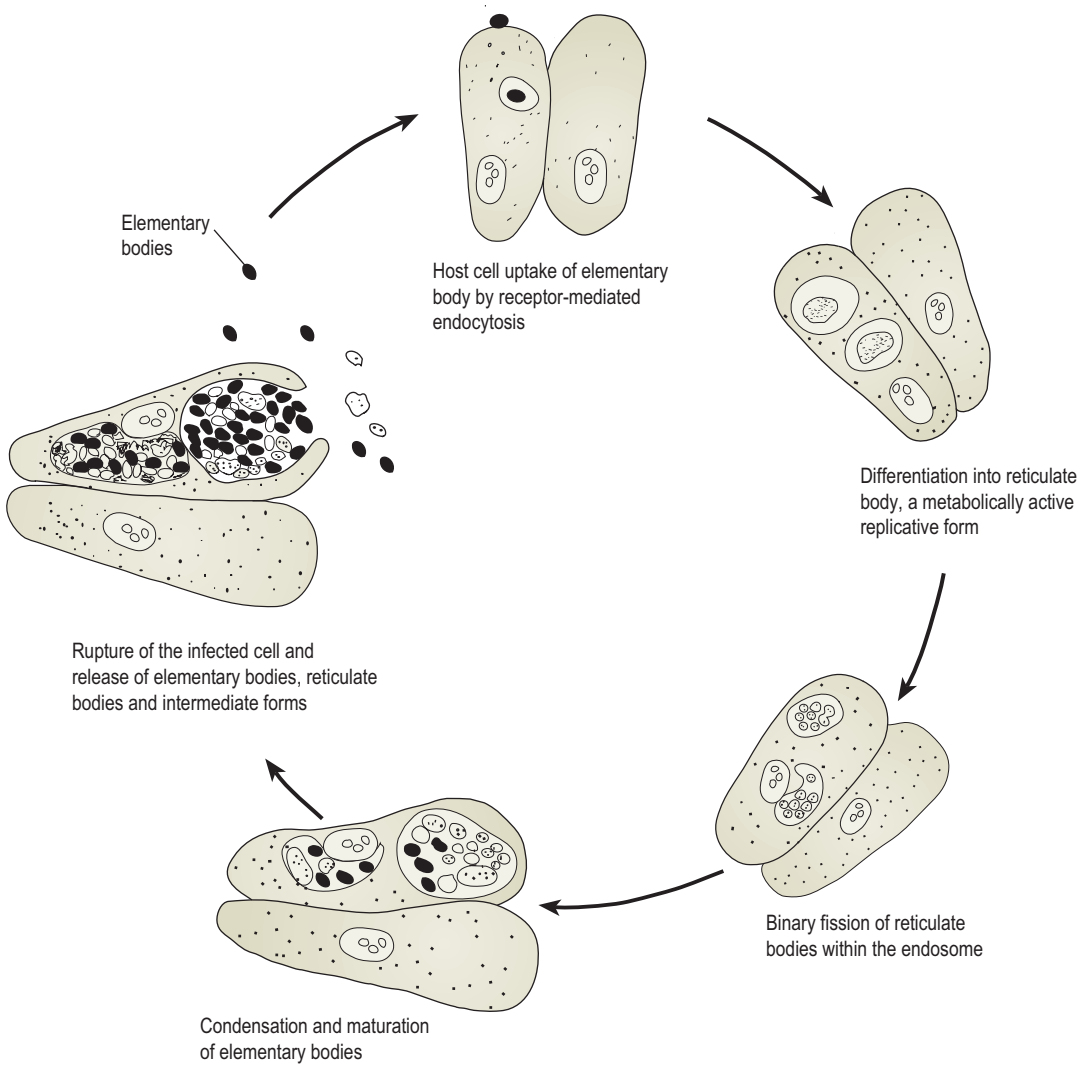

Figure 33.1 Representation of the life cycle of chlamydiae.

natural site for *C. psittaci* and *C. pecorum* infection in birds and mammals. Intestinal infections are frequently subclinical and persistent. Faecal shedding of the organism becomes intermittent over time but is typically prolonged.

Pathogenesis and Pathogenicity

Many chlamydial infections are asymptomatic, particularly where the infections are localized to superficial epithelial or mucosal surfaces, and may persist for long periods while inducing little protective immunity. Chronic, recurrent infections may provide an opportunity for repeated stimulation of the host immune system with particular chlamydial antigens. In a number of diseases such as trachoma or pelvic inflammatory disease in humans the pathology encountered is more severe than would be expected from infection alone. Chlamydiae possess a number of heat shock proteins which are similar to heat shock proteins in other bacteria and to a number of human mitochondrial proteins. It is believed that chronic stimulation of the immune system with these proteins contributes significantly to the delayed type hypersensitivity responses associated with tubal or conjunctival scarring in pelvic inflammatory disease and trachoma, respectively. Gamma interferon is produced in response to chlamydial infection and has been shown to be chlamydiastatic. It is likely that gamma interferon is important in helping to control primary chlamydial infections. However, there is also evidence that gamma interferon is responsible

Table 33.1 Chlamydial infections of importance

Species	Host	Significance of infection
Chlamydophila (*Chlamydia*) *psittaci*	Avian species	Pneumonia, air sacculitis Intestinal infection, diarrhoea Conjunctivitis Pericarditis Encephalitis
	Humans	Psittacosis/ornithosis
Chlamydophila (*Chlamydia*) *abortus*	Sheep	Enzootic abortion of ewes (EAE)
	Goats	Abortion
	Cattle	Abortion
	Pigs	Abortion
Chlamydophila (*Chlamydia*) *pecorum*	Sheep	Intestinal infection Conjunctivitis Polyarthritis
	Cattle	Sporadic bovine encephalomyelitis Polyarthritis Metritis
	Koalas	Urogenital infection Conjunctivitis
Chlamydophila (*Chlamydia*) *felis*	Cats	Conjunctivitis (feline pneumonitis)
Chlamydophila (*Chlamydia*) *caviae*	Guinea pigs	Guinea pig inclusion conjunctivitis (GPIC)
Chlamydophila (*Chlamydia*) *pneumoniae*	Humans	Respiratory tract infection Atherosclerosis
	Horses	Respiratory tract infection
	Koalas	Respiratory tract infection
Chlamydia trachomatis Three biovars: trachoma (serovars A, B, Ba or C), urethritis (serovars D-K) and lymphogranuloma venereum (serovars L1, L2, L3)	Humans	Trachoma, inclusion conjunctivitis of infants Non-specific urethritis Respiratory disease of infants Proctitis Lymphogranuloma venereum Arthritis
Chlamydia suis	Pigs	Intestinal tract infection
Chlamydia muridarum	Mice	Respiratory tract infection

for the induction of a state of latency or persistence in chlamydial infections which in turn may be responsible for up-regulation of heat shock protein expression (Ward 1995).

Clinical Infections

A very wide range of species of animals, including invertebrates, are known to be susceptible to infection with chlamydiae (Vanrompay et al. 1995). In mammals and birds, both the severity and the type of disease produced by chlamydiae are highly variable, ranging from clinically inapparent infections to local infections of epithelial surfaces to systemic infections spread by the bloodstream (Table 33.1). In mammalian species chlamydiae have been associated with intestinal infection, gastritis, pneumonia, abortion, urogenital infection, mastitis, polyarthritis, polyserositis, conjunctivitis, encephalomyelitis and hepatitis. In avian species chlamydiae are associated with generalized infection, pneumonia, air sacculitis, pericarditis, encephalitis, diarrhoea and conjunctivitis. A single clinical presentation usually predominates in an outbreak or series

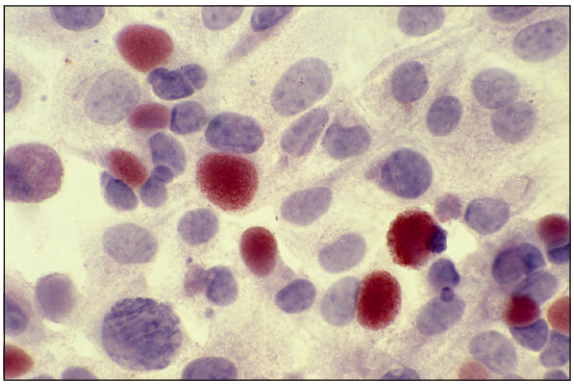

Figure 33.2 Immunoperoxidase staining with haematoxylin counter stain. *Chlamydophila* (*Chlamydia*) *abortus* (enzootic abortion of ewes) intracellular inclusions (red-brown) in McCoy cells. (×400)

of related clinical cases. The factors influencing the clinical signs and their severity include:

- Species or strain of chlamydia. In ruminants, *C. abortus* has a tropism for the trophoblast cells of the placenta and is an important cause of abortion, whereas infections with *C. pecorum* are frequently inapparent. There are at least six avian serotypes of *C. psittaci* labelled A through F. The syndromes and host range of particular serotypes are fairly specific: serotype A, psittacine birds; serotype B, pigeons; serotype C, ducks; serotype D, turkeys; serotype E, pigeons and ratites; and one isolate of serotype F from a psittacine bird (Andersen 1991, 1997). The serotypes can be distinguished using serovar-specific monoclonal antibodies or the PCR-RFLP technique (Vanrompay et al. 1997). Three further genotypes are generally accepted: genotype E/B, group of isolates from ducks, turkeys and pigeons; and two mammalian isolates, WC in cattle and M56 in muskrats (Geens et al. 2005). Based on sequence analysis of the *ompA* gene a further six genotypes of *C. psittaci* have been described (Sachse et al. 2008).
- Species of host. Chlamydial strains are associated with specific diseases in particular hosts. Interspecies transmission is uncommon. Disease in a secondary host may be similar as occurs when *C. abortus* is transmitted from sheep to catttle or it may be more severe as can occur when *C. abortus* transmits from sheep to pregnant women.
- Age, sex and physiological state. Lambs usually acquire *C. abortus* infections early in life on endemically infected farms. However, clinical signs do not become apparent until the animal is pregnant. Clinical signs are rare in infected rams.
- Route of infection and degree of exposure to chlamydiae. *Chlamydophila* (*Chlamydia*) *pecorum* is associated with conjunctivitis, arthritis and inapparent intestinal infection in sheep.
- Environment and management practices. Enzootic abortion of ewes is a disease of lowland, intensively managed flocks where indoor lambing facilitates the spread of infection to neonates and in-contact ewes.

Chlamydial abortion is described in cattle, sheep and goats. Enzootic abortion of ewes (EAE) is a major cause of economic loss in intensive sheep production. Chlamydial abortion is regarded as a serious problem in many sheep-rearing areas of the world. However, despite serological and cultural evidence of *C. abortus* infections in sheep, the disease appears to be extremely rare in countries such as Australia. Outbreaks of chlamydial polyarthritis have been reported in feedlot lambs in both the USA and Australia. In other countries this condition in lambs tends to be sporadic.

Sporadic bovine encephalomyelitis has been reported from the USA, Japan and Israel. The disease is characterized by inflammation of vascular endothelium throughout the body. However, nervous signs tend to predominate. The prevalence of clinical disease in infected herds is usually low.

The first feline isolate of *C. psittaci* was obtained from a cat with pneumonia and the disease was named feline pneumonitis. The majority of subsequent isolations have been from cats with mild upper respiratory tract infections and conjunctivitis. Affected cats recover spontaneously but tend to suffer recurrent episodes of conjunctivitis at intervals of 10–14 days. Psittacosis (ornithosis) is an acute or chronic infection of wild and domestic birds, characterized by respiratory, intestinal and systemic signs. It occurs worldwide and is transmissible to other animals, including man. Respiratory discharges and faeces are infective. A carrier state can exist after infection and stress may provoke recurrence of disease. The term 'psittacosis' refers to the infection in psittacine birds and 'ornithosis' to the disease in non-psittacine species. The clinical signs are similar in all birds, although chronic infections tend to occur more frequently in psittacine birds.

Chlamydophila (*Chlamydia*) *psittaci* strains are transmissible to man with varying efficiency. Isolates of *C. psittaci* are considered the most zoonotic of the chlamydial pathogens of animals but human infections may also be acquired following contact with aborting ewes (*C. abortus*) or cats with conjunctivitis (*C. felis*). Human infections acquired from psittacine species are termed psittacosis while those acquired from other avian species are termed ornithosis. There is no significant difference in the clinical course of either condition. Infection is usually acquired following inhalation of aerosols with infections varying from inapparent to severe systemic disease. Pulmonary involvement is common. Infected humans typically have

a cough, headaches, fever and myalgia. Severe cases may develop meningitis or meningoencephalitis. Death is rare provided adequate treatment is received. Diagnosis is usually based on the collection of paired serum samples and the demonstration of a significant rise in antibody titre by CFT or immunofluorescence. Psittacosis is a notifiable disease in many European countries as well as the USA and Australia. *Chlamydophila abortus* is a serious and potentially life-threatening zoonosis in pregnant women (Johnson et al. 1985, Buxton 1986). Pregnant women should avoid all contact with lambing ewes.

Laboratory Diagnosis

The history, clinical signs and pathology may be indicative of certain distinctive chlamydial infections such as feline chlamydiosis, sporadic bovine encephalomyeitis or enzootic abortion of ewes.

Specimens

The type of specimens suitable for diagnosis will vary depending on the clinical presentation and the diagnostic procedure being used. Specimens for isolation of the organism should be placed in a suitable transport medium based on sucrose-phosphate-glutamate medium supplemented with foetal calf serum, aminoglycoside antibiotics and an antifungal agent (Spencer & Johnson 1983). Chlamydiae are thermolabile and samples should be kept at 4°C. Longer-term storage can be achieved by holding samples at −70°C although each cycle of freezing and thawing will reduce the titre of the organism.

- **Abortion**: confirmation of a diagnosis of EAE is most easily accomplished by microscopic examination of stained smears from affected cotyledons or chorion for evidence of chlamydial EBs. If the placenta is unavailable, smears may be made from vaginal swabs taken within one to two days of abortion, or from the wet fleece of an aborted foetus. Culture is more sensitive than direct microscopy on stained smears and may be used to diagnose EAE from uterine discharges or foetal tissues in the absence of foetal membranes (Johnson et al. 1983).
- Suitable samples for isolation of chlamydiae include a small piece of affected cotyledon, vaginal swabs, foetal lung and foetal liver. These should be placed in chlamydial transport medium (Appendix 2) and held at 4°C.
- **Polyarthritis**: aspirated synovial fluid should be collected.
- **Conjunctivitis**: a conjunctival swab is an acceptable specimen.
- **Systemic infection**: samples of lung, liver and spleen should be submitted.

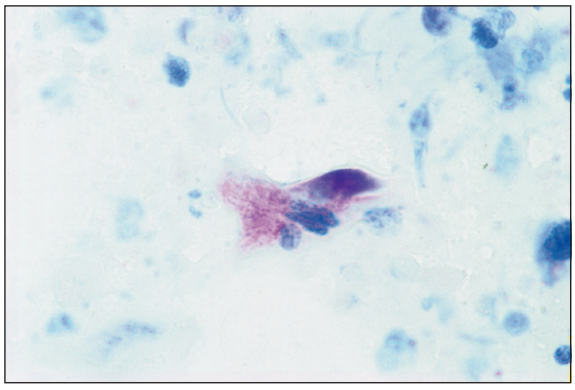

Figure 33.3 *Chlamydophila* (*Chlamydia*) *psittaci* in a MZN-stained smear from a bird with psittacosis. (×1000)

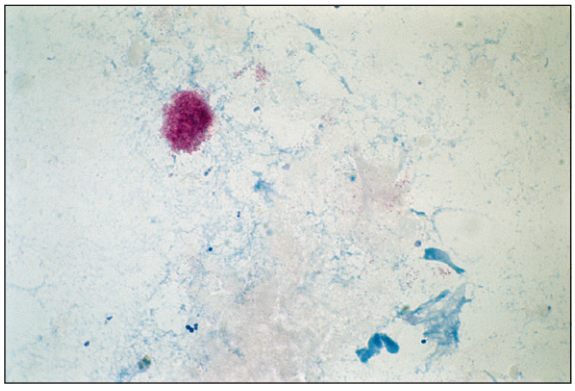

Figure 33.4 *Chlamydophila* (*Chlamydia*) *abortus* in a MZN-stained cotyledonary smear from a ewe with enzootic abortion (EAE). (×1000)

- Paired serum samples should be collected for serology, from both affected and clinically normal animals in the herd or flock.

Direct microscopy

It is possible to detect EBs in smears or tissue sections by the use of either chemical stains (Figs. 33.3 and 33.4) or immunological staining techniques such as immunofluorescence or immunoperoxidase (Szeredi & Bacsadi 2002). The latter staining methods are more sensitive than chemical stains and can be highly specific if based on monoclonal antibodies. In stained smears *Brucella* species and *Coxiella burnetii*, which are also MZN-positive, may look very similar to chlamydiae but can be differentiated by immunological staining, serologically or following isolation of the organism. Suitable chemical stains include:

- Modified Ziehl–Neelsen (MZN) stain (Appendix 1). The EBs tend to be in clumps and stain red against a blue background (methylene blue counter-stain) or a

green background, if malachite green is used as the counter-stain. If the MZN-stained smears are examined under darkfield microscopy, the EBs appear as bright green, coccal structures.
- Methylene blue stain (Appendix 1). If the stained smear is examined under darkfield illumination, the EBs are revealed as refractile, yellow-green bodies surrounded by a halo (Dagnall & Wilsmore 1990).
- Method of Macchiavello (Appendix 1). The EBs stain red against a blue background.
- Method of Castaneda (Appendix 1). The EBs are stained blue with a reddish background.
- Giemsa stain (Appendix 1). This stain is useful for staining smears prepared from conjunctival scrapings, particularly those from cats with feline pneumonitis. Infected conjunctival epithelial cells contain basophilic intracytoplasmic aggregates of *C. felis*.

Antigen detection

Several commercial kit sets, predominantly using ELISA methodology, have been developed for the detection of *C. trachomatis*. Many of these kit sets are based on detection of chlamydial lipopolysaccharide (LPS), a common antigen possessed by all chlamydial species, and consequently can be used to detect the LPS of other chlamydial species (Wilsmore & Davidson 1991, Wood & Timms 1992, Vanrompay et al. 1994).

Isolation and cultivation

Three laboratory systems can be used:

1. Mouse inoculation: this technique was used in the past, but currently has little place in a routine diagnostic laboratory.
2. Inoculation of embryonated hens' eggs (six to seven days old) via the yolk sac route: this method will detect all chlamydiae but it is laborious and prone to bacterial contamination.
3. Cell culture: a number of continuous cell lines are susceptible to chlamydial infection such as McCoy, baby hamster kidney-21, L929 and Vero. Chemical treatment of the cells with cycloheximide (1 µg/mL), 5-iodo-2-deoxyuridine (80 µg/mL), cytochalasin B (2 µg/mL), emetine (1 µg/mL) or diethylaminoethyl-dextran (20 µg/mL) and centrifugation of the sample onto the monolayer greatly enhance the sensitivity of the isolation procedure. After two to three days' incubation at 37°C the monolayer is fixed, stained as described above and examined for the presence of chlamydial inclusions (Figs. 33.5, 33.6 and 33.7). The use of antibiotics in the tissue culture medium to which chlamydiae are sensitive (oxytetracycline, erythromycin, penicillin, tylosin) should be avoided.

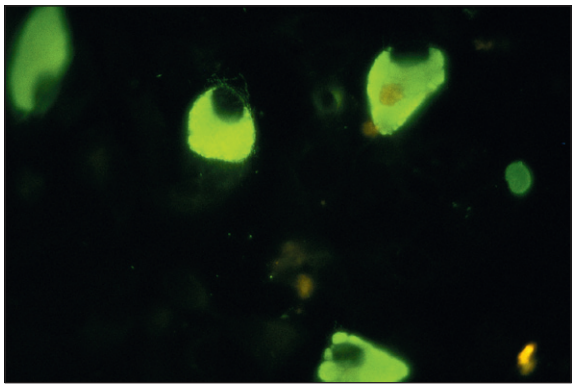

Figure 33.5 *Chlamydophila* (*Chlamydia*) *abortus* inclusions in McCoy cells. (Direct FA technique, ×400)

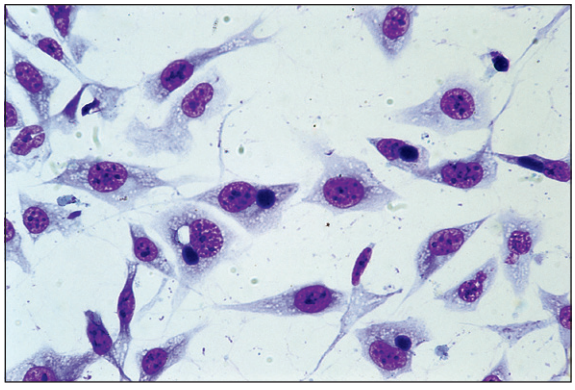

Figure 33.6 *Chlamydophila* (*Chlamydia*) *abortus* inclusions in McCoy cells. (Giemsa stain, ×400)

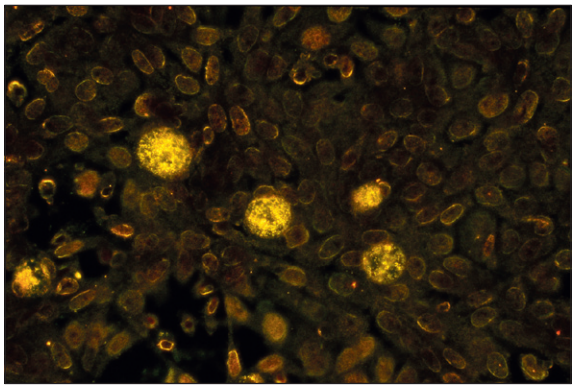

Figure 33.7 *Chlamydia trachomatis* in McCoy cells demonstrating autofluorescence of the inclusions (yellow) under darkfield illumination. (Giemsa stain, ×400)

Serology

A number of tests capable of detecting chlamydial antibody are in use:

- Complement fixation test (CFT): this is the standard serological test used. However, it is time-consuming and not particularly sensitive. The modified direct CFT is generally used for avian sera.
- Enzyme-linked immunosorbent assay (ELISA). This assay is generally considered more sensitive than the CFT but lacking species-specificity (Jones et al. 1997).
- Indirect immunofluorescence and microimmunofluorescence test. These tests are sensitive and specific but time-consuming.

On account of the widespread distribution and often subclinical nature of chlamydial infection in sheep, it is necessary to demonstrate an unusually high or rising antibody titre associated with the occurrence of abortion. Interpretation of results is complicated by the fact that many of the available serological tests primarily detect antibodies against chlamydial LPS and therefore will not differentiate between infections caused by different chlamydial species nor will they distinguish between responses to vaccination and infection. In addition, there is cross-reactivity between the LPS of chlamydiae and that of certain other Gram-negative bacteria such as *Acinetobacter* species. Complement fixation test titres less than 1/32 are generally considered to be non-specific. Recent efforts to produce improved serological assays have largely concentrated on the development of more sensitive yet specific assays based on ELISA methodology (Salti-Montesanto et al. 1997, Buendia et al. 2001, Hoelzle et al. 2004, Livingstone et al. 2005). In particular, efforts have been directed to diagnosing *C. abortus* in sheep by the use of recombinant proteins (Wilson et al. 2009). Current assays do not distinguish between vaccinated and infected animals.

Molecular Diagnosis

Conventional and real time polymerase chain reaction techniques have been developed for the detection of chlamydial DNA in samples (Hewinson et al. 1997, Messmer et al. 1997, Laroucau et al. 2001, Sachse & Hotzel 2003, Dean et al. 2005, Geens et al. 2005, Gutierrez et al. 2012). It is possible to distinguish different chlamydial species present in samples by the use of specific primers, restriction fragment length polymorphism and probes (Sheehy et al. 1996, Vanrompay et al. 1997, Everett & Andersen 1999, Pantchev et al. 2009). DNA microarray technology is available for the detection and identification of chlamydiae (Sachse et al. 2005, Ehricht et al. 2006, Sachse et al. 2008).

REFERENCES

Andersen, A.A., 1991. Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the micro-immunofluorescence test. Journal of Clinical Microbiology 29, 707–711.

Andersen, A.A., 1997. Two new serovars of *Chlamydia psittaci* from North American birds. Journal of Veterinary Diagnostic Investigation 9, 159–164.

Buendia, A.J., Cuello, F., Delrio, L., et al., 2001. Field evaluation of a new commercially available ELISA based on a recombinant antigen for diagnosing *Chlamydophila abortus* (*Chlamydia psittaci* serotype 1) infection. Veterinary Microbiology 78, 229–239.

Buxton, D., 1986. Potential danger to pregnant women of *Chlamydia psittaci* from sheep. Veterinary Record 118, 510–511.

Dagnall, G.J.R., Wilsmore, A.J., 1990. A simple staining method for the identification of chlamydial elementary bodies in the foetal membranes of sheep affected by ovine enzootic abortion. Veterinary Microbiology 21, 232–239.

Dean, R., Harley, R., Helps, C., et al., 2005. Use of quantitative real-time PCR to monitor the response of *Chlamydophila felis* infection to doxycycline treatment. Journal of Clinical Microbiology 43, 1858–1864.

Ehricht, R., Slickers, P., Goellner, S., et al., 2006. Optimized DNA microarray assay allows detection and genotyping of single PCR-amplifiable target copies. Molecular and Cellular Probes 20, 60–63.

Everett, K.D., Andersen, A.A., 1999. Identification of nine species of the *Chlamydiaceae* using PCR RFLP. International Journal of Systematic Bacteriology 49, 803–813.

Everett, K.D., Bush, R.M., Andersen, A.A., 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. International Journal of Systematic Bacteriology 49, 415–440.

Geens, T., Dewitte, A., Boon, N., et al., 2005. Development of a *Chlamydophila psittaci* species-specific and genotype specific real-time PCR. Veterinary Research 36, 787–797.

Greub, G., 2010a. Minutes of the Subcommittee on the Taxonomy of the Chlamydiae. International Journal of Systematic and Evolutionary Microbiology 60, 2691–2693.

Greub, G., 2010b. Minutes of the Subcommittee on the Taxonomy of the Chlamydiae. International Journal of Systematic and Evolutionary Microbiology 60, 2694.

Gutierrez, J., O'Donovan, J., Proctor, A., et al., 2012. Application of

quantitative real-time polymerase chain reaction for the diagnosis of toxoplasmosis and enzootic abortion of ewes. Journal of Veterinary Diagnostic Investigation 24, 845–853.

Hatch, T.P., 1996. Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? Journal of Bacteriology 178, 1–5.

Hewinson, R.G., Griffiths, P.C., Bevan, B.J., et al., 1997. Detection of *Chlamydia psittaci* DNA in avian clinical samples by polymerase chain reaction. Veterinary Microbiology 54, 155–166.

Hoelzle, L.E., Hoelzle, K., Wittenbrink, M.M., 2004. Recombinant major outer membrane protein (MOMP) of *Chlamydophila abortus, Chlamydophila pecorum,* and *Chlamydia suis* as antigens to distinguish chlamydial species-specific antibodies in animal sera. Veterinary Microbiology 103, 85–90.

Johnson, F.W.A., Clarkson, M.J., Spenser, W.N., 1983. Direct isolation of the agent of enzootic abortion of ewes (*Chlamydia psittaci*) in cell cultures. Veterinary Record 113, 413–414.

Johnson, F.W.A., Matheson, B.A., Williams, H., et al., 1985. Abortion due to infection with *Chlamydia psittaci* in a sheep farmer's wife. British Medical Journal 290, 592–594.

Jones, G.E., Low, J.C., Machell, J., et al., 1997. Comparison of five tests for the detection of antibodies against chlamydial (enzootic) abortion of ewes. Veterinary Record 141, 164–168.

Kuo, C.C., Stephens, R.S., Bavoil, P.M., et al., 2011. Genus I, *Chlamydia* Jones, Rake and Stearns 1945, 55. In: Kreig, N.R., Staley, J.T., Brown, D.R., et al. (Eds.), Bergey's Manual of Systematic Bacteriology, second edn. vol. 4. Springer, New York, pp. 846–865.

Laroucau, K., Souriau, A., Rodolakis, A., 2001. Improved sensitivity of PCR for *Chlamydophila* using pmp genes. Veterinary Microbiology 82, 155–164.

Livingstone, M., Entrican, G., Wattegedera, S., et al., 2005. Antibody responses to recombinant protein fragments of the major outer membrane protein and polymorphic outer membrane protein POMP90 in *Chlamydophila abortus*-infected pregnant sheep. Clinical and Diagnostic Laboratory Immunology 12, 770–777.

Messmer, T.O., Skelton, S.K., Moroney, J.F., et al., 1997. Application of a nested, multiplex PCR to psittacosis outbreaks. Journal of Clinical Microbiology 35, 2043–2046.

Pantchev, A., Sting, R., Bauerfeind, R., et al., 2009. New real-time PCR tests for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila abortus* from tissue samples. Veterinary Journal 181, 145–150.

Ruhl, S., Casson, N., Kaiser, C., 2009. Evidence for *Parachlamydia* in bovine abortion. Veterinary Microbiology 135, 169–174.

Sachse, K., Hotzel, H., 2003. Detection and differentiation of chlamydiae by nested PCR. Methods in Molecular Biology 216, 123–136.

Sachse, K., Hotzel, H., Slickers, P., et al., 2005. DNA microarray-based detection and identification of *Chlamydia* and *Chlamydophila*. Molecular and Cellular Probes 19, 41–50.

Sachse, K., Laroucau, K., Hotzel, H., et al., 2008. Genotyping of *Chlamydophila psittaci* using a new DNA microarray assay based on sequence analysis of ompA genes. BMC Microbiology 8, 63.

Salti-Montesanto, V., Tsoli, E., Papavassiliou, P., et al., 1997. Diagnosis of ovine enzootic abortion, using a competitive ELISA based on monoclonal antibodies against variable segments 1 and 2 of the major outer membrane protein of *Chlamydia psittaci* serotype 1. American Journal of Veterinary Research 58, 228–235.

Sheehy, N., Markey, B., Gleeson, M., et al., 1996. Differentiation of *Chlamydia psittaci* and *C. pecorum* strains by species specific-PCR. Journal of Clinical Microbiology 34, 3175–3179.

Spencer, W.N., Johnson, F.W.A., 1983. Simple transport medium for the isolation of *Chlamydia psittaci* from clinical material. Veterinary Record 113, 535–536.

Stephens, R.S., Myers, G., Eppinger, M., et al., 2009. Divergence without difference: phylogenetics and taxonomy of Chlamydia resolved. FEMS Immunology and Medical Microbiology 55, 115–119.

Szeredi, L., Bacsadi, A., 2002. Detection of *Chlamydophila* (*Chlamydia*) *abortus* and *Toxoplasma gondii* in smears from cases of ovine and caprine abortion by the streptavidin-biotin method. Journal of Comparative Pathology 127, 257–263.

Vanrompay, D., Van Nerom, A., Ducatelle, R., et al., 1994. Evaluation of five immunoassays for detection of *Chlamydia psittaci* in cloacal and conjunctival specimens from turkeys. Journal of Clinical Microbiology 32, 1470–1474.

Vanrompay, D., Ducatelle, R., Haesebrouck, F., 1995. *Chlamydia psittaci* infections: a review with emphasis on avian chlamydiosis. Veterinary Microbiology 45, 93–119.

Vanrompay, D., Butaye, P., Sayada, C., et al., 1997. Characterization of avian *Chlamydia psittaci* strains using omp1 restriction mapping and serovar-specific monoclonal antibodies. Research in Microbiology 148, 327–333.

Voigt, A., Schofl, G., Saluz, H.P., 2012. The *Chlamydia psittaci* genome: a comparative analysis of intracellular pathogens. PLOS ONE 7, e35097.

von Bomhard, W., Polkinghorne, A., Lu, Z.H., et al., 2003. Detection of novel chlamydiae in cats with ocular disease. American Journal of Veterinary Research 64, 1421–1428.

Ward, M.E., 1995. The immunobiology and immunopathology of chlamydial infections. APMIS 103, 769–796.

Wilsmore, A.J., Davidson, I., 1991. 'Clearview' rapid test compared with other methods to diagnose chlamydial infection. Veterinary Record 128, 503–504.

Wilson, K., Livingstone, M., Longbottom, D., 2009. Comparative evaluation of eight serological assays for diagnosing *Chlamydophila abortus* infection in sheep. Veterinary Microbiology 135, 38–45.

Wood, M.M., Timms, P., 1992. Comparison of nine antigen detection kits for diagnosis of urogenital infections due to *Chlamydia psittaci* in koalas. Journal of Clinical Microbiology 30, 3200–3205.

FURTHER READING

Andersen, A.A., 2008. Avian chlamydiosis. In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, sixth edn. OIE, Paris, France, pp. 431–442.

Longbottom, D., Coulter, L.J., 2003. Animal chlamydioses and zoonotic implications. Journal of Comparative Pathology 128, 217–244.

Rodolakis, A., Salinas, J., Papp, J., 1998. Recent advances on ovine chlamydial abortion. Veterinary Research 29, 275–288.

Sachse, K., Vretou, E., Livingstone, M., et al., 2009. Recent developments in the laboratory diagnosis of chlamydial infections. Veterinary Microbiology 135, 2–21.

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Chapter 34

Rickettsiales and *Coxiella burnetii*

The members of the *Rickettsiales* are minute, non-motile, pleomorphic, obligate intracellular Gram-negative bacteria. They are rods or coccobacilli, visible under the light microscope and varying in size from 0.3–0.6 µm in length. They are aerobic and divide by binary fission. The order comprises two families of veterinary significance. The members of the *Rickettsiaceae* (Family) have cell walls similar to those of other Gram-negative bacteria, but those belonging to members of the *Anaplasmataceae* lack a peptidoglycan layer. They all stain poorly with basic aniline dyes, such as those of the Gram stain, but they stain well with Giemsa and other Romanowsky stains. A few of the *Rickettsiaceae* will grow in conventional inert media, but most require living cells for propagation and are cultured in the yolk sac of fertile eggs or in tissue culture.

Significant re-classification of the order has occurred in recent years (Dumler et al. 2001), much of it based on DNA sequencing information and in particular 16S and 23S rRNA gene sequence comparisons. The original tribe structure, the *Rickettsiaceae* family, has been eliminated, while members of the former tribe *Ehrlichieae* have been placed in the family *Anaplasmataceae*. The family *Rickettsiaceae* now comprises two genera, *Rickettsia* and *Orienta* while the family *Anaplasmataceae* comprises five genera; *Anaplasma, Ehrlichia, Neorickettsia, Aegyptianella* and *Wolbachia*. The status of the genus *Aegyptianella* is somewhat uncertain with recent data suggesting a close relationship to the genus *Anaplasma*. Several species have been reassigned including *Cowdria ruminantium* to the genus *Ehrlichia* and *Ehrlichia (Cytoecetes) phagocytophila* to the genus *Anaplasma*. The genera *Haemobartonella* and *Eperythrozoon*, formerly assigned within the *Anaplasmataceae*, contain haemotrophic bacteria that lack a cell wall which have been shown to be closely aligned with species in the *Mycoplasma pneumoniae* group. These microorganisms now represent a new clade within the genus *Mycoplasma* (Neimark et al. 2001, 2002). The bacteria formerly known as HGE (human granulocytic ehrlichiosis) agent, *Ehrlichia equi* and *Ehrlichia phagocytophila* have been unified under the single species name of *Anaplasma phagocytophilum*. *Coxiella burnetii*, the cause of Q fever, is now known to be closely related to *Legionella* species and *Francisella tularensis* and has been re-classified to the gamma subgroup of the proteobacteria within the order *Legionellales*. For convenience, *Coxiella burnetii* is dealt with in this chapter as a separate section.

Natural Habitat

Members of the *Rickettsiales* are essentially parasites of arthropods, replicating in the cells of the gut before spreading to other organs such as salivary glands and ovaries. This requirement for an invertebrate vector distinguishes these organisms from most other bacteria. Some can be passed transovarially in ticks (*R. rickettsii*) and mites but others, such as *Ehrlichia canis, Anaplasma phagocytophilum* and *Ehrlichia ruminantium* are passed transstadially but not transovarially. *Neorickettsia* species infect a number of fluke species and all stages of the life cycle of the fluke are infectious. A number of pathogenic species, such as *Ehrlichia canis* and *Anaplasma marginale* persist in the host in a latent form.

Pathogenesis

Infection typically occurs as a result of the bite of an affected arthropod or following ingestion of infected flukes in the case of salmon poisoning and Potomac horse fever. The types of cells parasitized and disease pathogenesis varies with the rickettsial species (Table 34.1). Toxins,

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Table 34.1 Families and genera of the order *Rickettsiales* of veterinary importance and cell types targeted

Family	Genus	Principal cells parasitized in host
Rickettsiaceae	*Rickettsia*	Vascular endothelium
Anaplasmataceae	*Anaplasma*	Erythrocytes, platelets and leukocytes
	Aegyptianella	Erythrocytes
	Ehrlichia	Leukocytes
	Neorickettsia	Leukocytes

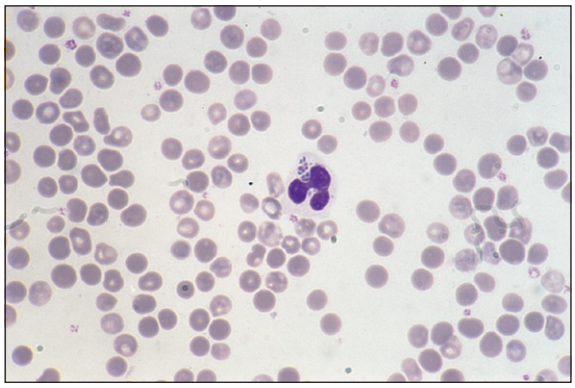

Figure 34.1 Leishman-stained blood smear from a sheep showing a neutrophil containing *Anaplasma* (*Ehrlichia*) *phagocytophilum*. (×1000)

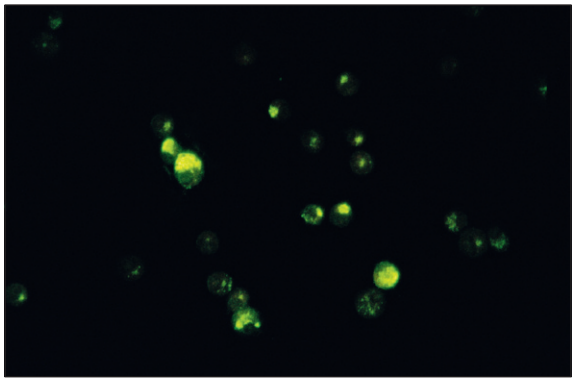

Figure 34.2 *Neorickettsia risticii* in mouse macrophages. (Direct FA technique, ×1000)

haemolysins and endotoxin-like lipopolysaccharides are known to be produced by some members of the group. *Rickettsia rickettsii* produces a phospholipase which aids escape from the phagosome soon after engulfment. Once in the cytosol nutrient acquisition and replication by binary fission is possible. The clinical signs of Rocky Mountain spotted fever are due to damage to the capillary endothelial cells, resulting in a necrotizing vasculitis and perivasculitis. Haemorrhages, thrombosis, oedema, loss of plasma and shock may follow. *Ehrlichia* species have a predilection for leukocytes and manage to replicate inside phagosomes by inhibiting fusion of the phagosome with a lysosome. When mammalian erythrocytes are parasitized, as in anaplasmosis or aegyptianellosis, anaemia probably results from the immune clearance of the altered erythrocytes. *Anaplasma phagocytophilum* and *A. bovis* are capable of surviving and multiplying within granulocytes while *A. platys* targets platelets. The principal diseases, hosts, vectors and geographical distribution of pathogens of veterinary importance in the *Rickettsiales* are summarized in Table 34.2.

Laboratory Diagnosis

Specimens

These will vary with the disease, but usually include unclotted blood or affected tissue for smears, direct immunofluorescence, isolation or PCR. Paired serum samples for serology are appropriate in many of the diseases; indirect immunofluoresence tends to be the gold standard serological assay.

Direct microscopy

As members of the *Rickettsiales* stain poorly with the Gram procedure, Giemsa and other Romanowsky stains, such as Gimenez, Macchiavello and Leishman (Fig. 34.1), as well as fluorescent antibody (FA) staining (Fig. 34.2) are most widely used for both blood and tissue smears. A number of blood smears may have to be examined as the agents may only appear in the blood periodically.

Isolation and cultivation

This is often difficult and may not be necessary for a diagnosis. Members of *Rickettsiales* generally require living cells for replication. Species of *Ehrlichia* and *Anaplasma* which parasitize monocytes tend to grow comparatively readily in the yolk sac of chick embryos. Alternatively, mammalian or tick cell cultures or laboratory animals (mice or guinea pigs) may be used. To date *Ehrlichia* and *Anaplasma* species which target granulocytes and *Anaplasma* species that affect red blood cells have not been cultivated in vitro.

Table 34.2 Rickettsial pathogens of veterinary significance

Pathogen	Host	Vector	Geographical distribution	Disease
Rickettsia rickettsii	Humans, dogs	Ticks (principally *Dermacentor* species)	Western hemisphere	Rocky Mountain spotted fever
Anaplasma marginale	Ruminants	Hard ticks	Tropical and subtropical regions	Anaplasmosis (gall sickness)
A. ovis	Small ruminants	Hard ticks	Tropical and subtropical regions	Anaplasmosis
A. phagocytophilum	Ruminants, horses, humans	*Ixodes* species of tick	Worldwide	Tick-borne fever, equine and human granulocytic ehrlichiosis
A. bovis	Cattle	Ticks (*Hyalomma* species)	Africa, South America, Middle East, Asia	Bovine ehrlichiosis
A. platys	Dogs	Ticks suspected	Americas, Mediterranean, Middle East	Infectious cyclic thrombocytopenia
Ehrlichia canis	Dogs	Ticks (*Rhipicephalus* species)	Tropical and subtropical regions	Canine monocytic ehrlichiosis
E. ewingii	Dogs, humans	Ticks (*Amblyomma* species)	USA	Canine granulocytic ehrlichiosis
E. ruminantium	Ruminants	Ticks (*Amblyomma* species)	Sub-Saharan Africa, Caribbean	Heartwater
'E. ovina'	Sheep	Ticks	Africa, Asia, Middle East	Ovine ehrlichiosis
'E. ondiri'	Cattle	Ticks suspected	Highlands of East Africa	Bovine petechial fever
Neorickettsia helminthoeca	*Canidae*, bears	Ingestion of salmonid fish infested with the infected metacecariae of the fluke *Nanophyetus salmincola*	West coast of North America	Salmon poisoning disease
'N. elokominica'	*Canidae*, bears, racoons	As for *N. helminthoeca*	As for *N. helminthoeca*	Salmon fever
N. risticii	Horses	Complex infection cycle involving an intermediate snail reservoir and a trematode vector. Transmission to horses believed to occur following ingestion of aquatic insects infested with infected metacercariae	USA, Europe	Potomac horse fever
Aegyptianella pullorum	Birds	Ticks (*Argus* species)	Africa, Asia, Mediterranean	Aegyptianellosis

Identification

This is usually based on the animal species, clinical signs, demonstration of the organisms in specimens and using specific serological tests. Specific PCR primers have been described for many species (Sirigireddy et al. 2006). Where an isolate has been obtained, sequence analysis of genes such as the citrate synthase gene (*gltA*), 16S rRNA gene and outer membrane protein genes is the preferred method of identification. The laboratory diagnosis of the principal rickettsial diseases is summarized in Table 34.3.

Table 34.3 Laboratory diagnosis of rickettsial diseases of veterinary significance

Pathogen	Disease	Laboratory diagnosis
Rickettsia rickettsii	Rocky Mountain spotted fever	Serological assays, usually indirect immunofluorescence or ELISA, can be used to detect IgM or IgG. Isolation in embryonated hens' eggs or tissue culture is possible but time-consuming. Rickettsial DNA may be successfully detected in canine or tick tissues using PCR
Anaplasma marginale	Anaplasmosis (gall sickness)	Giemsa-stained blood smears may reveal intracytoplasmic, reddish-violet pleomorphic forms (0.3 to 1.0 μm) near the periphery of erythrocytes. Up to 50% of erythrocytes may be affected. Sensitive PCR methods have been described. Recommended serological assays include competitive ELISA and card agglutination. The complement fixation test is considered to be of low sensitivity
A. phagocytophilum	Tick-borne fever, equine and human granulocytic ehrlichiosis	Up to 70% of neutrophils in Giemsa-stained blood smears may contain intracytoplasmic purplish-blue morulae during the febrile period. A sensitive and specific PCR assay is available. Indirect imunofluoresence can be used to detect a rising antibody titre
A. platys	Infectious cyclic thrombocytopenia	Examination of stained blood smears for the organism in platelets is unreliable. A PCR assay has been described. Seroconversion can be detected by indirect immunofluorescence
Ehrlichia canis	Canine monocytic ehrlichiosis	Purple-staining morulae may be detected in mononculear cells in Giemsa-stained preparations of the buffy coat. Indirect immunofluorescence test can be used to detect seroconversion. Isolation in a canine macrophage cell line is possible but time-consuming. A specific and sensitive PCR assay has been described
E. ewingii	Canine granulocytic ehrlichiosis	Morulae may be present in neutrophils. A PCR assay is available
E. ruminantium	Heartwater	Giemsa-stained smears from brain tissue may reveal purple-staining coccal or short bacillary forms in the cytoplasm of vascular endothelial cells of capillaries in the brain. A PCR assay has been described. Serological assays can be used to demonstrate seroconversion but cross-reactivity with other *Ehrlichia* species occurs
Neorickettsia helminthoeca	Salmon poisoning disease	The presence of fluke eggs (*Nanophyetus salmincola*) in the faeces of affected animals is suggestive. Detection of intracytoplasmic rickettsial bodies in macrophages in lymph node aspirates. Demonstration of seroconversion by indirect immunofluorescence or complement fixation test
N. risticii	Potomac horse fever	Demonstration of the organism in the cytoplasm of monocytes in stained blood films. A rising antibody titre detected by indirect immunofluorescence or ELISA is suggestive. Amplification of DNA from blood or faeces by PCR
Aegyptianella pullorum	Aegyptianellosis	Examination of Giemsa-stained blood smears for violet-reddish spherical and ring forms in erythrocytes

Antibiotic Susceptibility

Testing for antibiotic susceptibility is difficult in the *Rickettsiales*. Clinical trials indicate that tetracyclines or chloramphenicol are the drugs of choice. A comparatively long course of therapy is required to prevent relapses. Long-term medication of feed with tetracyclines has been used to eliminate the carrier state with *Anaplasma marginale* and *A. caudatum*.

COXIELLA BURNETII

Q fever or 'Query Fever', first described in Australia, acquired its name due to the fact that the cause was unknown at the time. It is an occupational disease of farmers, abattoir workers and veterinary surgeons. The aetiological agent, *Coxiella burnetii*, was discovered and described as rickettsial in nature in 1937. Although the

lifestyle and parasitic strategies of *C. burnetii* are similar to those of rickettsiae and chlamydiae phylogenetic studies, largely based upon sequence analysis of the 16S rRNA gene, indicate that the genus *Coxiella* belongs to the gamma subdivision of *Proteobacteria* in the order *Legionellales* with the closest related genera being *Legionella, Francisella* and *Rickettsiella*. It is an obligate, intracellular, pleomorphic bacterium (0.2 to 0.4 μm wide by 0.4 to 1 μm long) with a membrane similar to that of Gram-negative bacteria. Two forms are described: a spore-like, small-cell variant (SCV) which corresponds to the metabolically inactive extracellular form of the bacterium and a large-cell variant (LCV) which corresponds to the metabolically active intracellular form and a sylvatic cycle involving reservoir, wild, mammal species and ticks sometimes gives rise to transmission to domestic animals and humans.

Natural Habitat

Infection can occur in a wide variety of animals, particularly cattle, sheep, goats, dogs and cats. The organism is shed in milk, urine and faeces. In addition, large numbers are shed at parturition with the amniotic fluids and placentas of infected animals. The extracellular form of the bacterium is resistant to heat, desiccation, pH, ultraviolet radiation and a wide range of disinfectants. Environmental survival for long periods favours wind-borne transmission, sometimes over long distances. Complete inactivation of this bacterium may not occur even after exposure to pasteurization temperatures of 63°C for 30 minutes or 72°C for 15 seconds. Ticks and other arthropods may become naturally infected and in turn facilitate a sylvatic cycle in reservoir wildlife species. Occasionally ticks are responsible for transmission to domestic animals and man.

Pathogenesis

Aerosol transmission is important in domestic animals and man. The principal target cells are monocytes and macrophages, *C. burnetii* replicates in the acid environment of the phagolysosome. These cells are responsible for the dissemination of the microorganism around the body. Infection may localize in the liver, heart, reproductive tract and mammary glands. Infections in domestic animals and humans are usually mild or inapparent. Sporadic abortions have been reported in sheep, goats, cattle and cats. Risk factors for development of disease include immunosuppression, pregnancy and cardiac valve defects. Acute Q fever presents in humans as a flu-like disease with fever, headaches and myalgia. Cases of granulomatous hepatitis have also been described. Endocarditis is the most common presentation in chronic Q fever in humans.

Laboratory Diagnosis

The organisms cannot be stained using the Gram-stain; Giemsa or Gimenez staining is generally employed. Modified Ziehl–Neelsen staining of placental or vaginal discharge smears may reveal characteristic clumps of red coccobacilli, which must be differentiated from *Chlamydophila abortus*. Immunofluorescent staining is sensitive and specific. Isolation of the organism is possible in the yolk sac of five to seven-day-old embryonated hens' eggs or in tissue culture but must be carried out under biosafety level 3 conditions. Susceptible cell lines include chick embryo fibroblasts and Vero cells. The shell vial culture technique with centrifugation of the inoculum onto the monolayer is used for isolation from clinical specimens. Polymerase chain reaction assays for the detection of *C. burnetii* DNA are available. Diagnosis is often based on serology. Suitable tests for the demonstration of seroconversion include microimmunofluorescence, complement fixation, microagglutination, indirect immunofluorescence and ELISA. Cross-reactions have been described with *Legionella* species and with *Bartonella* species and can make interpretation of serological results difficult. Indirect immunofluorescence employing phase I and phase II antigens is currently considered the most reliable serological assay. *Coxiella burnetii* displays antigenic variation analogous to the smooth-rough variation of enteric bacteria. The natural, infectious phase is phase I, while phase II is not very infectious and occurs following in vitro cultivation. In acute Q fever cases antibodies to phase II antigens are generally detectable within a few weeks of the onset of illness. High levels of antibodies to phase I antigens are indicative of chronic Q fever as these antibodies take longer to appear and result from continued exposure to the bacterium.

REFERENCES

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., et al., 2001. Re-organization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. International Journal of Systematic and Evolutionary Microbiology 51, 2145–2165.

Neimark, H., Johansson, K.E., Rikihisa, Y., et al., 2001. Proposal to transfer some members of the genera

Haemobartonella and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of 'Candidatus Mycoplasma haemofelis', 'Candidatus Mycoplasma haemomuris', 'Candidatus Mycoplasma haemosuis' and 'Candidatus Mycoplasma wenyonii'. International Journal of Systematic and Evolutionary Microbiology 51, 891–899.

Neimark, H., Johansson, K.E., Rikihisa, Y., et al., 2002. Revision of haemotrophic *Mycoplasma* species names. International Journal of Systematic and Evolutionary Microbiology 52, 683.

Sirigireddy, K.R., Mock, D.C., Ganta, R.R., 2006. Multiplex detection of *Ehrlichia* and *Anaplasma* pathogens in vertebrate and tick hosts by real-time RT-PCR. Annals of the New York Academy of Sciences 1078, 552–556.

FURTHER READING

Cohn, L.A., 2003. Ehrlichiosis and related infections. Veterinary Clnics of North America: Small Animal Practice 33, 863–884.

Madigan, J.E., Pusterla, N., 2000. Ehrlichial diseases. Veterinary Clinics of North America: Equine Practice 16, 487–499.

Maurin, M., Raoult, D., 1999. Q fever. Clinical Microbiology Reviews 12, 518–553.

Torioni de Echaide, S., Knowles, D.P., McGuire, T.C., et al., 1998. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. Journal of Clinical Microbiology 36, 777–782.

Woldehiwet, Z., 2006. *Anaplasma phagocytophilum* in ruminants in Europe. Annals of New York Academy of Science 1078, 446–460.

Woldehiwet, Z., 2008. Immune evasion and immunosuppression by *Anaplasma phagocytophilum*, the causative agent of tick-borne fever of ruminants and human granulocytic anaplasmosis. Veterinary Journal 175, 37–44.

Chapter 35

The Mycoplasmas (class: mollicutes)

The mollicutes are the smallest procaryotic cells capable of self-replication. The genomes of *Mycoplasma* and *Ureaplasma* species may be as small as 600 kb compared to the average bacterial genome of approximately 3000 kb. These organisms lack the genetic ability to form a cell wall and are enclosed in a plasma membrane composed of protein, glycoprotein, glycolipid and phospholipid. As there is no rigid cell wall, the mollicutes are plastic and pleomorphic. Cell forms include cocci, spirals, filaments and rings. Some divide by binary fission while others have a reproductive cycle involving the break-up of elongated forms into round forms. The minimal reproductive unit is about 0.3 µm in diameter, but because the cells are pliable they are able to pass through a 0.22 µm membrane filter. They characteristically form fried-egg-shaped microcolonies that grow into agar media. Most are facultative anaerobes or microaerophiles except for the *Anaeroplasma* species that are anaerobic.

Because of the inability of the mollicutes to synthesize peptidoglycan they stain poorly with the Gram stain. Better staining is obtained using Giemsa and other Romanowsky stains. The first organism in the class Mollicutes to be isolated was *Mycoplasma mycoides* subsp. *mycoides*, the cause of contagious bovine pleuropneumonia (CBPP). As a result the collective term pleuropneumonia-like organisms (PPLOs) was once used for *Mycoplasma* organisms. The differential features of the genera are shown in Table 35.1. Species of the genera *Mycoplasma* and *Ureaplasma* are of significance in animals. Members of the genus *Acholeplasma* may be found in clinical specimens but are not considered to be pathogenic for animals. Organisms in the genera *Haemobartonella* and *Eperythrozoon*, which were previously grouped with the rickettsial organisms, have been reclassified as members of the *Mycoplasma* genus. These organisms parasitize red blood cells (Fig. 35.1) and are known as the haemotropic mycoplasmas or are sometimes referred to by the trivial name 'haemoplasmas'.

Natural Habitat

The mollicutes occur worldwide as free-living saprophytes or as parasites of animals. Both pathogenic and non-pathogenic species are found as commensals on the mucous membranes of the upper respiratory, intestinal and genital tracts, on articular surfaces and in the bovine mammary gland. Outside the host, the pathogenic species can survive in microenvironments, protected from sunlight, for several days.

Pathogenesis

The most important species of mycoplasmas and the diseases that they cause in poultry, other domestic animals and laboratory animals are listed in Table 35.2. The mycoplasmas tend to be fairly host-specific although it has been suggested that interspecies transmission can occur and may be of pathogenic importance in immunosuppressed hosts (Pitcher & Nicholas 2005). In addition to the mycoplasmas shown in Table 35.2, there are many species isolated from birds and animals, whose disease status is at present uncertain. These are listed in Table 35.3.

The parasitic mycoplasmas tend to adhere firmly to the mucous membranes of the host and some species have been shown to affix to cells by specific attachment structures. A number of surface proteins play a role in adhesion, including the P26 antigen and variable surface proteins of *M. bovis* (Caswell & Archambault 2007), variable lipoprotein haemagglutinin of the avian mycoplasmas (Noormohammadi 2007) and P116 of *M.*

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Table 35.1 Differential features of the genera of the mycoplasmas (mollicutes)

Genus	Cholesterol requirement	Habitat	Other features
Mycoplasma	+	Animals, humans	Many are animal pathogens Optimal pH 7.5 Microcolonies 0.1–0.6 mm in diameter
Ureaplasma	+	Animals, humans	Some associated with disease Optimal pH 6.0. Produce urease Microcolonies 0.01–0.05 mm in diameter, called 'T-mycoplasmas' (T = tiny)
Acholeplasma	-	Animals, soil, sewage	Many saprophytic, role in disease uncertain Microcolonies 0.1–1.0 mm in diameter
Spiroplasma	+	Plants, insects	Helical and motile forms
Entomoplasma	+	Plants, insects	
Mesoplasma	-	Plants, insects	
Asteroleplasma	-	Rumen of cattle and sheep	Commensals Anaerobic
Anaeroplasma	+	Rumen of cattle and sheep	Commensals Anaerobic

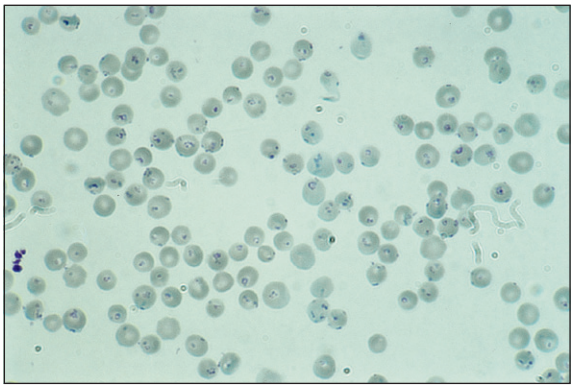

Figure 35.1 Leishman-stained blood smear from a sheep with *Mycoplasma* (*Eperythrozoon*) *ovis* parasitizing the red cells. (×1000)

hyopneumoniae (Seymour et al. 2010). Although the production of a cytotoxin (CARDS toxin) has been identified in the human pathogen, *Mycoplasma pneumoniae* (Kannan & Baseman 2006, Techasaensiri et al. 2010), production of specific toxins has not been demonstrated for pathogenic mycoplasmas of animals. It is considered that many of the toxic effects on host cells result from the action of toxic metabolic products which diffuse into the tissues of the host following adherence of the organisms. These metabolic products include H_2O_2 and other reactive oxygen species. Enzymes such as proteases and haemolysins may also be involved in the production of tissue damage.

Variable surface proteins (Vsps) have been identified in many mycoplasmal species and these proteins play a role in adhesion and colonization and, most importantly, in the evasion of the humoral response of the host. The production of a number of different Vsps has been demonstrated in many of the major mycoplasmal pathogens of animals, including *Mycoplasma mycoides* subspecies *mycoides* (Persson et al. 2002), *M. bovis* (Sachse et al. 2000) and the avian pathogens *M. gallisepticum* and *M. synoviae* (Noormohammadi 2007). The similarity between some mycoplasmal antigens and host cell antigens may also contribute to persistence of mycoplasmas in the host because of the inability to induce an effective immune response due to a failure in antigen recognition. However, the sharing of antigenic determinants between the organism and host cells may also lead to the development of autoimmune disease. It is thought that immunological mechanisms may be involved in the destruction of red cells in cats infected with *Mycoplasma haemofelis* although direct damage by the organism may also play a role. Important virulence factors of *Mycoplasma* species are listed in Table 35.4.

Mycoplasma species can affect may tissues of the animal body. Some pathogenic species have a predilection for mesenchymal cells lining joints and serous cavities. The respiratory tract and lungs are frequent sites of infection. Mycoplasmas are capable of destroying the cilia of cells in the respiratory tract, thus predisposing to secondary bacterial invasion. Latency can occur and various stresses predispose to mycoplasmal diseases. The infections are

Table 35.2 Mycoplasmas causing significant disease in domestic and laboratory animals

Species	Disease
Poultry	
Mycoplasma gallisepticum	Chickens: chronic respiratory disease Turkeys: infectious sinusitis Infection in game birds and imported Amazon parrots
M. synoviae	Chickens and turkeys: infectious synovitis
M. meleagridis	Turkeys: *Mycoplasma meleagridis* disease (MM disease), air sacculitis and bursitis in young birds
M. iowae	Turkey poults: air sacculitis, stunting and leg deformities. Mortality of turkey embryos can occur
M. anatis	Ducks: sinusitis
Pigs	
M. hyorhinis	Chronic progressive arthritis and polyserositis in three- to 10-week-old pigs
M. hyosynoviae	Mycoplasmal polyarthritis in 12–24-week-old pigs
M. hyopneumoniae	Enzootic ('virus') pneumonia of pigs
M. suis	Mild anaemia, poor growth rates
Cattle	
M. mycoides subsp. *mycoides* (small colony type)	Contagious bovine pleuropneumonia (CBPP)
M. bovis	Mastitis, arthritis, pneumonia, genital infections, abortion
M. bovigenitalum	Vaginitis, arthritis, mastitis, seminal vesiculitis
Ureaplasmas including *U. diversum*	Vulvovaginitis, pneumonia
M. dispar	Pneumonia (calves)
M. californicum	Mastitis
M. canadense	Mastitis
M. bovoculi	A predisposing cause of infectious bovine keratoconjunctivitis (*Moraxella bovis* infection)
M. wenyonii	Mild anaemia
Goats	
M. capricolum subsp. *capripneumoniae*	Contagious caprine pleuropneumonia (CCPP)
M. mycoides subsp. *capri*	Mastitis, arthritis, keratitis, pneumonia and septicaemia syndrome
M. putrefaciens	Mastitis, arthritis
Sheep	
M. ovipneumoniae	Pneumonia
M. ovis	Haemolytic anaemia of varying severity
Sheep and goats	
M. agalactiae	Contagious agalactia
M. conjunctivae	Keratoconjunctivitis
M. capricolum subsp. *capricolum*	Mastitis, arthritis, keratitis, pneumonia and septicaemia syndrome
Acholeplasma oculi	Keratoconjunctivitis
Horses	
Mycoplasma felis	Pleuritis (a commensal that can enter the pleural cavity after severe exercise)
Dogs	
M. cynos	Pneumonia (part of 'kennel cough' complex)
M. haemocanis	Usually mild or subclinical anaemia but more severe signs in splenectomized animals
Cats	
M. felis	Conjunctivitis
M. haemofelis	Feline infectious anaemia
Rats and mice	
M. neurolyticum	Rolling disease
M. pulmonis	Pneumonia
M. arthritidis	Polyarthritis

frequently chronic or low grade. Infections are either endogenous or exogenous. Transmission is usually by aerosol, venereal or vertical and the important avian mycoplasmas are egg-transmitted. Transmission of haemotropic mycoplasmas occurs through fighting and biting arthropods.

Laboratory Diagnosis

Specimens

Mycoplasmas are fragile and specimens must be kept refrigerated and delivered to a laboratory within 24–48 hours of collection. The specimens should be obtained

Table 35.3 ***Mycoplasma*** **species of minor or uncertain disease potential**

Poultry	Sheep and goats	Dogs
M. cloacae	*M. adleri*	*M. canis*
M. gallinaceum	*M. arginini*	*M. edwardii*
M. gallinarum	*M. auris*	*M. maculosum*
M. gallopavonis	*M. cottewii*	*M. molare*
M. glycophilum	*M. yeatsii*	*M. opalescens*
M. imitans		*M. spumans*
M. pullorum		
Pigs	**Horses**	**Cattle**
M. flocculare	*M. equigenitalium*	*M. alvi*
M. hyopharyngis	*M. equirhinis*	*M. arginini*
M. sualvi	*M. fastidiosum*	*M. bovirhinis*
A. axanthum	*M. salivarium*	*M. verecundum*
A. granularum	*M. subdolum*	*A. laidlawii*
	A. equifetale	*A. modicum*
	A. hippikon	*A. vituli*
	A. laidlawii	
	A. multilocale	
	A. parvum	
Guinea pigs	**Cats**	
M. caviae	*M. feliminutum*	
M. cavipharyngis	*M. gateae*	
A. cavigenitalium		

Table 35.4 Virulence attributes of pathogenic ***Mycoplasma*** **species (the production of every pathogenic factor listed has not been demonstrated for all the pathogenic species)**

Virulence factor	Comments
Capsular polysaccharide	Exact role unknown, aids in persistence and dissemination of the organism
Lipoproteins	Function in adhesion, stimulate the release of pro-inflammatory cytokines
Adhesins	Protein adhesins have been identified in some mycoplasmal species. Sialyl moieties important in adhesion also
Variable surface proteins	Role in evasion of host antibody response, in colonization and adhesion and in modulation of the host immune response
Toxic metabolic pathway products	These products include H_2O_2 and reactive oxygen species which induce toxic damage to host cells
Heat shock proteins	Role in adhesion to host cells
Biofilm	Some *Mycoplasma* species have the ability to produce biofilms although the genes commonly associated with biofilm formation in other bacterial species are lacking

from animals at an early stage in the clinical disease. The samples may include mucosal scrapings, tracheal exudates, aspirates, pneumonic tissue from the edge of the lesion, cavity or joint fluids and mastitic milk. Swabs should be submitted in transport medium (see Appendix 2). A simple transport medium for mastitic milk samples is the sample itself with 5 mg/mL ampicillin. This is kept at room temperature which will allow replication of the mycoplasmas in the specimen during transportation. A milk sample without antibiotics should also be collected for the isolation of any bacterial pathogens present.

Direct microscopy

Fragility, pleomorphism and weak staining by various methods make direct examination of stained smears of little value for the diagnosis of most mycoplasmal diseases. Leishman- or Giemsa-stained blood smears may be used to demonstrate *Mycoplasma haemofelis* on the surface of erythrocytes (Fig. 35.2). However, daily blood sampling may be necessary due to the cyclical nature of the parasitaemia. Fluorescent antibody techniques to identify *Mycoplasma* species in clinical specimens have been developed. These tests require the production of specific antibody

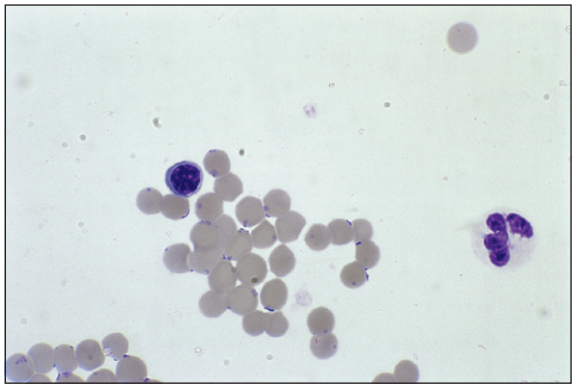

Figure 35.2 Leishman-stained blood smear from a cat with *Mycoplasma* (*Haemobartonella*) *haemofelis* on red blood cells. The rubricyte (top left) is indicative of red cell regeneration.

reagent and considerable skill is needed as non-specific fluorescence may be observed in some tissues. An indirect fluorescent antibody test on smears from pleural fluid of cattle affected with contagious bovine pleuropneumonia is described as satisfactory (Thiaucourt 2008).

Isolation

Culture media

Mycoplasmas are fastidious organisms and most require specific growth factors, an isotonic medium and the absence of inhibitory substances for growth. The basic medium is a good-quality beef infusion with supplements. *Mycoplasma* and *Ureaplasma* species require cholesterol and this is usually supplied by 20% horse serum. Serum from individual animals can vary in growth-promoting properties; pooled serum from several animals is preferred. Other growth factors are catered for by the addition of yeast extract, DNA and possibly nucleotides. The water for the medium should be of a quality similar to that used for tissue culture. Penicillin can be added to discourage the growth of Gram-positive bacteria and thallium acetate to inhibit fungi and Gram-negative bacteria. The final pH of the medium is adjusted to between 7.2 and 7.8 for *Mycoplasma* species. Ureaplasmas require the addition of urea and a final pH of 6.0. Thallium acetate is omitted from *Ureaplasma* media as it is harmful to them. Arginine is inhibitory to *M. bovoculi* while *M. dispar* may be sensitive to some penicillin G or benzyl penicillin preparations.

The basic medium and supplements can be bought commercially. Media usually take the form of broths or solid agar, although semisolid agar and diphasic media are also used. The mycoplasmal transport medium (Appendix 2) can also make a suitable broth medium for isolation. This can be converted to an agar medium by substituting 28 g of PPLO agar (Difco) for the 16.8 g of PPLO broth. A modification of Hayflick's medium for ureaplasmas is given in Appendix 2. *Acholeplasma* species will usually grow on media suitable for *Mycoplasma* species. Special media may have to be prepared for the mycoplasmas that are particularly difficult to culture, such as *M. dispar* and *M. meleagridis*.

The agar media are poured into small Petri dishes of about 60 mm diameter. Care must be exercised when selecting the brand of Petri dish used as poor growth has been reported when using certain types of plastic Petri dishes. Strict quality control measures are required for the media to obtain optimal growth of these fastidious organisms. Each new batch of basic medium and supplements should be pre-tested with a laboratory control strain to ensure that the medium sustains growth and does not contain any substances inhibitory to the mycoplasmas.

Inoculation of culture media

For routine isolation of mycoplasmas, the specimen should be inoculated into two broths and onto two plates of agar (one suitable for mycoplasmas and the other for ureaplasmas). The inoculation technique will vary according to the nature of the specimen:

1. Fluid materials such as foetal fluids and exudates can be inoculated directly into broth medium and spread over the surface of the agar medium.
2. Some specimens such as semen, joint fluids and tissues may contain inhibitors for mycoplasmas. Both undiluted specimen and 10-fold dilutions in mycoplasmal broth (up to 10^{-6}) should be cultured.
3. With tissues, such as pieces of lung, a freshly cut surface on a block of the tissue can be moved across the surface of an agar plate to inoculate it. Alternatively, the tissue can be homogenized in broth, 10-fold dilutions made and broth cultures inoculated and incubated.

The inoculated agar plates are incubated in a humid atmosphere at 37°C. It is advisable to inoculate and incubate duplicate plates, one aerobically and one under 5% CO_2 and 95% N_2. A candle jar may be satisfactory. The plates should be examined after 48 and 96 hours' incubation. The plates are viewed under a stereoscopic microscope or under the low-power objective of a light microscope, for the characteristic mycoplasmal 'fried-egg' microcolonies (Figs 35.3 and 35.4). The cultures can be regarded as negative if no microcolonies are seen after 14 days' incubation. Tubes of broth, incubated aerobically at 37°C, are checked daily for growth. When a slight turbidity is seen (or a colour change if the broth contains phenol red), loopfuls of the broth can be used to inoculate appropriate agar media.

Obtaining a pure culture

A pure culture of the isolated *Mycoplasma* should be obtained before carrying out identification techniques. The microcolonies grow into the agar (Fig. 35.5) and it is difficult to obtain cells from the colonies on an

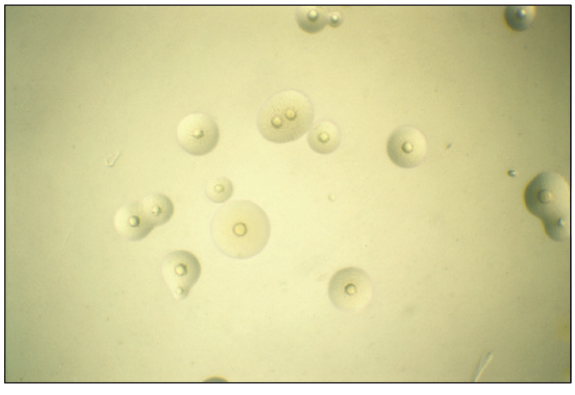

Figure 35.3 Unstained mycoplasmal microcolonies illustrating the characteristic appearance. (×10)

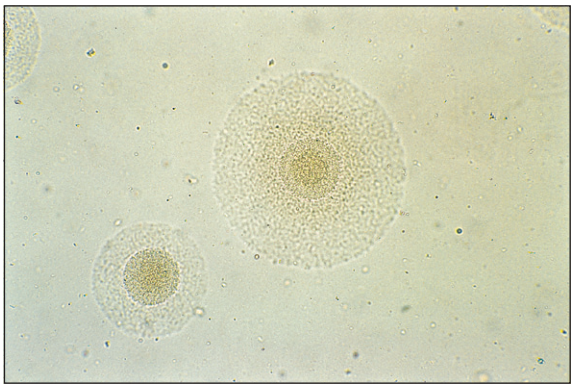

Figure 35.4 Unstained microcolonies of a *Mycoplasma* species showing the typical 'fried-egg' morphology. (×25)

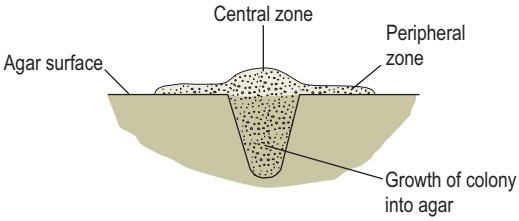

Figure 35.5 Section through a mycoplasmal colony showing surface and sub-surface growth.

inoculation loop, so different methods have to be used for subculture. An agar plate should be selected with well-separated colonies. Individual colonies are removed by cutting out a small block of agar, containing the colony of interest, with a sterile scalpel. The block is transferred to a tube with 2–3 mL of broth and incubated at 37°C for 48 hours or longer. The broth culture, when showing turbidity, is taken into a sterile syringe and passed through a 0.45 µm membrane filter. The filtrate is diluted with fresh broth, 1 : 10 and 1 : 100, and a loopful of each dilution is inoculated onto agar medium and the plates incubated. This procedure should be repeated three times. At least four colonies should be used as not all microcolonies will subculture.

Another method is to take a block of agar containing one colony and place it colony-side downwards on an agar plate. The agar block is pushed back and forth over the agar surface and the inoculated plate incubated for 48–72 hours. This procedure is repeated twice more.

Once a pure culture has been obtained, normal subculturing can be carried out using similar techniques, either from agar-to-agar or agar-broth-agar. Detailed procedures for the isolation and identification of the mycoplasmal diseases listed by the OIE (World Organisation for Animal Health) are available in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anon. 2012).

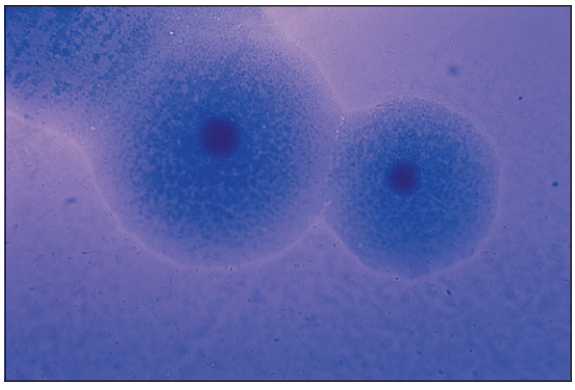

Figure 35.6 Mycoplasmal microcolonies. (Dienes' stain, ×25)

Identification

Differentiation from bacterial L-forms

Bacteria that have temporarily failed to form cell walls (L-forms) can produce microcolonies similar to those of the mycoplasmas. The failure to form cell walls is often due to the bacterium having been exposed to penicillin or other antibacterial agents that affect cell wall formation. Differentiation between mycoplasmal and bacterial L-form microcolonies may be necessary and can be carried out as follows:

- Subculturing the suspected bacterial L-form on media without antibacterial substances should cause reversion of the L-form with the formation of normal-sized bacterial colonies. Up to five subcultures may be necessary.
- Staining microcolonies with the Dienes' stain is useful for easier visualization of microcolonies but this also aids in the differentiation between mycoplasmal and bacterial L-form microcolonies. Mycoplasmal colonies retain the Dienes' stain indefinitely, whereas bacterial L-form microcolonies tend to decolourize in about 15 minutes. Staining microcolonies with the Dienes' stain is carried out as follows:

  A block of agar containing microcolonies is placed, colony-side upwards, on a microscope slide. A light film of Dienes' stain is placed on a cover slip and allowed to dry. This is then put, stain-side downwards, on the microcolonies on the agar block. The preparation is examined under the low-power objective of a light microscope. The denser centre of the microcolonies, that grow down into the agar, stain dark blue. The less dense peripheral zone, representing surface growth, stains light blue (Fig. 35.6). The Dienes' stain can be bought commercially; the formula is given in Appendix 1.

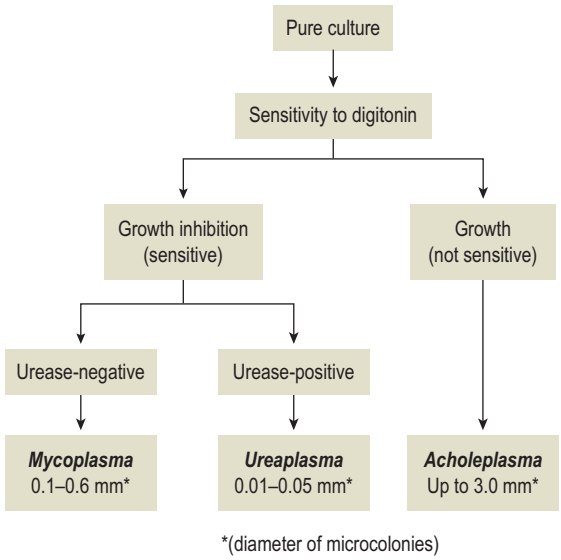

Figure 35.7 Differentiation of the three mycoplasmal genera isolated from animals

Identification of the genus

Differentiation between the three genera of veterinary importance is shown in Fig. 35.7.

1. *Sensitivity to digitonin*: this test reflects the requirement of cholesterol for growth. *Mycoplasma* species and *Ureaplasma* species are sensitive to digitonin while *Acholeplasma* species are not. Sterile filter paper discs (6 mm diameter) are saturated with 0.025 mL of digitonin (1.5% w/v in ethanol) and allowed to dry. An agar medium that gives good growth of the *Mycoplasma* under test is flooded with a broth culture of the organism and the surplus liquid removed. The surface of the plate is allowed to dry and the digitonin disc placed on the agar surface. The plate is incubated and the test can be read when microcolonies are detectable. The zone of inhibition around the disc should be 5 mm or more from the edge of the disc to indicate sensitivity.
2. *Modified urease test*: the ureaplasmas are able to produce the enzyme urease. To test for this activity a mixture of equal parts of 10% urea and 0.8% manganese chloride is applied directly onto 40-hour-old microcolonies. A positive result is indicated by an immediate colour reaction on the surface of the *Ureaplasma* colonies. The colour reaction goes from light to dark brown and finally to black due to the deposition of manganese on the surface of the colony.
3. *Size of the microcolonies*: this may also be an aid to the differentiation of the three genera isolated from animals.

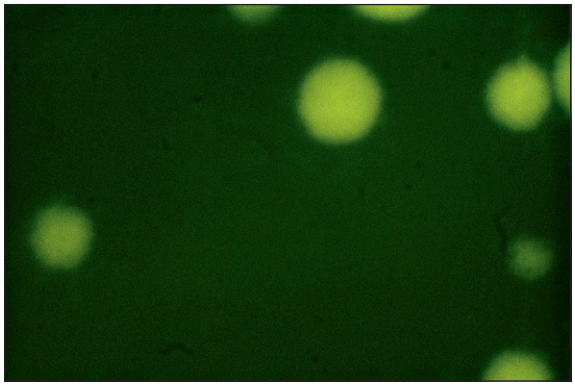

Figure 35.8 Microcolonies of *Mycoplasma bovis*. (Direct FA technique, ×25)

Identification of species

Knowledge of the animal species and disease process will often suggest a particular mycoplasmal species. Precise identification of the species often requires sophisticated techniques and cultures are usually submitted to reference laboratories for final identification. The following methods are examples of the techniques that can be used to identify mycoplasmal species:

- Fluorescent antibody (FA) staining to identify *M. dispar* and ureaplasmas in bronchial epithelium of calves with pneumonia.
- FA technique (direct and indirect) for staining mycoplasmal colonies (Fig. 35.8). This is the best method of recognizing mixed cultures and the method is used commonly with the avian mycoplasmas.
- Enzyme-linked immunoperoxidase used on frozen sections of porcine bronchial epithelium to detect *M. hyopneumoniae*.
- Agar gel diffusion tests using known antisera to identify prepared mycoplasmal antigen from broth cultures.
- ELISA and complement fixation test (CFT) for antigen identification using known antisera.
- PCR-based methods of identification have been developed for the identification of most species.
- Biochemical tests such as glucose fermentation, arginine hydrolysis, phosphatase activity and reduction of tetrazolium.
- Metabolic inhibition tests: essentially neutralization tests in which specific antisera prevent the utilization of a particular substrate.
- Growth inhibition tests using specific sera on filter paper discs.

Serology

A number of serological tests for the detection of antimycoplasmal antibodies are available and include:

- Rapid plate agglutination tests (coloured antigen) for screening poultry for the major mycoplasmal diseases.
- Haemagglutination-inhibition test for avian mycoplasmas.
- Indirect haemagglutination tests for *M. bovis* and *M. bovigenitalium*.
- Agar gel diffusion tests for avian mycoplasmas using known antigen.
- Complement fixation test for the control of contagious bovine pleuropneumonia and for screening pigs for enzootic pneumonia.
- ELISA techniques, usually indirect ELISAs, have been developed for detection of antibodies in many *Mycoplasma* infections of animals. Although the CFT was previously the principal test used for the purposes of international trade, ELISA tests are now prescribed tests for this purpose also.
- Latex agglutination test to detect antibodies to the mycoplasmas causing disease in goats.

Antibiotic Susceptibility Testing

Susceptibility testing using disc diffusion methods is not suitable for these organisms and broth microdilution methods are most commonly used. Application of flow cytometry for the determination of MIC of antimicrobial agents against animal *Mycoplasma* organisms has been reported (Assunção et al. 2007). The E-test method is satisfactory for *Mycoplasma* species which grow on solid media. At present, there are no universally accepted interpretive criteria for determination of susceptibility of mycoplasmal isolates in animals. Thus, susceptibilities to different antimicrobial agents are usually based on CLSI values for common veterinary pathogens (CLSI 2008).

Antimicrobial Resistance

Antimicrobial resistance to commonly used veterinary therapeutic agents has been studied in several *Mycoplasma* species. Ayling et al. (2005) tested *Mycoplasma mycoides* subsp. *mycoides* SC isolates from African countries against a range of 21 antimicrobial agents and found that oxytetracycline and tilmicosin showed the lowest MICs, with low values for erythromycin and enrofloxacin also. Isolates were resistant to tylosin. Susceptibility rates of *M. bovis* have been reported by several workers. High levels of resistance to tilmicosin, erythromycin, ampicillin and ceftiofur in both the US and Europe were recorded by Rosenbusch et al. (2005) whereas resistance to the tetracyclines was found mainly in Europe. Maes et al. (2008) suggested that although resistance of *M. hyopneumoniae* to tetracyclines, macrolides, licosamides and fluoroquinolones has been reported, improvements in clinical disease are achieved following the use of most antimicrobial agents.

Specific-Pathogen-Free (SPF) Programmes

Flocks of poultry (chickens and turkeys) free from the major avian mycoplasmas and pig herds free from *M. hyopneumoniae*, have been established in some countries with specific-pathogen-free programmes. There are usually two phases in these programmes—detection of infections and culling or isolation of affected pigs or birds, followed by monitoring of the flocks or herds for continued freedom from infection.

Strain Typing

Typing of *Mycoplasma* organisms is frequently carried out by enzymatic digestion of extracted DNA using *Sma*I, followed by pulsed-field gel electrophoresis (PFGE). However, digestion using *Bln*I and *BamH*I was reported to give better discrimination of *M. synoviae* strains (Harada et al. 2009). Variable number tandem repeat (VNTR) analysis and random amplified polymorphic DNA techniques may also be used. Isolates of *M. ovipneumoniae* with a number of different PFGE and RAPD patterns were identified within individual flocks in a UK study, suggesting introduction of strains by asymptomatic carrier animals (Parham et al. 2006). VNTR analysis detected greater variation in *M. agalactiae* isolates than PFGE typing and was considered a useful technique for outbreak tracing by McAuliffe et al. (2008). Amplified fragment length polymorphism analysis has been used for typing *Mycoplasma* species also.

Molecular Diagnosis

PCR-based techniques have been developed both for direct detection of *Mycoplasma* organisms in clinical specimens and for identification of isolates obtained by culture. Multiplex PCR techniques for the detection and identification of several *Mycoplasma* species which affect a particular animal species are also available, for example, avian mycoplasmas or those affecting small ruminants. PCR procedures for the OIE-listed *Mycoplasma* diseases are described in the relevant sections of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anon. 2012). Several PCR procedures for the detection and differentiation of *M. bovis* are described, including real-time PCR (Cai et al. 2005). These assays may be used on clinical samples such as milk or lung tissue. Owing to the current inability to culture the haemotropic mycoplasmas in vitro, PCR assays are the best methods for diagnosis of infection with these species. Real-time PCR has been developed by a number of authors which can differentiate between the three haemotropic mycoplasmas known to infect cats (Willi et al. 2007).

REFERENCES

Anon, 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012. Available at: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/ (accessed: 22 December 2012).

Assunção, P., Antunes, N.T., Rosales, R.S. et al., 2007. Application of flow cytometry for the determination of minimal inhibitory concentration of several antibacterial agents on *Mycoplasma hyopneumoniae*. Journal of Applied Microbiology April 102 (4), 1132–1137.

Ayling, R.D., Bisgaard-Frantzen, S., March, J.B. et al., 2005. Assessing the in vitro effectiveness of antimicrobials against *Mycoplasma mycoides* subsp. *mycoides* small-colony type to reduce contagious bovine pleuropneumonia infection. Antimicrobial Agents and Chemotherapy 49 (12), 5162–5165.

Cai, H.Y., Bell-Rogers, P., Parker, L., et al., 2005. Development of a real-time PCR for detection of *Mycoplasma bovis* in bovine milk and lung samples. Journal of Veterinary Diagnostic Investigation 17, 537–545.

Caswell, J.L., Archambault, M., 2007. *Mycoplasma bovis* pneumonia in cattle. Animal Health Research Reviews 8, 161–186.

Clinical and Laboratory Standards Institute (CLSI), 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard, third ed. Document M31-MA3. CLSI (formerly National Committee of Clinical Laboratory Standards – NCCLS), Wayne, PA, USA.

Harada, K., Kijima-Tanaka, M., Uchiyama, M., et al., 2009. 2009 Molecular typing of Japanese field isolates and live commercial vaccine strain of *Mycoplasma synoviae* using improved pulsed-field gel electrophoresis and vlhA gene sequencing. Avian Diseases December 53 (4), 538–543.

Kannan, T.R. Baseman, J.B., 2006. ADP-ribosylating and vacuolating cytotoxin of *Mycoplasma pneumoniae* represents unique virulence determinant among bacterial pathogens. Proceedings of the National Academy of Sciences USA 103 (17), 6724–6729.

Maes, D., Segales, J., Meyns, T., et al., 2008. Control of *Mycoplasma hyopneumoniae* infections in pigs. Veterinary Microbiology 126 (4), 297–309.

McAuliffe, L., Churchward, C.P., Lawes, J.R., et al., 2008. VNTR analysis reveals unexpected genetic diversity within *Mycoplasma agalactiae*, the main causative agent of contagious agalactia. BMC Microbiology 8, 193.

Noormohammadi, A.H., 2007. Role of phenotypic diversity in pathogenesis of avian mycoplasmosis. Avian Pathology 36, 439–444.

Parham, K., Churchward, C.P., McAuliffe, L., et al., 2006. A high level of strain variation within the *Mycoplasma ovipneumoniae* population of the UK has implications for disease diagnosis and management. Veterinary Microbiology 118 (1–2), 83–90.

Persson, A., Jacobsson, K., Frykberg, L., et al., 2002. Variable surface protein Vmm of *Mycoplasma mycoides* subsp. *mycoides* small colony type. Journal of Bacteriology 184, 3712–3722.

Pitcher, D.G., Nicholas, R.A., 2005. *Mycoplasma* host specificity: fact or fiction? Veterinary Journal 170, 300–306.

Rosenbusch, R.F., Kinyon, J.M., Apley, M., et al., 2005. In vitro antimicrobial inhibition profiles of *Mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. Journal of Veterinary Diagnostic Investigation 17, 436–441.

Sachse, K., Helbig, J.H., Lysnyansky, I., et al., 2000. Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. Infection and Immunity 68, 680–687.

Seymour, L.M., Deutscher, A.T., Jenkins, C., et al., 2010. A processed multidomain *Mycoplasma hyopneumoniae* adhesin binds fibronectin, plasminogen and swine respiratory cilia. Journal of Biological Chemistry 285, 33971–33978.

Techasaensiri, C., Tagliabue, C., Cagle, M., et al., 2010. Variation in colonization, ADP-ribosylating cytotoxin, and pulmonary disease Severity among *Mycoplasma pneumoniae* Strains. American Journal of Respiratory and Critical Care Medicine 182, 797–804.

Thiaucourt, F., 2008. Contagious bovine pleuropneumonia. In: Anon. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012. Available at: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/ (accessed: 16 January 2013).

Willi, B., Boretti, F.S., Meli, M.L., et al., 2007b. Real-time PCR investigation of potential vectors, reservoirs, and shedding patterns of feline hemotropic mycoplasmas. Applied and Environmental Microbiology 73, 3798–3802.

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Chapter 36

Mastitis

Mastitis, inflammation of the mammary gland, can be clinical or subclinical and can be caused by physical or chemical agents. However, the majority of cases are infectious and usually caused by bacteria. Invasion of the mammary gland by microorganisms is characterized by an increased leukocyte count in the milk, the majority of cells being neutrophils. The definition of mastitis is based on the somatic cell count (SCC) of quarter foremilk. The criteria for definition of mastitis have changed from a somatic cell count threshold of 500,000 cells per mL, with or without the detection of pathogens, set by the International Dairy Federation in 1967, to the current threshold of 100,000 cells per mL (Hamann 2005). However, the acceptable limit for the number of somatic cells in milk may differ depending on the country and the requirements of milk-processing companies. Over 130 different microorganisms have been isolated from bovine mastitic milk samples but *Staphylococcus aureus*, streptococci and members of the *Enterobacteriaceae* are among the most common aetiological agents in cows and in other animal species.

Epidemiology

Infection of the mammary gland is almost always via the teat canal. In cows this usually occurs when the teat sphincter is slack, for a period of 20 minutes to two hours after milking. The pathogenic microorganisms are generally classified as either environmental or contagious. Organisms such as *Escherichia coli*, other coliforms and *Streptococcus uberis* are present in the environment of cows, particularly those that are housed, and contamination of teat ends may result in infection. The origin of infection of the contagious pathogens (principally *Staphylococcus aureus* and *Streptococcus agalactiae*) is the mammary gland of infected animals, the teat skin is of lesser importance. These pathogens are transmitted via the milking machine or milkers' hands.

Following entry to the mammary gland, the organisms establish and multiply. Adherence to mammary epithelium is an important virulence factor for contagious pathogens but is not essential for the pathogenesis of coliform mastitis. The principal cell involved in eliminating bacteria from the mammary gland is the neutrophil. Host factors affecting the level and speed of the neutrophil response to infection are important in determining the outcome and severity of disease. These and other factors affecting the occurrence of mastitis are outlined in Table 36.1.

Clinical Syndromes of Mastitis

The main clinical types of mastitis are:

- **Peracute**: swelling, pain, heat and abnormal secretion in the mammary gland are accompanied by signs of systemic disturbance such as fever, depression, anorexia, weakness and a rapid, weak pulse. The signs are those of a toxaemia or septicaemia. Gangrenous mastitis is included in this category.
- **Acute**: changes in the mammary gland are similar to those of peracute mastitis but the systemic signs are much less severe.
- **Subacute**: no systemic reaction and the changes in the gland are less marked.
- **Chronic**: there are no systemic signs and very few external signs of change in the udder, but abnormal secretion in the gland occurs intermittently.
- **Subclinical**: the infection in the mammary gland is detectable only by bacterial culture or by tests to

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Table 36.1 Bacterial, host and environmental factors influencing the occurrence of mastitis

Microbial factors	Host factors	Environmental factors
Adherence – particularly important for the contagious pathogens	Age: older cows (>4th lactation) are more susceptible; may be due in part to the speed and efficiency of the neutrophil response	Presence of large numbers of potential pathogens in the immediate environment of the animal, whether housed or at pasture. Although 'coliform' mastitis is more frequent in housed cows, *S. uberis* numbers in heavily used pasture are comparable to numbers detected in contaminated bedding
Iron-binding capacity – particularly important for *E. coli*	Genetics: considerable differences between and within breeds. There is a negative correlation between level of milk production and resistance to mastitis. Heritability of mastitis resistance is low	Management factors including feeding practices. Negative energy balance in high-yielding cows early postpartum may compromise the immune response
Endotoxin production (Gram-negative organisms)	Stage of lactation	Milking shed environment including poor milking technique and hygiene
Antiphagocytic capacity	Presence of teat lesions may predispose to inadequate milking or may harbour mastitis-producing bacteria	External trauma such as that arising from rough, muddy approaches to the milking shed or, with ewes, the suckling of large vigorous lambs
Survival in the immediate environment of the host	Immunological factors, including the efficacy of the phagocytic response and levels of lactoferrin, complement and immunoglobulins	Milking-machine malfunction or inadequate design
Ability to colonize the teat canal		

demonstrate a high leukocyte count in the milk. There is no obvious change in the appearance of the milk. *Staphylococcus aureus* is notorious for causing a high percentage (up to 50% of the herd) of subclinical infections in dairy herds with a staphylococcal mastitis problem.

MASTITIS IN DOMESTIC ANIMALS OTHER THAN CATTLE

Mastitis has the greatest prevalence and economic importance in dairy cows but it can be significant in other domestic animals, particularly in sheep, goats and pigs. Table 36.2 gives the most common aetiological agents in small ruminants, their source and the type of clinical syndrome that is usually caused by each agent.

Small Ruminants

Mastitis is not as common in small ruminants as it is in dairy cattle. It is estimated that the annual incidence of clinical mastitis is less than 5% with the prevalence of subclinical mastitis reported as ranging from 5 to 30%. Outbreaks of mastitis can occur in ewes housed for lambing, probably due to contamination of bedding from infected udder secretions. The prevalence of mastitis increases with the age of the ewe and with the number and weight of the lambs. Trauma from large, vigorously sucking lambs may predispose the ewe to mastitis. The importance of mastitis lies not only in deaths from peracute cases but also as a cause of depressed weaning weights and lamb mortality from starvation. The most important cause of subclinical mastitis in dairy sheep and goats is infection with coagulase-negative staphylococci (CoNS). These organisms are less pathogenic than *S. aureus* but can cause persistent subclinical mastitis and sometimes clinical mastitis also. *Staphylococcus epidermidis, S. xylosius, S. chromogenes* and *S. simulans* are the most frequently isolated CoNS in sheep while *S. caprae* is common in goats. *Mycoplasma* species are important causes of mastitis in endemic areas and cause marked loss of milk production and elevations in somatic cell count, in addition to affecting other body systems apart from the mammary gland. Lentiviral infections are not usually considered as classical intramammary pathogens as they seldom cause clinical signs or elevated SCC. However, they can cause progressive induration of the mammary tissue and obliteration of secretory tissue.

Pigs

In pigs, mastitis is almost always a disease of recently farrowed gilts or sows, usually in the first 48 hours postpartum. Coliforms (*Escherichia coli*, *Klebsiella* and *Enterobacter* species) most commonly cause peracute mastitis. Coliform mastitis can occur as an entity in itself or the bacteria may play a part in the mastitis-metritis-agalactia syndrome, most frequently seen in gilts. Mortality in piglets, from starvation, can be high.

Subacute mastitis occurs in older sows and can involve either staphylococci or streptococci. One or more glands

Table 36.2 Ovine and caprine mastitis: aetiological agents, source of infection and clinical types

Aetiological agent	Source	Clinical type
Staphylococcus aureus	Skin and mucous membranes, subclinical and chronic intramammary infections	Acute or peracute; responsible for a small percentage of subclinical infections
Streptococcus uberis	Skin, faeces, environment	Acute
Streptococcus agalactiae	Intramammary pathogen	Acute
Streptococcus suis	Infected animals, environment	Acute
Coagulase-negative staphylococci	Skin and mucous membranes, intramammary infections	Responsible for the majority of cases of subclinical mastitis
Enterobacteriaceae	Environment, bedding	Acute, sporadic
Trueperella pyogenes	Skin and mucous membranes	Acute and suppurative
Corynebacteria	Mucous membranes	Acute and suppurative
Mannheimia haemolytica	Mucous membranes, mouth, nasopharynx and tonsils; may survive in environment in cool moist conditions	Peracute (gangrenous) 'blue-bag'
Pasteurella multocida	Mucous membranes, mouth, nasopharynx and tonsils	Acute
Pseudomonas aeruginosa	Environment, water, can survive in some disinfectants	Acute or peracute
Aspergillus fumigatus	Mouldy forage, bedding	Acute, subacute, chronic granulomatous
Serratia marcescens	Environment, water, can survive in some disinfectants	Subacute, chronic, subclinical
Mycoplasma species causing the contagious agalactia syndrome of goats including: *M. capricolum* *M. agalactiae* *M. putrefaciens*	Mucous membranes of urogenital, upper respiratory, upper intestinal tracts and external ear canal	Acute

are affected and this may impair the ability of the sow to suckle and rear a large litter successfully.

Granulomatous lesions in the mammary glands are associated with chronic *Staphylococcus aureus* infections or may be caused by *Actinomyces* species or *Actinobacillus lignieresii*. Other bacteria such as *Fusobacterium necrophorum* and *Trueperella pyogenes* have also been reported as causing mastitis in sows. Clinically inapparent mammary abscesses occur quite commonly.

Horses

Mastitis in mares is comparatively rare, but it can be severe when it does occur. One or both mammary glands can be affected with a marked and painful swelling. There is fever, depression and a stiff gait may be seen or the mare may stand with hindlegs apart because of the discomfort. Gangrenous mastitis has also been reported in mares. Lancefield group C streptococci are the main aetiological agents but *Staphylococcus aureus,* coliforms, *Pseudomonas aeruginosa, Actinobacillus lignieresi* and *Corynebacterium pseudotuberculosis* have also been isolated from mastitic milk from mares. If a sick foal is not sucking, a distended and painful udder can mimic the signs of mastitis.

Dogs and Cats

Mastitis is uncommon in dogs and cats. It usually occurs within six weeks of parturition and may involve one or more mammary glands. Infectious agents gain access through the teat orifice, from penetrating wounds or as a result of haematogenous spread. *Staphylococcus aureus*,

S. pseudintermedius or beta-haemolytic streptococci are the most commonly isolated bacteria from the milk. The clinical syndrome can be peracute, acute or subacute.

Rabbits

Staphylococcus aureus is the most common cause of mastitis in rabbits. The bacterium may be introduced via splinters from wooden nest boxes, teeth of nursing young or from an unhygienic environment.

BOVINE MASTITIS

Aetiology

Some of the many microorganisms that can cause bovine mastitis are summarized in Tables 36.3 and 36.4. The source and type of mastitic syndrome is given for each pathogen. Formerly, the contagious pathogens caused the majority of the cases of bovine mastitis. Members of this group of pathogens, particularly *S. aureus*, are still a major problem in individual herds and in areas or countries where the regulatory bulk milk somatic cell count levels, or those accepted by the milk-processing industry, are less stringent. The implementation of control programmes over the last four decades has meant that the number of clinical cases of mastitis has decreased. In the UK the number of clinical cases has decreased from approximately 150 cases per 100 cows per year in the 1960s to between 47 and 65 cases in 2004–2005 (Wilson & Kingwill 1975, Bradley et al. 2007). The environmental pathogens, *Escherichia coli* and *Streptococcus uberis*, now account for a large proportion of clinical cases.

Coagulase-negative staphylococci and *Corynebacterium bovis* are quite commonly isolated from milk samples. *Corynebacterium bovis* causes a persistent infection of the teat duct epithelium and a mild but significant rise in the leukocyte cell count. Because *C. bovis* is susceptible to disinfectants used as teat-dips, it has been suggested that the presence or absence of the bacterium could be used to monitor the efficiency of the teat-dipping procedure in a herd. Coagulase-negative staphylococci are of low pathogenicity although some strains isolated from mastitis cases have invasive and toxin-producing ability (Burriel & Dagnall 1997, Anaya-Lopez et al. 2006). This group of organisms may cause problems with increased somatic cell count in herds in which other mastitis pathogens are well controlled. The most frequently isolated species are *S. chromogenes* and *S. hyicus*. It has been suggested that the presence of coagulase-negative staphylococci may be advantageous to the host as they tend to occupy attachment sites in the teat duct required by the coagulase-positive, pathogenic staphylococci.

Pathogenesis

Host and pathogen factors as outlined in Table 36.1 are important in influencing the outcome of intramammary infection and the type of clinical syndrome observed. In general, the contagious pathogens cause low-grade chronic

Table 36.3 Bovine mastitis: principal aetiological agents, usual source and clinical types

Aetiological agent	Usual source	Clinical type of mastitis
Staphylococcus aureus	Mammary gland of other cows, udder lesions, skin and mucous membranes	Subclinical, chronic, acute and peracute, including gangrenous mastitis. A high percentage of subclinical carriers can occur in a herd
Streptococcus agalactiae	Intramammary in the milk ducts	Acute or chronic with recurring clinical cases. Infection can occur in maiden heifers
S. dysgalactiae	Buccal cavity and genitalia of cattle	Acute
S. uberis	Skin, tonsils, vagina, faeces	Acute, can occur in dry period
Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes	Faeces, sawdust and other bedding. Disease of housed cows	'Coliform mastitis'. Peracute (toxaemia) usually occurs just after calving in cows with low somatic cell counts. Life-threatening. Acute, chronic and subclinical infections can also occur. Little or no fibrosis in udder of recovered animals
Trueperella pyogenes	Skin and mucous membranes	Peracute, suppurative mastitis
Trueperella pyogenes and *Peptoniphilus indolicus* (±other organisms)	Both part of normal flora. Infection thought to be fly-borne	'Summer mastitis'. Most common in dry cows and heifers. Foul-smelling udder secretion. Loss of quarter or death can occur

Table 36.4 Bovine mastitis: less common aetiological agents, usual source and clinical types

Aetiological agent	Usual source	Clinical type of mastitis
Streptococcus pyogenes	Human pathogen	Acute mastitis
S. pneumoniae	Human pathogen	Peracute with fever
S. equi subsp. *zooepidemicus*	Mucous membranes	Subacute or chronic
Enterococcus faecalis	Faeces and skin	Acute mastitis
Pseudomonas aeruginosa	Soil, water or faeces	Peracute (toxaemia) but can be chronic and persistent
Nocardia species	Soil	Sporadic. Acute at first, becoming chronic. Granulomas in udder tissue
Serratia marcescens	Soil and faeces	Peracute (toxaemia) or chronic coliform mastitis
Pasteurella multocida	Mucous membranes (upper respiratory tract)	Acute mastitis
Mannhemia haemolytica	Mucous membranes (upper respiratory tract)	Peracute and severe or acute
Mycoplasma bovis, M. bovigenitalium, other *Mycoplasma* species	Respiratory tract and mucous membranes	Acute with rapid onset. Most severe in recently calved animals. All quarters often affected. Dramatic drop in milk secretion but rarely any systemic reaction
Mycobacterium bovis	Metastasis from existing tuberculous lesion	Induration and hypertrophy of tissue. Can often palpate lesions in udder after milking
M. fortuitum *M. smegmatis*	Soil, but also associated with oil-based intramammary preparations	Severe mastitis. Cows are either culled or die
Fusobacterium necrophorum	Part of normal anaerobic flora of animals	Acute, secretion viscid and stringy. No fibrosis in udder tissue
Bacillus cereus	Associated with feeding brewers' grains or via intramammary preparations	Peracute or acute
Leptospira serovars Hardjo or Pomona	Water, wet soil or urine of subclinical excretors	Agalactia, self-limiting
Candida albicans (yeast)	Mucocutaneous or environmental	Acute but often self-limiting
Cryptococcus neoformans (yeast)	Often introduced via intramammary tubes	Acute mastitis. Milk is mucoid. Severe swelling of udder
Aspergillus fumigatus (mould)	Often introduced via intramammary tubes	Acute (abscess formation) or chronic
Prototheca zopfii or *P. wickerhamii* (algae)	Mud, soil, faeces or water. Ubiquitous in environment	Chronic. Very difficult or impossible to treat

infections whereas coliform infection is classically characterized by acute disease, severe clinical signs and more rapid clearance of the pathogen from the mammary gland if the animal survives. The streptococci, *S uberis* and *S. dysgalactiae*, usually cause mild clinical signs and may persist longer in the mammary gland than the coliform group of organisms.

Selected virulence attributes of the major mastitis pathogens that are likely to be important in the establishment and outcome of infection are summarized in Table 36.5.

Contagious Pathogens

Staphylococcal mastitis

Affected quarters often have large numbers of *Staphylococcus aureus* in the milk. The staphylococci are easily

Table 36.5 Selected virulence attributes of the major bovine mastitis pathogens likely to be of importance in successful infection of the mammary gland

Pathogen	Virulence factor	Virulence attribute
Contagious pathogens		
Staphylococcus aureus	Fibrinogen, fibronectin, collagen-binding proteins, Clumping factor A	Adhesion to mammary epithelial cells
	Capsule, protein A, biofilm formation, coagulase activity may be important	Antiphagocytic
	Stress proteins	Some stress proteins shown to have a role in survival within cells, however, the precise mechanisms are unclear
	Exoenzymes, haemolysins, leukocidins	Tissue damage
*Streptococcus agalactiae**	Various surface proteins, including lactoferrin-binding protein	Adhesion to mammary epithelial cells
	Capsule, surface proteins	Antiphagocytic
	Exoenzymes, haemolysins including the CAMP factor	Tissue damage
Mycoplasma bovis	Variable surface proteins, other proteins	Probable role in adhesion, evasion of immune response
Environmental pathogens		
Escherichia coli	Enterobactin iron acquisition system	Iron scavenging
	Enzymes for lactose metabolism	Lactose utilization allowing multiplication to levels of 10^8/mL of milk
	Capsule	Antiphagocytic
	Endotoxin	Tissue damage
Streptococcus uberis	*S. uberis* adhesion molecule	Adhesion to mammary epithelial cells
	Capsule	Antiphagocytic
	Plasminogen activator	Activation of plasmin and generation of essential amino acids from casein; may play a role in hydrolysis of host matrix proteins
	Hyaluronidase, factor similar to CAMP factor	Tissue damage
Streptococcus dysgalactiae	Surface proteins	Binding to host proteins such as fibrinogen, fibronectin, collagen, immunoglobulin G
	Hyaluronidase, fibrinolysin	May help in dissemination within the host tissues
	Unknown	Survival within mammary epithelial cells

*Most data relating to virulence factors of *S. agalactiae* are derived from studies of human strains and should be interpreted with caution.

transmitted during milking via the teat cups or milkers' hands. Following entry to the mammary gland, adhesins such as fibrinogen-binding protein and fibronectin-binding protein A are likely to be important in adherence to mammary epithelial cells (Castagliuolo et al. 2006). *Staphylococcus aureus* strains with enhanced ability to survive within both phagocytes and mammary epithelial cells are more likely to set up infection and to persist in the face of the host immune response and antimicrobial therapy. Biofilm formation is now considered to be a factor in persistent infections also (Melchior et al. 2006). The type of mastitis produced by *S. aureus* ranges from

subclinical to the peracute life-threatening forms, one of which is gangrenous mastitis. Gangrenous mastitis is caused by the action of alpha toxin (alpha haemolysin) that damages blood vessels, resulting in ischaemic coagulative necrosis of adjacent tissue. The affected quarter becomes purplish and cold and will eventually slough, if the animal survives the toxaemia. However, subclinical and chronic forms of mastitis are the most common and economically significant types of *S. aureus* mastitis worldwide. Both lead to a gradual replacement of secretory tissue with fibrous tissue and a subsequent loss of milk production by the affected quarter. The chronic and subclinical forms of mastitis respond poorly to antimicrobial therapy, the reasons for which include host, pathogen and treatment factors (Barkema et al. 2006). Host factors such as age of cow, cell count and duration of infection influence treatment outcome as does treatment duration. Pathogen factors affecting cure rates include the ability to survive intracellularly, biofilm formation and antimicrobial resistance. Apart from therapeutic considerations, the emergence of MRSA as a cause of mastitis is of public health concern, in particular the emergence of a divergent *mecA* gene in bovine clones of *S. aureus* and the possibility that cattle may serve as a source for the emergence of new MRSA strains in humans (Garcia-Alvarez et al. 2011, Shore et al. 2011).

Streptococcus agalactiae

Although no longer a common cause of mastitis in major dairying areas, *Streptococcus agalactiae*, may be a significant problem in individual herds. It is an obligate parasite of the bovine mammary gland. The organism can adhere to mammary epithelial cells but does not invade the interstitial tissue. Few data on specific virulence factors of mastitis-producing strains of *S. agalactiae* have been published and most information relates to human strains. The production of a capsule allowing protection against phagocytosis is likely to be of importance. *Streptococcus agalactiae* secretes a number of enzymes and haemolysins which have a probable role in destruction of mammary tissue. Following establishment of infection, there is rapid multiplication of the bacterium with a great outpouring of neutrophils into the ducts and damage to the ductal and acinar epithelium. Ducts are obstructed with cells and debris causing involution of the acini in the affected lobules. Fibrosis of interalveolar tissue occurs, resulting in a loss of secretory function.

Mycoplasmal mastitis

Although mycoplasmal mastitis can be clinically severe, there is rarely any systemic involvement. *Mycoplasma bovis* is the most important species involved and is particularly important in very large dairy herds. Cows of all ages and all stages of lactation can be affected, with those that have recently calved showing the most severe signs. There can be long-term persistence of the organisms in udders and subclinically affected animals are an important source of infection. Haematogenous spread between quarters occurs. The role of the known virulence factors of *M. bovis* in the pathogenesis of mastitis is not entirely clear. Surface proteins are likely to have a role in adherence and immune evasion in the mammary gland as has been shown for other host tissues. The secretion from an affected quarter appears fairly normal in the early stages of infection, but if the milk is allowed to stand, a deposit of fine, flaky material settles out leaving a turbid, whey-like supernatant fluid. Leukocyte counts in the milk are usually very high, often over 20 million cells/mL.

Environmental Mastitis

Coliform mastitis

Up to 65% of cases of *E. coli* mastitis occurring during the first two months of lactation are the result of infection acquired during the dry period (Smith et al. 1985). It is thought that the survival and multiplication of *E.coli* strains within the dry mammary gland is dependent in part on the ability of the pathogen to acquire iron (Hogan & Smith 2003). *Escherichia coli* and other coliforms can multiply rapidly in quarters with a low cell count. The immune response of the host is a major determinant of the outcome of infection and both the somatic cell count and the speed of recruitment of neutrophils into the mammary gland affect the course and severity of the mastitis episode (Shuster et al. 1996). The inflammatory reaction destroys a large proportion of the bacterial population. When Gram-negative bacteria die and lyse, endotoxin is released from the cell wall. The sudden liberation of endotoxin can result in a severe toxaemia that is life-threatening. Approximately 10% of clinical coliform cases result in severe systemic signs (Hogan & Smith 2003). Unlike infections with contagious pathogens, coliform mastitis tends to be of short duration with *E. coli* infections during lactation typically lasting less than 10 days (Todhunter et al. 1991). However, there is evidence that this pattern may be changing and persistent infections with *E. coli* may be becoming more frequent (Bradley & Green 2001).

Streptococcus uberis

This organism is a commensal of the tonsils, gastrointestinal and genital tract of cattle and is found on the coat and in the environment. Faecally contaminated bedding is an important source of infection but heavily used pasture may carry high levels of the pathogen also. The organism is classified as an environmental pathogen but a specific molecule involved in adherence to bovine mammary epithelial cells has been demonstrated (Almeida et al. 2006). *Streptococcus uberis* produces exoenzymes

including a plasminogen activator which converts plasminogen to plasmin. Plasmin is a protease which in turn can hydrolyse casein to peptides which the organism then uses for growth. Production of a capsule confers resistance to phagocytosis. Only a few infections result in systemic signs of fever and inappetance, with clinical signs usually limited to abnormalities in the milk.

Streptococcus dysgalactiae

Streptococcus dysgalactiae has been classified as both a contagious and environmental pathogen. It is found in the environment of cows as well as colonizing the tonsils and mucosa of the mouth and genital tract. It is able to persist within the mammary gland although the mechanism whereby it survives within mammary epithelial cells is unknown. It is isolated relatively infrequently from clinical and subclinical cases of mastitis, 1.5% and 0.4% respectively in a survey by Bradley et al. (2007), but is associated with 'summer mastitis' also.

'Summer mastitis'

This syndrome is seen in Europe, North America and Japan. It is a mixed infection of *Trueperella* (*Arcanobacterium*) *pyogenes* usually with *Peptoniphilus indolicus* but frequently other pathogens such as *S. dysgalactiae* and other anaerobic organisms are isolated. 'Summer mastitis' occurs in non-lactating heifers and cows at pasture in the summer months and tends to be more common during wet weather. It is thought to be fly-borne. A massive invasion of the mammary tissue via the teat canal results in a large proportion of the gland being affected at one time. There is a severe systemic reaction and loss of function of the entire quarter. The secretion from the affected quarter is foul-smelling, attributable to the activities of the anaerobic *P. indolicus*. If the cow survives, the quarter becomes extremely indurated and abscesses develop, later rupturing through the floor of the udder, often at the base of a teat. The pathogenic mechanisms of the organisms which contribute to the clinical manifestations of this disease have not been clarified. However, it is known that *T. pyogenes* expresses a potent cytolysin (pyolysin) and also a number of adherence factors, including neuraminidases and a collagen-binding protein and can survive within epithelial cells (Jost & Billington 2005).

Uncomplicated infections with *T. pyogenes* alone can also be serious with quarters so severely affected that there is a permanent loss of the quarter and sloughing can occur.

Pseudomonas aeruginosa mastitis

Infection by *Pseudomonas aeruginosa* can have a pathogenesis similar to coliform mastitis and a severe endotoxaemia can occur. However, the infection may also result in a subclinical mastitis with the pathogen persisting in the mammary gland.

Mastitis caused by *Nocardia* species

Nocardia species cause a destructive mastitis that can be acute initially, but is characterized by a granulomatous inflammation that leads to extensive fibrosis and formation of palpable nodules in the udder tissue. Once clinical changes are evident in the mammary gland there is little hope of successful treatment.

Infections by atypical mycobacteria and fungi

The atypical mycobacteria, such as *Mycobacterium fortuitum*, are thought to be accidentally introduced into the udder with oil-based antibiotics. Infections due to yeasts and moulds may also be introduced with intramammary antibiotics. Prolonged or repetitive use of antibiotics can aid their establishment in the udder. These infections tend to be chronic and refractory to treatment.

Infectious conditions of the skin of mammary glands

Traumatic or infectious conditions affecting the skin or subcutaneous tissue of the teats or udder may predispose the cow to mastitis. Painful lesions can lead to difficulty in applying the teat cups or result in the cow giving an incomplete let-down of milk. In the case of 'acne' or 'impetigo', caused by *Staphylococcus aureus*, the infection may provide a source of organisms that could cause mastitis if they gained entry to the gland through the teat orifice. The infectious conditions affecting the external surface of teats and udder are summarized in Table 36.6.

Diagnosis of Bovine Mastitis

Regulatory limits for bulk milk SCC (BMSCC) and industry requirements differ greatly between countries and thus cell count levels taken as significant vary. BMSCC is monitored regularly and levels of 100,000 cells/mL or less may be demanded by some processing companies. Somatic cell counts of greater than 200,000 cells/mL milk are frequently used to define subclinical mastitis in an individual animal.

Essentially, bovine mastitis should be regarded as a herd problem and the methods of investigation and diagnosis should reflect this fact. The diagnostic methods include:

- Total and leukocyte cell counts both for herds and individual cows.
- Indirect chemical tests.
- Microbiological investigation to determine:
  - The major pathogen(s) causing mastitis in a herd.
  - The percentage of subclinical carrier cows.

Table 36.6 Miscellaneous infectious conditions of the external surface of bovine udder and teats

Infectious agents	Disease	Clinical findings
Bovine parapoxvirus (*Poxviridae*)	Pseudocowpox	Starts as small inflammatory papule, usually on teats, that develops into a dark red scab.The central area desquamates leaving a ring- or horse-shoe-shaped scab. Heals in four to six weeks
Bovine herpesvirus 2 (*Herpesviridae*)	Bovine ulcerative mammillitis	The lesions are usually on teats but can spread to the udder. Lesions start as a local thickening of the skin, followed by severe oedema and erythema. Detachment of the epithelium occurs leaving a raw ulcerated surface. Uncomplicated cases heal in three to four weeks
Bovine papillomaviruses (*Papillomaviridae*)	Bovine papillomas Types 1 and 2	Cutaneous fibropapilloma that can be large and cauliflower-like. Usually occurs on head and neck but can be present on the skin of the udder
	Type 5	Teat fibropapilloma with a 'rice-grain' appearance, being flat and white
	Type 6	Teat papilloma or 'frond warts' that are thin and pedunculated
	Types 7, 8, 9, 10	Warts on teats and sometimes skin of udder
Bovine orthopoxvirus (*Poxviridae*)	Cowpox	Now a rare condition in cattle. In Britain the infection is more common in cats. A pustule occurs and ruptures. The exudate forms a scab covering an ulcerated area. The lesion may take several weeks to heal
Fusobacterium necrophorum (±*Staphylococcus aureus*)	'Black pox' or 'black spot'	Deep crater-shaped ulcers with raised edges and a central black spot. Almost always at the tip of the teat and usually invades the sphincter
S. aureus	'Udder impetigo'	Small pustules (2–4 mm diameter) that involve the subcutaneous tissue. Lesions are often situated at the base of the teats
Pithomyces chartarum	Facial eczema (mycotoxicosis)	Photosensitization with reddening of the teats and udder with eventual sloughing of the skin
Aphthovirus (*Picornaviridae*)	Foot-and-mouth disease	Vesicles on teats of milking cow
Vesiculovirus (*Rhabdoviridae*)	Vesticular stomatitis	Vesicles may occur on the teats of milking cows
Orbivirus (*Reoviridae*)	Blue tongue	Lesions may occur on the teats of milking cows

  - The antimicrobial susceptibility patterns of the major pathogens so that effective treatment can be administered.
- Clinical examination and detection of abnormal milk using a strip cup.

Cell counts on milk

Many cell-counting methods have been developed but electronic counting methods using equipment such as Coulter or Fossomatic® (Foss Electric) counters are the most common. These are total cell counts as both exfoliated epithelial cells and leukocytes are counted. If such equipment is not available, direct microscopic counting methods can be used (modified Breed's smear). In this case leukocytes can be counted directly. A known volume of milk (0.01 mL) is spread over 1 cm^2 on a microscope slide, defatted and stained by a methylene-blue-based stain. The microscope is calibrated and from an average number of leukocytes per field (counting 50 fields) the number of leukocytes/mL of milk can be calculated. If comparatively large numbers of pathogenic bacteria are present in the milk sample, these may also be seen in the stained smear (Fig. 36.1).

The Californian Mastitis Test (CMT) is an indirect cell count method for use on individual samples. This test can be used in the field or in the laboratory and is based on the quantity of DNA in the milk and hence the number

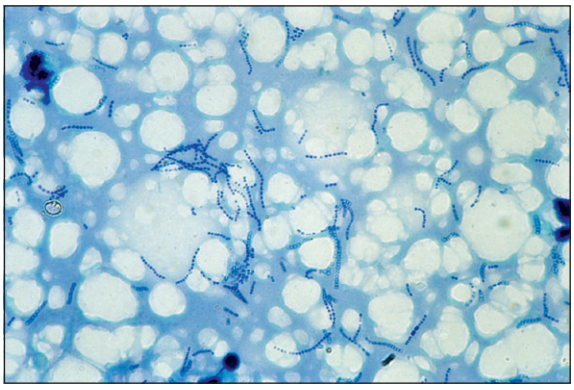

Figure 36.1 Newman stain showing chains of streptococci in a bovine mastitic milk sample. (×1000)

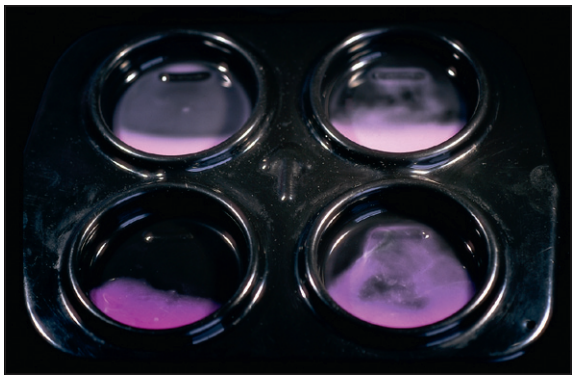

Figure 36.2 Californian mastitis test (CMT) designed as a 'beside-the-cow' test to detect subclinical mastitis. Interpretation: normal 0 (top left); positive 1+ (top right); positive 2+ (bottom right) and positive 3+ (bottom left).

Table 36.7 Correlation between the Californian mastitis test result and the somatic cell count

CMT score	Interpretation	Visible reaction	Total cell count (/mL)
0	Negative	Milk fluid and normal	0–200,000 0–25% neutrophils
T	Trace	Slight precipitation	150,000–500,000 30–40% neutrophils
1	Weak positive	Distinct precipitation but no gel formation	400,000–1,500,000 40–60% neutrophils
2	Distinct positive	Mixture thickens with a gel formation	800,000–5,000,000 60–70% neutrophils
3	Strong positive	Viscosity greatly increased. Strong gel that is cohesive with a convex surface	≥5,000,000 70–80% neutrophils

of leukocytes and other cells present. A squirt of milk from each quarter of the udder is placed in each of four shallow cups in the CMT paddle. An equal amount of commercial CMT reagent or 14% sodium lauryl sulphate (Teepol®, Shell) is added to each cup. A gentle circular motion is applied to the mixtures, in a horizontal plane and a positive gelling reaction occurs in a few seconds (Fig. 36.2). Table 36.7 gives the interpretation of the CMT scores (0–3), the visible reaction, the approximate total cell count and expected percentage of neutrophils in the total count (Schalm et al. 1971). The CMT gives a good indication of the leukocyte count of the milk. Other tests that depend on the development of gels are the Wisconsin and NAGase mastitis tests. The NAGase mastitis test is based on a cell-associated enzyme in milk, N-acetyl D-glucosaminidase, and is easily automated. High levels of the enzyme indicate a high cell count.

Leukocytes in milk samples disintegrate quite rapidly on storage so cell counts should be conducted within two hours of milk collection unless preservatives such as Bronopol® are added to the milk sample.

There are normal variations in cell counts on milk:

- Cows in early and late lactation have higher counts than those recorded in mid-lactation. However, the milk from all four quarters will be equally affected.
- Individual variation exists between cows and, normally, a cow will maintain a certain cell count level throughout life.
- The presence of *Corynebacterium bovis*, regarded as non-pathogenic, in the teat duct will cause a rise in the cell count.

In cases of chronic mastitis, the highest cell count is obtained from strippings at the end of milking.

The counts also vary depending on the pathogen present. Infections associated with *Streptococcus agalactiae* cause higher cell counts than those in staphylococcal mastitis.

Other tests to detect mastitis

There are a number of tests which are currently under investigation as possible online automated monitoring systems. They are based on changes which occur in milk following inflammation. An increase in electrical conductivity of the milk occurs in mastitis because of increased levels of sodium and chloride ions. Other tests are based on increases in compounds such as lactate or serum amyloid A.

Microbial investigation of mastitis

Milk sample collection

It is vital that a milk sample for microbiology is taken so as to ensure that the potential pathogen(s) in the sample came from the inside of the mammary gland and not from dust or faecal particles on the udder surface. It is not always possible for the veterinarian to collect the sample, but if the farmer is instructed in the correct procedure, good-quality samples can be obtained. It is essential to obtain a milk sample before the cow has been treated with either intramammary or systemic antimicrobial agents. The main points in a good collection technique are to: clean and dry the teat, then wipe the teat thoroughly twice with 70% ethyl alcohol, paying particular attention to the teat orifice; allow the alcohol to dry between applications; carry out the collection as swiftly as possible; hold the sterile collection bottle nearly horizontal and keep the lid in the crook of the little finger so that the lid does not become contaminated.

A composite milk sample is satisfactory unless it is necessary to investigate the quarters separately. The first stream of milk, from the teat canal, usually has a higher cell count and bacterial population than that in the mammary gland. As the results from the examination of this 'fore-milk' are more a reflection of the conditions existing within the teat than in the mammary gland itself, it is usually recommended that the first few squirts of milk from each quarter be discarded. The milk sample should, ideally, be kept refrigerated from the time of collection to the time of bacteriological examination. For practical purposes, particularly when farmers are collecting samples over time from a number of cows, it is possible to freeze milk samples and submit them in bulk to the laboratory for analysis. If mycoplasmal mastitis is suspected a simple transport medium is the milk sample itself with ampicillin added at 5 mg/mL. The milk sample, in this case, is held at the ambient temperature to allow mycoplasmal growth during transportation. A second milk sample should be submitted, without the ampicillin, as a check for other mastitis-producing pathogens.

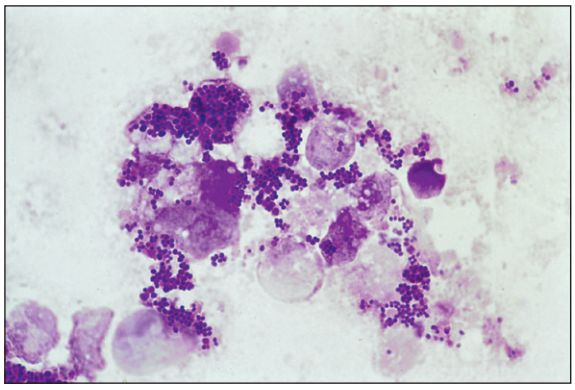

Figure 36.3 *Staphylococcus aureus* in a bovine mastitic milk sample. (Gram stain, ×1000)

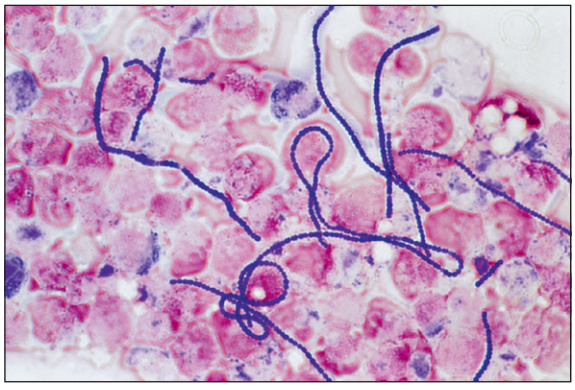

Figure 36.4 Streptococcal chains in a bovine mastitic milk sample. (Gram stain, ×1000)

Direct microscopy

The milk sample can be centrifuged and a stained smear made from the deposit. A Gram-stain is used routinely to detect Gram-positive pathogens such as staphylococci (Fig. 36.3), streptococci (Fig. 36.4) and will also reveal yeasts, such as *Candida albicans*, that are stained deeply by crystal violet. A MZN-stained smear can be made if *Nocardia asteroides* is suspected and a ZN-stained smear for the rare cases when 'acid-fast' bacteria such as *Mycobacterium fortuitum* or *M. bovis* (Fig. 36.5) are present.

Culture

Most of the bacterial pathogens causing mastitis grow on ox or sheep blood agar. A MacConkey No.2 agar plate is streaked in parallel to detect coliforms, *Enterococcus faecalis* and any other Gram-negative bacteria that are able to grow on the medium. Edwards medium is highly selective for

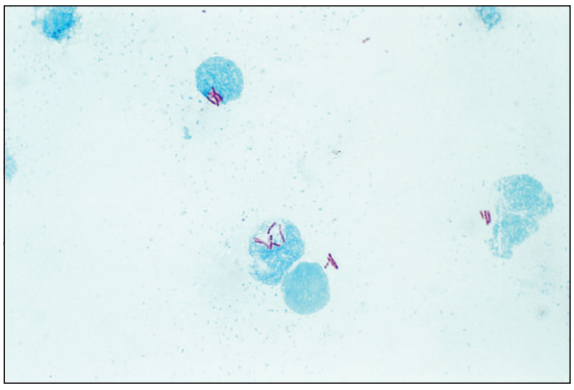

Figure 36.5 *Mycobacterium bovis* in a bovine milk sample from a case of tuberculous mastitis. (ZN stain, ×1000)

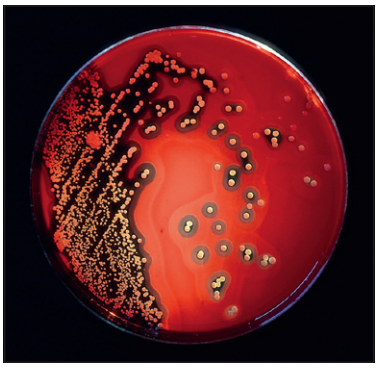

Figure 36.6 Sheep blood agar inoculated with a bovine mastitic milk sample giving an almost pure culture of *S. aureus*. Note the characteristic 'target' haemolysis.

streptococci and also acts as an indicator medium for haemolysis and for the hydrolysis of aesculin. A Sabouraud dextrose agar plate can be inoculated if a fungal pathogen is suspected. However, pathogens such as *Candida albicans* and *Aspergillus fumigatus* form colonies on blood agar at 37°C in 2–3 days, if there is little or no competition from faster-growing bacteria.

If a large number of milk samples are to be cultured on a herd basis, quarter-plating (Chapter 2) the samples on aesculin blood agar, alone, is satisfactory. This medium is not selective and will support the growth of the majority of the bacterial pathogens. Aesculin blood agar consists of blood agar with 0.05–0.1% aesculin added as a sterile solution, when the blood agar base has cooled to 50°C.

The inoculated plates are incubated aerobically, unless specific anaerobes such as *Fusobacterium necrophorum* or *Peptoniphilus indolicus* are being sought. All the mastitis-producing microorganisms will grow at 37°C and it is advisable to incubate the plates for up to five days to accommodate the slow-growing fungi and bacteria such as *Nocardia asteroides*.

Identification

The main characteristics and tests for the presumptive identification of the mastitis-causing pathogens are covered and summarized in Table 36.8. For more detailed information the relevant chapter on the bacterium or fungus should be consulted.

Staphylococcus aureus

1. Colonial appearance: round, shiny, golden-yellow colonies surrounded by a zone of double-haemolysis on blood agar (Fig. 36.6). There is no growth on most formulations of MacConkey agar, especially those with added crystal violet. No growth occurs on Edwards medium.
2. Gram-stained smear: Gram-positive cocci.
3. Coagulase test (Fig. 7.11): this test is necessary to ensure that the isolate is coagulase-positive and therefore a pathogenic strain. Commercial systems for the identification of clumping factor, protein A and, in some cases, capsular polysaccharides are available. These latex agglutination tests include tests such as Staphaurex and Staphaurex Plus (Murex Diagnostics Limited) and Pastorex Staphplus (Sanofi).
4. Purple agar with 1% maltose: as a presumptive check that the isolate is *S. aureus* (Fig. 7.13).
5. Antibiotic susceptibility test: many *S. aureus* strains are resistant to penicillin and to other commonly used antibiotics, so a susceptibility test is necessary.
6. Commercial identification systems such as API Staph (bioMérieux) are available.

Mastitis-producing streptococci

1. Colonial appearance: small, translucent colonies at 24 hours' incubation on blood agar with alpha-haemolysis, beta-haemolysis or gamma-haemolysis (Figs 8.1 and 8.2).
2. Growth on Edwards medium: all the streptococci are able to grow on this selective medium. *Streptococcus uberis* (Fig. 36.7) and *Enterococcus faecalis* (Fig. 36.8) hydrolyse aesculin but *S. agalactiae* (Fig. 36.9) and *S. dysgalactiae* (Fig. 36.10) do not. Aesculin hydrolysis on Edwards medium is indicated by a darkening of the medium and colonies (Fig. 36.11). This can be seen more clearly under ultraviolet light (Wood's lamp) (Fig. 36.12). The aesculin in the medium causes the agar itself to have a dull-blue glow.
3. Gram-stained smear: scattered Gram-positive cocci. Streptococci are not usually seen in chains from colonies on a solid medium.

Table 36.8 Bacteria capable of causing bovine mastitis showing some of their main characteristics leading to a presumptive identification

Mastitis-causing bacteria	Gram reaction	Shape	Catalase	Oxidase	Haemolysis	Growth on MacConkey agar	Aesculin hydrolysis (Edwards medium)	CAMP test	Lancefield group	Other characteristics and confirmatory tests
Streptococcus agalactiae	+	C	–	–	β,γ,α	–	–	+	B	CAMP test positive
S. dysgalactiae	+	C	–	–	α	–	–	–	C	Alpha haemolytic, CAMP negative
S. uberis	+	C	–	–	α,γ	–	+	–	–	Aesculin splitter, no growth on MacConkey
Enterococcus faecalis	+	C	–	–	α,γ	+	+	–	D	Pinpoint red colonies on MacConkey agar. Aesculin hydrolysis
S. pyogenes	+	C	–	–	β	–	–	–	A	Susceptible to bacitracin (0.04 unit disc)
S. pneumoniae	+	C	–	–	α	–	±	–	–	Susceptible to optochin. Often mucoid
S. equi subsp. *zooepidemicus*	+	C	–	–	β	–	–	–	C	Trehalose –, sorbitol+, lactose+, maltose+(–)
Staphylococcus aureus	+	C	+	–	+	–	Golden-yellow pigment; double-zoned haemolysis; coagulase+ and ferments maltose on purple agar base plus 1% maltose			
Escherichia coli	–	R	+	–	±	+	'IMViC' test+/+/–/–. Metallic sheen on EMB agar. Usually motile. Occasionally mucoid, often haemolytic			
Klebsiella pneumoniae	–	R	+	–	–	+	Mucoid colonies, non-motile, 'IMViC' test –/–(+)/+/+			
Enterobacter aerogenes	–	R	+	–	–	+	Mucoid colonies, motile, 'IMViC' test –/–/+/+			
Serratia marcescens	–	R	+	–	–	+	Red pigment at 25°C, some strains at 37°C			
Pseudomonas aeruginosa	–	R	+	+	±	+	Greenish-blue pigment, fruity smell			
Trueperella pyogenes	+	R	–	–	+	–	Small colonies, hazy haemolysis. Pits Loeffler serum slope			
Nocardia asteroides	+	F	+	–	±	–	Powdery white colonies, adherent to medium. MZN+. Requires three to four days' incubation. Growth on Sabouraud agar			
Pasteurella multocida	–	R	+	+	–	–	Colonies with sweetish smell. Non-haemolytic, indole+ and no growth on MacConkey agar			
Mannheimia haemolytica	–	R	v	+	+	+	No smell. Haemolytic, indole- and red, pinpoint colonies on MacConkey agar			
Bacillus cereus	+	R	+	–	+	–	Forms endospores. Wide zone of haemolysis. Large, flat, dry and granular colonies			

C = coccus, R = rod, F = filamentous, + = positive reaction, ± = most strains positive, (+) = some strains positive, v = strains vary, – = negative reaction, 'IMViC' = indole, methyl red, Voges–Proskauer and citrate tests. MZN = modified Ziehl–Neelsen stain

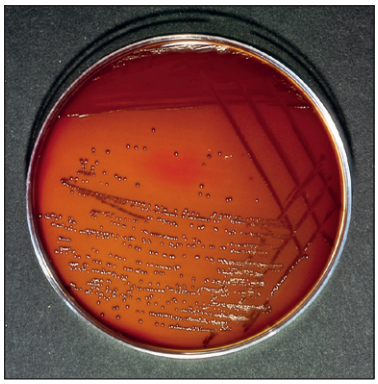

Figure 36.7 *Streptococcus uberis* on Edwards medium: alpha-haemolytic and aesculin splitting.

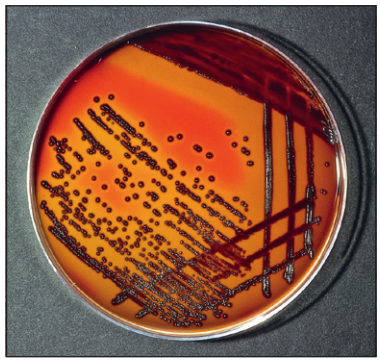

Figure 36.8 *Enterococcus faecalis* on Edwards medium: alpha-haemolytic and aesculin splitting.

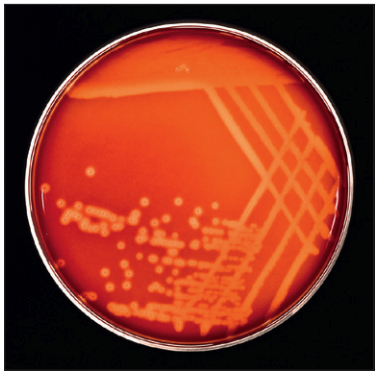

Figure 36.9 *Streptococcus agalactiae* on Edwards medium: beta-haemolytic but non-aesculin splitting.

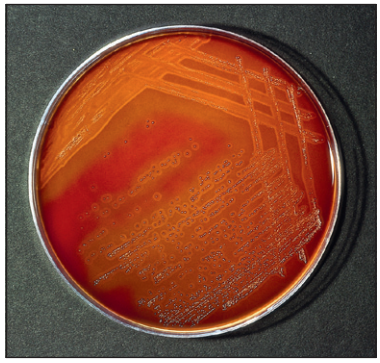

Figure 36.10 *Streptococcus dysgalactiae* on Edwards medium: alpha-haemolytic and non-aesculin splitting.

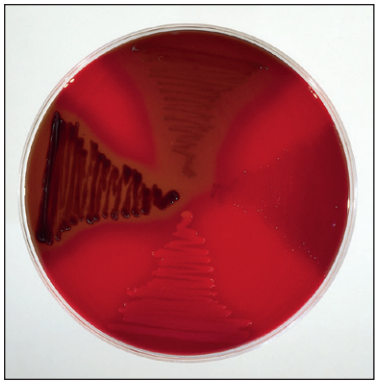

Figure 36.11 Aesculin hydrolysis on Edwards medium (under ordinary light): *Enterococcus faecalis* (left) and *S. uberis* (top) split aesculin, whereas *S. dysgalactiae* (right) and *S. agalactiae* (bottom) failed to do so.

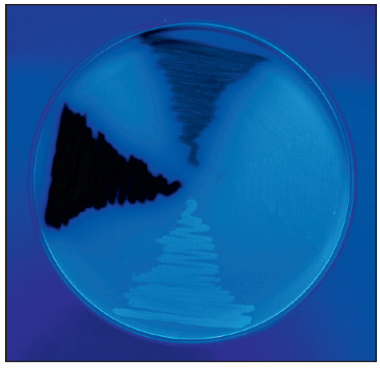

Figure 36.12 Wood's lamp (UV light) aids the detection of aesculin hydrolysis in Edwards medium: *Enterococcus faecalis* (left) and *S. uberis* (top) split aesculin, whereas *S. dysgalactiae* (right) and *S. agalactiae* (bottom) failed to do so (compare with Figure 36.11 showing the same plate under ordinary light).

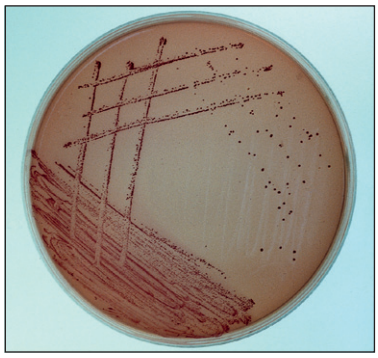

Figure 36.13 *Enterococcus faecalis* on MacConkey agar showing pinpoint red colonies. This is one of the few Gram-positive bacteria able to tolerate the bile salts in this medium.

Figure 36.14 CAMP test on sheep blood agar with *Staphylococcus aureus* as the horizontal streak. *Streptococcus agalactiae* (top right) gives a positive CAMP reaction with 'arrow-head' enhancement of the partial haemolysis caused by the staphylococcal beta-haemolysin. *Streptococcus dysgalactiae* (bottom right) gives no reaction, while the weak reactions of *S. uberis* (bottom left) and *E. faecalis* (top left) can easily be distinguished from the positive reaction of *S. agalactiae*.

4. Catalase test: streptococci are catalase-negative whereas the staphylococci are catalase-positive.
5. Growth on MacConkey agar: *Eenterococcus faecalis* and some of the other Lancefield Group D streptococci are able to tolerate the bile salts in MacConkey agar and grow as red, pinpoint colonies (Fig. 36.13).
6. CAMP test: only *S. agalactiae* gives a sharp arrow-head enhancement of haemolysis caused by the beta-haemolysin of *Staphylococcus aureus* (Fig. 36.14).
7. Lancefield Grouping: this is not usually necessary but can be carried out by a latex agglutination kit (Fig. 8.4) that covers Groups A, B, C, D and G. *Streptococcus uberis* does not belong to a Lancefield Group, but if the isolate is in a pure culture from a carefully taken milk sample, and corresponds to the characteristics in Table 36.8, then a presumptive identification can be made.
8. Optochin and bacitracin susceptibility: if the mainly human pathogens, *S. pyogenes* or *S. pneumoniae* are suspected, bacitracin or optochin susceptibility tests could be carried out. Group A streptococci (*S. pyogenes*) are susceptible to bacitracin (Fig. 8.15) but the other beta-haemolytic streptococci are resistant. *Streptococcus pneumoniae* is susceptible to optochin (Fig. 8.16) but the other alpha-haemolytic streptococci are not.
9. Commercial systems are available for the identification of streptococci, such as API 20Strep© (bioMérieux).
10. Antibiotic susceptibility test: some of the mastitis-producing streptococci have become resistant to penicillin, so an antibiotic susceptibility test on the isolate is advisable. This has to be carried out on a blood agar plate as the streptococci grow poorly, or not at all, on media without blood or serum.

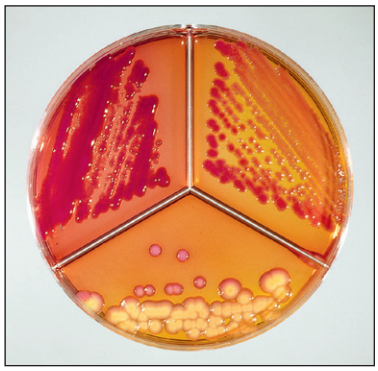

Figure 36.15 A mucoid isolate of *E. coli* (left), *Enterobacter aerogenes* (right) and *Klebsiella pneumoniae* (bottom) on MacConkey agar. All are lactose fermenters but colonies of *E. coli* are invariably a more vivid pink.

Coliforms

Escherichia coli, *Klebsiella pneumoniae* and *Enterobacter aerogenes* are the members of the *Enterobacteriaceae* most commonly involved in bovine mastitis and less commonly *Serratia marcescens*.

1. Colonial appearance: *Klebsiella pneumoniae*, *E. aerogenes* and very occasionally strains of *E. coli* have mucoid colonies (Fig. 36.15). More commonly *E. coli* is non-mucoid and the colonies are round, discrete and bright-pink (lactose fermenting) on MacConkey agar (Fig. 17.19). *Escherichia coli* is the only coliform that may be haemolytic on blood agar (Fig. 17.18) although this is a variable characteristic. The coliforms do not grow on Edwards medium.

2. Gram-stained smear: medium-sized Gram-negative rods.
3. Oxidase test: many Gram-negative bacteria are oxidase-positive but members of the *Enterobacteriaceae* are exceptional in being oxidase negative.
4. Motility test in semisolid medium (Chapter 2): to distinguish between *K. pneumoniae* and *E. aerogenes* that both characteristically have mucoid colonies. *Klebsiella pneumoniae* is non-motile but *E. aerogenes* is motile.
5. 'IMViC' test: used for the presumptive identification of *E. coli* which is invariably indole+/MR+/VP–/citrate– (Fig. 17.21). Table 36.8 shows the corresponding reactions for *K. pneumoniae* and *E. aerogenes*.
6. Pigment production: *Serratia marcescens* produces the distinctive red pigment, prodigiosin, at 25°C and less reliably at 37°C (Fig. 17.44). The pigment can be seen in the colonies on MacConkey agar as the bacterium would otherwise have pale colonies of a non-lactose fermenter.
7. Commercial identification systems, such as API 20E® (bioMérieux) are available for the *Enterobacteriaceae*.
8. Antibiotic susceptibility test: this is extremely important for members of the *Enterobacteriaceae* because of the possibility of transferable multiple drug resistance.

Trueperella pyogenes

1. Colonial appearance: after 24 hours' incubation the colonies on blood agar are so small that they are difficult to see but there will be a hazy haemolysis along the streak lines. After longer incubation, the small colonies are visible surrounded by a hazy type of haemolysis (Fig. 36.16). No growth occurs on MacConkey agar or Edwards medium.
2. Gram-stained smear: pleomorphic Gram-positive rods (Fig. 10.6).
3. Catalase test: *Trueperella pyogenes* is catalase-negative.
4. Loeffler serum slope: pitting of the slope by *T. pyogenes* in 24–48 hours (Fig. 10.15) gives a presumptive identification.
5. Antibiotic susceptibility test: the test is difficult to read with this slow-growing bacterium; however, it is usually sensitive to penicillin.

Pseudomonas aeruginosa

1. Colonial appearance: on blood agar *P. aeruginosa* produces large, flat colonies, usually haemolytic, with the green-blue pigment, pyocyanin, most obvious in areas of heavy growth (Fig. 18.1). The colonies have a characteristic fruity odour. On MacConkey agar the bacterium is a non-lactose fermenter, but the green-blue pigment is often superimposed on what would otherwise be pale colonies (Fig. 18.3). There is no growth on Edwards medium.
2. Gram stained smear: medium-sized Gram-negative rods.
3. Oxidase test: *Pseudomonas aeruginosa* is strongly oxidase-positive. This helps to distinguish it from the oxidase-negative coliforms.
4. Pyocyanin-enhancing medium: as pyocyanin is unique to *P. aeruginosa*, demonstration of this pigment gives a good presumptive identification of this bacterium. The pyocyanin-enhancing medium (Fig. 36.17) is useful for strains of the organism that are poor producers of the pigment.
5. Antibiotic susceptibility test: this is essential for *P. aeruginosa* as the organism is notoriously drug-resistant (Fig. 18.9).

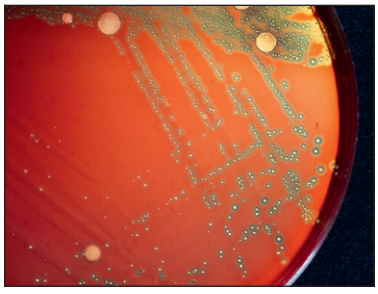

Figure 36.16 Sheep blood agar incubated with a mastitic milk sample yielding an almost pure growth of *Trueperella pyogenes*: tiny colonies even after 72 hours' incubation, with a hazy type of beta-haemolysis.

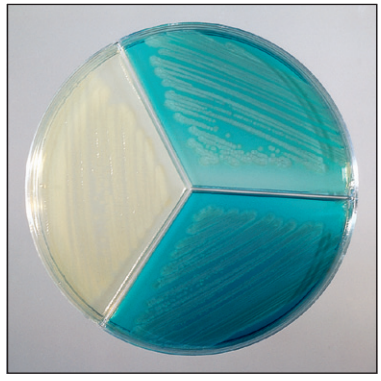

Figure 36.17 *Pseudomonas* strains on '*Pseudomonas* agar P' which enhances pyocyanin production. No pyocyanin is detectable from the strain on the left, while the strains on the right are good pyocyanin producers.

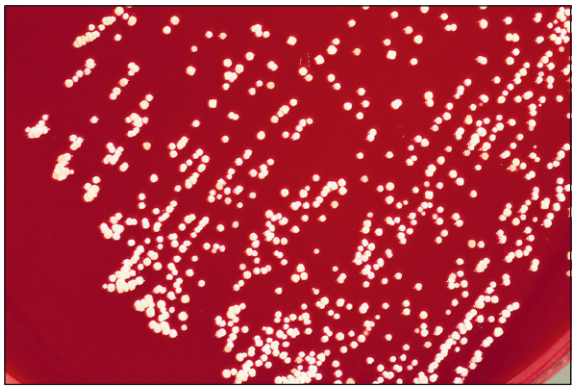

Figure 36.18 White powdery colonies of *Nocardia asteroides* on sheep blood agar after 96 hours' incubation.

Nocardia *species*

In chronic cases palpable granulomas are often present in the udder tissue.

1. Direct microscopy: *Nocardia asteroides* is slow-growing and difficult to culture, especially if the animal has been treated with antibiotics. A MZN-stained smear on the deposit from a centrifuged milk sample may be the best way to reach a diagnosis. The appearance is similar to that shown in Fig 10.16.
2. Colonial appearance: after three to four days' incubation, white, powdery colonies, that are embedded in the agar, are seen (Fig. 36.18). Some strains are haemolytic. The colonies of *N. asteroides* have no smell which distinguishes them from *Streptomyces* species, which have similar colonies (Fig. 10.20) but a pungent earthy odour. *Streptomyces* species are common contaminants on plates that have been left at room temperature for a few days.
3. Growth on Sabouraud dextrose agar: *Nocardia asteroides* and some *Streptomyces* species have the unusual ability of being able to grow on Sabouraud agar (Fig. 10.19), which is a selective medium for fungi.

Pasteurella *species*

1. Colonial appearance: *Pasteurella multocida* has medium-sized, shiny (sometimes mucoid), non-haemolytic colonies that have a pinkish tinge on blood agar (Fig. 21.4). The colonies have a delicate, but highly characteristic, sweetish odour. *Mannheimia haemolytica* is haemolytic and the colonies on blood agar are similar to those of a beta-haemolytic *Streptococcus* (Fig. 21.6). *Pasteurella multocida* will not grow on MacConkey agar but *M. haemolytica* grows as small, red, pinpoint colonies (Fig. 21.7) not unlike those of *Enterococcus faecalis*. No growth occurs on Edwards medium.
2. Gram-stained smear: Gram-negative rods that tend to be coccobacillary.
3. Catalase test: the pasteurellae are catalase-positive and this distinguishes them from streptococci.
4. Oxidase test: both *P. multocida* and *M. haemolytica* are oxidase-positive, whereas members of the *Enterobacteriaceae* are oxidase-negative.
5. Indole production: *Pasteurella multocida* is a good indole producer (this and other characteristics are summarized in Table 36.8). For further details of biochemical reactions *see* Chapter 21.
6. Commercial identification systems such as API 20NE® (bioMérieux) can be used.

Bacillus cereus

1. Colonial appearance: large, flat, granular, haemolytic colonies on blood agar (Fig. 14.4). It will not grow on MacConkey agar or Edwards medium.
2. Gram-stained smear: large Gram-positive rods, some of which may be sporing. The endospores are seen as clear, unstained, oval areas within the mother cells.
3. See Chapter 14 for confirmatory tests.

Mycoplasma *species*

Special media are required for the isolation of *Mycoplasma* species (Chapter 35). Indications that mycoplasmas are involved in an outbreak of mastitis would include the fact that no major pathogens could be isolated by routine cultural methods and by the clinical signs. There are severe changes to the mammary glands but usually no systemic involvement.

Mycobacteria

Infection of the udder by mycobacteria is rare except in countries where bovine tuberculosis is still relatively common. If a mycobacterial infection is suspected, possibly due to finding palpable lesions in the udder, about 50 mL of milk should be carefully collected. There are usually relatively few mycobacteria present, so the milk sample is centrifuged to concentrate the bacteria in the deposit. This deposit is used to prepare a ZN-stained smear (Fig. 36.5) and for culture or molecular detection methods (Chapter 11).

Leptospiral agalactia

This is not a typical mastitis although *Leptospira* serovars Hardjo or Pomona are present in the mammary glands. The presumptive diagnosis of this condition is based on the history and clinical signs. Confirmation of diagnosis is described in Chapter 31.

Fungal pathogens

These potential pathogens are ubiquitous and usually introduced accidentally into the udder via the nozzle of

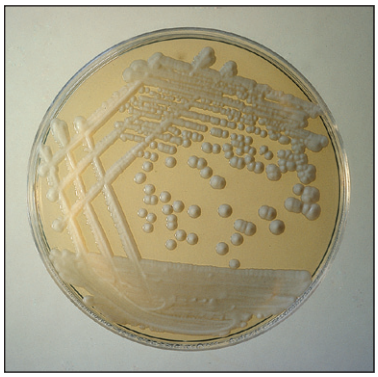

Figure 36.19 *Candida albicans* on Sabouraud agar growing as white, high convex, shiny colonies with a pleasant beer-like smell.

Figure 36.20 *Aspergillus fumigatus* on Sabouraud agar: blue-green powdery colony.

intramammary antibiotic tubes. Because fungi are ubiquitous, a repeat milk sample might be advisable. A large number of fungi have been isolated from mastitic milk samples and these include *Candida albicans* (Fig. 36.19), *Cryptococcus neoformans, Aspergillus fumigatus* (Fig. 36.20), *Trichosporon* and *Saccharomyces* species. For identification methods, consult the relevant chapter in the Mycology section.

Prototheca *species*

Prototheca zopfii and *P. wickerhamii* are achlorophyllic algae but they form bacteria-like colonies on blood agar at 25–37 °C, after 48–72 hours' incubation. The colonies are small and greyish-white (Fig. 36.21). Microscopic examination reveals large sporangia (8–25 µm) containing four to eight daughter cells (Fig. 36.22). They are best demonstrated in wet preparations or in smears stained by the Wright or Giemsa methods. These algae are widespread in water, soil, mud and cattle faeces. A repeat milk sample might be warranted to check that the algae did in fact

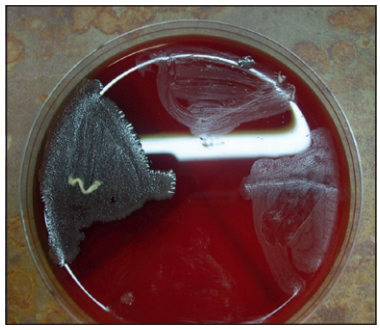

Figure 36.21 *Prototheca zopfii* on blood agar quarter plate (right hand side).

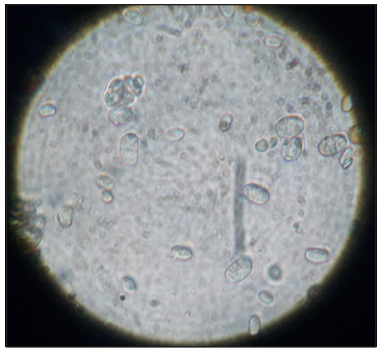

Figure 36.22 Sporangia of *Prototheca zopfii*. (wet preparation, × 400)

come from inside the mammary gland. Species identification can be performed using commercially available carbohydrate assimilation test kits. The prognosis is poor for this type of mastitis, as there is little response to treatment and the algae can persist in the udder tissue for over 100 days. Affected animals are a danger to the other cows in the herd and immediate culling should be advised.

Molecular diagnosis

There have been a number of publications describing molecular methods for the detection of mastitis-producing bacteria in milk, reviewed by Cai et al. (2003). Multiplex methods which can differentiate between a number of organisms may be particularly useful. Gillespie and Oliver (2005) describe a multiplex PCR which can detect and differentiate between the principal Gram-positive mastitis pathogens. Several PCR-based methods which can detect both Gram-positive and Gram-negative pathogens have also been described, with detection and identification of up to 11 different organisms (Lee et al. 2008, Shome et al. 2011, Ajitkumar et al. 2012). Commercial kits for PCR detection of mastitis pathogens are now available. Koskinen et al. (2010) evaluated one such real-time PCR kit and

found it more sensitive than conventional culture for detection of the major mastitis pathogens, with results available within four hours. However, further work is required in order to clarify interpretation of these tests in which more than one organism is detected in a clinical sample. The quantitative nature of real-time PCR test results will be of benefit in separating pathogens from likely contaminants in samples. PCR-based procedures are also particularly useful in the diagnosis of mastitis caused by organisms which are difficult to culture such as the mycoplasmas. Boonyayatra et al. (2012) developed a real-time PCR method combined with PCR-restriction fragment length polymorphism for the detection and identification of three mycoplasmal species causing bovine mastitis. Mastitis pathogens are not detected in a proportion of samples submitted to diagnostic laboratories. PCR-based methods are likely to detect the causative organism in some of these samples; Bexiga et al. (2011) detected a major mastitis pathogen using PCR procedures in 47% of samples in which there was no growth on conventional culture.

Molecular typing of mastitis-causing organisms can provide valuable epidemiological information, as illustrated in the review by Zadoks et al. (2011). Typing studies confirmed the discovery that many infections with *E. coli* originate in the dry period and that recurrent infections with the same strain of *E. coli* occur. Molecular epidemiological investigation of *S. aureus* mastitis strains within herds revealed that although one high-prevalence strain usually predominated, several other strains were usually present at low prevalence, indicating that not all infections are the result of cow-to-cow transmission.

Investigation of Mastitis Problem Herds

An indication that a dairy herd has a mastitis problem may come either from the owner or from milk factory records that show elevated cell counts, in the bulk milk tank samples, on three consecutive occasions. The following points are a guide to the investigation of the problem so that successful control measures can be suggested, based on the key factors of mastitis control (Box 36.1). Control of mastitis is based on prevention of new infections through teat dipping and hygiene standards that minimize transfer of infection via the milking machine and elimination of old infections.

- The milking machine must be checked thoroughly to ensure that it is functioning correctly. This could be carried out, and a report obtained, before visiting the farm for further investigations.
- It is advantageous to arrive at the farm about an hour before milking time. The herd records of mastitis cases, factory cell counts and herd production can be examined. The design and general hygiene in the milking shed should be noted. The owner could be questioned about the general herd management, treatment of mastitis cases, and culling rate for cows with persistent mastitis.
- During milking the following points should be observed: the milking technique, whether teat-dipping is being carried out efficiently and the general level of hygiene in the shed.
- Milk samples should be taken from any new cases of mastitis and random samples from about 10–20% of the herd for bacteriological examination. The samples should be obtained before the milker has washed the udder or carried out any other pre-milking procedure. While taking the milk samples the udder and teats can be examined for lesions. Evidence of hyperkeratosis of the epithelium at the teat orifice, or ecchymosis at the end of the teat, that might indicate excessively high vacuum levels in the past, should be noted.

Box 36.1 **Key factors for mastitis control**

- Milking machine
- Herd management
  - General
  - Milking shed (parlour)
- Teat dipping routine
- Treatment
  - Clinical cases
  - Dry-cow therapy
- Culling
  - Cows with chronic or persistent mastitis

Laboratory investigation of the milk samples should provide the following information:

1. The major pathogen(s) causing the mastitis.
2. The percentage of subclinical cases of mastitis in the herd.
3. The antibiotic susceptibility/resistance patterns of the pathogens. These will provide a guide to effective treatment of clinical cases and dry-cow therapy.

Treatment

The drug of choice in the treatment of mastitis is one to which the bacteria are sensitive and which achieves high concentrations in the mammary gland without provoking tissue changes. The intramammary route for the administration of antimicrobial drugs to cows with mastitis is usually practical and convenient.

The antimicrobial agent should be distributed well throughout the mammary gland and maintain sufficient

concentrations to clear the bacteria from the tissue. The formulation of the intramammary preparation, including the amount of drug present and the physical properties of the carrier influences the distribution and persistence in the mammary tissue. The distribution of drugs, following intramammary administration, occurs principally by passive diffusion. It is also a suitable route for administration of long-acting antimicrobial preparations at 'drying off' as part of a mastitis control programme. Dry-cow therapy is designed to eliminate infections present in the udder and prevent new infections during the dry period.

Successful treatment of clinical mastitis often requires a history of the herd, isolation, identification and susceptibility pattern of the bacteria involved and relevant information on the milking machine and on the milking routine. The quality of milk samples submitted for susceptibility testing are central to the information provided by the diagnostic laboratory. Although treatment may have to proceed before laboratory reports are at hand, it should, if necessary, be immediately revised as soon as susceptibility results are available.

An antimicrobial preparation for intramammary use should not be selected on the basis of the broadest possible spectrum as it is the **susceptibility of the bacterium being treated that should determine selection**. Penicillin G is frequently the most effective antibiotic against *Streptococcus* species, *Trueperella pyogenes* and susceptible *Staphylococcus aureus*. Treatment of *S. agalactiae* and *S. dysgalactiae* with penicillin G or a semisynthetic penicillin is generally more than 95% effective. *Streptococcus uberis* and enterococcal infections respond less favourably to this treatment.

Results of treatment of staphyloccal mastitis are highly variable and depend to a large extent on the duration of infection, the susceptibility pattern of the bacteria, the degree of inflammation at the time of treatment, the extent of tissue damage and the stage of lactation. Many *S. aureus* isolates produce penicillinase (beta-lactamase) which renders penicillin G and similar antibiotics ineffective. Cloxacillin and nafcillin are effective against such bacteria and clavulanic acid acts as an inhibitor of beta-lactamase. Cephalosporins and erythromycin are likely to have good activity against beta-lactamase-producing *S. aureus*. Third- and fourth-generation cepalosporins should not be used as first-choice treatments for mastitis as their use may encourage the development of resistance. Although *S. aureus* isolates may be susceptible in vitro to a selected antibiotic, poor penetration into chronic lesions can allow survival of bacteria. Therapeutic success may be achieved by continuing treatment for longer than 72 hours and by infusing intramammary preparations at more closely spaced intervals but recovery rates are poor, 30 to 60%.

The benefits of treatment of coliform mastitis with antimicrobials is questionable as the major clinical effects relate to endotoxin activity. This form of mastitis may proceed to severe toxaemia, in which case prompt treatment with antibiotics such as the cephalosporins or fluoroquinolones and supportive fluid therapy may be indicated. Both local and systemic therapy may be beneficial in coliform mastitis. When the infection is peracute and life-threatening, systemic treatment and stripping-out the quarters every two hours can aid in the elimination of both bacteria and endotoxin.

Mastitis due to *Nocardia* species, fungi or algae are generally unresponsive to treatment. Early recognition of the aetiological agent and immediate culling is the most appropriate approach.

Selection of intramammary preparations requires consideration of the spectrum of activity, distribution throughout the mammary gland, frequency of administration, cost and milk withholding-time. Table 6.3 should be consulted for the range of antimicrobial drugs suitable for treatment of bacteria causing mastitis. A narrow-spectrum drug, such as penicillin, is most appropriate for streptococcal infections and penicillin-susceptible staphylococci. Susceptibility testing is an essential step in mastitis control programmes, especially when dealing with long-standing infections in well-managed dairy herds. Antimicrobial therapy is only one aspect of mastitis control and, apart from dry-cow therapy, probably the least rewarding of the measures used in mastitis control programmes.

REFERENCES

Ajitkumar, P., Barkema, H.W., De Buck, J., 2012. Rapid identification of bovine mastitis pathogens by high-resolution melt analysis of 16S rDNA sequences. Veterinary Microbiology 155 (2–4), 332–340.

Almeida, R.A., Luther, D.A., Park, H.M., et al., 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). Veterinary Microbiology 115, 183–191.

Anaya-Lopez, J.L., Contreras-Guzman, O.E., Carabez-Trejo, A., et al., 2006. Invasive potential of bacterial isolates associated with subclinical bovine mastitis. Research in Veterinary Science 81, 358–361.

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Journal of Dairy Science 89 (6), 1877–1895.

Bexiga, R., Koskinen, M.T., Holopainen, J., et al., 2011. Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial

culturing. Journal of Dairy Research 78 (1), 49–55.

Boonyayatra, S., Fox, L.K., Besser, T.E., et al., 2012. A PCR assay and PCR-restriction fragment length polymorphism combination identifying the 3 primary *Mycoplasma* species causing mastitis. Journal of Dairy Science 95 (1), 196–205.

Bradley, A.J., Green, M.J., 2001. Adaptation of *Escherichia coli* to the bovine mammary gland. Journal of Clinical Microbiology 39, 1845–1849.

Bradley, A.J., Leach, K.A., Breen, J.E., et al., 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Veterinary Record 160, 253–258.

Burriel, A.R., Dagnall, G.J., 1997. Leukotoxic factors produced by staphylococci of ovine origin. Microbiology Research 152 (3), 247–250.

Cai, H.Y., Archambault, M., Gyles, C.L., 2003. Molecular genetic methods in the veterinary clinical bacteriology laboratory: current usage and future applications. Animal Health Research Reviews 4 (2), 73–93.

Castagliuolo, I., Piccinini, R., Beggiao, E., et al., 2006. Mucosal genetic immunization against four adhesins protects against *Staphylococcus aureus*-induced mastitis in mice. Vaccine 15 24 (20), 4393–4402.

García-Álvarez, L., Holden, M.T., Lindsay, H., et al., 2011. Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infectious Diseases 11 (8), 595–603.

Gillespie, B.E., Oliver, S.P., 2005. Simultaneous detection of mastitis pathogens, *Staphylococcus aureus, Streptococcus uberis,* and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. Journal of Dairy Science 88 (10), 3510–3518.

Hamann, J., 2005. Diagnosis of mastitis and indicators of milk quality. In: Hogeveen, H. (Ed.), Mastitis in Dairy Production: Current Knowledge and Future Solutions. Wageningen Academic Publishers; Wageningen, Netherlands, pp. 82–90.

Hogan, K.L., Smith, J., 2003. Coliform mastitis. Veterinary Research 34, 507–519.

Jost, B.H., Billington, S.J., 2005. *Arcanobacterium pyogenes:* molecular pathogenesis of an animal opportunist. Antonie Van Leeuwenhoek 88 (2), 87–102.

Koskinen, M.T., Wellenberg, G.J., Sampimon, O.C., et al., 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. Journal of Dairy Science 93 (12), 5707–5715.

Lee, K.H., Lee, J.W., Wang, S.W., et al., 2008. Development of a novel biochip for rapid multiplex detection of seven mastitis-causing pathogens in bovine milk samples. Journal of Veterinary Diagnostic Investigation 20 (4), 463–471.

Melchior, M.B., Vaarkamp, H., Fink-Gremmels, J., 2006. Biofilms: a role in recurrent mastitis infections? Veterinary Journal 171, 398–407.

Schalm, O.W., Carroll, E.J., Jain, N.C., 1971. Bovine Mastitis. Lea and Febiger. Philadelphia, USA, pp. 136–140.

Shome, B.R., Das Mitra, S., Bhuvana, M., et al., 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. Journal of Applied Microbiology 111 (6), 1349–1356.

Shore, A.C., Deasy, E.C., Slickers, P., 2011. Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent *mecA, mecI, mecR1, blaZ,* and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy 55 (8), 3765–3773.

Shuster, D.E., Lee, E.K., Kehril, M.E., 1996. Bacterial growth, inflammatory cytokine production and neutrophil recruitment during coliform mastitis in cows within ten days of calving compared with cows at midlactation. American Journal of Veterinary Research 11, 1569–1575.

Smith, K.L., Todhunter, D.A., Schoenberger, P.S., 1985. Environmental pathogens and intramammary infection during the dry period. Journal of Dairy Science 68 (2), 402–417.

Todhunter, D.A., Smith, K.L., Hogan, J.S., et al., 1991. Gram-negative bacterial infections of the mammary gland in cows. American Journal of Veterinary Research 52 (2), 184–188.

Wilson, C.D., Kingwill, R.G., 1975. International Dairy Federation Annual Bulletin 85, 422–438.

Zadoks, R.N., Middleton, J.R., McDougall, S., et al., 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal of Mammary Gland Biology and Neoplasia 16 (4), 357–372.

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Section 3

Mycology

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Chapter 37

Introduction to the pathogenic fungi

GENERAL CHARACTERISTICS OF THE FUNGI

Fungi have a eucaryotic cell type and are therefore insensitive to most bacterial antibiotics. They have nuclei with well-defined nuclear membranes, mitochondria and networks of microtubules. They are non-photosynthetic, usually non-motile and possess a cell wall external to the plasma membrane. Although their optimum pH is about 6, they can tolerate more acidic conditions. They are strict aerobes with an optimum temperature for growth of 20–30°C, but the pathogenic fungi causing systemic mycoses can tolerate 37°C. The fungi are comparatively slow-growing on laboratory media, the zygomycetes and *Aspergillus* species may show growth in two to three days but the incubation time for some of the dermatophytes may be as long as three to five weeks. The fungi can be divided into moulds and yeasts. Moulds are filamentous, growing apically and forming lateral branches. The branching filaments or hyphae are 2–10 µm in diameter. The hyphae in most moulds have cross-walls or septa but the zygomycetes rarely produce septa and are termed non-septate. The branching hyphae grow to form a tangled interlacing mass known as the mycelium. Moulds usually form large fluffy colonies on laboratory media and produce aerial fruiting hyphae that bear asexual spores. Yeasts are oval, spherical or elongated cells, about 3–5 µm in diameter, and form moist colonies that are usually larger than, but not unlike bacterial colonies. The yeasts reproduce by budding or by both budding and spore formation. The terms 'mould' and 'yeast' have no taxonomic significance and are not mutually exclusive. Some of the fungal pathogens are dimorphic, being yeasts or yeast-like in animal tissues and when grown on enriched media at 37°C but are moulds in their natural environment and when grown on media at 25°C. Yeasts such as *Candida albicans* can grow in animal tissue as elongated cells, joined together, that resemble septate hyphae and are known as pseudohyphae.

CLASSIFICATION OF THE FUNGI

The kingdom *Fungi* comprises more than 250,000 species of which perhaps a few hundred are of pathogenic importance for animals and humans. The kingdom is divided into six phyla: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Zygomycota*, *Microsporidiomycota* and *Glomeromycota*. Members of *Chytridiomycota* and *Glomeromycota* are not known to be of veterinary or medical importance. However, chytrids cause infections of the epidermis of frogs, interfering with their ability to respire across the skin. These infections have been implicated in the dramatic worldwide die-off of amphibians in recent times.

The members of the different phyla are primarily characterized by different methods of sexual reproduction. Traditionally fungi with no known sexual stage have been formally grouped in the phylum *Deuteromycota*, also referred to as the Fungi Imperfecti. Increasingly, molecular methods are being used to place fungal species, for which only the asexual form is known, in one of the recognized phyla without the need to discover a sexual form. The preferred term for fungi that are not known to have a meiotic stage is mitosporic fungi. For many years a dual system of names has been used, one for the asexual form (the anamorph) and one for the sexual form (the teleomorph). For example, the teleomorph of the yeast *Cryptococcus neoformans* is *Filobasidiella neoformans*, one of the few pathogenic fungi in the basidiomycetes. Most of the

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pathogenic fungi, previously in the Fungi Imperfecti, have been found to be ascomycetes when the sexual state is found. Several pathogenic fungi are better known by their anamorph name because it is often the asexual form that is found in pathological situations. However, the usefulness of this dual naming system is increasingly being questioned and it is expected that eventually a single name, that of the teleomorph, will only be recognized and used. A major re-organization of fungal classification based on molecular phylogenetic analyses has been proposed (Hibbett et al. 2007). Two new phyla have been added, *Blastocladiomycota* and *Neocallimastigomycota*, while the subkingdom *Dikarya* containing *Ascomycota* and *Basidiomycota* has been created. The status of the phylum *Zygomycota* is currently in doubt and may be broken up into a number of new phyla in the future. These proposals have been adopted in the Dictionary of the Fungi (Kirk et al. 2008) and Index Fungorum (www.indexfungorum.org accessed 31 December 2012).

A small number of fungal-like agents such as *Pythium insidiosum* and *Rhinosporidium seeberi* are traditionally included in texts on fungal diseases because they produce elements in tissue that resemble fungal elements and may form yeast-like colonies on fungal media. *Pythium insidiosum* is a member of the kingdom *Chromista* (also known as *Stramenopila* or *Heterokonta*), while *Rhinosporidium seeberi* belongs to a novel group of aquatic protistan parasites at the boundary between fungi and animals, in the class *Mesomycetozoea*, kingdom *Protozoa* (Mendoza et al. 2002). *Pneumocystis carinii*, an occasional cause of pneumonia in immunosuppressed dogs and horses (*P. jiroveci* is associated with terminal pneumonia in severely immunocompromised people), was considered a protozoa but has in recent years been shown to belong to the kingdom *Fungi*, phylum *Ascomycota*.

GENERAL FEATURES OF FUNGAL INFECTIONS

A few of the dermatophytes are considered to be obligate parasites but the majority of the pathogenic fungi are found widespread in the environment as saprophytes or present as commensals associated with animals and humans. Most are therefore opportunistic pathogens and predisposing factors often contribute to the establishment of fungal infections. These include an upset in the normal flora of the host by prolonged administration of antibiotics, immunosuppression, concurrent infections, breaks in the skin or mucous membranes, perpetually moist areas of skin or the exposure to a large infective dose, such as with *Aspergillus fumigatus* spores in brooder pneumonia of chicks. Fungal diseases do not usually assume epidemic proportions except in certain instances such as ringworm. No exotoxins or endotoxins have conclusively been shown to be produced, but mycotoxicoses occur due to animals ingesting preformed toxic metabolic products, termed mycotoxins, produced during fungal growth in animal feedstuffs.

Chronic fungal infections lead to a granulomatous reaction that resembles the host's response to a foreign body or to an actinomycete infection. Immunity to fungal infections is considered to be more dependent on cell-mediated than antibody-mediated activity. Antibodies are produced in most mycoses, but the antibodies do not appear to be protective. Hypersensitivity can develop to a particular fungus in the infected or exposed host and may lead to skin rashes in humans. Hypersensitivity responses form the basis of various diagnostic skin tests such as the use of histoplasmin for the diagnosis of histoplasmosis. The mycoses are sometimes divided into deep (systemic), subcutaneous and superficial mycoses. Several pathogenic fungi such as *Candida albicans* and *Aspergillus fumigatus* are capable of both superficial and deep infections. Systemic mycoses are uncommon and associated with opportunistic infections by saprophytic fungi which have been facilitated by predisposing factors such as host debilitation, immunosuppression, exposure to huge numbers of spores or disruption of normal flora due to prolonged antibiotic therapy. Subcutaneous mycoses are characterized by localized involvement of the dermis and subcutis by a range of fungal species. The term phaeohyphomycosis refers to infection with dematiaceous (pigmented) fungi while the term mycetoma (sometimes referred to as eumycetoma to distinguish it from mycetomas associated with actinomycete infections) indicates a tumour-like granulomatous lesion involving cutaneous and subcutaneous tissues. Superficial mycoses may be grouped into dermatomycoses and dermatophytoses. The former conditions refer to opportunistic infections of the skin or mucocutaneous junctions by fungi such as *Candida* species or *Malassezia pachydermatis*. Dermatophytes such as *Trichophyton* species and *Microsporum* species invade and destroy keratinized structures and are notable for being both easily transmitted and zoonotic.

GENERAL METHODS FOR THE DIAGNOSIS OF THE MYCOSES

History, clinical signs, gross pathology, and in a few cases intradermal skin tests, are all of value in the diagnosis of fungal infections backed by a laboratory investigation of clinical specimens. These investigations include direct microscopy, isolation and identification of the pathogen. The identification is based on colonial characteristics, examination of the fruiting heads and spores and on the results of biochemical reactions in the case of the yeasts and certain of the dermatophytes.

Table 37.1 Summary of the methods employed for the direct microscopic examination of fungi

Technique	Use	Fungi
10–20% KOH wet preparations	Clears specimens to make fungi more visible. Examine under low and high-dry objectives or phase contrast	Fungal elements of most moulds and yeasts. Dimorphic fungi as yeast-like forms in tissue. Arthrospores on affected hairs for dermatophytes
Calcofluor white (0.1%)	Fluorescence of fungal elements under fluorescence microscope. Visualization of fungi made easier	Detection of most fungal elements in wet preparations and in tissue sections
India ink or nigrosin	Wet preparation with cerebrospinal fluid or clear exudates	*Cryptococcus neoformans,* to demonstrate the characteristically large capsule
Gram or methylene blue stain	Fixed smears of tissues or exudates	Yeast cells such as *Candida albicans* as well as any bacteria that are present. *Cryptococcus neoformans* stains poorly by these methods
Fluorescent antibody technique	Frozen sections or fixed smears	Available in specialized laboratories for some of the dimorphic fungi such as *Blastomyces dermatitidis*
Periodic acid-Schiff (PAS) + counter-stain (haematoxylin)	Frozen or paraffin-embedded histological sections from biopsies or tissues	Most fungal elements can be demonstrated in tissues by this method. The fungi stain pink. Any tissue reaction caused by the fungal invasion can also be observed
Methenamine silver stain + counter-stain	Frozen or paraffin-embedded histological sections from biopsies or tissues	Most fungal elements in tissues will be stained a dark brown by this method and are easy to see. Visualization of any internal structures may be harder than with the PAS-haematoxylin stain
Wright or Giemsa stain	Fixed bone marrow smears or impression smears from biopsies	Demonstration is limited to *Histoplasma capsulatum*

Direct Microscopic Examination of Clinical Specimens

Table 37.1 indicates methods used for the examination of fungal elements in clinical specimens and Table 37.2 gives a brief summary of the diagnostic features of some of the fungi found in veterinary diagnostic samples. The specimens suspected of containing fungal pathogens range from hairs and skin scrapings for dermatophytes to exudates, biopsies and tissues. Because many of the potentially pathogenic fungi are ubiquitous it is important to take tissue for histopathology whenever possible so as to demonstrate fungal hyphae or yeast cells actually invading the tissue, often invoking a tissue reaction. If there is a correlation between the fungus that is isolated and the histopathological findings there can be greater confidence in the diagnosis of the disease or condition.

Histopathological sections can be made from biopsies or from tissues. Frozen sections are prepared from fresh tissue while more permanent tissue sections are made from material fixed in 10% formalin. These sections can be stained by such methods as the periodic acid-Schiff (PAS), that will differentially stain the fungal elements pink or by silver impregnation stains, such as Gomori's methenamine silver stain where the fungal elements will stain dark brown or black. Calcofluor white powder (Fluorescent Brightener 20, Sigma Chemical Co.) is a cotton brightener which binds to chitin in the fungal cell walls. On excitation with light of wavelength 350 nm the bound calcofluor white fluoresces blue-green. As a working solution (0.1% w/v) it can be used mixed with exudates, incorporated into 10% KOH solutions or employed to stain histological sections (see Appendix 1 for details). Wright or Giemsa stains can be used on impression smears from biopsies or bone marrow for *Histoplasma capsulatum*. A Gram stain or simple methylene blue stain is useful for many of the yeasts. India ink or nigrosin wet preparations are used to demonstrate the characteristically large capsule of *Cryptococcus neoformans* (Box 37.1). This and other comparatively rapid methods involving wet preparations to visualize fungi in diagnostic specimens are described below. To clear and clarify the specimens, so that the fungal elements can be seen, 10–20% potassium hydroxide (KOH) and other chemicals are used (Box 37.2).

Modifications to the KOH wet mount method

1. *DMSO plus KOH*: penetration and clarity of specimens can be improved by the addition of

Table 37.2 Morphological features of pathogenic fungi in diagnostic specimens

Fungus	Techniques	Summary of diagnostic features
Aspergillus fumigatus	KOH, calcofluor white, periodic acid-Schiff (PAS) or silver impregnation stains	Septate hyphae, dichotomous branching at a 45° angle. Hyphae 3–6 μm and rarely up to 12 μm in diameter. Tissue reaction is granulomatous or necrotizing, but may not occur in an immunosuppressed host. May see distorted fruiting heads if fungus spreads into an air space in the body
Zygomycetes: *Rhizopus, Mucor, Rhizomucor, Absidia* and *Mortierella* spp.	KOH, calcofluor white, PAS or silver impregnation stains	Large, bulging, non-septate hyphae that can be twisted and fragmented. About 10–20 μm in diameter (range 3–25 μm) with irregular branching. The invading hyphae of *Mortierella wolfii* tend to be finer (2–12 μm diameter) than the other zygomycetes
Candida albicans	Gram stain, KOH, PAS or silver impregnation stains	Budding cells, oval or round, 3–4 μm diameter. Pseudohyphae may be present in tissue; these have regular points of constriction between individual elongated yeast cells. They must be distinguished from moulds with septate hyphae
Malassezia pachydermatis	Gram stain, methylene blue, KOH or calcofluor white	Bottle-shaped, small yeast (1–2 × 2–4 μm). Unipolar budding and reproduction is by bud-fission in which the bud detaches from the mother cell by a septum
Cryptococcus neoformans	India ink, KOH, PAS or Mayer's mucicarmine stain	Spherical budding yeast cells, 2–15 μm diameter, usually surrounded by a large capsule. Produces pinched-off buds, sometimes multiple. Cells vary greatly in size in a single preparation. Encapsulated pseudohyphae are very occasionally seen
Blastomyces dermatitidis	KOH, calcofluor white, FA technique, PAS or silver-impregnation stains	Large, budding yeast 8–15 μm (range 2–30 μm) in diameter with very thick walls. Buds are connected by a broad base. Intracytoplasmic contents are usually evident
Histoplasma capsulatum	Wright, Giemsa, PAS or silver impregnation stains	Small, budding yeast, spherical to oval, 2–5 μm, intracellular in monocytic cells. A clear halo can be seen around the darker staining cell. Buds are single with narrow bases. The fungus is difficult to detect in unstained preparations
Coccidioides immitis	PAS and silver-impregnation stains, KOH + calcofluor white	Large spherules present in tissue. When mature, up to 200 μm in diameter and contain numerous non-budding endospores (2–5 μm). Immature spherules vary in size and do not contain endospores
Sporothrix schenckii	Gram stain or KOH on exudates. PAS or silver-impregnation stains on biopsies	Small, cigar-shaped yeasts, 2–6 μm. May exhibit multiple budding. Only a small number are usually present in exudates and they may be hard to see
Dermatophytes: *Microsporum* and *Trichophyton* spp.	KOH, KOH + calcofluor white, DMSO + KOH, blue-black ink + KOH	Septate hyphae (2–3 μm diameter) surround affected hairs and fragment into arthrospores. Some hyphae may still be present but more usually a sheath of refractile round arthrospores (2–8 μm diameter) is present. These arthropores must not be confused with fat globules or hair-pigment granules (melanosomes)
Fungi in mycetomas	KOH, calcofluor white, PAS and silver-impregnation stains	Irregular granules, 0.5–3.0 mm and variously coloured, are present in biopsies or scrapings. Within crushed granules are intertwined hyphae (2–5 μm) with swollen cells (15 μm or more) at the periphery
Fungi in chromoblastomycoses	KOH, calcofluor white, PAS and silver-impregnation stains	Single-celled or clustered, spherical (4–12 μm), thick-walled bodies and darkly pigmented (sclerotic) bodies. Hyphae may be present (2–6 μm) and are seen in skin scrapings and aspirates
Pneumocystis carinii	Giemsa stain, immunocytochemistry and methenamine silver stain	Trophic, cystic and spore forms may be found in lung tissue and bronchoalveolar lavage fluid of affected animals

Box 37.1 **India ink or nigrosin preparations (See Appendix 1 for preparation of the solutions)**

- Place a drop of India ink or nigrosin on a microscope slide
- Add a loopful of cerebrospinal fluid or clear exudate suspected of containing *Cryptococcus neoformans* to the India ink or nigrosin and mix well
- Place a cover slip on the mixture and press down gently. Blot-up any excess fluid
- Examine under the low and high-dry objectives of the light microscope

Box 37.2 **KOH wet mount**

- Place one to two drops of 10–20% KOH on a microscope slide and add a small amount of the specimen to the drop of KOH and mix well
- Gently pass the slide through a low flame of a Bunsen burner to hasten clearing (do not boil or over-heat). This is an optional step
- Place a cover slip on top of the preparation and press down gently
- Allow to stand for one to two hours, or even overnight in a moist chamber; the time required will depend on the density of the specimen. The KOH will partially digest the proteinaceous debris
- Examine under phase contrast or under the low- and high-dry objectives of a light microscope

dimethyl sulphoxide (DMSO) to the KOH. A formulation of 20% KOH and 36% DMSO is used.

2. *Blue-black ink to stain fungal elements*: the fungi take up the dye (ink) selectively and the specimen is satisfactorily cleared by the 10% KOH. The working solution is given in Appendix 1.
3. *Calcofluor white and KOH*: 1 drop of 0.1% calcofluor white and 1 drop of 10% KOH are placed on the microscope slide and a small amount of the specimen is added to it and a cover slip placed on top. After clearing the preparation is examined under a fluorescence microscope with maximal excitation occurring around 350 nm. The fungal elements will fluoresce blue-green making them easier to see.

Isolation and Subculture of Fungi

Media for fungi

The media designed for isolating pathogenic fungi from clinical specimens need to be inhibitory against the faster-growing bacteria and more rapidly growing contaminant fungi. Sabouraud dextrose agar is the medium most commonly used. It has a pH of 5·6 and is therefore inhibitory to bacteria while supporting the growth of fungi that are acid-tolerant. The medium may be made more selective by the addition of chloramphenicol (antibacterial). Cycloheximide (actidione) has an antifungal action against some contaminant fungi. However, cycloheximide can also be inhibitory for some of the pathogenic fungi such as the zygomycetes, *Aspergillus* species, *Cryptococcus neoformans* and some of the dimorphic fungi such as *Blastomyces dermatitidis*. It is advisable to use media with and without the selective agent cycloheximide, especially when attempting to isolate the dimorphic fungi. Enriched medium, such as brain-heart infusion agar with 5% blood, is used for the dimorphic fungi when incubated at 37°C as this aids conversion to the yeast phase of growth.

Emmons' modification of Sabouraud dextrose agar with a pH of 6·9 has been suggested for the dermatophytes as although they tolerate pH 5·6 they prefer a pH nearer neutrality. Yeast extract is added to the medium to provide growth factors needed by some of the *Trichophyton* species and also chloramphenicol (0·05 g/L) and cycloheximide (0·4 g/L). The selective agents can be obtained commercially as an antibiotic supplement such as Oxoid SR75. Both chloramphenicol and cycloheximide have the advantage of being stable at autoclaving temperatures. Sabouraud dextrose agar and Emmons' modification can be obtained from commercial firms such as Difco, BBL and Oxoid. Table 37.3 summarizes the media, incubation temperatures and times for some of the pathogenic fungi.

Using an incubation temperature of 37°C is in itself a selective procedure. Many of the potentially pathogenic fungi, such as *Aspergillus* spp., prefer lower incubation temperatures but fungi that invade animal tissue can tolerate 37°C. This incubation temperature will deter many of the non-pathogenic contaminant fungi.

The dermatophytes are slow-growing and need a lengthy incubation period so there is the danger of agar media drying up in conventional plastic Petri dishes. This can be overcome by several methods:

- By taping the plastic Petri dishes. However, as fungi are strict aerobes the tape should be removed and replaced daily to supply the fungus with the required oxygen.
- By pouring the medium, in 20–25 mL volumes, into sterile glass Petri dishes. The greater depth of agar helps to retain the moisture. These can also be taped if necessary.
- The medium can be poured as slopes in 30 mL universal bottles. These are inoculated and incubated with loose caps. The disadvantage is that any suspect fungi that grow on the slopes are more difficult to examine or to subculture.

Table 37.3 Summary of the most appropriate media and cultivation conditions for some of the pathogenic fungi

Fungal pathogen	Suitable media	Incubation temperature	Incubation time
Aspergillus species	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L)	37°C	1–4 days
Zygomycetes	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L)	37°C	1–4 days
Candida albicans	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L)	37°C	1–4 days
Cryptococcus neoformans	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L)	37°C	1–2 weeks
Dermatophytes	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L) or Emmon's Sabouraud agar + yeast extract + chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L)	25°C	2–6 weeks
Dimorphic fungi (yeast phase)	Brain-heart infusion agar +5% blood +/– chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L)	37°C	1–4 weeks
Dimorphic fungi (mould phase)	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L) +/– cycloheximide (0.4 g/L)	25°C	1–4 weeks
Fungi causing subcutaneous mycoses	Sabouraud dextrose agar +/– chloramphenicol (0.05 g/L)	25°C	2–3 weeks

Inoculation of media

Culture media for the isolation of yeasts can be streaked with an inoculum from the specimen as for bacterial cultures. When attempting to isolate a mould, the surface of the agar should be cross-hatched in about five sites with a sterile scalpel to a depth of about 2 mm, the cuts being at right angles to each other. The specimens, such as small bits of tissue, scabs or hairs can then be gently pushed into the agar at these cross-hatched areas. If the specimens are forced into the agar surface there is the danger of the agar splitting during incubation.

Subculturing fungal colonies

Yeasts can be subcultured, and pure cultures obtained, in the same manner as for bacteria. With moulds different techniques are needed:

- *If the mould colony is sporing*: a mould colony usually starts to produce spores from the centre outwards and it is often the spores that give a colony its characteristic colour. An inoculating loop can be made slightly moist and sticky by pushing it into a portion of sterile agar, and can then be used to collect spores from the colony. The inoculum is introduced just under the surface of the agar in the centre of the new plate if the fungus is a rapid grower, or in four segments of the plate if the fungus is a slow grower or produces small colonies.
- *If a mould colony is not sporing*: the staling phase and then death of a fungal colony starts with the hyphae in the centre of the colony. It is therefore important to subculture hyphae from the edge of a colony. A small block of agar (about 5 mm^2) is cut out of the centre of the subculture plate with a sterile scalpel and the agar block discarded. Using the same scalpel, a similar-sized block of agar is cut, including fungal hyphae, from the edge of the colony to be subcultured. The agar block containing hyphae is transferred to the subculture plate and placed snugly, mould-side up, into the previously cut hole. The cut hyphae will regenerate and grow out from the surface of the block.

Identification of Pathogenic Fungi

The specific methods for the identification of individual pathogenic fungi are given in the following mycology chapters. Molecular methods are increasingly being developed for species identification and specific nucleic acid probes are available for the rapid identification of dimorphic fungi. In general, more emphasis is placed on

morphological structures than is the case when identifying bacteria. The procedures include:

- The direct microscopic appearance of the fungus in the clinical specimen. Useful differentiating features for vegetative hyphae include the presence or absence of septa, either hyaline (colourless) or dematiaceous (pigmented), characteristic hyphal structures such as spiral or racquet-shaped hyphae.
- The size and morphology of the colony and the type of pigmentation on the obverse and reverse sides.
- The microscopic appearance of the fruiting heads and spores from mould colonies and the morphology of the yeasts and the type of budding (Table 37.4).
- Biochemical tests with yeasts and, to a more limited extent, with some moulds.
- Other tests specific to the particular fungus such as the germ-tube test for *Candida albicans*.

Methods for the examination of the microscopic aspects of fungal colonies

- **Use of the dissecting microscope**: the culture plate with the mould colony to be examined is placed under the microscope with its lid removed. The pattern of mycelial growth and presence and characteristics of the fruiting heads can often be seen. For example, a greenish, velvety colony is often either an *Aspergillus* or a *Penicillium* species, and the differentiation can usually be made on the morphology of the fruiting head under this low magnification. However, the results should be checked by wet mount preparations that are examined under the light microscope.
- **Wet mount method**: mycology needles are used to remove a small portion of the colony to be studied, including a little of the underlying agar. The sample is taken halfway between the centre and the periphery of the colony. With a yeast colony a little of the growth can be taken with an inoculating loop. The fungal growth is transferred to a drop of lactophenol cotton blue (LPCB) stain (see Appendix 1 for the formula) on a microscope slide. A cover slip is applied and pressure used directly over the colony fragment (an eraser on the end of a pencil is useful for this) to spread out the hyphae and other structures. The fungal structures are stained by the LPCB dye. The preparation is examined under the low and high-dry objectives of the light microscope. The disadvantage of this method is that it is difficult to preserve the continuity between the spores, fruiting structures and hyphae, due to the rigorous treatment these delicate structures receive during the preparation of the wet mount. This can be overcome to a certain extent by the 'sticky-tape' method and more so by the use of the slide culture technique.
- **Sticky tape preparation**: clear tape must be used such as Sellotape©, Scotch© brand tape No. 600 or an equivalent. Frosted tape is unsuitable. A 6 cm length of 2 cm wide tape is taken and held by the thumb and middle finger, with the index finger in the centre of the loop that is held sticky-side downwards. The adhesive side is then pressed firmly down, with the index finger, on the centre of the colony to be examined. The fruiting heads and spores stick to the tape and can be gently pulled from the mat of mycelium. The inoculated tape is placed over a drop of LPCB on a microscope slide. The tape is pulled taut and the free sticky ends folded over each end of the slide. The tape acts as a cover slip and the preparation can be examined under the light microscope.
- **Slide culture technique**: although this method is not suitable for making a rapid diagnosis, it is excellent for demonstrating the fruiting heads and attachment of spores in a relatively undisturbed state. This may be necessary for the definite identification of some fungal pathogens (Box 37.3; Fig. 37.1).

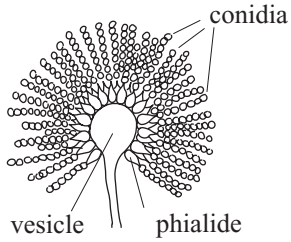
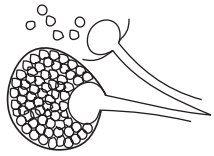
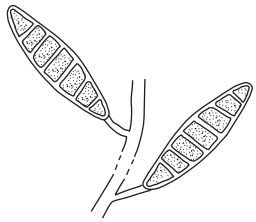
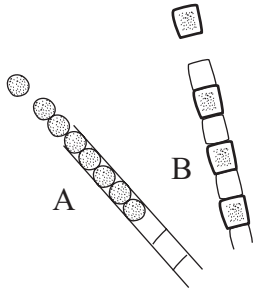
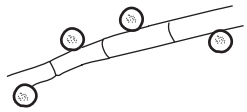
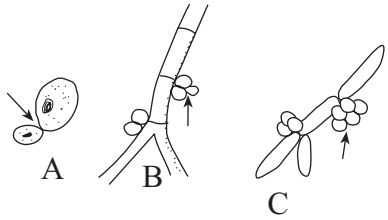
Serological Tests for Fungal Diseases

This aspect of the diagnosis of the animal mycoses has not received much attention due to difficulties such as the comparative rarity of the diseases themselves, the expense of the serological reagents and the interpretation of the results in animals. Serological tests are carried out for some of the mycotic diseases in humans and a few diagnostic reagents are available commercially. Where they are used, serological tests are mentioned in the following mycology sections on specific fungal pathogens.

Commonly Encountered Fungi on Laboratory Media

The main significance of these saprophytic contaminants is that they must be recognized and distinguished from pathogenic fungi. Some of these common contaminants are also potential pathogens. A presumptive identification, to a generic level, can often be made on the basis of the colonial appearance and the microscopic characteristics of the fruiting heads and spores (Figs 37.2 and 37.3). Figure 37.4 shows the microscopic aspects of some of the commonly encountered fungi. Table 37.5 corresponds with Figure 37.4 and briefly describes the colonial and microscopic characteristics of the fungi that are illustrated. Table 37.6 explains a few of the commonly used mycological terms.

Table 37.4 Microscopic aspects of asexual spores

Type of asexual spore	Description	
Phialconidia	Spores produced from phialides, a special flask-like portion of the conidiophore	
Sporangiospores	These spores are produced by zygomycetes and are borne within a sporangium. Release occurs following rupture of the mature sporangium	
Macroconidia	Large multi-celled spores produced in culture by dermatophytes	
Microconidia	Small spores produced by certain dermatophytes	
Arthrospores (arthroconidia)	Formed by the fragmentation of a hypha. These spores may be formed successively (A) as occurs in dermatophytes, or with empty cells in-between (B) as occurs in *Coccidioides immitis*	
Chlamydospores (chlamydoconidia)	Thick-walled, resistant spores that contain storage products and are formed during unfavourable environmental conditions by the direct differentiation of hyphae	
Blastospores (blastoconidia)	Spores produced by budding from a mother cell (A), hyphae (B) or from pseudohyphae (C)	

Box 37.3 **Slide culture technique**

- A bent glass rod is placed in a glass Petri dish with a circular piece of filter paper at the bottom. A microscope slide and a cover slip (22 mm^2) are also placed in the Petri dish. This is then wrapped in brown paper and autoclaved
- A small block of agar is cut from an Emmons' Sabouraud dextrose agar plate previously poured to a depth of about 4 mm. The block (18 mm^2) can be cut with a sterile scalpel or with the open end of a sterile test tube (18 × 150 mm) pushed into the agar. The block can be expelled from the test tube by gently heating the tube causing expansion of the enclosed air. The block of agar is placed in the centre of the microscope slide in the Petri dish. The slide itself is raised from the bottom of the dish by the glass rod. All procedures must be carried out aseptically
- Spores or a small portion of the fungal colony to be studied are inoculated into four points in the side of the agar block. The sterile cover slip is then placed on top of the agar block with a pair of sterile forceps (Fig. 37.1)
- The circular filter paper at the bottom of the dish is moistened with sterile distilled water and the lid replaced. The preparation is incubated at the optimum temperature of the fungus. The fungus should grow on the inoculated sides of the agar block and under the edges of the cover slip. Examine regularly to make sure the preparation does not become dry and to judge when sufficient growth has occurred and fruiting bodies have formed at the edge of the cover slip
- Remove the cover slip from the agar block and place it, fungal side down, on a drop of LPCB on a microscope slide. Gently press down on the cover slip and blot away any excess stain. For a semi-permanent mount the cover slip can be generously 'ringed' with a clear nail polish
- If the fungus has grown down from the agar block onto the microscope slide, this can serve as a second mount. Remove and discard the agar block and place a drop of LPCB stain on the area of fungal growth and apply a cover slip. Examine under the light microscope

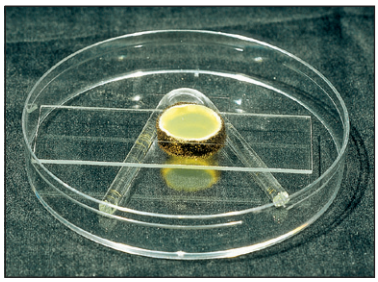


Figure 37.1 Slide culture technique: agar plug inoculated with *Aspergillus niger*.

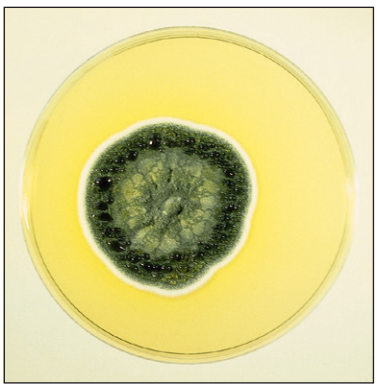


Figure 37.2 *Penicillium* species on Sabouraud agar, six days.

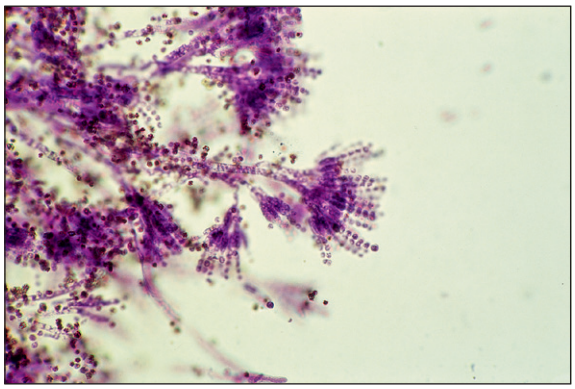


Figure 37.3 *Penicillium* species showing characteristic arrangement of conidiophores, phialides and conidia. Slide culture preparation. (×400)

Safety Aspects in Mycology

Many of the fungi causing disease in animals are also pathogenic for humans. Great care should be exercised when handling material and cultures suspected of containing pathogenic fungi, particularly *Coccidioides immitis*, which produces highly infective arthrospores at 25°C and 37°C that easily form an aerosol. *Cryptococcus neoformans* and the dimorphic fungi, such as *Blastomyces dermatitidis*, can cause serious disease in man. Ideally an approved biological safety hood should be used for all mycological procedures.

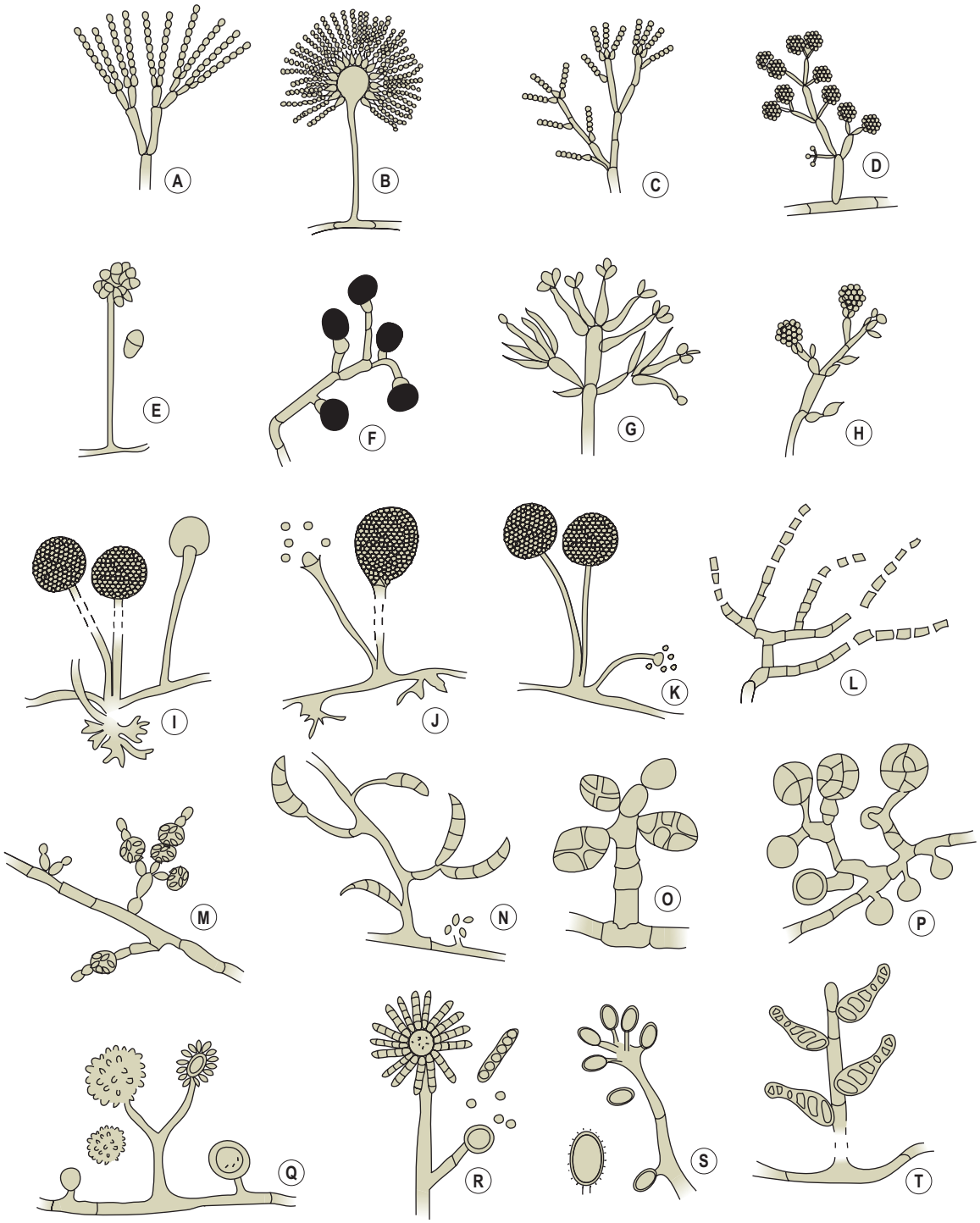


Figure 37.4 Microscopic aspects of some genera of the common contaminating fungi. A *Penicillium*, B *Aspergillus*, C *Paeciliomyces*, D *Trichoderma*, E *Trichothecium*, F *Nigrospora*, G *Verticillium*, H *Gliocladium*, I *Rhizopus*, J *Absidia*, K *Mucor*, L *Geotrichum*, M *Scopulariopsis*, N *Fusarium*, O *Stemphylium*, P *Epicoccum*, Q *Sepedonium*, R *Syncephalastrum*, S *Sporotrichum*, T *Helminthosporium*.

Table 37.5 Colonial and microscopic characteristics of the common contaminating fungi (illustrated in Figure 37.4)

Genus	Colonial appearance	Microscopic appearance
(A) *Penicillium* species	Usually blue-green and velvety, occasionally species have other colours and textures	Brush-like arrangement of fruiting head. Conidiophores have secondary branches (metulae) bearing whorls of phialides from which the smooth or rough and round conidia (2.5–5.0 μm) are borne
(B) *Aspergillus* species	Often bluish-green and velvety but shades of yellow, brown or black occur depending on the species	Conidiophore unbranched and rising from a foot cell. A swollen vesicle is produced at the tip of the conidiophore and from this arise the phialides or metulae and then phialides. The latter produce chains of round conidia (2–5 μm)
(C) *Paeciliomyces* species	Flat surface, powdery or velvety, yellowish-brown or light pastel shades of pink, violet or grey-green	Resembles *Penicillium* but the phialides are more elongated and taper into a long slender tube. The conidia are elliptical or oblong and occur in chains
(D) *Trichoderma* species	Greenish and cottony, becoming powdery with a lawn-like growth	Conidiophores are short and often branched at wide angles. Phialides are flask-shaped and the conidia are clustered at the tips of the phialides
(E) *Trichothecium* species	Pink or orange-pink and woolly	Conidiophores are long and unbranched. Conidia (8–10 × 12–18 μm) are smooth, two-celled and pear- or club-shaped. They are produced in alternating directions and remain side by side. The conidia are similar to those produced by *Microsporum nanum*
(F) *Nigrospora* species	Compact and woolly, white at first but black areas appear due to the production of black, globose conidia. The reverse is black	Short conidiophores that swell and then taper to the point of conidia formation. Conidia are large (10–14 μm), black, round but slightly flattened
(G) *Verticillium* species	Powdery or velvety, white becoming brown, pink, yellow, red or green, and spreading	Conidiophores are simple or branched and in whorls. Phialides are very elongated with a pointed apex; while conidia are oval to cylindrical, single-celled, and appear in clusters at the ends of phialides but are easily disturbed
(H) *Gliocladium* species	White at first, centre becoming green. Fluffy-to-granular and lawn-like	Conidiophores and phialides are similar to *Penicillium* but the conidia from several phialides clump together to form large green balls
(I) *Rhizopus* species	Fills Petri dish in five days with dense grey, woolly mycelium. Sporangia can often be seen as small black dots	Rhizoids are nodal. Sporangiophores are long, usually branched, and terminate in dark round sporangia (60–350 μm). Stolons connect the groups of sporangiophores
(J) *Absidia* species	Rapid growth of woolly, white-to-grey mycelium filling the Petri dish	Rhizoids are internodal and not very obvious. Sporangiophores are often branched and widen (forming an apophysis) just below the columella. Sporangia are pear-shaped (20–120 μm). A short collarette often remains when the sporangial wall disintegrates
(K) *Mucor* species	Rapid spread of colony over the surface of the agar but growth is low. White turning grey or yellowish	No rhizoids. Sporangiophores often branched and bear round sporangia (60–300 μm)
(L) *Geotrichum* species	Whitish, flat, moist and yeast-like with a granular surface. Some strains produce short, white, cottony aerial hyphae	The septate mycelium fragments into arthrospores (arthroconidia), which are formed consecutively and become round. No blastoconidia are produced

Continued

Table 37.5 Colonial and microscopic characteristics of the common contaminating fungi (illustrated in Figure 37.4)—cont'd

Genus	Colonial appearance	Microscopic appearance
(M) *Scopulariopsis* species	White and glabrous, becoming powdery and some shade of yellow, buff or brown	Fairly short conidiophores and the conidia-bearing cells can be cylindrical. Conidia are comparatively large (4–9 μm), thick-walled and spiny when mature
(N) *Fusarium* species	Tendency to produce delicate rose-pink, lavender or purple pigments seen on obverse and reverse of the colony	Produces large (3–8 × 11–70 μm), banana-shaped macroconidia from phialides, and simple conidiophores can bear small (2–4 × 4–8 μm), one- to two-celled conidia singly or in clusters resembling those of *Acremonium* species
(O) *Stemphylium* species	Black, almost yeast-like colony with white aerial hyphae	Conidioiphores are simple, occasionally branched, and bearing muriform conidia
(P) *Epicoccum* species	Yellow to orange at first, becoming black with age. A diffusible pigment (yellowish or red) may colour the agar	Clusters of conidiophores bear round to pear-shaped muriform conidia (15–30 μm), which are dark brown and warty
(Q) *Sepedonium* species	White and waxy, becoming fluffy and yellow with age	Simple or branched conidiophores bearing large (7–17 μm), round, rough, knobby conidia. Rather similar to *Histoplasma capsulatum* but no microconidia are produced and *Sepedonium* species do not convert to a yeast form
(R) *Syncephalastrum* species	Rapidly fills the Petri dish with a white cottony growth that turns dark grey with age	An occasional septum is seen. Short branched sporangiophores have swollen tips bearing chains of spores enclosed in tubular sporangia (4–9 × 9–60 μm). Rhizoids can be present
(S) *Sporotrichum* species	Velvety to granular surface, white becoming tan, pinkish, yellow or orange. Reverse is tan	Short, simple conidiophores that bear single-celled, ovoid, yellow conidia. The conidia tend to retain a portion of conidiophore after separation. Clamp connections present at the septa
(T) *Helminthosporium* species	Cottony and dark grey to black. Reverse is black	Unbranched conidiophores that are brown, slightly curved, with conidia forming along the sides. The latter are large, dark, multicelled and club-shaped

Table 37.6 A short glossary of mycological terms

Term	Explanation
Adiaspores	Spores that increase in size without replication
Aerial hyphae	Hyphae above the agar surface. They often produce fruiting structures
Anamorph	Asexual form of a fungus
Anthropophilic	Describes fungi that usually infect humans only
Apical	Located at the tip of a pointed extremity
Arthrospore (arthroconidium)	An asexual spore formed by the fragmentation of a hypha. The resulting spores can be rectangular, barrel-shaped, or can become rounded
Ascocarp	A mycelial sac within which are formed asci and ascospores of the ascomycetes
Ascospore	The sexual spore of the ascomycetes, which develop in a sac-like structure termed an ascus

Table 37.6 A short glossary of mycological terms—cont'd

Term	Explanation
Ascus	A sac-like structure usually containing eight ascospores that are produced during the sexual reproduction of an ascomycete
Basidiospore	The sexual spore of the basidiomycetes, which are produced on club-shaped structures termed basidia
Blastospore (blastoconidium)	An asexual spore produced by a budding process along hyphae or pseudohyphae
Chlamydospore (chlamydoconidium)	A thick-walled, resistant spore formed by the direct differentiation of the mycelium, contains storage products and typically formed in unfavourable environmental conditions
Clavate	Club-shaped
Columella	The sterile, inflated end of a sporangium extending into a sporangium
Conidia (sing. conidium)	Asexual fungal spores that are abstricted in various ways from a conidiophore
Conidiophore	Specialized aerial hypha bearing conidia
Dermatophytes	A fungus belonging to the genera *Microsporum, Trichophyton* and *Epidermophyton* (human), which has the ability to infect skin, hair, nails or claws of animals and man; 'ringworm fungi'
Dichotomous	Describes where hyphae are divided into two equal branches with the same diameter as that of the original hypha, seen in *Aspergillus* spp.
Dimorphic	Having two morphological forms, such as the dimorphic fungi which have a mould and yeast phase
Ectothrix	Outside the hair shaft
Endothrix	Inside the hair shaft
Fusiform	Spindle-shaped
Geophilic	Fungi whose natural habitat is the soil (literally 'soil-loving')
Germ tube	The tube-like process from a germinating spore that develops into a hypha. In the case of *Candida albicans* it is a tube extruded from the yeast cell that develops into a pseudohypha
Hyphae (sing. hypha)	Filaments that collectively make up the mycelium of a fungus
Hyaline	Usually refers to hyphae that are colourless or transparent
Imperfect state	Asexual state. The phase of a life cycle in which there is no sexual reproduction
Internodes	Areas on a stolon between the points where sporangiophores are borne
Macroconidium (pl. macroconidia)	A large conidium, usually multicelled, as opposed to a smaller conidium which is called a microconidium
Microconidium	The smaller of the two types of conidia (see above)
Mycelium	A mat of intertwined and branching hyphae
Mycetoma	Fungal-induced, tumour-like granulomatous lesion of cutaneous and subcutaneous tissues with signs of swelling, draining sinuses and granules in exudates
Mycosis (pl. mycoses)	A disease produced by a fungus
Mycotoxicosis (pl. mycotoxicoses)	A disease caused by the ingestion of preformed toxic fungal metabolites produced while the fungus is growing on foodstuffs
Nodal	Denoting the area on a stolon where sporangiophores are borne
Perfect state	Sexual stage

Continued

Table 37.6 A short glossary of mycological terms—cont'd

Term	Explanation
Phaeohyphomycosis	Subcutaneous mycosis associated with a dematiaceous (dark-walled hyphae) fungus
Phialide	Special flask-like portion of the conidiophore from which the conidia are borne
Phialoconidia	Asexual conidia produced from phialides
Pseudohyphae (sing. pseudohypha)	Filaments formed by elongated yeast cells that have failed to separate from each other
Rhizoid	Root-like branched hyphae extending into the substrate
Saprophyte	An organism that obtains nutrients from dead organic matter
Sclerotium (pl. sclerotia)	A hard, compact mass of mycelium. It represents the resting stage of fungi such as *Claviceps purpurea*
Septate	Describes hyphae that are divided by cross-walls
Spherule	A thick-walled, closed, usually spherical structure enclosing asexual spores
Sporangiophore	A specialized hyphal branch bearing a sporangium
Sporangiospore	An asexual spore borne within a sporangium
Sporangium	A closed structure enclosing sporangiospores produced by cleavage
Sterigmata	Specialized projections borne on a vesicle and producing conidia
Stolon	A horizontal hypha that sprouts where it touches the substrate and often produces rhizoids and sporangiophores at that point
Stroma	A cushion-like mat of fungal elements
Teleomorph	Sexual form of a fungus
Vegetative	Refers to hyphae involved in food absorption as opposed to producing spores
Verrucose	Covered with wart-like projections
Vesicle	A bladder-like structure
Zoophilic	Fungi that infect animals
Zoospore	Asexual, motile spore
Zygospore	A thick-walled, sexual spore produced through the fusion of two gametangia (side projections produced from two compatible hyphae). Produced by the zygomycetes

REFERENCES

Hibbett, D.S., Binder, M., Bischoff, J.F., et al., 2007. A higher level phylogenetic classification of the fungi. Mycological Research 111, 509–547.

Kirk, P.M., Cannon, P.F., Minter, D.W., et al., 2008. Dictionary of the Fungi, tenth ed. CABI, Wallingford, UK.

Mendoza, L., Taylor, J.W., Ajello, L., 2002. The class *Mesomycetozoea:* a heterogeneous group of microorganisms at the animal-fungal boundary. Annual Reviews in Microbiology 56, 315–344.

FURTHER READING

Sukura, A., Saari, S., Jarvinen, A.-K., et al., 1996. *Pneumocystis carinii* pneumonia in dogs – a diagnostic challenge. Journal of Veterinary Diagnostic Investigation 8, 124–130.

Chapter 38

The dermatophytes

The dermatophytes are a group of closely related, septate fungi that require and use keratin for growth. They tend to be confined to the superficial integument including the outer stratum corneum of the skin, nails, claws and hair of animals and man. The classical lesions are circular and known as ringworm. Traditionally the dermatophytes are placed in the Deuteromycota or Fungi Imperfecti in three anamorphic (asexual or imperfect state) genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. However, the teleomorphic (perfect or sexual) state has been described for some and they are classified in the genus *Arthroderma*, phylum *Ascomycota*.

Over 30 species of dermatophytes are known. Those affecting animals are placed in one of two genera, *Microsporum* or *Trichophyton*. *Epidermophyton floccosum* is principally a human pathogen. The dermatophyte species affecting animals are described as ectothrix as the septate hyphae invading the skin and hairs fragment into arthrospores and these form a sheath around the infected structures. Macroconidia and microconidia are produced in the non-parasitic state in laboratory cultures. The *Microsporum* species tend to produce spindle (Fig. 38.1) or boat-shaped (Fig. 38.2) macroconidia whereas those of the *Trichophyton* species are usually elongated, cigar-shaped with almost parallel sides (Fig. 38.3). The macroconidia of *M. nanum* are unique in being round and usually two-celled (Fig. 38.4). Figure 38.5 gives the main points of differentiation between the two genera. The colonies of many of the dermatophytes are pigmented and both the obverse and reverse of the colonies should be examined to assist identification.

Natural Habitat

The geophilic (soil-loving) dermatophytes inhabit the soil and can exist there as free-living saprophytes. *Microsporum gypseum* and *M. nanum* are examples of geophilic dermatophytes that can also cause lesions in animals and in man. The zoophilic dermatophytes are obligate pathogens, primarily parasitizing animals but also capable of infecting humans. Humans are the main host for the anthropophilic dermatophytes and these very rarely cause ringworm in animals and are not considered in this chapter. Some dermatophytes have become adapted for survival in the skin of specific host animals, for example:

- *Microsporum canis*: cats
- *Microsporum persicolor*: voles
- *Trichophyton mentagrophytes* var. *erinacei*: European hedgehogs
- *Trichophyton mentagrophytes* var. *mentagrophytes*: rodents
- *Trichophyton verrucosum*: cattle.

These dermatophytes generally give rise to subclinical or inapparent infections in the host animal, although they can also produce clinical lesions. Zoophilic species tend to be associated with a particular animal species. However, infection can also occur from reservoirs such as rodents (*T. mentagrophytes*), hedgehogs (*T. erinacei*; Fig. 38.6), soil (*M. gypseum*) and fomites such as bedding, grooming gear and harness containing hairs with infective arthrospores. Whilst growing on keratinized structures these fungi rarely produce macroconidia and rely instead on the production of arthrospores for transmission from host to host. Arthrospores can remain viable on shed hairs and skin particles for at least six to 12 months. The reservoir, transmission and site of the lesions can often be related to the actual dermatophyte involved. This is especially evident in the horse and the dog and is an argument in favour of attempting to isolate and identify the dermatophyte in an infection. Table 38.1 lists the main dermatophytes affecting animals, the main hosts of each and their geographical distribution.

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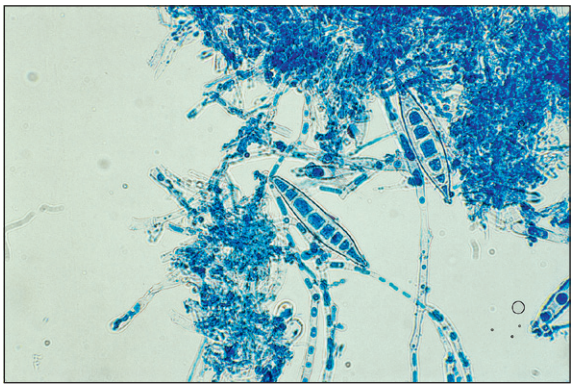


Figure 38.1 *Microsporum canis*: spindle-shaped macroconidia. (LPCB, ×400)

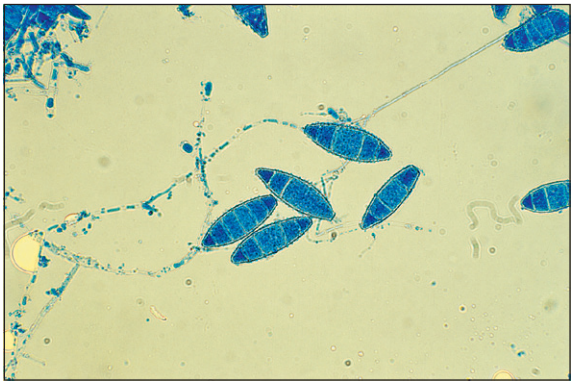


Figure 38.2 *Microsporum gypseum*: macroconidia. (LPCB, ×400)

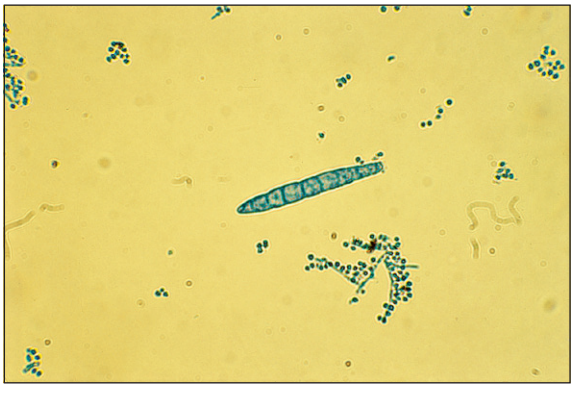


Figure 38.3 *Trichophyton mentagrophytes*: numerous microconidia and a macroconidium. (LPCB, ×400)

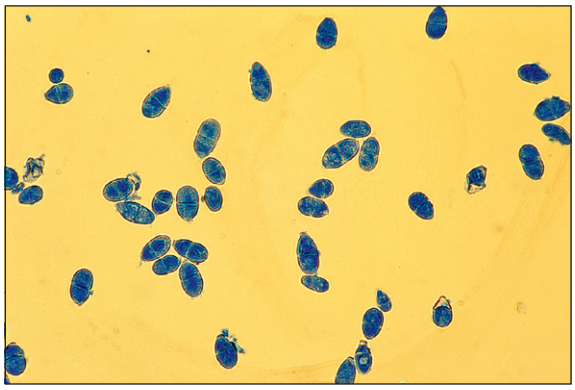


Figure 38.4 *Microsporum nanum*: macroconidia. (LPCB, ×400)

Pathogenesis

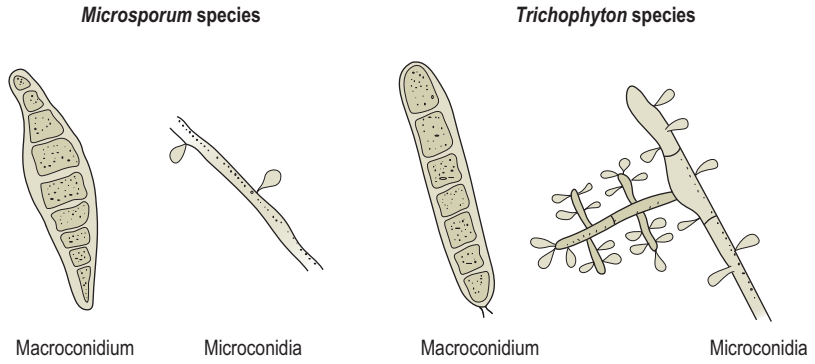
Infective arthrospores germinate within six hours of adhering to keratinized structures. Minor trauma of the skin and dampness may facilitate infection. The ability of the dermatophytes to hydrolyse keratin causes damage to the epidermis, hair shafts, hair follicles and feathers. The nature of the lesions is affected by the virulence of the fungus and the immunological response of the host. Very young and very old animals as well as debilitated or immunosuppressed individuals are most susceptible to infection. The host mounts an inflammatory response to the fungal metabolic products that is harmful to the fungus, so the dermatophyte moves away peripherally towards normal skin. The result is the commonly seen circular lesions (ringworm) of alopecia with healing at the centre and inflammation at the edge (Fig. 38.7). There appears to be a balanced host–parasite relationship where a dermatophyte has become adapted to a specific host animal. While these animals may not show lesions they can act as a reservoir of infection. The manifestations of dermatophyte infections can vary and may be summarized as:

- Subclinical or inapparent infections
- Classical round ringworm lesions
- Serious generalized lesions that may be complicated by mange mites or by secondary bacterial infection, in particular by *Staphylococcus aureus* or *S. pseudintermedius*
- Nodular or tumourous lesions called kerions, seen most commonly in dogs.

Laboratory Diagnosis

Preliminary examination: Wood's lamp

Microsporum canis, *M. canis* var. *distortum*, *M. audouinii* (human) and *M. ferrugineum* (human) produce certain metabolites when growing on hairs and skin that will



Macroconidium	Large thick-walled and divided into many cells by transverse septa. Tend to be spindle- or boat-shaped.	Few or absent in some species. If present they are elongated and cigar- or pencil-shaped. The walls are thin and smooth. Divided by septa into 3–8 cells.
Microconidia	Relatively few or absent. If present they are tear-shaped and borne singly on the hyphae.	Usually numerous and borne singly along the hyphae or in grape-like clusters.

Figure 38.5 Microscopic differentiation of the dermatophyte genera affecting animals.

Figure 38.6 *Trichophyton erinacei*: muzzle alopecia in a terrier known to worry hedgehogs.

fluoresce a vivid apple-green under the ultraviolet light of a Wood's lamp. The animal itself can be examined with the lamp in a dark room and the site of the lesions will fluoresce. The technique is particularly useful for suspected inapparent infections in kittens that almost always involve *M. canis*. The infected areas are often the face, front paws and abdominal area of these kittens. Alternatively, the lamp can be used to examine plucked hairs or skin scrapings taken from lesions. It is estimated that about 50% of *M. canis* infections give this fluorescence, so negative cases should always be submitted for further laboratory examinations. If a topical ointment has been applied to the lesion, this can sometimes lead to spurious fluorescence.

Specimens

The following points should be noted:

- Hairs should be plucked from the lesions, never cut with scissors, as the basal portion of the hairs often contains the most useful diagnostic material. Any stubby or damaged-looking hairs that might be present should be collected.
- Scab material should be obtained from the edge of the lesion as this is the site where the dermatophyte is most likely to be viable. A blunt scalpel blade is used to scrape until blood is just drawn. The scrapings and the scalpel blade should be submitted with adherent material. This specimen will also be useful to detect any mange mites that may be present.
- A paper envelope can be held under the lesion when scrapings are being taken to catch the scab material, scurf and damaged hairs. The specimens can be submitted to the laboratory in the envelope (inside additional wrapping) as they tend to remain drier and less contaminated in paper, compared to a glass or plastic container.
- Scrapings and clipping from claws should be taken from as near the base as possible.
- In suspected inapparent infections, if the site of the dermatophyte infection has not been detected following a Wood's lamp examination, the animal should be brushed, using a brush that can be

Table 38.1 Dermatophytes of veterinary significance

Species	Hosts	Geographical Distribution	Colonial Appearance	Microscopic Appearance
Microsporum canis (var. *canis*)	Cats, dogs, important cause of ringworm in humans	Worldwide	Growth rapid. Surface white and silky at centre with bright yellow periphery. Reverse side bright yellow or orange	Usually abundant macroconidia. They are spindle-shaped and mature spores end in a distinct knob. Cells 6–15, size 8–20 × 40–150 µm. Few microconidia
M. canis var. *distortum*	Dogs	North America, New Zealand, Australia	Growth fairly rapid. Surface white to tan and reverse white or yellowish tan. Colony is velvety to fluffy with a tendency to form radial grooves	Usually abundant macroconidia that are distorted in shape, thick-walled and multicellular. Size 12–27 × 30–60 µm. Numerous microconidia
M. canis (syn. *M. equinum*)	Horses	Worldwide	Slow growth. Surface white and velvety to finely powdery. Reverse salmon or buff	Macroconidia rare, resemble shortened *M. canis* macroconidia, size 5–15 × 18–60 µm
M. gypseum	Horses, dogs, rodents	Worldwide (in soil)	Fairly rapid growth. Colony is flat, powdery with a fringed border. Obverse is buff to cinnamon-brown and reverse pale yellow to tan or occasionally red. Odour similar to a mouse colony	Abundant macroconidia. Boat-shaped with rounded ends and thick, rough walls. Cells 4–6, size 8–12 × 30–50 µm
M. nanum	Pigs	North and South America, Europe, Australasia (in soil)	Colony is flat, white and cottony at first, later granular and buff-coloured	Abundant macroconidia, pear-shaped with spiny walls. Cells 1–3, size 4–8 × 12–18 µm
M. gallinae	Chickens, turkeys	Worldwide	Rapid growth. Surface white to pinkish, velvety and folded. Reverse strawberry-pink, diffusible pigment	Abundant macroconidia, fusiform with blunt spatulate tips. Walls smooth and thick. Cells 2–10, size 6–8 × 15–50 µm
Trichophyton equinum	Horses	Worldwide	Fairly rapid growth. Colony initially flat, white and fluffy but later velvety with central folding. Cream to tan in colour, reverse is yellow to reddish-brown	Macroconidia are rare. Slightly club-shaped, smooth, thin-walled with 3–5 cells. Abundant microconidia. Chlamydospores are abundant in old cultures
Trichophyton equinum var. *autotrophicum*	Horses	New Zealand, Australia	Colony at first white with a raised centre, later white to buff with folded centre. Reverse is yellow becoming dark rose-red	Macroconidia not reported

Table 38.1 Dermatophytes of veterinary significance—cont'd

Species	Hosts	Geographical Distribution	Colonial Appearance	Microscopic Appearance
T. mentagrophytes var. *mentagrophytes*	Rodents, dogs, horses and many other species	Worldwide	Rapid growth. Two colony forms: 1. Granular, obverse cream, reverse buff-tan to dark-brown; 2. Downy, white and woolly with older colonies becoming cream-tan, reverse varies from white through yellow to reddish-brown	Macroconidia cigar-shaped, thin-walled. Cells 3–7, size 4–8 × 20–50 µm. Abundant microconidia in grape-like clusters
T. mentagrophytes var. *erinacei*	European hedgehogs, dogs	Europe, New Zealand	Rapid growth. Colony finely granular and flat with raised centre. Fringed subsurface border. Obverse white to cream, reverse brilliant yellow	Macroconidia uncommon. Irregular shape and size, smooth, thin-walled. Cells 2–6
T. mentagrophytes var. *quinckeanumi*	Mice	Australia, North America, Europe	Colony initially white and fluffy, becoming downy and deeply folded. Reverse is deep yellow becoming orange-brown	Macroconidia rare. Smooth, thin-walled, cigar- to club-shaped. Cells 4–6
T. simii	Monkeys, poultry, dogs	Brazil, Guinea, India (in soil)	Rapid growth. Finely granular colony with diffuse margin, white to pale or rose-buff. Reverse white and later reddish-brown	Abundant macroconidia, cylindrical to fusiform in shape. Cells 3–10, size 6–11 × 35–85 µm
T. verrucosum	Cattle	Worldwide	Very slow-growing. Small, white, velvety, heaped and folded colony. Obverse white or whitish-grey and occasionally yellow-ochre, reverse is white	Macroconidia very rare but characteristic chains of chlamydospores

sterilized or discarded after use. The hairs and scurf are collected in a container held under the animal.

- Where the specimens tend to be very contaminated by bacteria and saprophytic fungi, particularly in the case of pigs, consideration might be given to wiping the lesions with 70% alcohol and then allowing the area to dry thoroughly before collecting the specimens.

Direct microscopy

The KOH wet preparation method or modifications (see Chapter 37) is used for hairs, scabs or claw scrapings. The preparation is examined under the low-power objective, with the condenser lowered slightly, focusing on any abnormal-looking hairs. The high-dry objective is used to visualize the round, refractile arthrospores surrounding the hair or on pieces of scab material (Fig. 38.8). Occasionally the septate hyphae of the dermatophyte can be seen forming chains of arthrospores. Care must be taken not to mistake normal skin or hair structures such as fat globules or hair pigment granules (melanosomes) for arthrospores. Arthrospores vary slightly in size depending on the dermatophyte involved; those of *T. verrucosum* are particularly large (about 5–6 µm in diameter) and easy to see. Mange mites, if present, will be visible in the cleared KOH preparations.

If skin lesions from cattle, sheep or horses are being examined and scab material or scurfy pieces of skin are available, a Gram- or Giemsa-stained smear should be made from them and examined for *Dermatophilus congolensis*.

Isolation

Even if either the Wood's lamp examination or the direct microscopy for arthrospores have proved to be positive, it is still useful to attempt the isolation and identification of the dermatophyte for transmission and control aspects. A culture medium for dermatophytes must cater for the few that require specific growth factors. These can be satisfied by the use of commercially available trichophyton media,

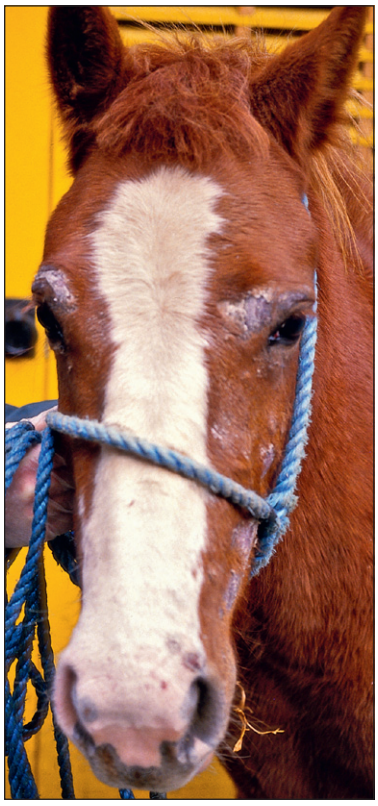


Figure 38.7 *Trichophyton equinum*: typical ringworm lesions in a horse (bridle area).

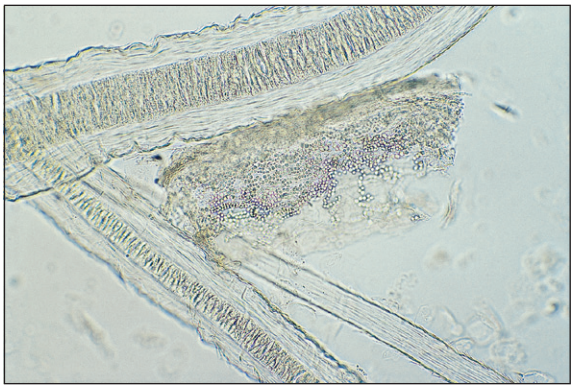


Figure 38.8 *Trichophyton verrucosum*: infected bovine hair with arthrospores. (10% KOH, ×400)

developed for the *Trichophyton* species. The control medium is known as trichophyton agar 1 (T1) and is a casein basal agar. Growth factors are added as required:

- *Trichophyton verrucosum*: requires thiamine or thiamine and inositol (trichophyton agar 3).
- *Trichophyton equinum*: requires nicotinic acid (trichophyton agar 5) whereas *T. equinum* var. *autotrphicum* does not (trichophyton agar 1).
- *Microsporum gallinae*: thiamine stimulates growth (trichophyton agar 3).

A practical alternative is to prepare a medium on which all the dermatophytes that are likely to affect animals will grow. Such a medium is prepared as follows:

- Emmons' Sabouraud dextrose agar (pH 6.9)
- Yeast extract 2–4% (to supply specific growth factors)
- Chloramphenicol 0.05 g/L (antibacterial)
- Cyclohexamide 0.4 g/L (to inhibit some faster-growing fungi).

A light inoculum of hairs and skin scrapings can be scattered over the surface of the agar and gently pressed down on the medium with a swab or sterile forceps. The dermatophyte cultures are incubated aerobically at 25°C. The plates should be examined twice weekly and not discarded as negative for three weeks, for most of the dermatophytes. The plates should be kept for five weeks in the case of *T. verrucosum*. Some of the more rapidly growing dermatophytes, such as *M. canis*, may be recognized after four to six days' incubation. *Trichophyton verrucosum* is unusual among the dermatophytes in growing well at 37°C (*T. mentagrophytes* will also tolerate 37°C). When attempting to isolate this dermatophyte duplicate cultures can be made, one plate being incubated at 25°C and the other at 37°C. The plates may be taped to prevent the agar drying, but as the dermatophytes are strict aerobes the tape should be removed and replaced once daily. *Trichophyton mentagrophytes* hydrolyses urea when grown on Christensen urea agar.

Dermatophyte test medium (DTM), which can be obtained commercially, is a selective and differential medium for dermatophytes containing the pH indicator phenol red. The dermatophytes produce alkaline metabolic products changing the medium from yellow to red. The medium is also changed, but usually more slowly, by some saprophytic fungi, yeasts and bacteria. It is not advisable to use this medium alone for primary isolation as its colour obscures the characteristic pigmentation of dermatophytes that is useful for species identification.

Identification

Usually a dermatophyte can be identified by the animal host from which it was isolated, the colonial appearance and the microscopic characteristics of the colonies. If there is any doubt about a particular isolate, a subculture on an agar slant should be submitted to a mycology reference laboratory.

Colonial appearance

Considerations such as rate of growth, texture and pigmentation of the obverse and reverse sides of the colony

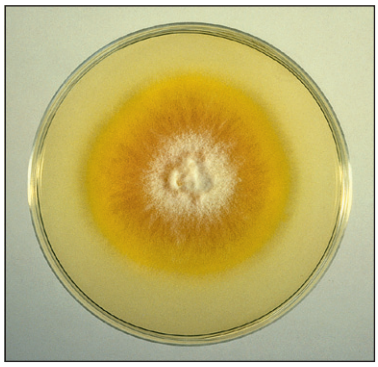


Figure 38.9 *Microsporum canis* on Sabouraud agar, 10 days.

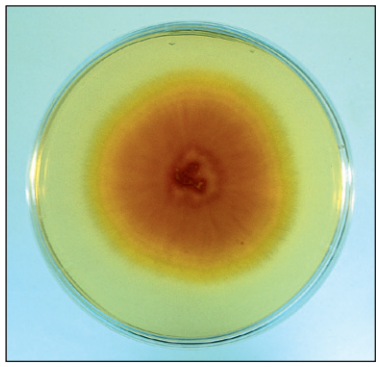


Figure 38.10 *Microsporum canis* on Sabouraud agar, 10 days. Reverse.

Figure 38.11 *Microsporum gypseum* on Sabouraud agar, 12 days.

Figure 38.12 *Microsporum gypseum* on Sabouraud agar, 12 days. Reverse.

should be noted. The characteristics of the colonies of dermatophytes commonly affecting animals are given in Table 38.1 and illustrated (Figs 38.9 to 38.22).

Microscopic appearance of the colony

The lactophenol cotton blue (LPCB) stain (Appendix 1) is suitable for staining wet preparations for examination of fungal structures. Figure 38.5 indicates the microscopic appearance of the macroconidia for the dermatophytes commonly affecting animals. The macroconidia of *Microsporum* species are generally spindle- or boat-shaped with rough, thick walls. The macroconidia of *Trichophyton* species are far less numerous in culture and have an elongated, cigar or pencil shape. The walls are thin and smooth. Macroconidia are extremely rare in the cultures of *T. verrucosum* but chlamydospores forming chains are a characteristic feature.

Hair perforation test

This test is used mainly in medical mycology as an aid to distinguish *T. mentagrophytes* from *T. rubrum* and atypical *M. canis* from *T. equinum* (Box 38.1). *Trichophyton mentagrophytes* and *M. canis* have the ability to invade the hair shaft and produce conical perforations of the hair, seen in LPCB preparations as wedge-shaped areas (Fig. 38.23). *Trichophyton rubrum* and *M. canis* do not penetrate the hair but grow on the surface. The conventional method, given in many of the standard medical textbooks, involves adding sterile hairs to a moist piece of filter paper in a

Box 38.1 Hair perforation test

- Collect hairs from a young child with fair hair
- Layer the sterile hairs on a 3–5-day-old subculture of the dermatophyte under test and incubate at 25°C
- Examine the hairs daily from seventh day of incubation onwards by mounting a few hairs in lactophenol cotton blue and examining them microscopically, using the low and high-dry objectives

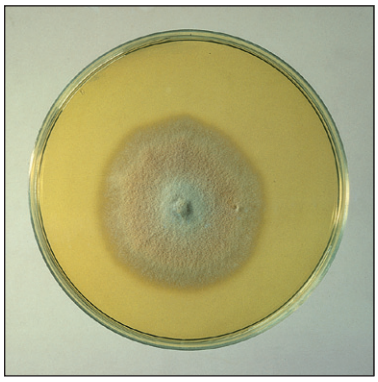


Figure 38.13 *Microsporum nanum* on Sabouraud agar, 13 days.

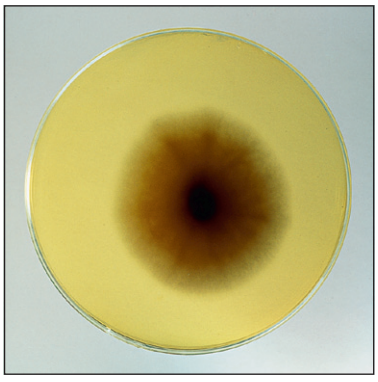


Figure 38.14 *Mrichophyton nanum* on Sabouraud agar, 13 days. Reverse.

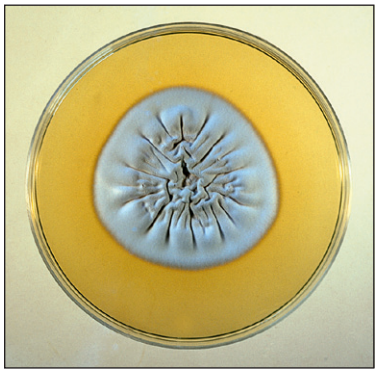


Figure 38.15 *Trichophyton equinum* on Sabouraud agar, 15 days.

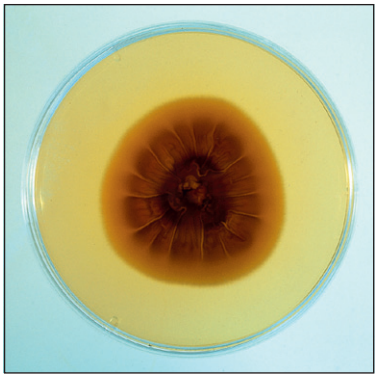


Figure 38.16 *Trichophyton equinum* on Sabouraud agar, 15 days. Reverse.

Figure 38.17 *Trichophyton erinacei* on Sabouraud agar, 12 days.

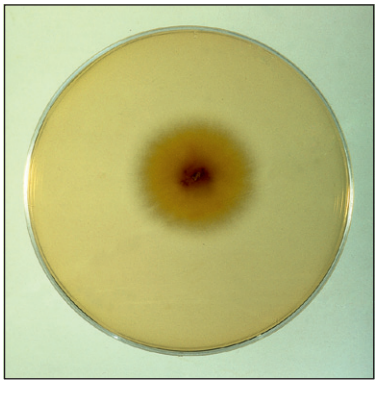


Figure 38.18 *Trichophyton erinacei* on Sabouraud agar, 12 days. Reverse.

Figure 38.19 *Trichophyton mentagrophytes* on Sabouraud agar, 12 days. Granular-type colony.

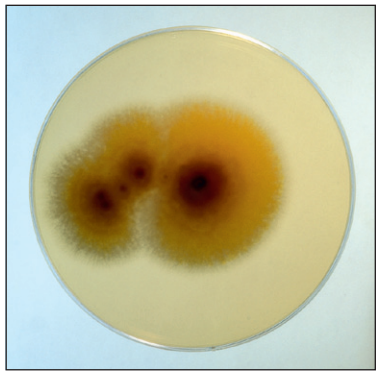


Figure 38.20 *Trichophyton mentagrophytes* on Sabouraud agar, 12 days. Reverse.

Figure 38.21 *Trichophyton verrucosum* on Sabouraud agar, 21 days.

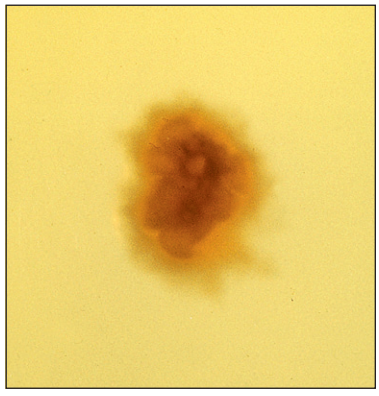


Figure 38.22 *Trichophyton verrucosum* on Sabouraud agar, 21 days. Reverse.

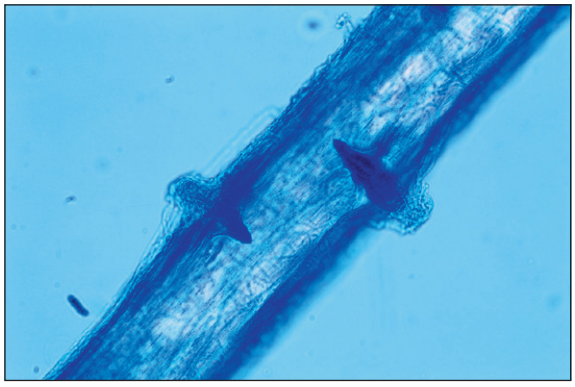


Figure 38.23 *Trichophyton mentagrophytes*: illustrating in vitro hair penetration by this dermatophyte seen as wedge-shaped, dark-blue areas. (LPCB, ×400)

Petri dish and adding a portion of the fungal colony to the hairs. However, the authors have found that a more reproducible and convenient method is as described in Box 38.1.

Histological sections

Fungal structures may be visible in stained sections of skin lesions or pseudomycetomas. Suitable staining techniques include PAS and methenamine silver.

Molecular techniques

A number of DNA-based techniques have been developed for the detection of fungal DNA in dermatological specimens (Turin et al. 2000, Gutzmer et al. 2004, Nardoni et al. 2007) and for use in identifying isolated fungi (Liu et al. 2000, Kamiya et al. 2004).

REFERENCES

Gutzmer, R., Mommert, S., Kuttler, U., et al., 2004. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. Journal of Medical Microbiology 53, 1207–1214.

Kamiya, A., Kikuchi, A., Tomita, Y., et al., 2004. PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. Journal of Dermatological Science 34, 35–48.

Liu, D., Coloe, S., Baird, R., et al., 2000. Application of PCR to the identification of dermatophyte fungi. Journal of Medical Microbiology 49, 493–497.

Nardoni, S., Franceschi, A., Mancianti, F., 2007. Identification of *Microsporum canis* from dermatophytic pseudomycetoma in paraffin-embedded veterinary specimens using a common PCR protocol. Mycoses 50, 215–217.

Turin, L., Riva, F., Galbiati, G., et al., 2000. Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. European Journal of Clinical Investigation 30, 511–518.

FURTHER READING

Chermette, R., Ferreiro, L., Guillot, J., 2008. Dermatophytoses in animals. Mycopathologia 166, 385–405.

Graser, Y., Kuijpers, F.A., El Fari, M., et al., 2000. Molecular and conventional taxonomy of the *Microsporum canis* complex. Medical Mycology 38, 143–153.

Kanbe, T., 2008. Molecular approaches in the diagnosis of dematophytosis. Mycopathologica 166, 307–317.

Weitzman, I., Summerbell, R.C., 1995. The dermatophytes. Clinical Microbiological Reviews 8, 240–259.

Chapter 39

Aspergillus species and *Pneumocystis carinii*

ASPERGILLUS SPECIES

More than 190 species of *Aspergillus* have been formally recognized, most are harmless saprophytes. It is estimated that *Apergillus fumigatus* is responsible for 90–95% of aspergillosis infections in animals. Other *Aspergillus* species that occasionally cause infections are *A. niger*, *A. flavus*, *A. terreus*, *A. deflectus*, *A. flavipes* and possibly *A. nidulans*. *Aspergillus flavus* is more commonly involved in aflatoxicosis. Taxonomically the genus is placed in the phylum *Ascomycota*. For many species the sexual stage has not been identified. The sexual reproductive cycle of *Aspergillus fumigatus* was observed for the first time in recent years and the teleomorph named *Neosartorya fumigata* (O'Gorman et al. 2008).

The *Aspergillus* species are rapidly growing, aerobic moulds with septate hyphae. Many have highly coloured colonies that range from bluish-green through yellow to black due to the profuse production of pigmented spores (conidia). The chains of small (2–3 µm) oval or spherical conidia are borne from the tips of phialides radially positioned over the surface of the swollen tip (vesicle) of the aerial hypha (conidiophore) which develops at right angles from specialized hyphal foot cells (Fig. 39.1). *Aspergillus* species can cause disease in several ways, they can be invasive, cause mycotoxicoses and are involved in allergic reactions in humans.

Natural Habitat

The aspergilli are ubiquitous and can be isolated from soil, air and decomposing organic matter. They are worldwide in distribution. They are common laboratory contaminants due to the presence of their spores in dust and air.

Pathogenesis

Aspergillus fumigatus produces haemolysins, proteolytic enzymes and other toxic factors, in particular gliotoxin, a mycotoxin which has immunosuppressive properties. Infection is acquired from environmental sources, generally by inhalation or ingestion. It is an opportunistic pathogen depending on impaired, overwhelmed or by-passed host defences to permit hyphal invasion of tissues. In pulmonary infections, following spore inhalation, suppurative exudates accumulate in the bronchioles. The spores are small enough to be carried to the terminal parts of the bronchial tree. Mycelial growth may extend into blood vessels, leading to vasculitis, thrombus formation and dissemination to other parts of the body. Granulomas can develop in many body organs and are visible as yellowish-grey nodules. If *A. fumigatus* breaks out into an air space in the body, such as the air sacs in chickens, distorted fruiting heads can be formed (Fig. 39.2). Table 39.1 indicates the diseases that can be caused in animals by *A. fumigatus*. On rare occasions, other opportunistic fungi (*Paecilomyces* species, *Penicillium* species, *Pseudallescheria boydii* and *Scedosporium apiospermum*) may be isolated from lesions similar to those associated with *Aspergillus* species (Watt et al. 1995). Spores of *Aspergillus fumigatus* present in organic stable dust may act as an allergen and have been associated with cases of chronic obstructive pulmonary disease in horses.

Laboratory Diagnosis

Specimens

Specimens could include pneumonic lung, granulomatous nodules, centrifuged mastitic milk, foetal lesions, foetal stomach contents, cotyledons, ear swabs, skin

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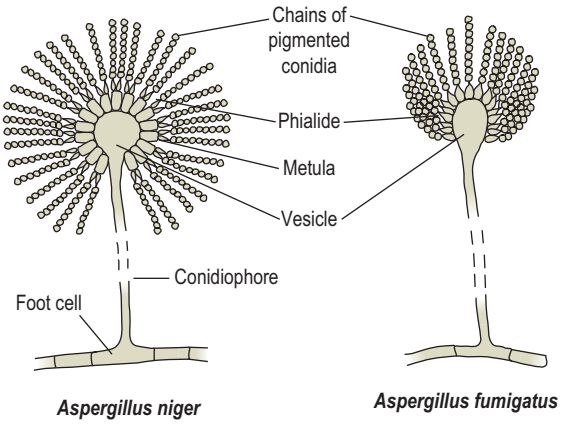

Figure 39.1 Comparison of the fruiting heads of *Aspergillus niger* and *Aspergillus fumigatus*.

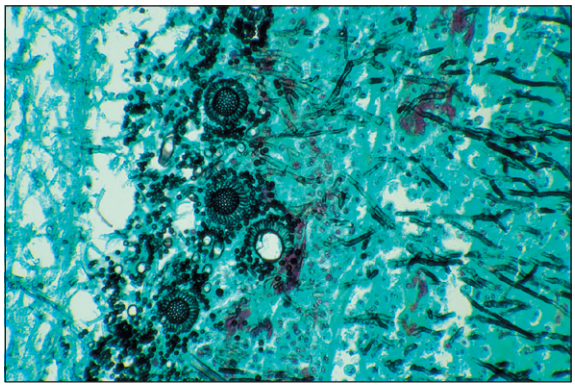

Figure 39.2 *Aspergillus fumigatus*: infected air sac of a swan showing septate hyphae and fruiting heads. (Methenamine silver stain, ×400)

Table 39.1 Diseases caused by *Aspergillus* species in veterinary species

Host	Disease	Comments
Cattle	Mycotic abortion	Sporadic occurrence, thickened, leathery placenta and raised plaques on skin of foetus
	Mycotic mastitis	Chronic, progressive infection associated with use of contaminated intramammmary antibiotic tubes
	Intestinal aspergillosis	Described occasionally in calves
	Mycotic pneumonia	Rare, in housed calves
Horses	Guttural pouch mycosis	Sporadic, often unilateral, necrotizing infection of guttural pouch. May involve carotid blood vessels and glossopharyngeal nerve
	Nasal granuloma	Presents as nasal discharge and interference with breathing
	Keratomycosis	May follow on from ocular trauma
	Intestinal aspergillosis	Described occasionally in foals
Dogs	Nasal aspergillosis	Sporadic occurrence, usually in young to middle-aged dolichocephalic breeds. Persistent nasal discharge. May involve turbinate bones and paranasal sinuses
	Otitis externa	Occurs as part of mixed infection
	Systemic aspergillosis	Rare, recorded in German Shepherd breed particularly. Osteomyelitis is often a feature
Birds	Brooder pneumonia	Respiratory infection that occurs in newly hatched chicks exposed to high numbers of spores in incubators
	Aspergillosis	Pneumonia and air sacculitis in birds exposed to aerosols of spores from contaminated litter or feed. May occur in outbreaks where birds are stressed, for example, penguins kept in warm environments. Infection may become disseminated to other internal organs

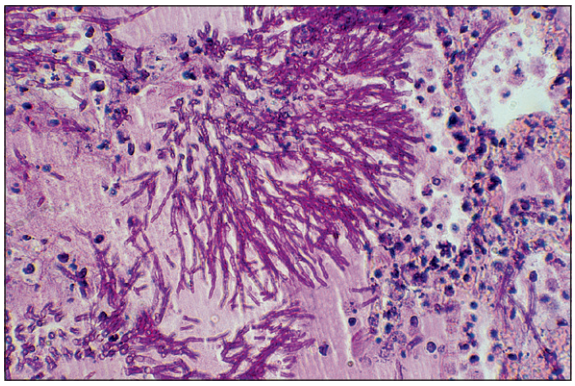

Figure 39.3 *Aspergillus fumigatus*: equine mycotic pneumonia. (PAS stain, ×400)

scrapings and biopsies from nasal granulomas and fungal plaques in the guttural pouch. Due to the ubiquitous nature of aspergilli, tissue for histopathology should be obtained where possible. For example, in the case of a nasal granuloma, the significance of culturing *A. fumigatus* from a nasal discharge would be difficult to interpret, whereas a more confident diagnosis could be made if invading, septate hyphae were seen in a histological section from a biopsy, together with the isolation of *A. fumigatus*. If the biopsy tissue is very small, it can be wrapped in a piece of paper before placing it in the 10% formalin so that it can be more easily found again.

Direct microscopy

Tissue scrapings and other material can be examined after clearing in 10% KOH or by modifications of this method (see Chapter 37). Histopathological sections should be prepared and examined wherever possible. These are stained by the methenamine silver stain or PAS stain (Fig. 39.3) and septate hyphae invading the tissue should be noted.

Isolation

Sabouraud dextrose agar, with and without 0.05 g/L chloramphenicol, is used. The aspergilli are sensitive to cycloheximide. The surface of the agar should be cross-hatched to a depth of about 2 mm in four to five well-separated areas on the plate. Small pieces of tissue (about one-quarter the size of a finger nail) are placed on the cross-hatched areas and gently pushed into the agar. The inoculated plates for *A. fumigatus*, or other aspergilli causing a systemic mycosis, are incubated aerobically at 37°C for up to five days although usually the colonies will appear in two to three days. *Aspergillus fumigatus* can tolerate temperatures up to 45°C but some of the other aspergilli, that are occasionally involved in superficial mycoses, may not grow at 37°C, so an additional plate could be inoculated and incubated at 25°C.

Identification

Mycologists base the speciation of the aspergilli on colony pigmentation; the size and length of the conidiophores; the shape and size of the vesicles; presence or absence of metulae; position of phialides; size, shape and appearance of the conidia; the length of the chains of spores and other criteria. This requires considerable expertise. However, if the culture is pure and correlates with the histopathological findings, then a presumptive identification can be made of the aspergilli known to be pathogenic based on their colonial and microscopic appearance. If there is doubt, the culture should be referred to a mycology reference laboratory.

Colonial morphology

- ***Aspergillus fumigatus***: white fluffy colony when it first appears, rapidly becoming velvety or granular and a bright bluish-green in colour (Fig. 39.4). Older colonies can assume a smoky battleship-grey colouration.
- ***Aspergillus niger***: white at first when very young but soon developing a black pepper effect as the black conidia are produced (Fig. 39.5). The reverse remains buff or cream-coloured. This distinguishes *A. niger* from the dematiaceous moulds.
- ***Aspergillus flavus***: cottony aerial mycelium when young but soon becomes a yellow-green with a sugary texture (Fig. 44.2).
- ***Aspergillus terreus***: white becoming a cinnamon-buff and sugary in texture as the profuse sporulation occurs.

Microscopic appearance

Mounts are made in lactophenol cotton blue (LPCB) from the colony. The characteristic fruiting heads indicate the genus but rather more experience is required for speciation.

- ***Aspergillus fumigatus***: conidiophores are moderate in length and have a characteristic 'foot cell' at their bases. The vesicles are dome-shaped and the upper one-half to two-thirds bear phialides from which long chains of globose, spiny, green conidia (2–3 µm) are borne. These chains tend to sweep inwards (Figs 39.1 and 39.6).
- ***Aspergillus niger***: this has very large fruiting heads that look like small black balls under the dissecting microscope. The spherical vesicle bears large metulae to which the smaller phialides are attached (Fig. 39.1). The conidia are black and rough.

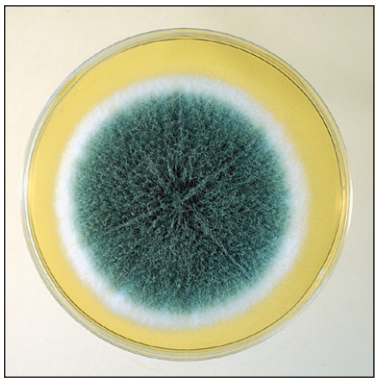

Figure 39.4 *Aspergillus fumigatus* on Sabouraud dextrose agar, five days.

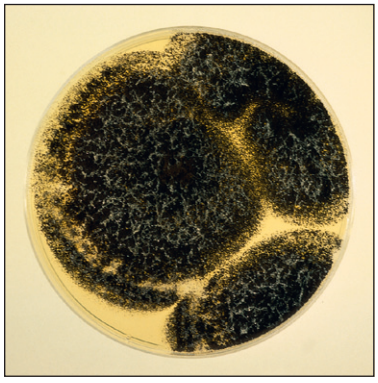

Figure 39.5 *Aspergillus niger* on Sabouraud dextrose agar, five days.

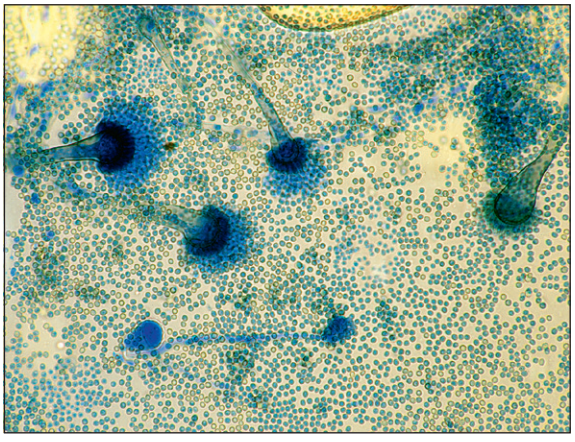

Figure 39.6 *Aspergillus fumigatus*: conidiophores and conidia. Transparent adhesive tape preparation. (LPCB, ×400)

- ***Aspergillus flavus***: the vesicles are round with sporulation over the entire surface. Phialides alone or phialides and metulae are present. The conidia are 3–5 µm in diameter, yellowish, elliptical or spherical and become spiny with age.
- ***Aspergillus terreus***: the vesicles are small and dome-shaped and both phialides and metulae are present. The conidia (2–3 µm diameter) are elliptical. Aleuriospores are produced on submerged hyphae.

Serology

Assays based on ELISA, AGID and counterimmunoelectrophoresis for the serological diagnosis of aspergillosis are available. The interpretation of positive antibody results may not always be easy because the antigens of the aspergilli are widespread in the environment. A commercial human ELISA for the detection of galactomannan, a major cell wall antigen of *Aspergillus* species, has been used as an aid to the diagnosis of aspergillosis in avian species (Cray et al. 2009).

Molecular techniques

Techniques such as PCR have been developed for the rapid detection and identification of *Aspergillus fumigatus* in clinical specimens (O'Sullivan et al. 2003).

PNEUMOCYSTIS CARINII

There are two species in the genus: *P. jiroveci* (human isolates) and *P. carinii* (animal isolates). *Pneumocystis carinii* represents a heterogeneous cluster of organisms known as special forms (*formae speciales*), each restricted to a particular host species, for example *P. carinii* f. sp. *equi* for equine strains. Due to the current lack of an in vitro system of propagation the life cycle of this organism has not been fully determined. The proposed life cycle involves asexual and sexual phases. The normal habitat is thought to be the lung and while the natural reservoir is unknown there is evidence that the organism is present in young, clinically normal humans and animals. *Pneumocystis carinii* is an opportunist pathogen capable of causing severe pneumonia in immunocompromised individuals. Most cases in domestic animals have occurred in horses and dogs. Foals suffering from combined immunodeficiency disease are particularly susceptible. Biopsies, bronchoalveolar lavage material and histopathological specimens are suitable for diagnosis. The Giemsa stain will demonstrate the trophic, cyst and spore forms of the organism. Immunocytochemical staining methods may be used to specifically identify the organism. Molecular techniques are available for detection of the organism (Peters et al. 1994).

REFERENCES

Cray, C., Watson, T., Rodriguez, M., et al., 2009. Application of galactomannan analysis and protein electrophoresis in the diagnosis of aspergillosis in avian species. Journal of Zoo and Wildlife Medicine 40, 64–70.

O'Gorman, C.M., Fuller, H.T., Dyer, P.S., 2008. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature 457, 471–474.

O'Sullivan, C.E., Kasai, M., Francesconi, A., et al., 2003. Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. Journal of Clinical Microbiology 41, 5676–5682.

Peters, S.E., Wakefield, A.E., Whitwell, K.E., et al., 1994. *Pneumocystis carinii* pneumonia in thoroughbred foals: identification of a genetically distinct organism by DNA amplification. Journal of Clinical Microbiology 32, 213–216.

Watt, P.R., Robins, G.M., Galloway, A.M., et al., 1995. Disseminated opportunistic fungal disease in dogs: 10 cases (1982–1990). Journal of the American Veterinary Medical Association 207, 67–70.

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Chapter 40

The pathogenic yeasts

Yeasts are common in the environment, often found on plants or associated with plant materials. They are also found as commensals of the skin and mucous membranes of animals. Some species cause opportunistic infections, facilitated by factors such as immunosuppression or antimicrobial therapy which upsets the resident flora on mucosal surfaces. Asexual reproduction is characterized by the production of blastoconidia, also known as buds or daughter cells. A number of species are known to produce a range of forms while causing infection. *Candida albicans* tends to a filamentous growth pattern when grown on media with low concentrations of glucose in an atmosphere of elevated CO_2 concentration. Lesions caused by *C. albicans* may contain budding yeasts, pseudohyphae and hyphae.

CANDIDA ALBICANS

There are more than 200 species of *Candida* but only *C. albicans* is commonly associated with disease in animals. *Candida albicans* grows as a budding yeast cell, oval and 3.5–6.0 × 6.0–10.0 µm in size, on agar cultures and in animal tissues. Pseudohyphae are also produced in animal tissue by elongation of individual yeast cells that fail to separate. These can be mistaken for septate hyphae of moulds. Thick-walled resting cells, known as chlamydospores, are produced in vitro on certain media such as cornmeal Tween 80 agar. *Candida albicans* will grow on ordinary media over a wide range of pH and temperatures. At both 25°C and 37°C it produces white, shiny, high-convex colonies in 24–48 hours.

Natural Habitat

Candida albicans is a commensal of mucocutaneous areas, particularly of the intestinal and genital tracts of many animal species and humans. Most infections are endogenous in origin and predisposing causes, such as immunosuppression, prolonged antibiotic therapy, intercurrent infection and malnutrition, can initiate infections. In cattle the yeast is often introduced into the udder on the nozzle of tubes of intramammary antibiotics. *Candida albicans* is worldwide in distribution.

Pathogenesis

Neuraminidase and proteases may play a part in virulence and cell wall glycoproteins have an endotoxin-like activity. Candidal surface proteins facilitate adhesion to matrix proteins. The production of extracellular, cytotoxic phospholipases and proteases is thought to aid tissue invasion and correlate with virulence. Phospholipase activity is concentrated at the growing tips of hyphae. Infections caused by *C. albicans* frequently involve overgrowth of resident *C. albicans* on mucous membranes. The yeast form is thought of as the form responsible for colonization of epithelial surfaces whereas tissue penetration and invasion is mediated by transition to the hyphal form. Granulomatous lesions are rare and inflammatory responses are predominantly neutrophilic. In severe, chronic infections, such as infection of the crop in chickens, the wall of the crop becomes thickened and covered by a corrugated pseudomembrane of yellowish-grey necrotic material giving it the characteristic 'terry-towelling' effect. Infections due to *C. albicans* have been given several names including

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Table 40.1 Diseases and main hosts of *Candida albicans*

Hosts	Diseases
Birds	Crop mycosis ('thrush') may affect the crop, mouth or oesophagus causing stunting and high mortality in young birds
Horses	Gastric ulceration in foals. Genital infections have been described in adults
Cattle	Pneumonic, enteric (gastro-oesophageal ulcers, rumenitis) and generalized candidiasis. Infection seen in calves following prolonged antibiotic therapy. A mild and self-limiting mastitis may occur in cows. Abortion has also been reported
Pigs	Gastro-oesophageal ulcers
Dogs	Mycotic stomatitis occurs in pups. Genital tract infections in bitches. Cutaneous candidiasis has been described. Generalized infections occur rarely
Cats	Mycotic stomatitis in kittens. Pyothorax, rare cases due to *C. albicans*

moniliasis, candidosis and candidiasis. Table 40.1 lists the diseases caused by *C. albicans* in animals. There have been reports of mastitis in cattle being caused by other *Candida* species including *C. tropicalis, C. pseudotropicalis, C. parapsilosis, C. guilliermondii, C. krusei* and *C. rugosa*.

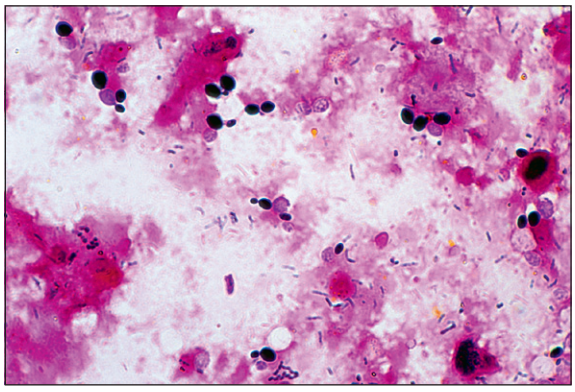

Figure 40.1 *Candidia albicans* in a faecal smear from a calf. (Gram stain, ×1000)

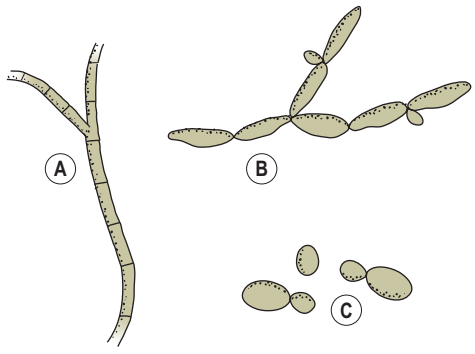

Figure 40.2 *Candidia albicans*: A hypha; B pseudohypha; C yeast cells budding.

Laboratory Diagnosis

Specimens

These may include scrapings from lesions, centrifuged milk samples and biopsy or tissue samples in 10% formalin for histopathology.

Direct microscopy

Candida albicans can be demonstrated in specimens by Gram-stained smears (Fig. 40.1), 10% KOH preparations, or in tissue sections stained by PAS-haematoxylin or methenamine silver stains. *Candida albicans* stains purple-blue with the Gram-stain. In tissue sections it appears as thin-walled, oval, budding yeast cells, hyphae and/or pseudohyphae (Fig. 40.2).

Isolation

Candida albicans grows well on blood agar or on Sabouraud dextrose agar with and without inhibitors. However, some of the other *Candida* species may be inhibited by cycloheximide. The plates are streaked with a small inoculum as for bacteria. The cultures are incubated at 37°C, aerobically, for up to five days. Isolation alone is not sufficient for a diagnosis of candidiasis. Presence of the organism, particularly psuedohyphae, in direct smears from lesion material is usually indicative.

Identification

Colonial appearance

Colonies of *C. albicans* usually appear in one to three days. They are white to cream, shiny, high-convex and have a pleasant 'beery smell'. They can attain a diameter of 4–5 mm (Fig. 40.3).

Microscopic appearance

A small amount of growth can be placed in lactophenol cotton blue as a wet preparation, or a Gram or methylene blue stain can be used on fixed smears from the colonies. *Candida albicans* produces thin-walled, budding yeast cells on Sabouraud dextrose agar or blood agar.

Figure 40.3 *Candidia albicans* on Sabouraud dextrose agar, five days.

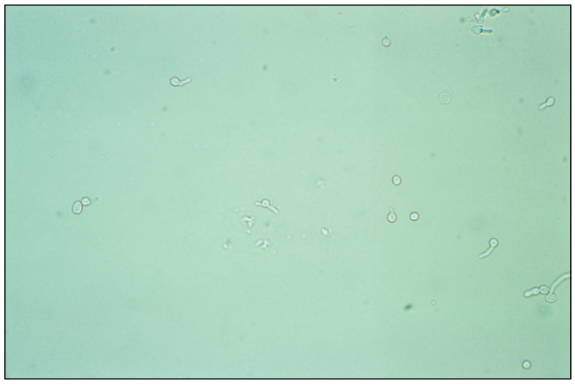

Figure 40.4 *Candidia albicans* forming germ tubes following two hours' incubation at 37°C in serum. (Unstained, ×400)

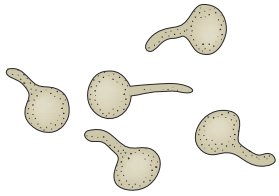

Figure 40.5 *Candidia albicans* producing germ tubes.

Demonstration of germ tubes

A small inoculum from an isolated colony is suspended in 0.5 mL of sheep, bovine, rabbit or human serum and is incubated at 37°C for two to three hours. A drop of the preparation is examined under phase contrast or the high-dry objective of the light microscope (with the condenser slightly lowered). Small tubes will be seen projecting from some of the yeast cells (Fig. 40.4). This is characteristic of *C. albicans* (Fig. 40.5). Some strains of *C. tropicalis* can occasionally produce pseudo-germ-tubes but only after three hours' incubation or more.

Figure 40.6 *Candidia albicans*: thick-walled terminal chlamydospores, pseudohyphae and two clusters of smaller blastospores. (Unstained, ×400)

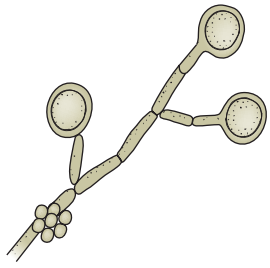

Figure 40.7 Thick-walled chlamydospores of *Candidia albicans*, blastospores also illustrated.

Chlamydospore production

A plate of cornmeal Tween 80 or chlamydospore agar is inoculated by making three parallel cuts in the medium 1.0 cm apart. The cuts are made at 45° to the surface to facilitate later microscopic examination. Subsurface inoculation is made as chlamydospore production is enhanced by lowered oxygen tension. The inoculated plates are incubated at 30°C for two to four days. A thin cover slip is placed on the surface of the agar and the preparation examined under the low and high-dry objectives for the thick-walled chlamydospores (8–12 µm) borne on the tips of pseudohyphae (Fig. 40.6). Clusters of smaller blastospores may also be present (Fig. 40.7).

Biochemical tests

Positive tests with the production of germ tubes and chlamydospores are sufficient for a good presumptive identification of *C. albicans*. For a definite identification of *C. albicans* and some of the other *Candida* species either conventional biochemical tests can be used or commercial systems such as API 20C, API-yeast-Ident (bioMérieux) and Uni-Yeast-Tek system (Remel). CHROMagar™ Candida

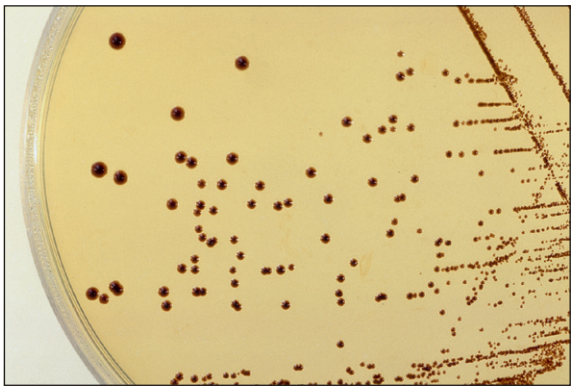

Figure 40.8 *Candidia albicans* on 'BiGGY' agar, seven days.

is a commercial agar designed for the differentiation of *C. albicans, C. tropicalis* and *C. krusei*.

BiGGY agar

Bismuth sulphite glucose glycine yeast (BiGGY) agar can be used for the isolation and/or presumptive identification of *Candida* species. Most bacterial contaminants are inhibited by the bismuth sulphite. *Candida albicans*, *C. kruseri* and *C. tropicalis* strongly reduce the bismuth sulphite to bismuth sulphide. *Candida albicans* gives smooth, circular, brownish colonies with a slight white fringe and no colour diffusion into the surrounding medium (Fig. 40.8). The colonies of *C. tropicalis* are similar but there is diffuse blackening of the medium after 72 hours. *Candida krusei* gives large, flat, wrinkled, silvery, brown-black colonies with a brown periphery and yellow diffusion into the surrounding medium.

Molecular techniques

Although molecular techniques for the identification of *Candida* species in clinical veterinary samples have been developed (Kano et al. 2002), they are largely used as research tools in mycology reference laboratories.

CRYPTOCOCCUS NEOFORMANS

Of the more than 30 species of *Cryptococcus*, only *C. neoformans* is pathogenic for animals and humans. It is a spherical to oval, thin-walled, budding yeast that varies greatly in diameter (2.5–10.0 µm). The cells are surrounded by a mucoid polysaccharide capsule that varies in thickness, but in animal tissues it is usually very large, the width of the capsule exceeding the diameter of the parent cell. Daughter cells are usually single and budded from the parent cell by a narrow neck. *Cryptococcus neoformans* is a member of the *Basidiomycota*. The teleomorph has only been observed in vitro. Four serotypes A–D (a hybrid AD serotype also occurs) are recognized based on capsular polysaccharide agglutination reactions. Serotype A strains are designated *C. neoformans* var. *grubii*, serotype D strains as *C. neoformans* var. *neoformans* while serotypes B and C are designated *C. neoformans* var. *gattii*. It has been proposed that *C. neoformans* var. *gattii* should be recognized as a separate subspecies, *C. gattii*, in the *C. neoformans* species complex. Two morphologically distinct teleomorphs, *Filobasidiella neoformans* produced by serotypes A and D and *F. bacillispora* produced by serotypes B and C, have been demonstrated. Nine molecular types of *C. neoformans* have been described. Cryptococcosis (European blastomycosis, torulosis) is a subacute or chronic infection that frequently involves the central nervous system, the respiratory system and the eye.

Natural Habitat

Cryptococcus neoformans var. *grubii* and var. *neoformans* have a worldwide distribution and are present in soil and dust. They have been isolated from the skin, mucous membranes and intestinal tract of normal animals and birds. The yeasts are concentrated in pigeon faeces that are rich in creatinine. The creatinine inhibits many other microorganisms but can be used by *C. neoformans*, which can survive in pigeon droppings for more than a year. *Cryptococcus neoformans* var. *gattii* is found mainly in association with decaying wood of eucalyptus trees.

Pathogenesis

The virulence of *C. neoformans* is largely associated with the anti-phagocytic and immunosuppressive capsule. Capsule formation is much reduced in environments that contain high salt and sugar concentrations resulting in a size of yeast that is readily aerosolized and capable of reaching the alveoli following inhalation. It is possible that basidiospores may be an important infectious form of the fungus. Capsule production is initiated once inside the tissues. Virulence is also thought to be associated with the production of phenol oxidase, which promotes the degradation of catecholamine and resultant accumulation of melanin in the yeast cell wall. Melanin is a potent free radical scavenger that may protect the cell from oxidants within phagolysosomes. Additional virulence factors are thought to be phospholipase, involved in membrane disruption, and mannitol, which interferes with phagocyte killing by scavenging hydroxyl radicals. The cryptococcal lesions, on gross examination, range from discrete granulomas to myxomatous neoplasms. They consist of capsular slime, yeast cells, some inflammatory cells and later histiocytes, epithelioid and giant cells. The histiocytes and giant cells often contain *C. neoformans*. The route of infection is usually respiratory, often with localization in the nasal

Table 40.2 Diseases and main hosts of *Cryptococcus neoformans*

Hosts	Diseases
Cats	Nasal cavity is primary site of infection resulting in rhinitis and nasal granulomas with possible spread to central nervous system. Ocular and cutaneous infections also described
Dogs	Less common than in cats. Target sites include respiratory tract, eye, skin and CNS
Horses	Nasal passage granulomas with nasal discharge. Less commonly lesions in skin or lungs
Cattle	Rare cause of mastitis with severe swelling and firmness of the mammary glands
Koala	Sporadic, respiratory infection with frequent dissemination to CNS

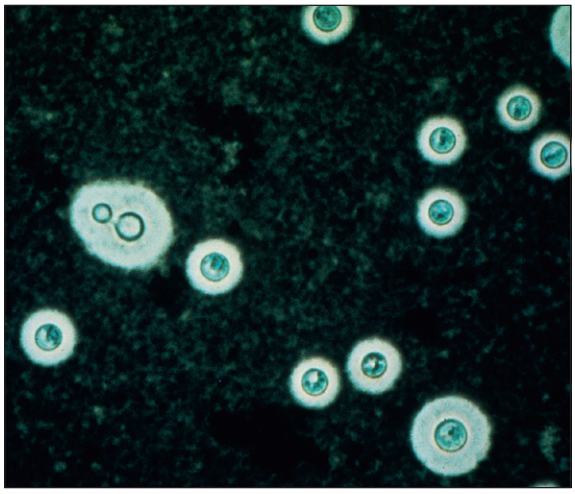

Figure 40.9 *Cryptococcus neoformans* in exudate. (Nigrosin stain, ×1000)

cavity or paranasal sinuses and later extension to the brain and meninges. Infection of the meninges can resemble tubercular meningitis. There can also be extension to the optic nerve resulting in blindness. Subcutaneous granulomas occasionally occur, often in the cervical or pedal regions. *Cryptococcus neoformans* can probably affect any mammal but cryptococcosis is most commonly seen in cats, dogs, cattle, horses and humans. Table 40.2 summarizes the diseases and conditions caused by *C. neoformans* in animals.

Laboratory Diagnosis

Great care should be exercised when handling material suspected of containing *Cryptococcus neoformans* as it can cause serious disease in humans. An approved biological safety hood should be used when carrying out procedures with materials or cultures thought to contain this yeast.

Specimens

Cerebrospinal fluid, lesions or exudates, mastitic milk, biopsies and tissues for histopathology should be collected.

Direct microscopy

India ink or nigrosin preparations can be made with cerebrospinal fluid or clear exudates (see Chapter 37). These stains will demonstrate the characteristic capsule (Fig. 40.9). Histological sections from biopsies of tissue from lesions can be stained by the PAS-haematoxylin stain. This will stain, or outline, the yeast cell but not the capsule which appears as a clear area surrounding the cell. In tissues Mayer's mucicarmine stain can be used to stain the capsule, the wall of the yeast and the capsule are stained red, this being diagnostic for *C. neoformans*. Alternatively, melanin can be demonstrated in cell walls using the Fontana-Masson stain on tissue sections.

Isolation

Cryptococcus neoformans will grow well on blood agar or on Sabouraud dextrose agar (without cycloheximide). The plates are streaked out as for bacteria and incubated aerobically at 37°C for up to two weeks. Capsular growth can be enhanced by culture on chocolate agar under 5% CO_2 at 37°C. The majority of the saprophytic *Cryptococcus* species are unable to grow at 37°C, whereas *C. neoformans* can grow at temperatures up to 40°C.

Identification

Colonial morphology

Colonial growth can be apparent in 48 hours or it may take nearly two weeks' incubation. The colonies are smooth, moist, shiny and become very mucoid with age. They are initially white but develop a yellowish shade. Mucoid yeast colonies are produced at 25°C and at 37°C indicating that it is a yeast and not a dimorphic fungus (Fig. 40.10).

Microscopic appearance

LPCB or nigrosin wet preparations should reveal a spherical, budding yeast surrounded by a capsule. Following growth on laboratory media the capsules are often not as large as those present around *C. neoformans* cells from animal tissue (Fig. 40.11).

Figure 40.10 *Cryptococcus neoformans* on Sabouraud dextrose agar incubated at 25°C (left) and 37°C (right).

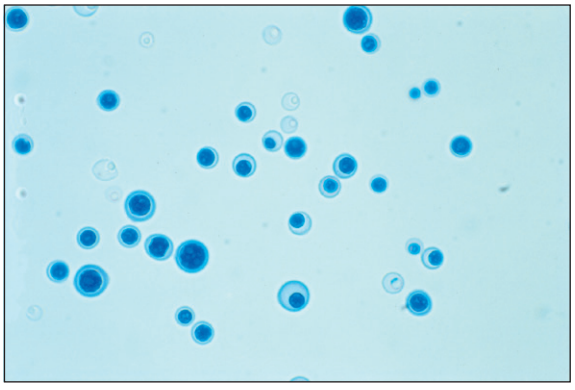

Figure 40.11 *Cryptococcus neoformans* from a culture. (LPCB, ×1000)

Ability to grow at 37°C

This differentiates *C. neoformans* from the majority of the *Cryptococcus* species.

Biochemical tests

- Urease production: *Cryptococcus* species will produce urease on a heavily inoculated Christensen's urea agar slope.
- Melanin production on Niger seed or birdseed agar: *Cryptococcus neoformans* is one of the few *Cryptococcus* species that can use creatinine and produce brown-pigmented colonies on media containing di- and polyphenolic compounds. The formula for the medium is given in Appendix 2 but it can be obtained commercially. The plates are heavily inoculated and then incubated aerobically at 37°C for at least a week. The dark-brown pigment develops first on the area of heaviest growth and later spreads to the remainder of the inoculated plate.
- Biochemical profile: the API 20C (bioMérieux) and Uni-Yeast-Tek (Remel) systems can be used to obtain a biochemical profile of the isolate for full identification.
- *Cryptococcus neoformans* var. *gattii* differs from *C. neoformans* var. *grubii/neoformans* in being able to utilize glycine as a sole source of nitrogen and in being resistant to the chemical canavanine.

Mouse inoculation

Mice can be inoculated intraperitoneally. If they do not die, the mice are euthanized after two weeks when gelatinous lesions are found in the abdominal cavity and possibly in the lungs. *Cryptococcus neoformans* is the only *Cryptococcus* species that is pathogenic for mice.

Immunology

Slide latex agglutination test and ELISA kits have been designed to demonstrate soluble capsular antigen in serum and cerebrospinal fluid. Indirect FA, ELISA and latex agglutination tests have been used to detect antibody. Antibody may not be consistently demonstrated due to its combination with circulating capsular antigens. However, the presence of antibody is considered a favourable sign indicating decreasing antigen levels.

Molecular techniques

A PCR-based assay for the detection of *C. neoformans* in feline specimens has been described (Kano et al. 2001).

Summary of the characteristics for the presumptive identification of *C. neoformans*

- Demonstration of a budding yeast with a large capsule
- Growth at 37°C; smooth, shiny colonies becoming mucoid
- Production of a brown pigment on birdseed agar
- Urease production.

These characteristics give a good presumptive identification of the yeast. Confirmation requires a larger range of biochemical tests.

MALASSEZIA PACHYDERMATIS (PITYROSPORUM CANIS)

Malassezia species, members of the *Basidiomycota*, occur as commensals on the oily areas of skin and ears of dogs, cats and probably other animals. They are lipophilic yeasts that

reproduce by unipolar budding. There are 20 species currently recognized in the genus. *Malassezia pachydermatis* is considered to be of most veterinary importance and is associated with otitis externa and seborrhoeic dermatitis in dogs. It is also associated with otitis and feline chin acne. Other species including *M. furfur, M. slooffiae, M. globosa* and *M. sympodialis* have been isolated from both healthy animals and otitis cases in dogs, cats and cattle. The yeast is small (1–2 × 2–4 µm) and bottle-shaped, reproducing by a process known as bud fission in which the bud detaches from the parent cell by the production of a septum. While the bud and parent cell are joined there is a wide base between them.

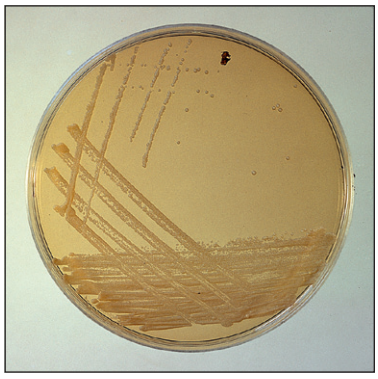

Figure 40.12 *Malassezia pachydermatis* on Sabouraud dextrose agar, 10 days.

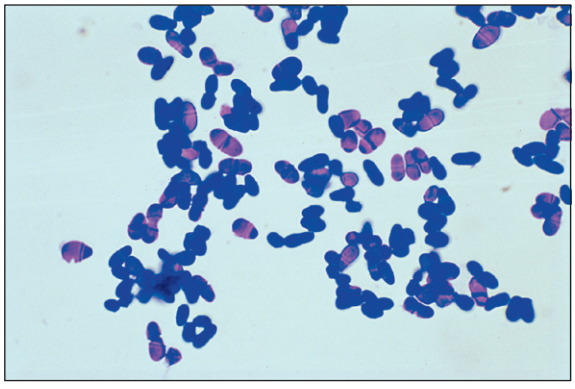

Figure 40.13 *Malassezia pachydermatis* from a culture. (Methylene blue stain, ×1000)

Pathogenesis

Disease caused by these organisms is associated with predisposing factors such as immunosuppression, allergic dermatitis, hairy or pendulous ears leading to high humidity and wax accumulation in the ear canal. The yeast cell wall contains zymogen, capable of activating the complement cascade which in turn may damage keratinocytes and give rise to inflammation and pruritis. Proteases and other enzymes produced by *M. pachydermatis* probably contribute to damage to the mucosa of the ear canal. Lipases act on sebum to create a favourable environment for the yeast. In turn, large numbers of the yeast induce excessive sebaceous secretion.

Laboratory Diagnosis

Recovery of these organisms from clinical specimens must be interpreted with caution due to their role as part of normal flora. The presence of one organism per oil-immersion field in conjunction with clinical signs can be considered significant (Chen & Hill 2005).

Direct examination

Gram-stained or methylene-blue-stained smears of exudate from dogs with otitis externa will often reveal large numbers of this yeast with its characteristic morphology. Suitable smears can be collected from skin lesions by the use of clear acetate strips, pressing a glass slide to the skin, performing a superficial skin scraping or vigorously swabbing the site. A presumptive identification can be made on the microscopic appearance alone but confirmatory culture is advisable.

Isolation

Discharge from ears or skin scrapings can be streaked out on Sabouraud dextrose agar and the culture incubated at 32–37°C for up to a week in a humid atmosphere. Colonies may be confined to the well of the plate but growth can be improved by placing a film of sterile olive or coconut oil on the surface of the agar before inoculation. Lipid supplementation is not a requirement for the growth of *M. pachydermatis* unlike the other members of the genus. The colonies are small, smooth and often have an odour not unlike a 'wet-dog' (Fig. 40.12). The typical bottle-shaped yeast cells with a wide septum between mother and daughter cells can be seen in stained smears from cultures (Fig. 40.13). *Malassezia* species are urease-positive. A suitable lipid-supplemented agar for the cultivation of lipid-dependent *Malassezia* species is Dixon's agar.

Molecular techniques

Techniques based on PCR have been developed for the identification and differentiation of *Malassezia* species (Mirhendi et al. 2005).

OTHER YEASTS THAT MAY OCCASIONALLY BE PATHOGENIC

Macrorhabdus ornithogaster

This avian gastric yeast is believed to be the cause of budgerigar wasting disease, a fatal condition characterized by chronic weight loss (Phalen 2005). The yeast cells can be demonstrated in faeces and proventricular scrapings using Gram's stain or Romanowsky stains. They are very large Gram-positive rods, measuring 1–5 µm by 20–90 µm, and were formerly referred to as 'megabacteria'. The organism can be found in the proventriculus of clinically normal birds but is capable of producing disease where predisposing factors such as overcrowding, poor hygiene and genetic predisposition exist. A definitive diagnosis of megabacteriosis requires postmortem examination and histopathology.

Trichosporon beigelii (cutaneum)

Trichosporon beigelii is a soil saprophyte producing yeast cells, pseudohyphae, true hyphae and arthrospores (Fig. 40.14). It has been recovered from a nasal granuloma and a bladder infection in cats, skin infections in horses and monkeys and mastitis in cattle and sheep. *Trichosporon capitum* has been reported as causing mastitis in cattle. These yeasts can be identified by the API 20C (bioMérieux) system. Growth is inhibited by the presence of cycloheximide.

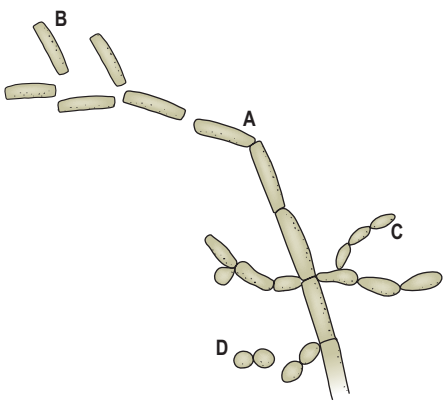

Figure 40.14 *Trichosporon beigelii*: A hypha; B arthrospores; C pseudohypha; D yeast cells.

Geotrichum candidum

Geotrichum candidum is a saprophytic mould but the colonial growth and odour are very yeast-like (Fig. 40.15). It is widespread in nature and its isolation is not necessarily significant. There is doubt regarding its ability to invade animal tissues. However, the fungus may be associated with intestinal infections especially if the animals have been on a prolonged course of antibiotic therapy. Cutaneous lesions and disseminated disease have been reported in dogs. The fungus does not produce conidiophores but the hyaline, septate hyphae fragment into rectangular, one-celled arthrospores that tend to remain in chains (Fig. 40.16). Most strains do not grow at 37°C with optimal growth occurring at 25°C.

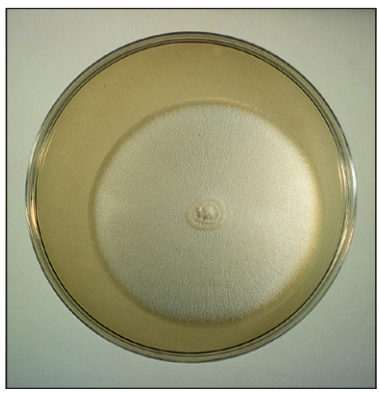

Figure 40.15 *Geotrichum* species on Sabouraud agar, five days.

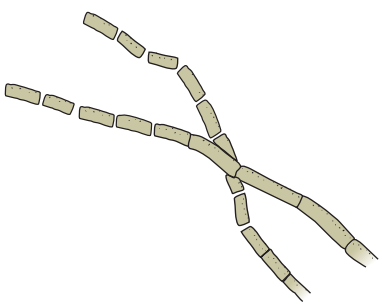

Figure 40.16 Arthrospores of *Geotrichum candidum*.

REFERENCES

Chen, T.-A., Hill, P.B., 2005. The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. Veterinary Dermatology 16, 4–26.

Kano, R., Fujino, Y., Takamoto, N., et al., 2001. PCR detection of the *Cryptococcus neoformans* CAP59 gene from a biopsy specimen from a case of feline cryptococcosis. Journal of Veterinary Diagnostic Investigation 13, 439–442.

Kano, R., Hattori, Y., Okuzumi, K., et al., 2002. Detection and identification of the *Candida* species by 25S ribosomal DNA analysis in the urine of candidal cystitis. Journal of Veterinary Medical Science 64, 115–117.

Mirhendi, H., Makimura, K., Zomorodian, K., Yamada, T., Sugita, T., Yamaguchi, H., 2005. A simple PCR-RFLP method for identification and differentiation of 11 *Malassezia* species. Journal of Microbiological Methods 61, 281–284.

Phalen, D., 2005. Diagnosis and management of *Macrorhabdus ornithogaster* (formerly megabacteria). Veterinary Clinics of North America, Exotic Animal Practice 8, 299–306.

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Chapter 41

The dimorphic fungi

The dimorphic fungi present two growth forms, a mould when growing saprophytically in the environment or when on culture media at 25–30°C, and a yeast or yeast-like form in animal tissues or when cultured on enriched media at 37°C. The mould or mycelial phase tends to be the more stable of the two. These fungi cause deep or systemic mycoses in animals and humans. Table 41.1 indicates the fungi included in this group and gives the main hosts and disease, the reservoir of infection and the geographical distribution. Three varieties of *Histoplasma capsulatum* are recognized, var. *capsulatum* (chiefly a New World pathogen), var. *duboisii* (an African human pathogen) and var. *farciminosum* (an Old World equine pathogen). However, phylogenetic studies have identified at least eight clades suggesting that the three varieties are phylogenetically meaningless (Kasuga et al. 2003). Teleomorphs have been identified for some of these fungi. The teleomorph of *B. dermatitidis* is *Ajellomyces dermatitidis* and the teleomorph of *H. capsulatum* is *Ajellomyces capsulatus* (phylum *Ascomycota*).

Laboratory Diagnosis

A summary of the diagnostic tests used to identify the dimorphic fungi is given in Box 41.1.

Safety aspects

All of the dimorphic fungi can cause disease in humans and should be treated with respect. Cultures of *Coccidioides immitis* in particular, represent a major biohazard for laboratory personnel because the arthrospores, produced on media at 25–37°C, can easily form an infective aerosol. The culture of this dimorphic fungus should either be avoided or appropriate precautions must be taken. These include the use of a biological safety cabinet when handling any material suspected of containing the organism, especially cultures. Cultures on slopes in screw-capped bottles are recommended and if culture plates are used these must be taped. The cultures of *C. immitis* should be covered with sterile water or saline before introducing an inoculation needle to prevent dispersion of the arthrospores. All microscopic preparations must be done in a biohazard cabinet. Cultures should be autoclaved as soon as the final diagnosis of *C. immitis* has been made. Isolates of *Coccidioides immitis* can be divided into 'Californian' and 'non-Californian' isolates, a second species has been proposed *Coccidioides posadasii* to distinguish the latter (Fisher et al. 2002).

Direct microscopy

Table 37.2 (see Chapter 37) and Figure 41.1 indicate the microscopic morphology of the dimorphic fungi in animal tissue and in cultures at 25°C and 37°C. Histopathological sections are most useful for demonstrating the yeast forms in animal tissues (Figs 41.2 and 41.3).

Yeast conversion of the dimorphic fungi

For full identification of these fungi, an attempt to convert them to the yeast phase should be made on enriched media at 37°C. This is possible with varying degrees of difficulty for all of them except *C. immitis*. The mould or mycelial phase is the more stable one. For the mould phase, inoculated plates of Sabouraud dextrose agar, with and without chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L), are incubated at 25°C. When suspicious colonies have grown (three to four days for *S. schenckii* and two to four weeks for the other fungi) a heavy subculture

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Table 41.1 Diseases and distribution of dimorphic fungi associated with disease in animals

Fungus	Disease(s)	Geographical distribution	Main host(s)	Usual habitat	Site of lesions
Sporothrix schenckii	Sporotrichosis (lymphangitis of limbs in horse)	Worldwide, more common in subtropical and tropical regions	Horses, dogs, cats, humans	Old wooden posts, rose thorns, dead vegetation, soil, moss	Subcutaneous nodules, lymphatics
Blastomyces dermatitidis (teleomorph: *Ajellomyces dermatitidis)*	Blastomycosis	Eastern regions of North America, sporadic cases in Europe, India, the Middle East	Dogs, cats, humans	Acidic soil rich in organic material	Primary lesions in lungs with metastases to skin and other organs
Coccidioides immitis and *Coccidioides posadasii*	Coccidioidomycosis	Semi-arid regions in southwestern USA, Central and South America	Dogs, horses, cats, humans	Soil of low-elevation deserts	Primary lesions in lungs with secondary lesions in bones
Histoplasma capsulatum var. *capsulatum*	Histoplasmosis	Mississippi and Ohio river valleys, sporadic cases in many other countries worldwide	Dogs, cats, humans	Nitrogenous soils enriched with bird or bat faeces	Primary lesions in lungs with dissemination to intestines and other organs
Histoplasma capsulatum var. *farciminosum*	Epizootic lymphangitis (African farcy)	Africa, Middle East, Asia	Horses, mules, donkeys	Soil	Skin, lymphatics, lymph nodes

Box 41.1 **Summary of the diagnostic tests for the identification of the dimorphic fungi**

- Direct microscopy:
  - wet mounts of exudates and tissues
  - histopathology on tissue sections
- Culture and demonstration of the mould phase at 25–30°C and the yeast phase on enriched medium at 37°C
- Microscopic appearance of cultures: fruiting structures and spores (colonies at 25–30°C) and yeast-forms (colonies at 37°C)
- Nucleic acid probes (available commercially) (Stockman et al. 1993)
- Exoantigen tests
- Immunological and serological tests
- Mouse inoculation

is made on brain-heart infusion agar plus 5–10% sheep blood on slopes in 30 mL screw-capped bottles. A few drops of sterile water can be placed in the bottle to provide moisture during incubation. The caps of the bottles are slightly loosened to allow oxygen to reach the cultures. The plates are incubated at 37°C and *S. schenckii* should show growth in three to five days but *B. dermatitidis* and *H. capsulatum* may require two to four weeks. The colonies are examined in lactophenol cotton blue preparations for yeast cells. *Coccidioides immitis* has experimentally been forced to produce the spherule phase in vitro using a liquid medium at 40°C, but it is a difficult technique and not carried out routinely. *Histoplasma farciminosum* needs slightly different techniques and is considered separately at the end of this section.

Colonial morphology

Sporothrix schenckii

At 25°C growth is visible in three to five days. Colonies are white to cream at first, becoming wrinkled with delicate aerial hyphae and then later turning dark and leathery (Figs 41.4 and 41.5). At 37°C colonies are yeast-like, smooth, soft and cream to tan in colour. Growth occurs in about three to five days.

Blastomyces dermatitidis

At 25°C growth occurs in about two to four weeks. The colonies are small and produce white, cottony aerial hyphae, becoming greyish or dark brown with age. They vary from a flat, dull colony to a heaped fungal mass with

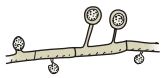
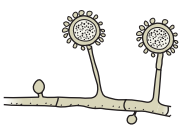
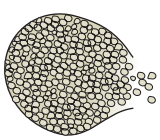
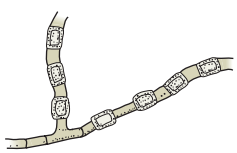
Dimorphic fungus	Animal tissue (37°C)	Culture (37°C) Brain-heart + 5% blood agar	Culture or environment (25°C) Sabouraud dextrose agar
Sporothrix schenckii	Cigar-shaped, budding yeast cells, that may occur within neutrophils. Usually very few present. (2–4 μm in diameter) Asteroid bodies may occur	Single or multiple-budding yeast cells, 2–4 μm in diameter	Fine branching hyphae with 2–3 μm pyriform conidia in flowerettes from short conidiophores. Conidia connected by thread-like process
Blastomyces dermatitidis	Large (8–10 μm) round or oval, thick-walled yeast cells. Buds on a broad base, single buds. Cytoplasmic granulation is often obvious	Large (8–10 μm), round or oval thick-walled yeast cells budding on a broad base	 Small (2–3 μm) oval or pear-shaped conidia borne on tips of short conidiophores on septate hyphae
Histoplasma capsulatum	Small (2–5 μm) budding yeast cells intracellular in phagocytic cells. The yeast cells are usually surrounded by a halo	Oval budding yeast cells (3–4 μm diameter) with a narrow neck between mother and daughter cells	 Two types of conidia; large (8–14 μm) tuberculate macroconidia that are sunflower-like and small tear-drop like microconidia
Coccidioides immitis	 Spherules (15–60 μm), the mature forms filled with endospores. No endospores in immature spherules	 Septate hyphae branching at right angles. With age the hyphae dissociate into barrel-shaped arthrospores. These are separated by clear, non-viable cells. Arthrospores are wider than the hyphae. Cannot be converted easily to spherule form in vitro	

Figure 41.1 Microscopic morphology of the dimorphic fungi.

hyphal tufts (Fig. 41.6). At 37°C the waxy, yeast-like colonies are wrinkled and cream to tan in colour. They can have radiating 'prickles' from the surface.

Histoplasma capsulatum *var.* capsulatum

At 25°C white to cream colonies with cottony aerial hyphae are seen. They turn grey to brown with age and require two to four weeks' incubation. The colonies are similar to those of *B. dermatitidis*. At 37°C the colonies are smooth, yeast-like and cream to tan in colour. The fungus has a tendency to revert to the more stable mycelial form.

Coccidioides immitis

At 25°C and 37°C delicate cobweb growth in three to 21 days occurs. It causes a greenish discolouration on blood agar. Colonies have fluffy areas alternating with areas adherent to the agar surface.

Microscopic appearance

Figure 41.1 summarizes the microscopic appearance of the dimorphic fungi in animal tissue and from cultures at 25°C and 37°C.

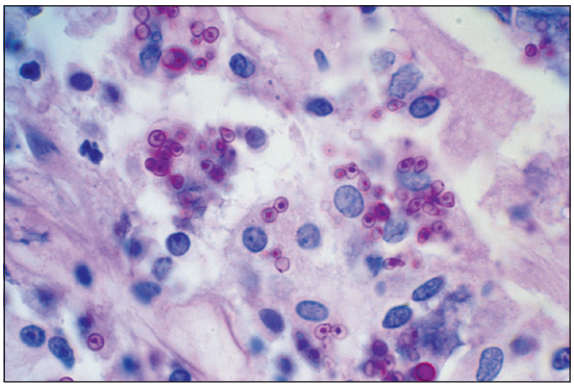


Figure 41.2 *Blastomyces dermatitidis* yeast form in tissue. (PAS-haematoxylin stain, ×1000)

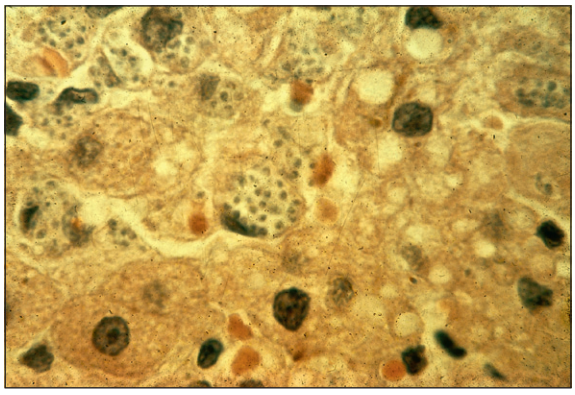


Figure 41.3 *Histoplasma capsulatum* yeast form in Kupffer's cells (dog's liver). (Silver stain, ×1000)

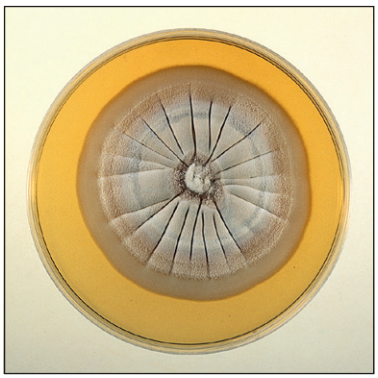


Figure 41.4 *Sporothrix schenckii* on Sabouraud agar at 25°C, 13 days.

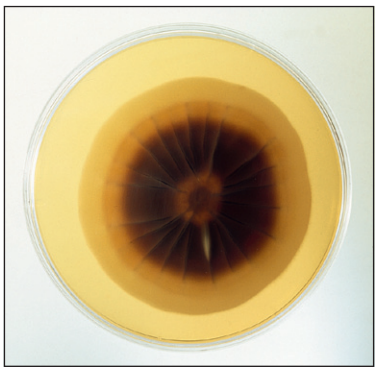


Figure 41.5 *Sporothrix schenckii* on Sabouraud agar, 13 days. Reverse.

Figure 41.6 *Blastomyces dermatitidis* on Sabouraud agar. (25°C, 16 days)

Sporothrix schenckii

Large numbers of yeast cells may be seen in methylene-blue-stained smears prepared using exudates from lesions in cats. Yeast cells tend to be sparse in exudates from other animal species. Yeast cells may also be visible in histopathological sections stained by PAS or methenamine silver techniques. Immunohistochemical staining may be used to specifically identify the yeast cells. The mould-form (Fig. 41.7) and yeast-form (Fig. 41.8) of *S. schenckii* are illustrated.

Blastomyces dermatitidis

Yeast cells may be demonstrated in cytological or histopathological preparations from lesions. Methylene blue or Giemsa stains are suitable for smears from exudates or aspirates.

Histoplasma capsulatum *var*. capsulatum

Yeast cells in macrophages may be visible in Giemsa-stained smears of exudates or aspirates. Histopathological

Figure 41.7 *Sporothrix schenckii* conidiophore and conidia. Culture incubated at 25°C. (LPCB, ×400)

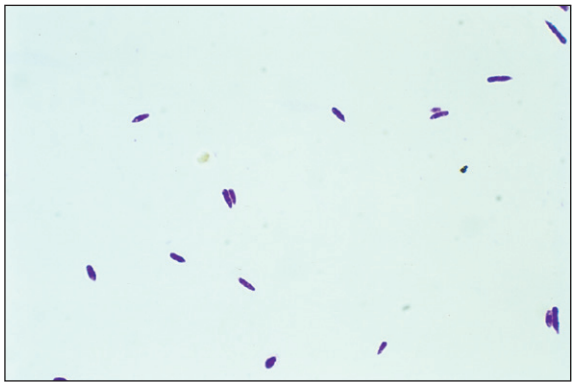


Figure 41.8 *Sporothrix schenckii* yeast cells. Culture incubated at 37°C. (LPCB, ×1000)

sections of affected tissues may demonstrate pyogranulomatous foci which contain yeast cells.

Coccidioides immitis

Exudates and aspirates cleared with 10% KOH or stained tissue sections are suitable for the demonstration of the characteristic spherules.

Molecular techniques

DNA probes are available commercially for identification of cultures of *Blastomyces dermatitidis, Coccidioides immitis* and *Histoplasma capsulatum* (Stockman et al. 1993). DNA amplification techniques have been widely used in human medicine for detection of dimorphic fungi. Nested PCR assays have been described for the diagnosis of blastomycosis and histoplasmosis in dogs (Bialek et al. 2003, Ueda et al. 2003). Molecular methods are available to distinguish *Coccidioides immitis* from *C. posadasii* (Tintelnot et al. 2007).

Exoantigen test

This test is used in some laboratories for *B. dermatitidis*, *H. capsulatum* and *C. immitis*. It is a relatively simple and rapid method for identification and if positive it obviates the necessity to convert the fungus to the yeast phase. The method is an immunodiffusion test that, using reference antisera, detects cell-free antigens (exoantigens) extracted and concentrated from a mycelial colony. Kaufman and Standard (1987) have described the technique. Positive control antisera can be obtained commercially (Immuno-Mycologics, Meridian Diagnostics and Nolan/Scott Biological Laboratories). *Blastomyces dermatitidis* has the exoantigen **A**; *H. capsulatum* has **h** and **m**; and *C. immitis* **HS**, **F** or **HL**.

Immunological tests

Coccidioides immitis infection gives a strong immunological response and the serological tests are more reliable than for the other mycoses. With *B. dermatitidis* many of the serological tests cross-react with the other dimorphic fungi and are of limited value. Table 41.2 summarizes the immunological tests, and their interpretation, used to diagnose mycoses caused by the main dimorphic fungi. Immunodiffusion kits are available commercially. An enzyme immunoassay for the detection of *Blastomyces dermatitidis* antigen in serum or urine has been described (Spector et al. 2008).

Mouse inoculation tests

All the dimorphic fungi cause lesions in mice. There is little need for animal inoculation now because of the availability of other specific confirmatory tests. However, mouse inoculation might be the only method of recovery of the fungi from very contaminated specimens.

- ***Sporothrix schenckii***: mice are inoculated intratesticularly. Orchitis develops in two to four weeks.
- ***Blastomyces dermatitidis***: mice or guinea pigs are inoculated intraperitoneally. Lesions may be found in liver, spleen, lungs and lymph nodes in three weeks.
- ***Histoplasma capsulatum* var. *capsulatum***: mice are inoculated intraperitoneally and the yeast-form can be recovered from the liver and spleen in two to four weeks.
- ***Coccidioides immitis***: intraperitoneal inoculation into mice. The mice are euthanized seven to 10 days postinoculation and nodules are found in the peritoneum, lungs and spleen. These nodules are examined for the characteristic spherules produced by the fungus.

Table 41.2 Immunological tests for dimorphic fungi

Fungus	Test	Comments
Sporothrix schenckii	Immunodiffusion, complement fixation test, latex agglutination	Antibodies are demonstrable only in the rare cases where systemic spread has occurred. Limited application in animals to date
	Immunofluorescence	For identification of yeast cells in exudates and tissues
Blastomyces dermatitidis	Skin test	Lacks sensitivity and specificity
	Immunodiffusion, complement fixation, ELISA, counter-immunoelectrophoresis	AGID and CFT are not considered to be sufficiently sensitive or specific
	Immunofluorescence	For identification of yeast cells in exudates and tissues
Coccidioides immitis	Skin test (coccidioidin)	Positive test may revert to negative as infection becomes disseminated and advanced, poor prognosis
	Immunodiffusion	Multiple bands tend to be associated with active infection whereas a single band is associated with chronic infection
	Complement fixation test	Antibody titre rises in disseminated disease and tends to remain high
	Latex agglutination test	Antibodies detected early in disease (IgM)
Histoplasma capsulatum var. *capsulatum*	Skin test (histoplasmin)	Positive reaction merely indicates exposure. Lack of reaction may be due to anergy
	Immunodiffusion	Erratic results obtained with animal sera. Of questionable usefulness
	Complement fixation test, latex agglutination	Useful in humans, reliability less certain with animal sera
	Immunofluorescence	For identification of yeast cells in exudates and tissues

HISTOPLASMA CAPSULATUM VAR. *FARCIMINOSUM*

About 90% of the cases of epizootic lymphangitis (African farcy) have been reported in horses and the remainder in mules and donkeys. The legs and neck are most commonly involved, displaying nodular, granulomatous and ulcerative lesions of skin, subcutaneous tissue and lymphatic vessels. The disease can become disseminated. The natural habitat, other than infected animals, remains unknown. Transmission is thought to occur mainly through breaks in the skin or via biting insects. The morphology and life cycle of the fungus is similar to that of *H. capsulatum* var. *capsulatum*.

Direct Microscopy

Wet mounts of pus or exudates or biopsies can be examined for the intracellular, pear-shaped, double-contoured yeast cells (2–4 µm). They are usually present inside macrophages or neutrophils. Budding usually occurs from the pointed end of the yeast cell.

Culture

The isolation of *H. farciminosum* is a difficult and slow process. Sabouraud dextrose agar, with and without antimicrobial agents, is inoculated with material taken aseptically from unruptured nodules and incubated at 25–30°C for two to eight weeks. For the conversion to the yeast phase, Hartley digest agar with 10% horse serum is inoculated and incubated at 37°C, under 20% CO_2 for two to eight weeks.

Identification

Colonial and microscopic appearance

At 25°C the colonies appear as minute grey flakes later becoming dry and very wrinkled. The colonies are often composed of sterile hyphae although very rarely chlamydospores (5–10 µm), arthrospores and blastospores are present. At 37°C the small, grey, flaky colonies are composed of yeast cells and some hyphae.

Immunology

The immunodiffusion test on mycelial extracts detects the **h** and **m** genus-specific exoantigens.

Skin sensitivity develops after exposure to the fungus and a skin test may be positive in the absence of clinical signs. An ELISA and an indirect fluorescent antibody test have been developed for the detection of antibodies.

Mouse inoculation

Mice can be inoculated intraperitoneally with a suspension of material suspected of containing the fungus. Impression smears from the liver and spleen, two to four weeks post inoculation, should reveal the yeast-form of the fungus.

ADIASPIROMYCOSIS

Adiaspiromycosis is a self-limiting respiratory infection seen in humans, rodents, dogs and a variety of wild mammals (Borman et al. 2009). The infection results from the inhalation of the conidia from the soil fungi *Emmonsia parva* (*Chrysosporium parvum* var. *parvum*) or *E. crescens (C. parvum* var. *crescens)*. The inhaled conidia do not replicate in the host's lungs but simply enlarge to form thick-walled adiaspores. In var. *parvum* these are up to 40 µm in diameter and up to 400 µm in var. *crescens.* These adiaspores could be confused with the spherules of *Coccidioides immitis.* Infections are usually discovered incidentally in the course of histopathological examination of lung tissue for other infections. The condition has been reported from Africa, Asia, Europe, New Zealand, North America and South America. On direct microscopy the two varieties may be distinguished by the relative sizes of the adiaspores and by the fact that the adiaspores remain uninucleate in *E. parva* but become multinucleate in *E. crescens.*

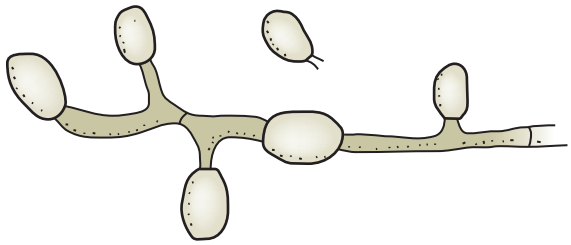


Figure 41.9 *Emmonsia* species from a mycelial culture (25°C) illustrating one-celled conidia (2–10 µm) with truncated base and broad basal scar.

Culture

Both varieties grow well on Sabouraud dextrose agar to produce mycelial colonies at 25°C. Strains vary considerably in appearance. Young cultures are colourless and smooth but become white or brown with white aerial hyphae. The reverse ranges from white to yellow or brown. Microscopically these mycelial cultures show septate hyphae and ovoid, one-celled, smooth or rough-walled conidia with a broadly truncated base and basal scar, often with a remnant of hyphal wall left attached (Fig. 41.9).

On brain-heart infusion blood agar at 37°C *E. crescens* produces adiaspores that are 25–400 µm in diameter with walls 70 µm thick. The incubation temperature must be raised to 40°C before *E. parva* will produce adiaspores. These are 10–25 µm in diameter with walls about 2 µm thick.

REFERENCES

Bialek, R., Cirera, A.C., Herrmann, T., et al., 2003. Nested PCR assays for the detection of *Blastomyces dermatitidis* DNA in paraffin-embedded canine tissue. Journal of Clinical Microbiology 41, 205–208.

Borman, A.M., Simpson, V.R., Palmer, M.D., et al., 2009. Adispiromycosis due to *Emmonsia crescens* in native British mammals. Mycopathologia 168, 153–163.

Fisher, M.C., Koenig, G., White, T.J., et al., 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov. previously recognized as the non-California population of *C. immitis*. Mycologia 94, 73–84.

Kasuga, T., White, T.J., Koenig, G., et al., 2003. Phylogeography of the fungal pathogen *Histoplasma capsulatum*. Molecular Ecology 12, 3383–3401.

Kaufman, L., Standard, P.G., 1987. Specific and rapid identification of medically important fungi by exoantigen detection. Annual Review of Microbiology 41, 209–225.

Spector, D., Legendre, A.M., Wheat, J., et al., 2008. Antigen and antibody testing for the diagnosis of blastomycosis in dogs. Journal of Veterinary Internal Medicine 22, 839–843.

Stockman, L., Clark, K.A., Hunt, J.M., et al., 1993. Evaluation of commercially available acridinium ester-labelled chemiluminescent DNA probes for culture identification of *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans* and *Histoplasma capsulatum*. Journal of Clinical Microbiology 31, 845–850.

Tintelnot, K., De Hoog, G.S., Antweiler, E., et al., 2007. Taxonomic and diagnostic markers for identification of *Coccidioides immitis* and

Coccidioides posadasii. Medical Mycology 45, 385–393.

Ueda, Y., Sano, A., Tamura, M., et al., 2003. Diagnosis of histoplasmosis by detection of the internal transcribed spacer region of fungal rRNA gene from a paraffin-embedded skin sample from a dog in Japan. Veterinary Microbiology 94, 219–224.

FURTHER READING

Brömel, C., Sykes, J.E., 2005. Histoplasmosis in dogs and cats. Clinical Techniques in Small Animal Practice 20, 227–232.

Brömel, C., Sykes, J.E., 2005. Epidemiology, diagnosis and treatment of blastomycosis in dogs and cats. Clinical Techniques in Small Animal Practice 20, 233–239.

Graupmann-Kuzma, A., Valentine, B.A., Shubitz, L.F., et al., 2008. Coccidioidomycosis in dogs and cats: a review. Journal of the American Animal Hospital Association 44, 226–235.

Chapter 42

The pathogenic *Zygomycetes*

Zygomycosis (older term phycomycosis) is an all-embracing term for an infection due to a fungus in the taxonomic Class *Zygomycetes*, phylum *Zygomycota*. Most of these fungi are saprophytes and widespread in the environment but some of them can be opportunistic pathogens. Clinical disease usually occurs if the host's defences are lowered or if large numbers of spores are ingested or inhaled. The hyphae, in cultures and in animal tissues, are wide (5–15 µm diameter), irregular and ballooning (Fig. 42.1). They lack septa except near fruiting structures and if there has been damage to hyphae or in older cultures. The sexual spores are thick-walled zygospores (Fig. 42.2) produced through fusion of gametangia, often from two different strains of the species.

The Class contains three Orders of veterinary significance – *Mucorales, Mortierellales* and *Entomophthorales*. Pathogenic species within the Order *Mucorales* are found within several genera including *Lichtheimia* (*Absidia*), *Mucor, Rhizopus, Rhizomucor, Saksenaea*. The Order *Mortierellales* contains one genus of veterinary significance, *Mortierella*. The Order *Entomophthorales* includes *Basidiobolus ranarum* and *Conidiobolus coronatus* both of which can cause disease in animals and humans.

THE MUCORACEOUS ZYGOMYCETES (ORDERS *MUCORALES* AND *MORTIERELLALES*)

The mucoraceous zygomycetes characteristically have rapidly growing colonies with greyish, woolly mycelium that fills the Petri dish in three to five days and often reaches and raises the lid of the plate (Fig. 42.3). An exception to this is *Mortierella wolfii* that has a white, low, velvety growth (Fig. 42.4). The aerial hyphae (sporangiophores) arise from stolons and usually end in a columella that is enclosed in the sac-like sporangium. Within the sporangium the asexual spores (sporangiospores) are formed. These spores can be colourless, yellowish or brown and the sporangia, packed with spores, often appear as dark, pinhead-sized dots within the woolly grey mycelium. As the spores mature, the wall of the sporangium becomes fragile and ruptures releasing myriads of spores (Fig. 42.5). The root-like rhizoids are produced by several genera and promote anchorage to the substrate (Fig. 42.6).

Natural Habitat

The mucoraceous zygomycetes are usually saprophytes and widespread in soil, vegetation and air. Specimens are often contaminated by them but cycloheximide, added to Sabouraud dextrose agar when their culture is not required, will inhibit their growth. *Mortierella wolfii* is not easy to find in the environment although it has been recovered, with difficulty, from soil associated with a silage stack and from debris in cattle yards. Most of the zygomycetes occur worldwide. Infection in cattle due to *M. wolfii* has been reported from New Zealand, Australia, the UK and North America.

Pathogenesis

Infection with these fungi is associated with immunodeficiency, corticosteroid treatment. prolonged administration of antibiotics and immunosuppressive viral infections such as feline leukaemia or feline panleukopaenia in cats. Members of the genera *Rhizopus, Rhizomucor* and *Lichtheimia* (*Absidia*) are thermotolerant and able to grow at core mammalian body temperature. The enzyme ketone

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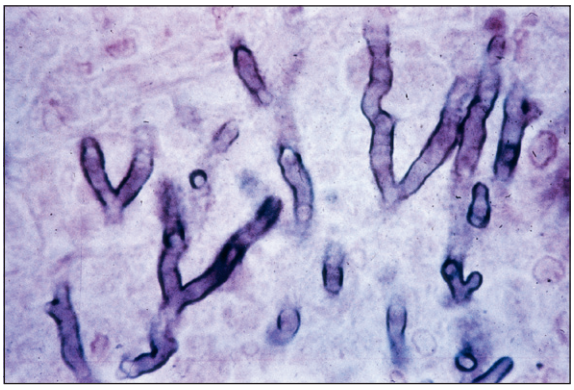

Figure 42.1 *Rhizopus* species non-septate hyphae in the wall of bovine rumen. (Silver stain, ×1000)

Figure 42.2 *Rhizopus* species: zygospore. (×400)

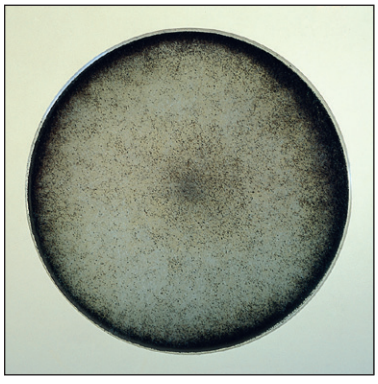

Figure 42.3 *Rhizopus* species on Sabouraud agar, seven days.

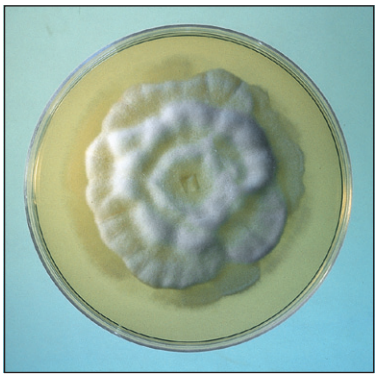

Figure 42.4 *Mortierella wolfii* on Sabouraud agar, four days.

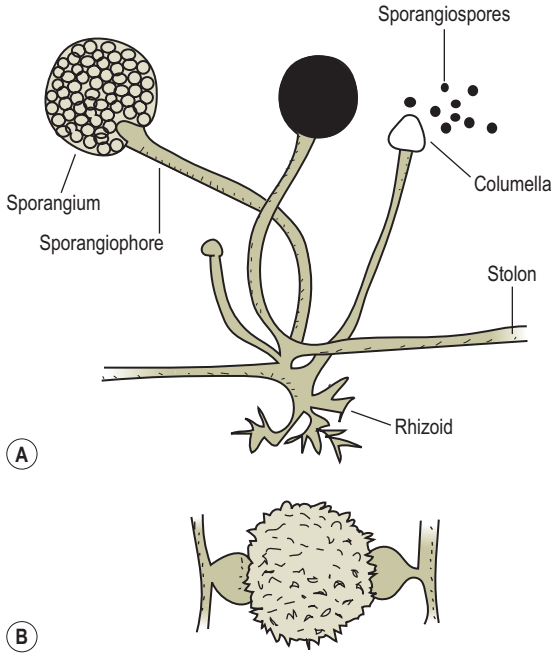

Figure 42.5 A mucoraceous zygomycete showing: A asexual fruiting structure, B sexual zygospore.

reductase is produced by *Rhizopus* species and facilitates the growth of this fungus in the acidic and glucose-rich environment of the rumen following grain overload. Proteases and lipases produced by *Rhizopus* species probably assist in the invasion of host tissues. *Lichtheimia* (*Absidia*) species produce siderophores that aid iron scavenging.

The route of exposure usually determines the clinical presentation. Inhalation of large numbers of spores may result in pulmonary disease while ingestion of mouldy feed may result in intestinal infection. Infection is thought

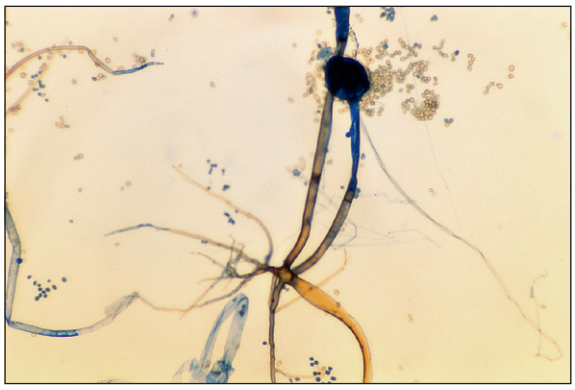

Figure 42.6 *Rhizopus* species: sporangiophore, collapsed sporangium, sporangiospores and a rhizoid. (LPCB, ×100)

Figure 42.8 Fungal plaques on a bovine foetus characteristic of mycotic abortion (*Mortierella wolfii*).

Figure 42.7 Bovine mycotic placentitis (*Mortierella wolfii*).

Figure 42.9 Bovine lung. Acute, diffuse, mycotic pneumonia (*Mortierella wolfii*).

to reach the placenta via the bloodstream from the respiratory or alimentary tract. The most common form of zygomycosis (sometimes referred to as mucormycosis) affects the lymph nodes of the respiratory and intestinal tracts. The lymph nodes may enlarge and show caseous necrosis. The internal organs can also be involved. This type of infection is most common in cattle, pigs and dogs. Young animals are more prone to intestinal tract infections, clinically manifested by diarrhoea. A granulomatous reaction, ulceration and caseous necrosis occur in the intestinal tract. In acute and severe infections the fungi characteristically display a pronounced angiotropism and invade blood vessels, causing a necrotizing vasculitis with thrombosis and haemorrhage.

Mortierella wolfii causes abortion in cattle and in about 5% of these animals an acute, fulminating pneumonia followed by death occurs within 48 hours of the abortion. Lesions are characteristic of mycotic abortion and resemble those seen in *Aspergillus fumigatus* infections. The placenta is thickened and 'wooden' in appearance (Fig. 42.7) and the foetus may have ringworm-like fungal skin plaques (Fig. 42.8). In fatal pneumonia cases the lungs are heavy, red and wet with most lobes affected (Fig. 42.9). A variable amount of fluid is present in the thoracic cavity. Other zygomycetes have been isolated from cases of abortion in cattle but, as these other fungi are more widely distributed than *M. wolfii*, fungal involvement should be confirmed by histological examination of cotyledons and foetal lesions. Table 42.1 summarizes the main hosts and diseases caused by the mucoraceous zygomycetes. Zygomycosis has also been reported in mink, guinea pigs, mice, poultry and exotic birds.

Laboratory Diagnosis

Specimens

Lesion biopsies or tissues from dead animals, cotyledons, foetal abomasal contents and uterine discharges can be collected. It is easy for the hyphae to become damaged and

Table 42.1 Diseases caused by the mucoraceous zygomycetes

Host	Disease
Cattle	Rumenitis, abomasal ulcers
	Abortion
	Mesenteric and mediastinal lymphadenitis
	Enteritis in calves
	Pneumonia following mycotic abortion due to *Mortierella wolfii*
Pigs	Gastric ulcers
	Mediastinal and submandibular lymphadenitis
	Enteritis in piglets
Horse	Abortion
Dogs	Enteritis
Cats	Enteritis
	Pneumonia

non-viable during sampling on account of their coenocytic hyphae. Tissue samples, in 10% formalin, should be taken when possible for histopathology.

Direct microscopy

Histological examination of tissue sections is needed for diagnostic confirmation of the involvement of these ubiquitous fungi. They are PAS-positive (pink staining). Alternatively methenamine silver staining can be used. The hyphae are broad (10–15 µm diameter), irregular, non-septate and branching. The hyphae of *M. wolfii* are somewhat finer (2–12 µm) but irregular. Fungal hyphae may also be seen in KOH wet preparations.

Culture

Sabouraud dextrose agar without cycloheximide or Emmons' modification can be used. The inoculated plates are incubated aerobically for up to 10 days although some colonial growth is usually seen in 48 hours. The species capable of causing systemic infections can tolerate 37°C. This temperature has the advantage of discouraging any of the purely saprophytic fungi. If the lesions are of a superficial nature a duplicate plate could be inoculated and incubated at 25°C. The zygomycetes will grow well on blood agar plates either as contaminants or as significant pathogens in cases of unsuspected mycotic infections. *Mortierella wolfii* does not compete well with the faster-growing bacteria on blood agar or with fungal contaminants on mycology plates. Sporulation of *Mortierella wolfii* and *Saksenaea vasiformis* occurs only on media deficient in certain nutrients.

Identification

At present there are no identification kits, nucleic acid probes or widely available immunological or serological means for identifying the zygomycetes. Accordingly there must be heavy reliance on histological examination of affected tissues and the colonial and microscopic appearance of the isolated zygomycete. Table 42.2 indicates the morphology of some of the species recognized as causing disease in animals. The cultures may lend themselves to identification at a generic level but subcultures could be submitted to a mycology reference laboratory for species confirmation. Suitable molecular techniques are gradually becoming available (Schwarz et al. 2006, Hata et al. 2008, Piancastelli et al. 2009).

THE ENTOMOPHTHORACEOUS ZYGOMYCETES (ORDER *ENTOMOPHTHORALES*)

The Order includes two genera *Basidiobolus* and *Conidiobolus* that contain recognized pathogens in humans and have also been recorded as causing rare infections in animals. In humans they are often responsible for subcutaneous mycoses (zygomycoses). In direct microscopy from specimens, broad, irregularly branching hyphae (5–18 µm), sparsely septate and enclosed by a sheath of eosinophilic material comprising immune complexes, tissue debris and fibrin (Splendore-Hoeppli phenomenon) will be seen. They exhibit an entirely different colonial appearance to that of the mucoraceous zygomycetes and grow in three to five days as flat, waxy colonies that often develop radial grooves. They become fuzzy with age and the hyphae often become septate in older cultures. Colonies are difficult to remove from the agar and the colour varies from tan to grey-brown. A unique feature is the production of a single spore that is forcibly discharged when mature. A brief description is given of the colonial and microscopic appearance of these two fungi but the identification should be confirmed by a mycology reference laboratory.

Natural Habitat

Basidiobolus and *Conidiobolus* species are saprophytes in soil and decaying vegetation. They may be found in the faeces of cold-blooded animals and insects. Their distribution is probably worldwide but tends to be concentrated in warmer climates.

Table 42.2 Colonial and microscopic morphology of some clinically significant mucoraceous zygomycetes

Fungus	Colonial morphology	Microscopic appearance
Lichtheimia (Absidia) corymbifera	Rapid growth. Woolly and white, becoming olive-grey. Fills Petri dish like *Rhizopus*. It is thermotolerant and grows at 45°C	Rhizoids are internodal (arising from stolons between the sporangiophores). Sporangiophores are long (450 μm) and branch repeatedly. Sporangia are pear-shaped (10–40 μm) and contain globose to oval spores (2–4 μm). Columella is conical and merges with a pronounced apophysis (swelling) below the sporangium
Mortierella wolfii	Colony white and velvety with a lobulated outline giving a rosette appearance. Spreads rapidly over the plate	Colonies on Sabouraud agar are sterile or produce very few sporangia. On hay or potato-carrot agar sporangia are produced more freely in two to 10 days. Rhizoids are nodal. Sporangiophores are 80–250 μm long and tapered, each bearing up to five branches. The lack of a columella is characteristic of the genus. The sporangia (15–48 μm) are globose, smooth and fragile. Spores are colourless and ovoid or kidney-shaped (2–5 × 6–12 μm)
Mucor circinelloides	Colonies spread right across Petri dish but growth is low. Pale grey or yellowish-brown at 37°C	No rhizoids. Branching or simple sporangiophores that are short. Sporangia are round (25–80 μm) and columella is variable in shape but is entirely within the sporangium. Spores are hyaline, ellipsoidal (4–7 μm) and smooth-walled
Rhizomucor pusillus, R. miehei	Rapidly growing, cottony colonies. White but surmounted by brown sporangia. Thermophilic with growth at 50–60°C	Rhizoids poorly developed and internodal. Sporangiophores arise from surface hyphae and usually branched. Sporangia are spherical (40–60 μm) and brown or grey. Columella shape is variable. Spores are hyaline (3–4 μm) and smooth-walled. *Rhizopus miehei* has sporangia with spiny walls
Rhizopus arrhizus (R. oryzae), R. microsporus	Coarse, rampant growth. Petri dish is filled in five days with dense, woolly mycelium. White at first, becoming greyish and surmounted by black pinhead-sized sporangia. Growth reaches lid of the plate and may raise it	Rhizoids are nodal (immediately under the sporangiophores), well-developed and usually obvious in wet mounts or even under the dissecting microscope. The long sporangiophores arise singly or in groups from nodes on the stolon. Sporangia (60–175 μm) are black, round and contain globose spores. The columella is hemispherical
Saksenaea vasiformis	Fast growing, downy, white colonies	Fails to sporulate on primary isolation media. Sporulation may be stimulated by use of nutrient-deficient agar such as cornmeal-glucose-sucrose-yeast extract agar. Darkly pigmented rhizoids. Sporangia are flask-shaped. The columella is prominent and dome-shaped. Small (3–4 μm), oblong spores

Pathogenesis

The route of entry is minor abrasions in the skin or nasal mucous membranes. Granulomatous lesions result from infection. Invasion of blood vessels is uncommon but spread via lymphatic vessels may occur. Disseminated disease is rare. Virulence factors include thermotolerance and tissue-degrading enzymes. *Conidiobolus coronata, C. incongruous* and *C. lamprauges* have been associated with nasal granulomas and chronic sinusitis, particularly in the horse. *Basidiobolus ranarum* has been associated with subcutaneous, ulcerative, granulomatous lesions, most commonly in dogs and horses.

Laboratory Diagnosis

Specimens

Biopsy or postmortem material should be submitted for culture and histopathology.

Direct microscopy

Histological examination of tissue sections may reveal the fungal hyphae and evidence of the Splendore-Hoeppli phenomenon. It can be difficult to visualize the fungal elements using routine fungal stains such as periodic-acid-Schiff (PAS) or Gomori methenamine silver (GMS).

Culture

Sabouraud dextrose agar without cycloheximide is suitable. They can tolerate temperatures of 37°C but tend to prefer temperatures of 25–30°C.

Colonial appearance

- ***Basidiobolus ranarum***: the colonies are thin, waxy and buff to grey. They become radially folded, greyish-brown and covered with white aerial hyphae on longer incubation. The reverse is white. Some strains have an earthy odour similar to that of *Streptomyces* species.
- ***Conidiobolus coronatus***: flat, waxy, buff or grey colonies becoming covered with white aerial hyphae. The colonies become tan to brown with age and the reverse is white. The Petri dish lid becomes covered with spores that have been forcibly ejected from the conidiophores.

Microscopic appearance

The main microscopic features are shown diagramatically in Figures 42.10 and 42.11.

- ***Basidiobolus ranarum***: hyphae are wide (8–20 µm) with a few septa that can become more numerous during sporulation. Sporangiophores, each bearing a single-celled sporangium, arise from the hyphae. Cleavage of the sporangial contents may occur leading to the production of several sporangiospores within the sporangium. When mature the sporangium is forcibly ejected together with fragments of the sporangiophore. Zygospores, formed by the conjugation of two adjacent hyphal cells, are common. They are thick, smooth-walled, 20–50 µm in diameter and have a prominent beak-like appendage on one side that is the remnant of a copulatory tube (Fig. 42.10).
- ***Conidiobolus coronatus***: the hyphae (10–25 µm) have few septa and give rise to sporophores which at their tips produce spherical spores 10–30 µm in diameter. At maturity the spores are forcibly ejected and bear a broad tapering projection at the site of previous attachment. Spores may develop short hair-like appendages or germinate to produce single or multiple hyphal tubes. These in turn can become sporophores each bearing secondary spores. A spore may also replicate by producing a number of short extensions that give rise to a corona of secondary spores (Fig. 42.11).

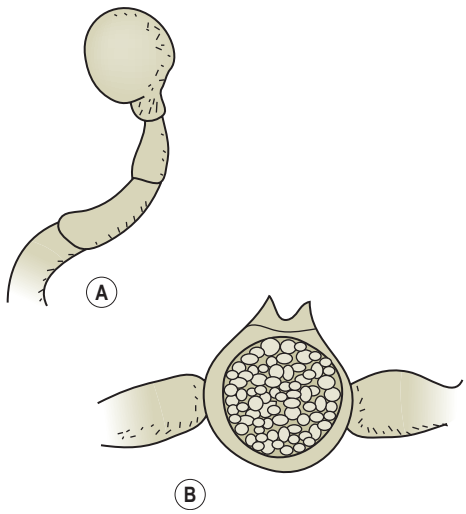

Figure 42.10 *Basidiobolus ranarum*: A single-celled sporangia produced on a hypha, B zygospore with beak-like appendage.

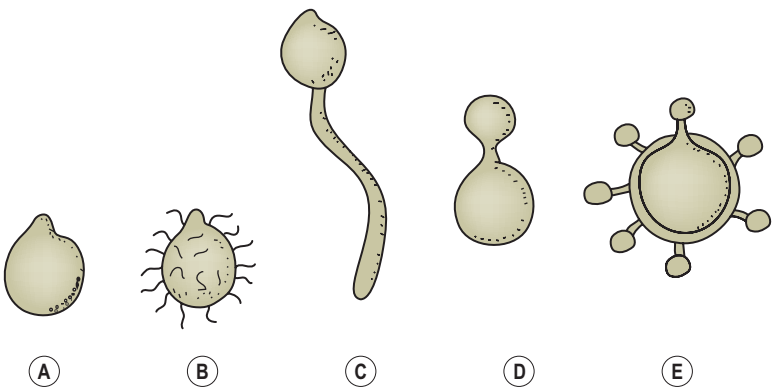

Figure 42.11 *Conidiobolus coronatus*: A conidium, B conidium with hair-like appendages, C germination of a conidium to form a hyphal tube, D conidium bearing a secondary conidium, E conidium bearing a corona of secondary conidia.

REFERENCES

Hata, D.J., Buckwalter, S.P., Pritt, B.S., et al., 2008. Real-time PCR method for detection of zygomycetes. Journal of Clinical Microbiology 46, 2353–2358.

Piancastelli, C., Ghidini, F., Donofrio, G., et al., 2009. Isolation and characterization of a strain of *Lichtheimia corymbifera* (ex *Absidia corymbifera*) from a case of bovine abortion. Reproductive Biology and Endocrinology 30, 138.

Schwarz, P., Bretagne, S., Gantier, J.C., et al., 2006. Molecular identification of zygomycetes from culture and experimentally infected tissues. Journal of Clinical Microbiology 44, 340–349.

FURTHER READING

Jensen, H.E., 1994. Systemic bovine aspergillosis and zygomycosis in Denmark with reference to pathogenesis, pathology and diagnosis. APMIS Supplement 42 (102), 4–48.

Taintor, J., Schumacher, J., Newton, J., 2003. Conidiobolomycosis in horses. Compendium on Continuing Education for the Practicing Veterinarian 25, 872–876.

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Chapter 43

Fungi causing subcutaneous mycoses

The subcutaneous mycoses are all transmitted in a similar manner. Fungi that are normally saprophytes in soil, vegetable debris, water or on plants become implanted in the skin due to trauma, with the subsequent development of a subcutaneous infection that is usually chronic. Occasionally there is spread of the infection with involvement of other organs. With the exception of sporotrichosis, which occurs worldwide, the subcutaneous mycoses are most common in tropical and subtropical regions. Table 43.1 summarizes the subcutaneous mycoses and gives the causative fungi, distribution, main hosts, type of lesions produced and the appearance of the fungi on direct microscopy of the specimen.

The phaeoid fungi involved in chromoblastomycosis and phaeohyphomycosis are dematiaceous or darkly pigmented due to the presence of melanin in their hyphal walls (Fig. 43.1). The actual fungal species isolated from a lesion often reflects its relative abundance in the particular geographical area. For example, *Phialohora verrucosa* is the most common cause of chromoblastomycosis in humans when the condition occurs in temperate regions. Dark sclerotic bodies (5–12 µm in diameter) are characteristic of chromoblastomycosis. These brown, thick-walled, multiseptate forms known as muriform bodies (sclerotic cells, Medlar bodies) are thought to represent an intermediate vegetative form, phenotypically arrested between a yeast and a mould (Fig. 43.2). The lesions of phaeohyphomycosis contain dematiaceous hyphae and yeast-like cells but no sclerotic bodies (Fig. 43.3). Chromoblastomycosis occurs uncommonly in humans, toads and frogs and is extremely rare in other animals. Phaeohyphomycosis occurs sporadically in cats, dogs, horses, cattle and goats, sometimes as a disseminated infection.

Mycetomas ('fungal tumours') can be caused by a range of fungi or by the procaryotic actinomycetes. One of the characteristics of mycetomas is the granules (0.5–3.0 mm) that occur in the pus from the lesions. In mycetomas caused by fungi (eumycetomas), the granules are composed of intertwined fungal hyphae 2–5 µm in width and inflammatory components. The filaments present in the actinomycotic granules are only 0.5–1.0 µm in width. True fungal mycetomas are rare in domestic veterinary species.

A number of fungus-like organisms are associated with rare, sporadic infections in animals and humans following contact with contaminated water:

- *Rhinosporidium seeberi*, causing rhinosporidiosis, has been cultured in vitro using a human rectal tumour cell line. The normal habitat of this novel aquatic protistan parasite is thought to be stagnant water. It is associated with chronic, polypous rhinitis characterized by the presence of large sporangia (spherules) containing numerous endospores in affected tissues (Fig. 43.4).
- *Pythium insidiosum* is an aquatic oomycete in the kingdom *Chromista* and the cause of cutaneous pythiosis. Biflagellate, motile zoospores are produced when plants in wet environments are colonized. These zoospores may opportunistically invade the damaged tissues of animals when they wade in stagnant water containing the organism. In plant and animal tissues aseptate hyphae which resemble those of zygomycetes develop. Cutaneous and intestinal forms of pythiosis are described. A number of other oomycetes, members of the genus *Lagenidium*, have been associated with a similar clinical picture in dogs and cats.
- *Lacazia* (*Loboa*) *loboi*, the aetiological agent of lobomycosis in humans and dolphins, is a

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Table 43.1 The subcutaneous mycoses

Disease	Fungus	Distribution	Host(s)	Lesions	Direct microscopy
Sporotrichosis *See* Chapter 41	*Sporothrix schenckii*	Worldwide, most common in tropical and subtropical regions	Horses, dogs, cats and humans	Ulcerating cutaneous nodes that follow lymphatic vessels, often on the hind legs of horses. Ulcers heal but re-erupt. Lymphatics become thickened. In dogs and cats dissemination to other organs may occur	Single or multiple budding, cigar-shaped yeast cells (2–4 μm). Low numbers of organisms in clinical specimens
Epizootic lymphangitis (African farcy) See Chapter 41	*Histoplasma farciminosum*	Africa, Asia, Middle East	*Equidae*	Chronic pyogranulomatous infection of equine skin and lymphatics. Regional lymph nodes are involved and dissemination can occur	Intracellular, pear-shaped, double-contoured yeast cells (2–4 μm). Usually inside mononuclear cells or neutrophils. Budding occurs most commonly at the pointed end of the cells
Subcutaneous zygomycosis *See* Chapter 42	*Basidiobolus ranarum, Conidiobolus coronatus*	Africa, Asia, Caribbean, South America. Some cases in USA	Humans and rarely in animals	*Basidiobolus ranarum* is associated with lesions on limbs and body. *Conidiobolus coronatus* tends to cause a rhinofacial infection. Nodular subcutaneous lesions are produced. If untreated the lesions are slowly progressive	Broad, irregularly branching, hyphae (5–18 μm), sparsely septate and enclosed in eosinophilic material (Splendore-Hoeppli phenomenon)
Chromoblastomycosis	*Fonsecaea pedrosoi, F. compacta, Phialophora verrucosa, Cladophialophora carrionii, Rhinocladiella aquaspersa*	Africa, Australia, South America and Japan. *Phialophora verrucosa* in temperate regions	Humans and rarely in animals	Legs and feet are most commonly affected. Lesion begins as a nodule but growth becomes large and cauliflower- or wart-like. The initial and satellite growths remain localized and persist for many years	Dark sclerotic bodies (5–12 μm) are present. They are thick-walled, muriform (divided by vertical and horizontal septa), and are usually chestnut brown. In the crusts, dematiaceous (dark), septate, branched hyphae may be seen
Phaeohyphomycosis	*Wangiella dermatitidis, Exophiala jeanselmei, Phialophora verrucosa, Aureobasidium pullulans, Alternaria* species, *Cladophialophora bantiana, Cochliobolus spicifera, Ulocladium* species, *Moniliella suaveolens, Stemphylium* species	Tropical regions mainly	Humans, cats, dogs, horses, cattle and goats	The superficial lesions are similar to those in chromoblastomycosis but systemic infections can occur involving a wide range of tissues	Dematiaceous hyphae and yeast-like cells, or both, may be present. The hyphae are regular or irregular with swollen ends. No sclerotic bodies are seen

Mycetomas	*Pseudallescheria boydii, Exophiala jeanselmei, Curvularia geniculata, Madurella mycetomatis*	Africa, Asia, South and Central America. Occasional cases in some temperate regions	Humans and more rarely in cattle, horses, dogs and cats	Mycetomas can be caused by both fungi and actinomycetes. Characterized by granulomatous swellings with sinus tracts discharging exudates containing granules. Slowly progressive and can involve adjacent tissue	Granules in the pus are small (0.5–3.0 mm), irregularly shaped and of various colours. Microscopically, the granules consist of broad (2–5 μm), intertwined hyphae and swollen cells (15 μm or more) at periphery
Rhinosporidiosis	*Rhinosporidium seeberi*	Tropics and subtropics	Humans, horses, cattle, mules, dogs, goats and some wild water fowl	Granulomatous, mucocutaneous infection. Large polyps, tumours or wart-like lesions on nasal and ocular mucous membranes. The growths are highly vascularized, sessile or pedunculated. Associated with nose rings in bulls	Large sporangia (up to 200–300 μm diameter) that contain numerous endospores. Release of endospores provokes a marked pyogranulomatous reaction
Cutaneous pythiosis (swamp cancer)	*Pythium insidiosum*	Tropical and subtropical regions mainly	Humans, horses, dogs, cats and calves	The skin lesions are pyogranulomatous or fibrogranulomatous. The disease is chronic and progressive. In the horse the lesions are large (up to 45 cm), discharging swellings, usually on extremities, ventral trunk or head. Yellow, necrotic masses termed 'kunkers' or 'leeches' can be removed intact from the granulomas. Nasal mucosa can be involved. Intestinal pythiosis is the more common form in the dog	Masses of hyphae (4 μm diameter) are seen in histological sections. These are mixed with a variety of inflammatory cells such as macrophages, epithelioid cells and giant cells, forming necrotic masses
Lobomycosis (keloidal blastomycosis)	*Lacazia (Loboa) loboi*	Dolphins off Florida coast and in Surinam River	Humans and dolphins	Lesions are nodular and keloidal in appearance. Early in infection they are freely moving, subcutaneous nodules, but later verrucose, nodular plaques are formed. The lesions spread by peripheral extension	Spherical, thick-walled yeast cells (5–12 μm diameter) are present. Buds are single but sequential budding can lead to chains of cells linked by tubular isthmuses

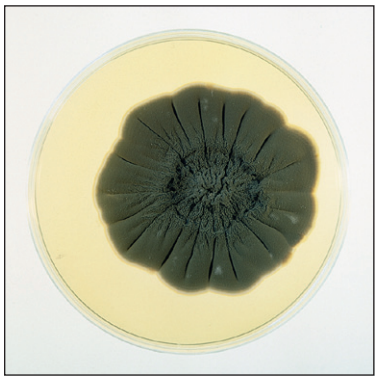

Figure 43.1 *Cladophialophora* species (a dematiaceous mould) on Sabouraud agar, five days.

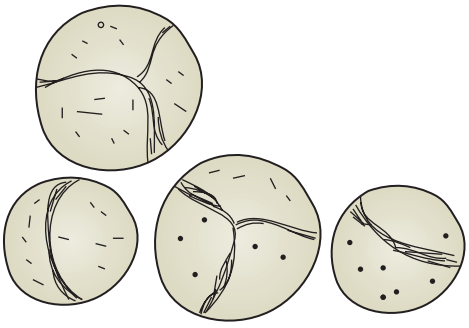

Figure 43.2 Muriform sclerotic bodies (5–12 µm in diameter) seen in chromoblastomycosis.

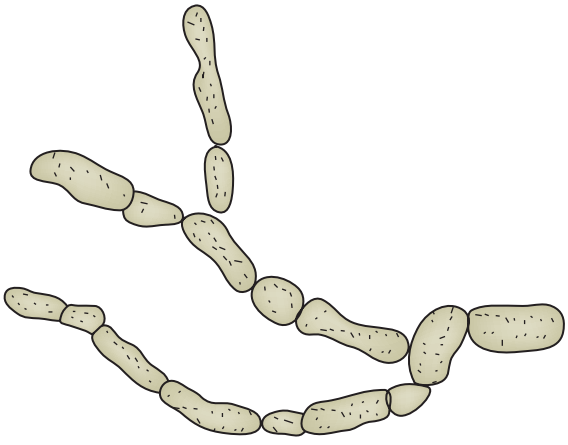

Figure 43.3 Dematiaceous hyphae (5–10 µm in diameter) as seen in an aspirate from a case of phaeohyphomycosis.

yeast-like organism related to *Paracoccidioides brasiliensis* but has not yet been grown in vitro. The organism, from lesions in dolphins, has been successfully passaged in mice by foot-pad inoculation.

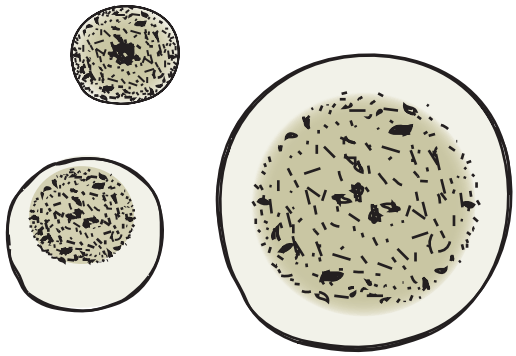

Figure 43.4 Sporangia of various sizes (6–300 µm in diameter) that occur in nasal polyps in rhinosporidiosis.

Laboratory Diagnosis

Specimens

Suitable specimens include punch biopsies, fine-needle aspirates and postmortem tissues.

Direct microscopy

Table 43.1 summarizes the subcutaneous mycoses and indicates the morphological appearance of the fungi on direct microscopy. Wet mounts of exudates or tissues and histopathological sections of biopsies or tissues, stained by the PAS or methenamine silver stains, should yield a great deal of information. The Masson-Fontana silver stain can be used to demonstrate the presence of melanin in the hyphae of phaeoid fungi. For practical purposes, the clinical lesions together with direct microscopy can give a presumptive diagnosis of most of the superficial mycoses.

Culture

Sabouraud dextrose agar, with and without antimicrobial agents, will support the growth of the normally saprophytic fungi involved in chromoblastomycosis, phaeohyphomycosis and mycetomas. A few are difficult to isolate and others are very slow-growing, requiring an incubation time of up to six weeks. An incubation temperature of 25–30°C is suitable.

Identification

For the fungi associated with chromoblastomycosis, phaeohyphomycosis and mycetomas the identification is based on the macroscopic and microscopic morphology of the isolated fungus. In the case of the dematiaceous fungi in particular, identification requires a considerable amount of experience. Slide cultures will often be necessary to clearly see the continuity between the conidia,

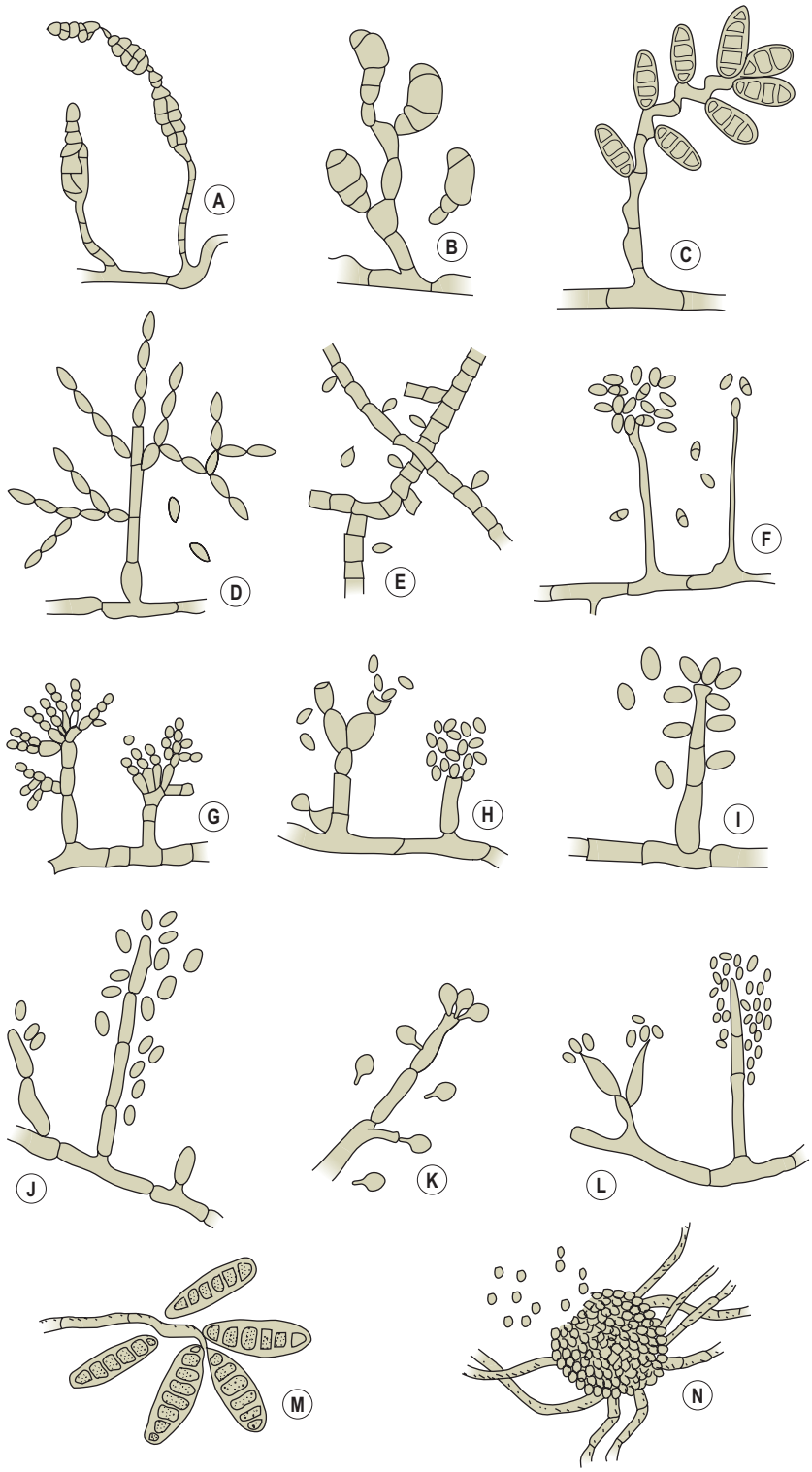

Figure 43.5 Fruiting heads of the genera of some fungi associated with subcutaneous mycoses. A *Alternaria*, B *Curvularia*, C *Cochliobolus*, D *Cladophialophora*, E *Aureobasidium*, F *Acremonium*, G *Fonsecaea*, H *Phialophora*, I *Rhinocladiella*, J *Wangiella*, K *Scedosporium*, L *Exophiala*, M *Exserohilum*, N *Phoma*.

conidia-bearing cells, conidiophores and vegetative hyphae.

The nomenclature is complicated. Fungi should be classified by sexual structures if they are produced and where possible the teleomorphic name (that of the sexual state) is used. However, a teleomorph may produce more than one asexual form (anamorph). For example, *Pseudallescheria boydii* is the teleomorph of both anamorphs *Scedosporium apiospermum* and *Graphium eumorphum*. But *S. apiospermum* is an anamorph that can be produced by several species of both *Pseudallescheria* and *Petriella*. The aid of a reference mycology laboratory should be sought for the identification of the cultures. Schematic drawings of the asexual fruiting heads of some of the more common fungi involved in chromoblastomycosis, phaeohyphomycosis and fungal mycetomas are shown in Figure 43.5. A summary of the colonial and microscopic features is given in Table 43.2.

Molecular techniques

A commercial DNA sequencing kit has shown promise in the identification of dematiaceous fungi (Hall et al. 2004). A nested PCR assay has been developed for the detection and identification of *Pythium insidiosum* (Grooters & Gee 2002). In addition, a species-specific DNA probe is available (Schurko et al. 2004).

Serology

An ELISA has been described for the serodiagnosis of pythiosis in dogs (Grooters et al. 2002).

Table 43.2 Colonial and microscopic characteristics of some fungi associated with the subcutaneous mycoses (illustrated in Figure 43.5)

Fungus	Species affected	Colonial appearance	Microscopic appearance
Alternaria species	Horse (*A. alternata*), cat (*A. infectoria*)	Dark greenish-black to grey-brown with a light border. Reverse is black	Hyphae are dark and septate. Conidia are large (7–10 × 23–34 μm), brown, muriform, club-shaped and occur singly or in chains
Curvularia species	Cow, dog, horse (*C. geniculata*), cat (*C. lunata*)	Olive-green to brown or black with a pinkish-grey woolly surface. Reverse is black	Conidiophores are simple or branched and bend at the point of attachment of each conidium. Conidia are large (8–14 × 21–35 μm), often four-celled and appear curved due to the swelling of a central cell
Cochliobolus (Bipolaris/ Drechslera) species	Cat, dog, horse, cow	Greyish-brown forming a matted centre and raised lighter periphery	Dark septate conidiophores with knobbly, bent appearance. Conidia are brown, thick-walled, oblong to cylindrical (6–12 × 16–35 μm) and contain four or more cells
Cladophialophora (*Cladosporium*) species	Cat, dog (*C. bantiana*)	Dark, velvety and olive green with a dark reverse	Conidiophores of varying lengths that produce long branching chains of brown, smooth-walled, oval, pointed conidia. The conidia are easily dispersed
Aureobasidium (*Pullularia*) *pullulans*	Dog	Mature colony is black, shiny, leathery and yeast-like with a lighter fringe. The reverse is black	Two types of hyphae are produced: 1. Thick, dark-walled and closely septate, some forming tubes that produce oval conidia; 2. hyaline hyphae producing conidia directly from the walls
Fonsecaea species	Cat (*F. pedrosoi*)	Slow-growing, velvety to woolly, olive to black colony. The reverse is dark	Dark, erect condiophores bearing masses of conidial chains. The conidia are one-celled, cask-shaped to round
Phialophora verrucosa	Cat	Mat-like to heaped and granular. The obverse is olive-grey and the reverse is black	Conidiophores, when present, are short and the phialides flask-shaped. Distinct collarettes are present at the apices of the phialides. The conidia (1–3 × 2–4 μm) are hyaline and occur in balls which may slip down the phialides

Table 43.2 Colonial and microscopic characteristics of some fungi associated with the subcutaneous mycoses (illustrated in Figure 43.5)—cont'd

Fungus	Species affected	Colonial appearance	Microscopic appearance
Scedosporium apiospermum (*Pseudallescheria boydii*)	Dog, horse	Rapidly growing, cottony, initially white but change to smokey-grey to brown	Conidia may occur singly or in clusters at the apices of short and long conidiophores. The conidia are obovate, truncate, subhyaline to light black
Exophiala species	Cat (*E. jeanselmei, E. spinifera*)	Moist, black and yeast-like. Reverse is black	The conidiophores are dark and simple, bearing clusters of oval conidia. The conidia-bearing cells are tapered to a narrow apex
Exserohilum rostratum	Cow	Dark grey to black cottony colony, reverse is black	Conidiophores have an uneven, thick-walled appearance and bear fusiform, septate conidia
Phoma glomerata	Goat	Powdery or velvety, greyish brown colony, reverse is brown	The asexual fruiting body, termed pycnidium, is dark and round with an opening and contains the conidia

REFERENCES

Grooters, A.M., Gee, M.K., 2002. Development of a nested polymerase chain reaction assay for the detection and identification of *Pythium insidiosum*. Journal of Veterinary Internal Medicine 16, 147–152.

Grooters, A.M., Leise, B.S., Lopez, M.K., et al., 2002. Development and evaluation of an enzyme-linked immunosorbent assay for the serodiagnosis of pythiosis in dogs. Journal of Veterinary Internal Medicine 16, 142–146.

Hall, L., Wohlfiel, S., Roberts, G.D., 2004. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. Journal of Clinical Microbiology 42, 622–666.

Schurko, A.M., Mendoza, L., de Cock, A.W., et al., 2004. Development of a species-specific probe for *Pythium insidiosum* and the diagnosis of pythiosis. Journal of Clinical Microbiology 42, 2411–2418.

FURTHER READING

Elad, D., 2011. Infections caused by fungi of the *Scedosporium/Pseudallescheria* complex in veterinary species. Veterinary Journal 187, 33–41.

Grooters, A.M., 2003. Pythiosis, lagenidiosis and zygomycosis in small animals. Veterinary Clinics of North America, Small Animal Practice 33, 695–720.

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Chapter 44

Mycotoxins and mycotoxicoses

Although moulds can grow on a wide range of organic matter including growing crops and stored feed, those of greatest significance in veterinary medicine produce secondary metabolites which are toxic for many animal species and man in low concentrations. Fungal toxins are referred to as mycotoxins and the diseases they produce are termed mycotoxicoses. Production of mycotoxins occurs as a result of normal fungal metabolism. No specific role for these metabolites in the life cycle of the fungus has been demonstrated. Some mycotoxicoses such as ergotism have been known since the Middle Ages while many other diseases arising from the ingestion of mycotoxin-contaminated pasture or feed have been recognized only in recent decades.

Mycotoxicoses are not infections but are acute or chronic intoxications produced by toxic metabolites of fungal origin. Many of the toxigenic fungi are widespread throughout the world and over 100 known species are capable of elaborating mycotoxins. Many of these fungi belong to the genera *Aspergillus*, *Fusarium* and *Penicillium*. More than one fungal species may produce the same mycotoxin while individual moulds may produce two or more different mycotoxins. Because taxonomic classification of fungi is based almost exclusively on morphological rather than physiological considerations, there is frequently little correlation between the pattern of toxin production by particular fungi and their phylogenetic classification. Toxin production only occurs under specific conditions of moisture, temperature, suitability of substrate and appropriate oxygen tension. The optimum conditions for toxin production are relatively specific for each fungus. *Fusarium sporotrichoides* elaborates its toxin at freezing temperatures while *Aspergillus flavus* requires a temperature of 25 °C. Only some strains of a single species such as *Aspergillus flavus* have the ability to produce toxins, even under favourable conditions. Some strains of fungi have a distinct preference for certain substrates and consequently there may be a regional prevalence of particular mycotoxicoses depending on the types of crops or pasture cultivated in the region.

The susceptibility of different crops to mould infection is governed by the presence of suitable substrates. The seed or kernel may be the preferred target of some fungi because of the ready availability of carbohydrates, while the fibrous part of the plant with its high cellulose content may be invaded by other fungi capable of using this substrate. Damage to the seed coat by insects, mechanical harvesting, severe frost or other factors may predispose crops to fungal attack. Insects may also serve as carriers of fungal spores.

Mycotoxicoses are diseases in which many factors interact. Animals vary widely in their susceptibility to mycotoxins. Younger animals tend to be more susceptible than adults. There is also considerable variation in species and individual susceptibility. The conditions which favour mycotoxin production and the factors which influence the severity of mycotoxicoses are summarized in Figure 44.1. Since mycotoxins are most likely to be concentrated in highest amounts in stored feeds, groups of animals at risk include poultry, pigs, dairy and feedlot cattle fed on contaminated feed. In some countries, particularly New Zealand, mycotoxicoses such as 'facial eczema' are associated with standing pasture and accordingly prevailing climatic conditions determine the occurrence of disease.

Characteristics of Mycotoxins

As mycotoxins are low-molecular-weight, non-antigenic substances in their naturally occurring forms, acquired immunity does not occur following exposure. Many of the major mycotoxins are heat-stable and consequently can

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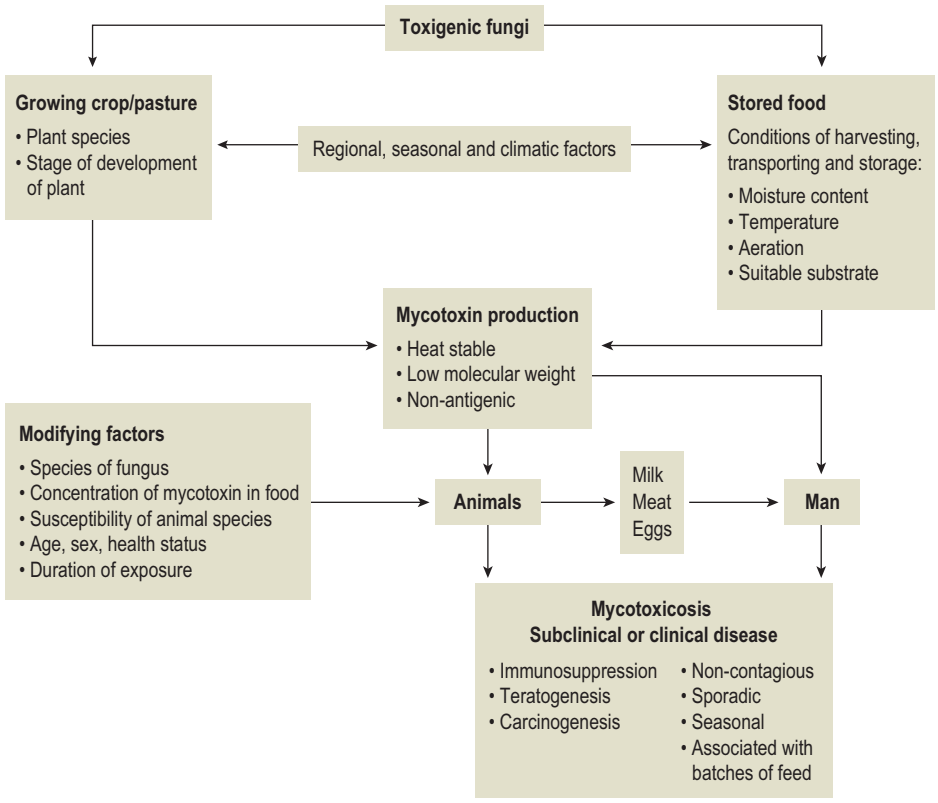

Figure 44.1 Factors influencing the production of mycotoxins on growing crops or stored foods and the occurrence of myctoxicoses in animals and man.

retain their toxicity after processing temperatures used for pelleting or other milling procedures. Each fungal toxin if present in the diet at a sufficient concentration usually affects specific target organs or tissues. Secondary mycotoxic disease may be more difficult to recognize because low levels of toxin intake may not result in a specific mycotoxicosis but in a heightened susceptibility to intercurrent infections due to immunosuppression. Some of the more important characteristics of mycotoxins are presented in Box 44.1.

Box 44.1 Summary of the characteristics of mycotoxins

- Secondary fungal metabolites
- Diverse group of compounds with a wide spectrum of toxic effects including immunosuppression, mutagenesis, carcinogenesis and teratogenesis
- Low-molecular-weight, heat-stable substances that are active at low dietary levels
- Non-antigenic; exposure does not induce a protective immune response
- May affect specific target organs or tissues such as the liver or central nervous system
- Human exposure may result from excretion in milk or accumulation in food-animal tissues

Mycotoxicoses

The severity of mycotoxicoses in animals and their clinical recognition is determined by many factors including the species of toxigenic fungus, the concentration of mycotoxin in the food, the age, sex and health status of the exposed animal, the target organ or tissue affected and the duration of exposure to contaminated feed. The features which characterize mycotoxic diseases include sporadic and seasonal occurrence, lack of transmissibility, association with certain batches of stored food or particular types of pasture, and disappointing response to drug treatment effective against infectious diseases (Box 44.2). Clinical and laboratory procedures may demonstrate pathological changes in the animal, characteristic of a particular intoxication. Clinical diagnosis can be complicated by the

Box 44.2 **Principal features of mycotoxicoses**

- Outbreaks are often seasonal and sporadic
- May be associated with particular batches of stored feed or certain types of pasture
- No evidence of transmission to in-contact animals
- Susceptibility can vary with the species, age and sex of the animals exposed
- Clinical presentation may be ill-defined
- Antimicrobial treatment is ineffective
- Recovery depends on type and amount of mycotoxin ingested and the duration of exposure to contaminated food
- Characteristic lesions in target organs of affected animals provide supporting diagnostic evidence
- Confirmation requires demonstration of significant levels of a specific mycotoxin in suspect feed or in tissues of affected animals

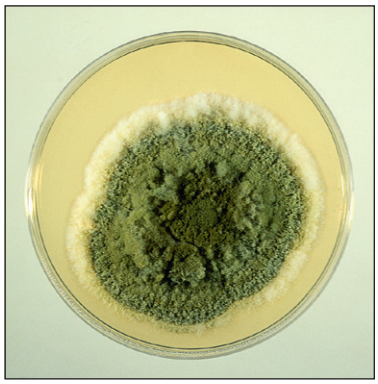

Figure 44.2 *Aspergillus flavus* (toxigenic strain) on Sabouraud dextrose agar, five days.

presence of a number of toxigenic fungal species on a food source. Diagnosis of a mycotoxicosis requires the demonstration of biologically effective concentrations of the fungal toxin in the feed available to the animal, or in the animal's tissues, secretions or excretions. Some advances have been made in the application of molecular techniques for the detection of toxigenic fungi (Seifert & Levesque 2004, Mule et al. 2005). Table 44.1 summarizes the principal features of those mycotoxicoses which can be recognized clinically. The level of subclinical and chronic disease associated with the consumption of fungal toxins in feed is difficult to quantify but is certainly of economic and public health significance.

Aflatoxicosis

Aflatoxins are a group of approximately 20 related toxic compounds produced by some strains of *Aspergillus flavus* (Fig. 44.2), *Aspergillus parasiticus* and a number of other *Aspergillus* species during growth on natural substrates including growing crops and stored food. These fungi are ubiquitous, saprophytic moulds which grow on a variety of cereal grains and foodstuffs such as maize, cottonseed and groundnuts. About half of the strains of *A. flavus* and *A. parasiticus* are toxigenic under optimal environmental conditions. High humidity and high temperatures during preharvesting, harvesting, transportation and storage, as well as damage to field crops by insects, drought and mechanical injury during harvesting favour the growth of *A. flavus* and toxin production. Although other fungi such as *Penicillium* species and *Rhizopus* species, are capable of producing aflatoxins, their relevance to livestock production has not yet been established. The name 'aflatoxin' derives from *Aspergillus*(a-), *flavus* (fla-) and toxin.

One of the first, well-documented outbreaks of aflatoxicosis occurred in East Anglia, England in l960 when more than 100,000 turkey poults died of an unknown disease ('turkey X disease'). Subsequently, it was demonstrated that these birds died from a toxin present in pelleted feed which formed a major part of their diet. A shipment of groundnut meal containing aflatoxin had been used as a protein supplement in the turkey rations. Examination of the incriminated groundnut meal revealed the presence of mould mycelia and thin-layer chromatography showed the presence of several compounds which fluoresced under ultraviolet light. The fungus was identified as *Aspergillus flavus* and the toxic metabolites were called aflatoxins. Since that time, numerous outbreaks of aflatoxicosis have been described worldwide.

Aflatoxins

Aflatoxins are a group of related difuranocoumarin compounds with toxic, carcinogenic, teratogenic and mutagenic activity. The four major aflatoxins are B_1, B_2, G_1 and G_2. Aflatoxin B_1 (AFB_1) is the most commonly occurring and also the most toxic and carcinogenic member of the group. These mycotoxins are named according to their position and fluorescent colour on thin-layer chromatograms, when viewed under ultraviolet light. AFB_1 and AFB_2 produce a blue and AFG_1 and AFG_2 a green fluorescence. Most of the other aflatoxins are metabolites formed endogenously in animals after ingestion or administration of aflatoxins. Aflatoxins are stable compounds in food and feed products and are relatively resistant to heat. They

Table 44.1 Mycotoxicoses of domestic animals

Disease	Fungus	Geographical distribution	Crop or substrate	Mycotoxin(s)	Species affected	Comments
Aflatoxicosis	*Aspergillus flavus, A. parasiticus*	Worldwide	Stored grain, maize, groundnuts, soybean	Aflatoxins B1, B2, G1, G2	Cattle, pigs, poultry, dogs, trout	Aflatoxins are hepatotoxic, immunosuppressive, carcinogenic and teratogenic. Clinical effects include ill-thrift, drop in milk yield, nervous signs in young animals
Diplodiosis	*Stenocarpella (Diplodia) maydis*	Southern Africa	Maize cobs	Diplonine	Sheep, cattle, horses, goats	Neurotoxic effects as well as stillbirths and neonatal deaths if pregnant animals are exposed
Ergotism	*Claviceps purpurea*	Worldwide	Seedheads of ryegrass and other grasses, cereals	Ergotamine, ergometrine, ergocristine	Cattle, sheep, deer, pigs, horses, poultry	Neurotoxicity and vasoconstriction. Clinical effects include convulsions, gangrene of extremities and agalactia
Facial eczema	*Pithomyces chartarum*	New Zealand, Australia, South Africa, South America, occasionally North America and Europe	Pasture litter	Sporidesmin	Sheep, cattle	Hepatotoxicity and biliary occlusion leading to photosensitization and jaundice
Fescue toxicity	*Neotyphodium coenophialum*	New Zealand, Australia, USA, Italy	Tall fescue grass	Ergovaline	Cattle, sheep, horses	Fescue foot: vasoconstriction and dry gangrene in cold weather; Summer fescue toxicosis: hyperthermia and agalactia
Fusarium toxicoses						
Oestrogenism	*Fusarium graminearum* and other *Fusarium* species	Worldwide	Maize, barley, pelleted cereals	Zearalenone	Pigs, cattle, sheep	Oestrogenic activity giving rise to hyperaemia and oedema of vulva, mammary development and reduced fertility

Leukoencephalomalacia	*Fusarium verticilloides* and other *Fusarium* species	South Africa, Egypt, Greece, USA	Maize	Fumonisins	Equidae	Liquefactive necrosis in cerebrum, neurological signs
Food refusal and emetic syndrome	*Fusarium graminearum* and other *Fusarium* species	Temperate countries	Cereals	Vomitoxin (Deoxynivalenol)	Pigs	Neurotoxicity, food refusal and emesis
Haemorrhagic syndrome	*Fusarium sporotrichioides, F. poae, F. tricinctum* and other *Fusarium* species	USA	Cereals	T-2 toxin, diacetoscirpenol	Cattle, pigs, poultry	Coagulopathy, immunosuppression, haemorrhages, necrotic lesions of epithelia
Mouldy sweet potato toxicosis	*Fusarium solani*	USA, Australia, New Zealand, Great Britain	Sweet potatoes	Derivative of 4-ipomeanol	Cattle	Interstitial pneumonia and pulmonary oedema
Mycotoxic lupinosis	*Diaporthe toxica*	Worldwide	Lupins	Phomopsins A, B, C, D, E	Sheep and occasionally other species	Hepatotoxic, acute cases present as hepatic encephalopathy, photosensitization and jaundice may also occur
Myrotheciotoxicosis	*Myrothecium* species	New Zealand, former USSR, parts of Europe	Ryegrass, white clover	Roridin	Sheep, cattle, horses	Sudden death, unthriftiness, abdominal pain
Ochratoxicosis	*Aspergillus ochraceus, A. alutaceus* and other *Aspergillus* species, *Penicillium verrucosum*	Worldwide	Barley, wheat, oats, rye	Ochratoxin A, B, C, D	Pigs, poultry	Nephrotoxin, liver toxin, immune suppressant, teratogen and carcinogen. Weight loss, polydipsia and polyuria

Continued

Table 44.1 Mycotoxicoses of domestic animals—cont'd

Disease	Fungus	Geographical distribution	Crop or substrate	Mycotoxin(s)	Species affected	Comments
Slaframine toxicosis	*Rhizoctonia leguminicola*	USA	Red clover in pasture and hay	Slaframine	Sheep, cattle, horses	Cholinergic effects such as salivation, lacrimation, urination, diarrhoea
Stachybotryotoxicosis	*Stachybotrys chartarum*	Europe, former USSR, South Africa	Hay, straw, stored cereals	Satratoxin, roridin, verrucarin	Horses, cattle, pigs, sheep	Cytotoxic and radiomimetic effect on tissues characterized by stomatitis, necrotic lesions in alimentary tract, haemorrhages
Tremorgens						Neurotoxicity characterized by incoordination, muscular tremors, convulsive episodes
Ryegrass staggers	*Neotyphodium (Acremonium) lolii*	New Zealand, Australia, USA, Europe	Perennial ryegrass	Lolitrem B	Cattle, sheep, deer, horses, pigs, poultry	
Penitrem staggers	Many *Penicillium* species and some *Aspergillus* species	Worldwide	Pasture, stored feed	Verruculogen, penitrem A, roquefortine	Many species, particularly ruminants	
Paspalum staggers	*Calviceps paspali*	New Zealand, Australia, Americas	Seedheads of paspalum grasses	Paspalinine, paspalitrems A, B, C	Cattle, sheep, horses	
Aspergillus clavatus staggers	*Aspergillus clavatus*	South Africa, China, Europe	Sprouted grains, millers' malt culms	Patulin	Cattle	

retain much of their activity after exposure to dry heat at 250°C and moist heat at 120°C but may be degraded by sunlight. They have a low molecular weight and are non-antigenic in their native state.

When growing in maize, *A. flavus* usually produces B_1 and B_2 aflatoxins, while *A. parsiticus* produces all four of the major aflatoxins. On soybeans, only low concentrations of AFB1 are produced by both species. Mould growth and toxin formation require a moisture content of the substrate greater than 15%, a temperature of at least 25°C and adequate aeration. Toxin formation can occur in a matter of hours when favourable conditions exist.

Biological effects of aflatoxins

The toxic effects of aflatoxins are dose-, time- and species-dependent. Mature ruminants are less susceptible to the effects of mycotoxins than young animals and monogastric animals. The toxins are absorbed from the stomach and metabolized in the liver to a range of toxic and non-toxic metabolites which are then excreted in urine and milk. The major biological effects of aflatoxins include inhibition of RNA and protein synthesis, impairment of hepatic function, carcinogenesis and immunosuppression. AFB_1 is bioactivated in the liver to a highly reactive intermediate compound which reacts with various nucleophiles in the cell and binds covalently with DNA, RNA and protein. After deliberate administration of AFB_1 there is marked interference with protein synthesis at the translational level which seems to correlate with disaggregation of polyribosomes in the endoplasmic reticulum. Many of the toxic responses observed in animals resulting from AFB_1 activity can be attributed to alterations in carbohydrate and lipid metabolism and interference with mitochondrial respiration.

The biological effects of aflatoxins, observed clinically, can be divided into two categories: short-term effects and long-term effects, depending on the dosage level and frequency of exposure to the toxin. Short-term effects include acute toxicity with clinical evidence of hepatic injury and nervous signs such as ataxia and convulsions. In acutely affected animals death may occur suddenly. Long-term consumption of low levels of aflatoxins probably constitutes a much more serious veterinary problem than acute, fulminating outbreaks of aflatoxicosis. With chronic aflatoxicosis there is reduction in efficiency of food conversion, depressed daily weight gain, decreased milk production in dairy cattle and enhanced susceptibility to intercurrent infections in most species due to immunosuppression. AFB_1 is also an extremely potent hepatocarcinogen in many species of animals.

The principal target organ of these mycotoxins in all species is the liver. Depending on the severity and duration of the intoxication, lesions in the liver may vary from acute swelling with hepatocellular necrosis and bile retention to cirrhosis and marked bile duct hyperplasia. Reduction of hepatic function and increased serum enzyme activities indicative of hepatic cell necrosis are common sequelae to aflatoxin-induced hepatic injury. Although not related to pyrrolizidine alkaloids, aflatoxins produce similar liver changes. As liver function is progressively altered, other effects such as coagulopathy, icterus, serosal and mucosal haemorrhages may occur. Acute hepatic failure and massive haemorrhage due to impaired blood clotting and increased capillary fragility leading to death, may occur with higher doses.

In addition to liver damage, higher doses may cause degenerative changes in the proximal tubules of the kidney. The thymus is also affected and AFB_1 induces thymic cortical aplasia leading to depressed cell-mediated responses. Humoral immunity appears to be affected minimally, but reduced complement levels and decreased phagocytic activity have been reported in aflatoxin-treated animals. Young animals are reported to be notably more susceptible to aflatoxin poisoning than mature animals of the same species. Young pigs, calves, turkey poults and ducklings are particularly susceptible to these fungal toxins.

Aflatoxins are extremely potent hepatocarcinogens in many animal species. AFB_1 is one of the most carcinogenic compounds known for the rat and for the rainbow trout. Experimentally, the carcinogenic activity of pure AFB_1 has been confirmed in rats, duck, pigs, trout and monkeys. Epidemiological studies of primary hepatocellular carcinoma in man indicate that aflatoxins are aetiologically involved. Hepatitis B virus and aflatoxins are believed to act synergistically as hepatocarcinogens in man. Teratogenic and embryotoxic effects of aflatoxins have been reported in chickens, Syrian golden hamsters, mice and pigs.

Diagnosis of aflatoxicosis: clinical aspects

Sporadic outbreaks of disease associated with a particular consignment of feed, accompanied by unthriftiness, inappetance and vague signs of illness may suggest the presence of a hepatotoxin in the rations. Clinical signs vary with the susceptibility of individual species to aflatoxins. Prominent signs in calves include blindness, circling, grinding of the teeth, diarrhoea and tenesmus. Elevated plasma aspartate aminotransferase, gamma-glutamyl transferase and alkaline phosphatase are likely findings. Terminally, convulsions may occur. Postmortem findings include a pale, firm fibrosed liver usually with centrilobular necrosis and bile duct proliferation. The kidneys of affected cattle may be yellow; ascites and oedema of the mesentery may be present. Blood coagulation defects are likely to occur when extensive liver damage is present. In dairy cattle, aflatoxins M_1 and M_2, hydroxylated metabolites of B_1 and B_2, are excreted in the milk and are of public health significance, if present in appreciable amounts. Milk containing AFB_1 proved hepatotoxic when

fed to ducklings. AFM_1 is stable in cheese made from naturally contaminated milk for at least three months. Other ruminants vary in their susceptibility to these toxins. Aflatoxicosis has been described in goats, but sheep appear to be very resistant and most of the aflatoxin administered to sheep appears to be degraded in the body.

Aflatoxicosis in pigs produces a range of clinical signs including drowsiness, inappetance, jaundice, weight loss and yellow urine. Changes in the activities of liver-specific enzymes usually parallel the extent of hepatic lesions. A depression of acquired immune responses has been observed in pigs with aflatoxicosis.

Ducklings are considered to be the avian species most susceptible to aflatoxins. Signs of acute disease include anorexia, poor growth rate, ataxia and opisthotonos, followed by death. In birds over three weeks of age, subcutaneous haemorrhages of legs and feet may be evident. In both acute and chronic aflatoxicosis, liver lesions are a common finding. Prolonged exposure to low levels of aflatoxins leads to marked nodular hyperplasia of the liver, bile duct proliferation, fibrosis and hepatocellular carcinoma. Ducklings have been used for biological assays because of their rapid response to aflatoxins.

Acute toxicity of aflatoxins in chickens and turkeys may be characterized by haemorrhage in many tissues, liver necrosis and jaundice. Aflatoxicosis increases the susceptibility of turkeys to pasteurellosis and salmonellosis. Chickens become more susceptible to coccidiosis and Marek's disease, presumably because of the immunosuppressive effect of aflatoxins. When layers are fed contaminated feed, aflatoxins are found in eggs, principally in the yolk.

Outbreaks of aflatoxicosis have been described in dogs scavenging garbage, and they appear to be particularly susceptible to AFB_1. Chronic aflatoxicosis is associated with loss of weight, jaundice and ascites. Lesions include subserosal and submucosal haemorrhages in the thoracic and peritoneal cavities and a yellow mottled liver.

Laboratory investigation of outbreaks

Diagnosis of aflatoxin poisoning in animals requires careful consideration of epidemiological factors, clinical signs in affected animals and postmortem findings. Laboratory examination of suspect material may assist in identifying potentially toxigenic fungi growing in food. Chemical identification of mycotoxins in food samples submitted and biological assays for toxicity are important confirmatory steps in field investigations. Cultural examination of food may show the presence of potentially toxigenic fungi (*A. flavus* and *A. parasiticus*) but this is not diagnostic of aflatoxicosis. Laboratory confirmation of mycotoxicoses requires the demonstration of toxigenic strains of *A. flavus* or *A. parasiticus* and of potentially toxic levels of mycotoxins in the food or tissues in conjunction with appropriate clinical or pathological findings. Concentrations of AFB_1 in excess of 100 µg/kg of feed are considered toxic for cattle.

The analytical procedures for detecting mycotoxins generally follow a standard pattern: sampling, extraction, clean-up, separation, detection, quantitation and confirmation. Mycotoxins are rarely uniformly distributed in natural products such as cereal grains. Aflatoxins are generally found in high concentrations at sites where toxigenic fungi have invaded the crop or stored feed. Accordingly, when investigating suspected field outbreaks of aflatoxicosis, a representative sample of the entire batch is necessary in addition to a sample from contaminated areas. A 5-kg sample, taken into a clean dry container should be labelled with relevant information and stored at −20 °C. Mycotoxin formation is continuous when temperature, moisture, aeration and substrate are favourable, therefore it is necessary to stop aflatoxin formation in the sample at the time of collection by freezing.

A number of rapid, economical analytical methods are available for determining aflatoxin levels in a wide range of agricultural food products. Thin-layer chromatography (TLC) is relatively inexpensive and a number of samples can be analyzed simultaneously. The chromatogram is viewed under ultraviolet light for blue or green fluorescent spots that agree in colour and location with internal and external standards (Fig. 44.3). Many different TLC methods have been reported including two-dimensional chromatography for differentiating co-extracted substances. Mini-column detection methods are employed for rapid screening procedures. Although more expensive, high-performance liquid chromatography (HPLC) is easier, faster and gives more sensitive and more reproducible results than TLC.

Although aflatoxins are low-molecular-weight substances, they can be conjugated to protein or polypeptide

Figure 44.3 Thin-layer chromatography plate of aflatoxins under ultraviolet light showing blue, B_2 (extreme left) and green, G_2 (extreme right) fluorescence.

carriers and subsequently used for immunization. A number of immunoassay methods are currently available including radioimmunassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

Biological assays for aflatoxins include bile duct proliferation in one-day-old ducklings, chick-embryo bioassay for $AFB_{1,}$ brine shrimp larvae tests, mutagenicity tests with different bacteria and trout-embryo bioassay for carcinogenicity.

Control and prevention of aflatoxicosis

Prevention of contamination at all stages of food production, storage and use is the preferred method of preventing aflatoxicosis. This can be accomplished by reducing fungal infections in growing crops, by rapid drying of harvested crops or by using effective antifungal preservatives. Effective methods have been devised for minimizing fungal damage of many food crops, for detection of aflatoxins at low levels and for detoxification of contaminated products. Quality control measures can only be applied, however, where the necessary level of technological services is available during harvesting, storage, food processing and distribution. The risk of aflatoxin contamination is often greatest in regions where detection measures are unavailable, particularly where climatic conditions favour mould damage to crops and stored food.

Decontamination strategies proposed include physical removal, thermal inactivation, irradiation, microbial degradation and chemical treatment of aflatoxin-contaminated feeds. Physical removal and chemical inactivation of toxins in contaminated feed have been used commercially in some countries. Acids, alkalis, aldehydes, oxidizing agents and selected gases have been used for degrading aflatoxins. Ammonia gas at elevated temperatures and pressures cleaves aflatoxin molecules and it is claimed that ammoniated feeds can be safely fed to animals. Suspect feed or aflatoxin-contaminated material that has been decontaminated should not be fed to dairy cattle because of the danger of AFM_1 transfer into milk; such feed is also unsuitable for young pigs, calves or turkey poults because of their high susceptibility to aflatoxin. High-affinity inorganic adsorptive compounds such as hydrated sodium calcium aluminosilicate or glucomannan-containing polymers added to the diet have been reported to bind aflatoxins and reduce their bioavailability and toxicity. In many countries legislation has been introduced to regulate the maximum acceptable levels of aflatoxins in animal feeds and human food. Levels of AFB_1 in the region of 100 μg/kg are considered toxic for cattle; for trout the limit is zero.

After harvesting and during shipment, storage or compounding of agricultural crops, growth of *A. flavus* and *A. parasiticus* is influenced by moisture levels, temperature, aeration, mould spore density, conditions of storage, particularly leakage of water into storage containers or condensation, and by biological heat and the chemical nature of the crop. The most critical environmental factors for aflatoxin production are moisture content, temperature and time. Where physical methods of storage are unsatisfactory, chemical preservatives should be considered. The addition of selected chemicals at harvesting can reduce fungal growth where other methods cannot be used. Various organic acids such as benzoic and propionic acid have been widely used as preservatives for stored agricultural products. However, it should be noted that sub-inhibitory levels of propionic acid may actually stimulate aflatoxin biosynthesis.

Diplodiosis

Diplodiosis is a neuromycotoxicosis of cattle and sheep grazing on harvested maize lands that contain mouldy cobs of maize. The fungus involved, *Stenocarpella* (*Diplodia*) *maydis*, which is responsible for stem and ear rot of maize, produces characteristic fruiting bodies or pycnidia on affected parts towards the end of the growing season containing the neurotoxin diplonine (Snyman et al. 2011). Ingestion of maize infested with this fungus produces a characteristic neurotoxic disease. Affected animals show ataxia, paresis, paralysis, lacrimation and salivation. The mortality rate may be high if grazing animals are not removed promptly from contaminated lands. Gross pathological changes are not usually present in diplodiosis although spongiform lesions may be found in the brains of affected animals.

Ergotism

Ergotism in animals results from the ingestion of grasses and cereals, particularly rye, infected with fungal species of the genus *Claviceps*, notably *Claviceps purpurea*. The fungus colonizes the seed head and the ovarian tissue is destroyed and replaced by a soft mycelial mat which enlarges, hardens and darkens to form sclerotia. The word ergot, which is the French term for a rooster's spur, accurately describes the compacted mass of hyphae that projects from the ear as a dark, purplish-black, misshapen replica of the original seed. Although ergotism is one of the oldest mycotoxic conditions known, with descriptions of the condition dating to biblical times, it was not until the middle of the 18th century that an association between the ingestion of rye infected with *Claviceps purpurea* and clinical ergotism was established. Major epidemics of human ergotism were recorded in central Europe from the 12th to the 18th centuries. Few epidemics of ergotism in the human population have been recorded in recent years as a result of modern methods of grain cleaning. Ergot poisoning of livestock from consumption of infected grain or grazing pastures containing grasses with ergotized seed heads is relatively common. Toxicity may be retained in silage.

Claviceps purpurea has a complex life cycle which takes a year to complete. The sclerotia overwinter on the soil and during the following spring they produce several long-stalked, mushroom-like stromata with globose heads containing perithecia that open to the surface. Within each perithecium, several cylindrical asci are formed, each containing thread-like ascospores. When susceptible grasses are flowering in the spring, the ascospores are forcibly discharged from the perithecia. The wind-borne ascospores germinate when they reach cereal rye, rye-grasses or other suitable grass flowers. The heaviest infestation occurs when germination of the over-wintered sclerotia coincides with the flowering of the grasses. The tissue of the ovary is replaced by a soft mycelial mat that becomes covered by layers of short conidiophores bearing one-celled conidia mixed with sticky exudate. At this stage insects may spread the conidia and cause secondary infections of other plants. The mycelial mats in infected ovaries enlarge, harden and darken to form sclerotia that completely replace the grain or grass seed and repeat the life cycle (Fig. 44.4).

Ergot alkaloids

The sclerotia or ergots contain the toxic alkaloids (Fig. 44.5). Ergot alkaloids are derivatives of lysergic and isolysergic acids. Although more than 40 alkaloids have been isolated from the sclerotia of *C. purpurea*, the major toxic alkaloids are ergotamine and ergometrine. In addition, the sclerotia contain a large number of amines and other compounds with physiological activity.

Two forms of ergotism are observed in animals, gangrenous and convulsive ergotism. The ergot alkaloids, particularly ergotamine, stimulate and then depress the central nervous system when taken in large amounts. They also exert a direct stimulatory effect on constrictor adrenergic nerves supplying arteriolar smooth musculature and inhibit prolactin secretion. When consumed in small amounts over long periods, they produce arteriolar spasm, capillary and endothelial damage resulting in vascular stasis, thrombosis, ischaemia and gangrene of the affected part. Convulsive ergotism, characterized by neurotoxicity, is an uncommon, acute form of the disease. Towards the

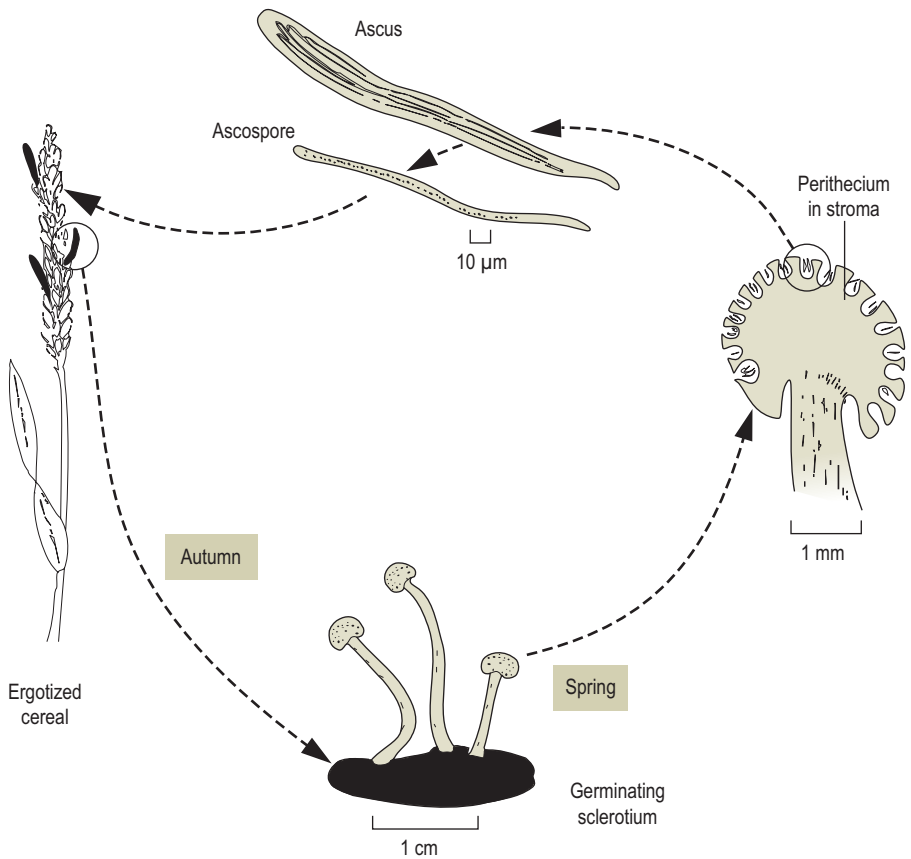

Figure 44.4 Life cycle of *Claviceps purpurea*.

Figure 44.5 Ryegrass with sclerotia of *Claviceps purpurea*. Arrow indicates a free sclerotium.

end of pregnancy, ergot alkaloids may exert an oxytocin-like effect on the pregnant uterus, but this response only occurs late in gestation. While abortions have been described in cattle consuming ergotized grass, it is doubtful if the mycotoxins produced by *C. purpurea* are responsible for abortion in this species. Premature births, low litter size and mummified foetuses have been attributed to chronic poisoning in pigs and experimental exposure to ergotamine has caused abortions and foetal deaths in sheep.

Clinical findings

Gangrenous ergotism affects most species of domestic animals. Gangrenous necrosis of the extremities – nose, ears, tail, teats and limbs – following arteriospasm, congestion and endothelial cell degeneration of the capillary bed is a common clinical finding in many species of animals. In poultry, the tongue, comb and wattles are frequently affected.

Cattle grazing ergotized pasture or fed contaminated grain or silage develop lameness and gangrene as a major clinical sign of ergot toxicity. Lameness may appear about two weeks after initial ingestion, depending on the concentration of alkaloids in the ergot and the quantity of ergot in the feed. Hind limbs are usually affected before forelimbs. An elevated body temperature and increased pulse and respiration rates may accompany the lameness. Affected limbs are often swollen with a defined line separating the normal tissue from the dry, gangrenous extremity (Fig. 44.6). A cold environment predisposes the extremities to gangrene. The affected part, which gradually loses sensation, may eventually slough. The tips of the ears or tail may become necrotic and the teats and udder may appear unusually pale. The nervous form of ergotism, attributed to *C. purpurea*, is uncommon in cattle, and is accompanied by muscular incoordination, tremors, blindness and convulsions. Dairy cattle on ergotized feed show a sharp drop in milk production.

Figure 44.6 Ergotism in a cow: a swollen right hind leg showing a line of separation and terminal gangrene. The left hind limb is unaffected.

Sheep with the acute form of ergotism may show nervous signs. Gangrenous ergotism seems to be uncommon in this species. Ulceration and necrosis of the tongue and the mucosa of the digestive tract may be observed in chronic forms of the disease. Classical signs of convulsive and gangrenous ergotism are not described in pigs. Chronic ergotism results in lack of udder development and agalactia in sows and the birth of small pigs. Low litter sizes, premature births and lack of vitality in both sows and piglets have been associated with the chronic form of this disease.

Diagnosis

Diagnosis of ergotism is based on the demonstration of sclerotia on pasture, in grains or in silage and on the signs of disease in affected animals. Extraction of ergot alkaloids and detection by chromatography, or biological testing may be necessary in suspect ground grain meals or in processed feeds.

Prevention of ergotism

The occurrence of ergotism on growing pasture or in grain is related to the susceptibility of the crop, climatic factors and agricultural practices. Sclerotia are stimulated to germinate by low winter soil temperatures and damp conditions. Ergot infestation of grain fields can be minimized by using clean seed, crop rotation and deep cultivation. Climatic factors such as cold wet springs, which prolong the flowering period tend to increase the level of primary infection. Ascospore dispersal is favoured by windy conditions and

maturation of the sclerotia is enhanced by warm summers. Frequent grazing or topping of pastures prone to ergot infection during the summer months will reduce flower head production and help prevent the disease.

Ergotized grain or pastures containing ergotized grasses should not be used for animal feeding. Separation of ergots from sound grain can be achieved by mechanical or flotation techniques when small batches of grain are contaminated. Growing ergot-resistant grains such as wheat, or barley, rather than rye may be advisable in areas where ergotism is a recurring problem.

Facial eczema

Facial eczema is a hepatogenous photosensitivity of sheep and cattle caused by the ingestion of conidia of *Pithomyces chartarum*. The mycotoxin, sporidesmin, is contained in the conidia which become dispersed by wind and water and adhere to pasture which is then ingested by grazing animals. The fungus is widely distributed and the disease has been reported in New Zealand, Australia, South Africa, several South American countries and in southern France. Not all isolates of the fungus are toxigenic.

Pithomyces chartarum grows saprophytically on dead plant material littering pasture. Field outbreaks of facial eczema typically occur after a period of warm, rainy weather, following a drought during the summer. The drought results in the death of grass leaves, which act as a substrate for the saprophytic growth of *P. chartarum*. Although the disease is commonly associated with ryegrass pastures the fungus is capable of growing on many kinds of dead leaf material. Climatic conditions which favour sporulation of the fungus have been accurately defined in New Zealand. Sporidesmin is produced in the fungal spores as *P. chartarum* grows in dead leaves at the base of the pasture and the spores are then carried up through the dead material by new grass growth, which is then consumed by grazing animals.

Sporidesmin produces liver damage and biliary occlusion which result in secondary photosensitization. Phylloerythrin, a photodynamic pigment that is a degradation product of chlorophyll produced in the digestive tract of ruminants by enteric organisms, is normally excreted in bile. As a result of the progressive liver damage, there is inadequate excretion of absorbed phylloerythrin and it accumulates in the blood. When the accumulated phylloerythrin reaches the peripheral circulation and skin, it becomes activated by absorbing energy from ultraviolet radiation of sunlight and photosensitization occurs. The liver damage also results in the accumulation in the blood of bilirubin leading to signs of jaundice. There is a latent period of 10–14 days between ingestion of sporidesmin and manifestation of photosensitivity. This interval corresponds to the time taken for the development of obliterative cholangitis and the subsequent retention of phylloerythrin.

Figure 44.7 Sheep with facial eczema (*Pithomyces chartarum*). Lesions of photosensitization on non-wool areas.

Clinical signs of facial eczema occur suddenly when animals are exposed to sunlight. In sheep the first observable signs are swelling and reddening of the exposed parts of the skin on the head, ears and lips (Fig. 44.7). The animals stop grazing when their lips become irritated. Exudation, with scab formation follows and eventually skin necrosis. Jaundice is invariably present. Affected animals have photophobia and seek out any shade available. Mortality can be high if photosensitive animals are not removed from sunlight. In cattle, apart from lesions on the head and non-pigmented parts of the body, the teats and udder may be severely affected.

Postmortem findings include jaundice and a swollen mottled liver with thickened bile ducts. Moderate to severe portal fibroplasia and bile duct proliferation are consistently present. Other findings are degenerative and haemorrhagic lesions in the gall and urinary bladders. Elevated serum liver enzymes are found in affected animals. Sporidesmin can be detected in bile, urine, plasma or whole blood using a competitive ELISA (Briggs et al. 1993).

Three important control measures have been used with success in New Zealand:

- Recognition and avoidance of toxic pastures
- Reduction in the numbers of toxic conidia in pastures by applying fungicides
- Management of animals to restrict intake of toxin and treatment of affected animals.

The accumulation of pasture litter can be controlled by pasture management techniques. Daily oral administration of zinc salts has been reported to reduce the toxic effects of sporidesmin.

Fescue Toxicity

Tall fescue grass (*Festuca arundinacea)* is widely cultivated in the USA and in parts of Australia and New Zealand. Cattle and sheep grazing pastures consisting mainly of this

grass may develop sporadic intoxication. Two diseases are associated with tall fescue grass, namely fescue foot and fescue summer toxicosis.

Fescue foot is a disease of cattle grazing fescue pastures in late autumn and early winter. Affected cattle develop ischaemic necrosis of the skin of the extremities including the fetlock, ears and tail. The hooves and portions of the tail may slough off and the disease is clinically indistinguishable from gangrenous ergotism. The herd incidence of disease may be as high as 10%. The poisonous effects of tall fescue grass are associated with the presence in the grass of the endophytic fungus, *Neotyphodium* (*Acremonium*) *coenophialum*, but other fungi may be involved also. The lesions are caused by a vasoconstrictive alkaloid, ergovaline, whose effects are similar to ergotism.

Summer fescue toxicosis is associated with high environmental temperatures. Clinical signs include reduced weight gain, a drop in milk production, a dull rough hair coat and elevated body temperature. The hyperthermia has been attributed to peripheral vasoconstriction by ergovaline. The disease has also been observed in animals fed on hay made from affected pastures.

Fusarium Toxicoses

The genus *Fusarium* is the largest single group of fungi with known toxigenic capability. Fungi of this genus and related organisms with sexual (perfect) reproduction produce a number of mycotoxins which are capable of causing a variety of diseases in animals. Because of their close association with plants and their relatively high water activity requirements for growth, fusaria are usually well established in a crop before harvesting and may cause many problems in cereals following a late harvest after a wet summer. Although some species such as *Fusarium moniliforme* are particularly associated with tropical and subtropical climates and others such as *F. sporotrichorides* with cold climates, many species occur in temperate parts of the world. Two general categories of toxins are produced by *Fusarium* species: the oestrogenic metabolites such as zearalenone (also referred to as F-2 toxin) and the trichothecene toxins. The fusaria generally produce mycotoxins at temperatures below those supporting optimal mycelial growth and some species have been reported to form several toxins. These fungi can germinate, grow and produce toxins in a wide variety of plants and feedstuffs. *Fusarium* species tend to produce highly coloured colonies, both obverse (Fig. 44.8) and reverse (Fig. 44.9) with banana-shaped macroconidia (Fig. 44.10).

Oestrogenism

This oestrogenic syndrome was first described in the USA more than 80 years ago. The disease, then termed vulvovaginitis, was associated with the consumption of mouldy maize by gilts. *Fusiarium graminearum* (teleomorph

Figure 44.8 *Fusarium* species on Sabouraud dextrose agar, seven days.

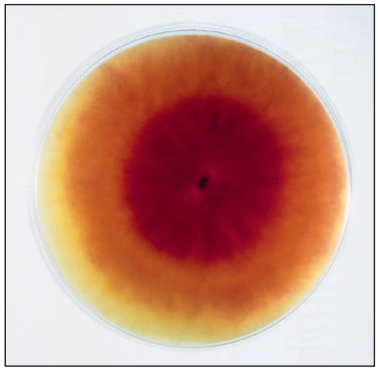

Figure 44.9 *Fusarium* species on Sabouraud dextrose agar, seven days. Reverse.

Figure 44.10 *Fusarium* species showing typical banana-shaped macroconidia. (LPCB, ×400)

Gibberella zeae) and other *Fusiarium* species growing on maize, barley and other grains produce zearalenone, a phenolic resocyclic acid lactone with oestrogenic activity. The target organ system is the reproductive tract and pigs are the animals most commonly affected by this mycotoxin, although other species may also be affected. Oestrogenic syndromes in pigs fed mouldy grain have been recognized worldwide. Although commonly included in articles on myctoxicosis, zearalenone is biologically active rather than toxic and is better described as a myco-oestrogen.

Infection of susceptible maize by *F. graminearum*, a phytopathogen, is favoured by cool, wet weather during the final stages of growth of the crop. The production of zearalenone in infected maize is enhanced by conditions of high moisture and alternating moderate and low temperatures. Sometimes *Fusiarium* fungi may be absent from the sample of feed but zearalenone may still be present as it is a stable compound.

Pigs are the most sensitive animals, especially sexually immature gilts. Clinical signs of oestrogenism occur about one week after consumption of contaminated feed and include hyperaemia and oedema of the vulva, enlargement of the mammary glands and in severe cases prolapse of the vagina and rectum due to mucosal irritation and straining. Although the morbidity rate is high and may approach 100%, the mortality rate is usually low. Affected sows may have anoestrus, pseudopregnancies, infertility, decreased litter size with small weak piglets that may have splayed limbs. In boars, swelling of the prepuce and atrophy of the testicles has been reported.

Porcine oestrogenism is sometimes erroneously referred to as 'vulvovaginitis'. The latter term is an incorrect description as there are usually no inflammatory changes in the genital tract and the alterations are principally physiological in nature due to interstitial oedema and proliferation of epithelium in the vagina and cervix. The thickened endometrium is oedematous and the submucosal glands are hyperplastic. Clinical signs usually disappear about seven days after withdrawal of contaminated feed.

Cattle appear to be much more resistant than pigs to the oestrogenic effects of cereals infected with *F. graminearum*, although zearalenone can lower the conception rate in heifers. Chickens appear to be minimally affected.

Zearalenone can be demonstrated in feeds by thin-layer or gas chromatography. Administration of extracts of zearalenone-contaminated feed to sexually immature female mice causes uterine engorgement and hypertrophy. Zearalenone is secreted into milk, if dairy cattle are fed *F. graminearum*-infected cereals and may be of public health concern. An ELISA has been developed for detecting zearalenone in urine and pasture samples.

Equine leukoencephalomalacia

Leukoencephalomalacia, also called mouldy corn poisoning, is a highly fatal neuromycotoxicosis of *Equidae* caused by the ingestion of maize infected with *Fusarium* species, particularly *Fusiarium verticillioides*. The disease is characterized by focal liquefactive lesions of the cerebral white matter.

The fungus infects maize in the field and produces several mycotoxins, in particular fumonisin B_1. The most common form of the disease is the neurotoxic form which is characterized by acute and severe neurological disease and is usually fatal. This form of the disease is called equine leukoencephalomalacia. Equine leukoencephalomalacia has been recognized in the USA since the latter part of the 19th century and subsequently in many other countries including South Africa, Egypt, China, Brazil and Argentina. Field outbreaks of disease are associated with the feeding of mouldy maize, particularly in wet seasons preceded by a drought.

The first signs of disease appear abruptly and consist of unthriftiness, reluctance to move backwards, muscular tremors, locomotory disturbance and ataxia. As the disease progresses, nervous signs become more pronounced and the animal walks into objects and later may become frenzied and run wildly into objects. Inability to swallow and paralysis of the lower lip may be evident. Death after two or three days may be preceded by recumbency and paddling limb movements. The pathognomonic lesions of this disease are one or more areas of liquefactive necrosis in the white matter of the cerebral hemispheres. Histopathologically, the encephalomalacic areas appear as large irregular empty spaces where the white matter of the brain has disintegrated. Perivascular haemorrhage and oedema may be present also.

Trichothecene toxicoses

Many *Fusarium* species produce a number of sesquiterpene metabolites called trichothecenes and some of these mycotoxins, especially vomitoxin (deoxynivalenol, DON), T-2 toxin and diacetoxyscirpenol (DAS) are implicated in mycotoxicoses in different species of animals. Although trichothecene toxicoses involve a broad spectrum of clinical syndromes, those that are most frequently recognized include food refusal and emetic syndrome and haemorrhagic syndrome. The degree to which these effects are manifested varies greatly among different species. Fusaria may produce several different mycotoxins depending on the substrate and growth conditions. Accordingly, multiple toxins may occur in the same batch of mouldy food. The pharmacological effects of trichothecenes centre on potent inhibition of eukaryotic protein synthesis. Consequently these toxins affect most cell types and often produce radiomimetic effects in tissues.

Food refusal and emetic syndromes

One of the most commonly occurring mycotoxins in animal feeds is vomitoxin. Pigs refuse to consume *F.*

graminearum-infected maize, barley or mixed feeds containing these cereals. Emesis may occur in animals consuming small quantities of infected cereals. Vomitoxin appears to be the principal mycotoxin responsible for the feed-refusal and emetic syndromes produced by this fungus although other tricothecenes such as T-2 toxin and DAS may also be involved. *Fusarium graminearum* also produces the oestrogenic mycotoxin, zearalenone. Emesis is apparently not due to local irritation of the gastric mucosa as parenteral administration of emetic extracts induces vomition, suggesting that the response is centrally mediated.

Haemorrhagic syndrome

Trichothecene intoxication can result in haemorrhagic syndromes in cattle, pigs and poultry. Toxic effects on the marrow, thrombocytopenia and other induced blood changes result in defective blood coagulation. Many of these changes are attributed to the inhibitory effects of trichothecenes on protein synthesis.

Other biological effects of trichothecene toxins

T-2 toxin and DAS are potent epithelial necrotizing agents causing necrosis and ulceration of epithelial surfaces of the skin, mouth and upper gastrointestinal tract following contact with contaminated feed. T-2 toxin has an immunosuppressive effect on growing pigs, affecting both T and B lymphocytes. Lymphocytopenia follows exposure to T-2-contaminated feed; serum protein levels decline, haemorrhagic lesions occur in the digestive tract and degenerative lesions are seen in the liver and kidneys. There is some evidence that T-2 toxin is both mutagenic and teratogenic.

Mycotoxic Lupinosis

Lupins (*Lupinus* species) are capable of causing two distinct forms of poisoning in livestock; one, a nervous syndrome caused by toxic alkaloids in the seeds and a second disease referred to as mycotoxic lupinosis caused by fungi growing on the plant. *Diaporthe toxica* (anamorph, *Phomopsis leptostromiformis*) is a phytopathogen (cause of stem blight) of certain *Lupinus* species which induces a hepatic syndrome in sheep and sometimes other species, called lupinosis. This disease has been reported in Australia, New Zealand, South Africa and in a number of European countries. The fungus invades the growing plant in the field and after death of the diseased plant, *D. toxica* continues to grow saprophytically on the dead plant tissue. Mycotoxins produced by the fungus, particularly during the saprophytic phase of growth, are potent hepatotoxins. Clinical signs of acute disease include inappetance, ruminal stasis and jaundice. Photosensitization may also occur. The most obvious postmortem findings in acute lupinosis are discoloration of the liver, jaundice and serosal haemorrhages. Bilary duct proliferation, fibroplasia of portal ducts and cirrhosis, are common histological findings. Five mycotoxins referred to as phomopsins, are produced by *D. toxica*. Phomopsin A, the principal mycotoxin has been characterized as a cyclic hexapeptide.

Myrotheciotoxicosis

Myrothecium species, particularly *Myrothecium roridum* and *Myrothecium verrucaria* are noted plant pathogens and produce toxic metabolites belonging to the trichothecene group. A high prevalence of these fungi on ryegrass and white clover plants has been reported in New Zealand. Clinical signs appear within 48 hours of introduction of animals to affected pastures and resemble those of non-specific chemical rumenitis. Depression, salivation, abdominal pain, haemoconcentration and dehydration are features of this disease. Postmortem findings include ruminal and abomasal distension with hyperaemia and desquamation of the mucosa and patchy ulceration. Microscopically there is widespread necrosis of the mucosa of the abomasum, reticulum and rumen. A syndrome similar to myrotheciotoxicosis is seen in cattle grazing Kikuyu grass (*Pennisetum clandestinum*); a mycotoxin may be responsible (Bourke 2007).

Ochratoxicosis and Citrinin Toxicosis

Several *Aspergillus* and *Penicillium* species, particularly toxigenic strains of *Aspergillus ochraceus, A. alutaceus* and *Penicillium verrucosum* produce ochratoxins, a group of related isocoumarin derivatives. Ochratoxin A is the principal nephrotoxic mycotoxin in this group. It impairs kidney function in many species, particularly pigs and poultry. Natural production of ochratoxin occurs primarily in spoiled, stored barley, wheat and maize in the northern hemisphere. Ochratoxin A is a stable compound which is only partially destroyed by heat processing and autoclaving. The primary target organ of ochratoxin is the kidney, but high doses may produce liver damage also. A condition known as 'mycotoxic nephropathy' has been recognized for many years in pigs in Scandinavia. The mycotoxin citrinin, which can also be produced by *A. ochraceus* as well as by *Penicillium citrinum, P. viridicatum* and *P. expansum*, is nephrotoxic. Citrinin, frequently found together with ochratoxin A in affected foodstuffs, can enhance the effects of ochratoxin A.

In pigs, clinical signs of chronic ochratoxicosis include reduced food intake, loss of body weight, depression, polydipsia and polyuria. The mortality rate is usually low. Macroscopically, the kidneys may be enlarged and pale with a mottled surface. Histologically, the renal lesions are characterized by degeneration of the proximal tubules and interstitial fibrosis of the cortex. Poultry

affected by ochratoxicosis show a depressed growth rate, coagulopathy and poor-quality eggshells. Postmortem findings include pale kidneys and liver, intestinal congestion and sometimes ruptured large intestines. Ruminants, especially adult ruminants, appear to be less susceptible to ochratoxicosis than monogastric animals. The flora of the adult rumen has been shown to degrade ochratoxin A.

Ochratoxins can cross the placental barrier in some species and exert a teratogenic effect. They are also immunosuppressive, particularly in relation to antibody production. Many of the biological effects of ochratoxin A relate to interference with protein synthesis at the translational level. Ochratoxin formation is primarily a grain storage problem and detection of these mycotoxins requires solvent extraction followed by thin-layer chromatography of separated fractions. Ochratoxins fluoresce yellow-green under ultraviolet light.

Slaframine Toxicosis

Slaframine is a cholinergic mycotoxin produced by the growth of *Rhizoctonia leguminicola* on red clover pasture or hay. Clinical manifestations in cattle and horses include excessive salivation ('slobbers'), urination, lacrimation and diarrhoea. Decreased milk production may occur in dairy cattle. Weight loss and abortion may also occur.

Stachybotryotoxicosis

Stachybotryotoxicosis is a trichothecene-induced disease produced by *Stachybotrys chartarum (S. atra)* growing on hay or straw. Most reports of this intoxication come from the former Soviet Union, eastern European countries and South Africa. The saprophytic, cellulose-degrading fungus, *S. chartarum*, growing on harvested crops produces mycotoxins during storage. Contaminated material is not only toxic when ingested but also when used as bedding as the conidia are toxic on contact or when inhaled. Horses, cattle, sheep and other species may be affected. The disease, which is best characterized in the horse, progresses in stages. After consumption of mould-infested hay or straw over several weeks, stomatitis, hyperaemia and necrosis of the oral mucosa and the tongue may be evident. The second stage may be asymptomatic with depression of leukocytes and thrombocytes. This is followed by a severe and progressive depression of leukocytes and thrombocytes, presumably due to toxic effects on haematopoietic tissues. There is impaired clotting with consequential haemorrhage.

Secondary bacterial infection results in an elevated temperature and death frequently results from haemorrhages and septicaemia. Cattle appear to be less susceptible to stachybotryotoxicosis than horses but otherwise the course of the disease is similar. Postmortem findings include haemorrhages and necrosis in many tissues. Necrosis is particularly evident along the gastrointestinal tract and ulcers may be present in the large intestine. Histopathological lesions include degenerative changes in the liver, kidney and myocardium. Atrophy and necrosis of lymphoid tissue and aplastic anaemia are also present.

At least five trichothecenes are produced by *S. chartarum* including satratoxins, verrucarin and roridin. These toxins are cytotoxic and have a marked radiomimetic effect especially on lymphoid tissues, bone marrow and epithelial tissue. Damage to these tissues results in leukopenia, anaemia, thrombocytopenia and immunosuppression. Under field conditions, poisoning is usually chronic following low level intake of toxins over a prolonged period. Disease outbreaks can be minimized by ensuring that straw and hay are kept dry during storage and by prompt removal of contaminated feed and bedding.

Tremorgens

A heterogeneous group of compounds called tremorgens produced by toxigenic fungi produce nervous signs when ingested or administered to animals. Many of these mycotoxins produce similar clinical signs, often without obvious morphological changes in nervous tissue. Signs frequently develop after excitement or strenuous exercise, while recovery is usually rapid following removal of the affected animals from the contaminated pasture or feed.

Ryegrass staggers

Annual ryegrass staggers in cattle and sheep has been described in Australia and South Africa. It results from the ingestion of seedheads of annual ryegrass (*Lolium rigidum*) containing the seed gall nematode *Anguina funesta* infected with the bacterium *Rathayibacter toxicus (Corynebacterium rathayi)*. The toxicity of the ryegrass is associated with the bacterial galls, the active principle being a toxin, sometimes termed corynetoxins. Accordingly, this disease is not caused by a mycotoxin but is due to tunicaminyluracil antibiotics. It is referred to here, however, because of the similarities of these tremorgenic syndromes involving grasses, regardless of their aetiology. The toxin is believed to interfere with glycosylation enzymes, leading to depletion of essential glycoproteins. Clinical signs are similar to paspalum staggers (referred to below) but convulsions and muscular twitching are more severe. During seizures affected animals tend to lie on their sides with opisthotonos and limbs rigidly extended. Lesions include perivascular oedema and occasional haemorrhages in the brain and meninges. Degenerative liver changes have been recorded in experimentally induced annual ryegrass toxicosis in sheep.

Tremors and ataxia in cattle, sheep, farmed deer and horses grazing perennial ryegrass (*Lolium perenne*) have been recorded in many countries particularly Australia, New Zealand, the USA and parts of Europe. Toxicity is associated with the presence of an endophytic fungus

Neotyphodium (Acremonium) lolii growing within the plant, especially in the leaves. The active tremorgens have been isolated from endophyte-infected ryegrass plants and chemically characterized as lolitrems. Perennial ryegrass staggers is usually associated with the grazing of ryegrass pastures but conserved forage may also induce the disease. Clinical signs include locomotor incoordination or abnormal staggering gait, stumbling and collapse followed by severe muscular spasms. Morbidity may be high with up to 80% of a flock or herd becoming affected after a few days on infected pasture; mortality is usually low. The disease often occurs in late summer or autumn when grass growth declines and the pasture is short causing the animals to graze close to the roots where more mycotoxin is present due to concentration of the growth of *N. lolii* in the older lower leaf sheaths. Deaths are uncommon if animals are promptly moved to safe pasture. No specific pathological tissue changes have been attributed to the mycotoxins causing perennial ryegrass staggers.

Penitrem staggers

Penicillium species such as *Penicillium crustosum* and *P. verruculosum* as well as some *Aspergillus* species including *A. fumigatus* and *A. flavus* produce a range of toxins associated with outbreaks of nervous diseases in domestic animals. The principal mycotoxins involved are penitrem A, verruculogen and roquefortine, which produce clinical signs similar to those of ryegrass staggers with tremors, ataxia and convulsive episodes in animals.

Paspalum staggers

Paspalum staggers is a neurotoxic syndrome of animals, particularly cattle and occasionally sheep and horses, grazing pastures or eating hay that contains dallis grass or other grasses of the genus *Paspalum* parasitized by *Claviceps paspali*. The fungus colonizes the ovaries of susceptible grasses in a manner similar to the colonization of rye grass by *Claviceps purpurea*. The mycotoxins involved are paspalinine and paspalitrems A, B, and C.

Infestation of the seed head is favoured by wet, humid summers and the disease occurs sporadically in several parts of the world. Affected animals show hyperexcitability, muscle tremors and uncoordinated movements. Signs become more pronounced with exercise. Severely affected animals show ataxia and may become recumbent with paddling of the limbs. The appetite is unaffected and although occasional animals may die from sustained seizures resulting in interference with respiration, or from misadventure, the majority of animals recover when removed from affected pasture. No specific lesions have been described for paspalum staggers. High mowing of toxic seed heads followed by raking or heavy grazing during spring and summer to prevent formation of seed heads are measures aimed at preventing this mycotoxicosis.

Aspergillus clavatus tremors

Aspergillus clavatus has been associated with hypersensitivity, muscle tremors and ataxia in cattle grazing sprouted wheat or fed contaminated millers' malt culms. This fungus is known to produce a number of mycotoxins such as patulin and cytochalasin E. Patulin is thought to be the main neurotoxin responsible for the clinical signs (Sabater-Vilar et al. 2004). The disease has been reported in China, South Africa and in several European countries. Clinical signs are not evident when animals are at rest. On exercise they may show ataxia, frothing from the mouth and knuckling of the hindlimbs. Changes described in the brain and spinal cord include degenerative changes in neurons and focal gliosis.

REFERENCES

Bourke, C.A., 2007. A review of kikuyu grass (*Pennisetum clandestinum*) poisoning in cattle. Australian Veterinary Journal 85, 261–267.

Briggs, L.R., Towers, N.R., Molan, P.C., 1993. Sporidesmin and ELISA technology. New Zealand Veterinary Journal 41, 220.

Mule, G., Gonzalez-Jaen, M.T., Hornok, L., et al., 2005. Advances in molecular diagnosis of toxigenic *Fusarium* species: A review. Food Additives and Contaminants 22, 316–323.

Sabater-Vilar, M., Maas, R.F., De Bosschere, H., et al., 2004. Patulin produced by an *Aspergillus clavatus* isolated from feed containing malting residues associated with a lethal neurotoxicosis in cattle. Mycopathologia 158, 419–426.

Seifert, K.A., Levesque, C.A., 2004. Phylogeny and. molecular diagnosis of mycotoxigenic fungi. European Journal of Plant Pathology 110, 449–471.

Snyman, L.D., Kellerman, T.S., Vleggaar, R., et al., 2011. Diplonine, a neurotoxin isolated from cultures of the fungus *Stenocarpella maydis* (Berk.) Sacc. that induces diplodiosis. Journal of Agricultural and Food Chemistry 59, 9039–9044.

FURTHER READING

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clinical Microbiology Reviews 16, 497–516.

Berry, C.L., 1988. The pathology of mycotoxins. Journal of Pathology 154, 301–3311.

Cheeke, P.R., Shull, L.R., 1985. Natural toxicants in feeds and poisonous plants. AVI Publishing Company, Inc., Westport, Connecticut, USA.

Coppock, R.W., Jacobsen, B.J., 2009. Mycotoxins in animal and human patients. Toxicology and Industrial Health 25, 637–655.

Fink-Gremmels, J., 2008. The role of mycotoxins in the health and performance of dairy cows. Veterinary Journal 176, 84–92.

Hollinger, K., Ekperigin, H.E., 1999. Mycotoxicosis in food producing animals. Veterinary Clinics of North America: Food Animal Practice 15, 133–165.

Humphreys, D.J., 1988. Veterinary Toxicology, third ed. Bailliere Tindall, London.

Kabak, B., Dobson, A.D.W., Var, I., 2006. Strategies to prevent mycotoxin contamination of food and animal feed: a review. Critical Reviews in Food Science and Nutrition 46, 593–619.

Kellerman, T.S., Coetzer, J.A.W., Naude, T.W., 1988. Plant poisoning and mycotoxicoses of livestock in Southern Africa. Oxford University Press, Cape Town, South Africa.

Leung, M.C.K., Díaz-Llano, G., Smith, T.K., 2006. Mycotoxins in pet food: a review on worldwide prevalence and preventative strategies. Journal of Agricultural and Food Chemistry 54, 9623–9635.

Marasas, W.F.O., Nelson, P.E., 1987. Mycotoxicology. The Pennsylvania State University Press, University Park, Penn.

Moss, M.O., 2008. Fungi, quality and safety issues in fresh fruits and vegetables. Journal of Applied Microbiology 104, 1239–1243.

Pier, A.C., 1981. Mycotoxins and animal health, Advances in Veterinary Science and Comparative Medicine 25, 185–243.

Richard, J.L., Thurston, J.R., 1986. Diagnosis of mycotoxicoses. Martinus Nijhoff Publishers, Dordrecht.

Seawright, A.A., 1982. Animal Health in Australia, Volume 2, Chemical and Plant Poisons. Australian Government Publishing Service, Canberra.

Section 4

Virology (including prions)

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Chapter 45

Parvoviridae

Parvoviruses (from the Latin word *parvus* meaning small) are unenveloped, icosahedral in symmetry and possess a linear genome of single-stranded DNA (Fig. 45.1). Their size ranges from 20–26 nm in diameter. The family *Parvoviridae* is divided into two subfamilies *Parvovirinae*, which includes viruses of vertebrates, and *Densovirinae*, members of which infect arthropods (Fig. 45.2). There are five genera within the subfamily *Parvovirinae*. Viruses of veterinary importance are generally contained within the genus *Parvovirus*. Two genera have recently been created: *Amdovirus* and *Bocavirus*. The genus *Erythrovirus* contains the human parvovirus B19, which causes erythema infectiosum ('fifth disease'), a common, self-limiting infection of children. The members of the genus *Dependovirus* are generally dependent on co-infection with a helper virus, usually an adenovirus, for efficient replication and are found in several animal species. Most dependoviruses are not associated with disease but duck parvovirus and goose parvovirus are not dependent on helper viruses for replication and have been associated with disease in Muscovy ducks and in geese (Derzsy's disease) respectively.

Parvoviruses replicate in the nucleus of host cells. Replication is closely associated with the host cell cycle, requiring the cell to pass through the S-phase and probably involving host DNA polymerases. Parvoviruses are unable to induce resting cells to enter the S-phase and therefore can only replicate in dividing cells. Helper viruses appear to promote cell division rather than becoming directly involved in parvovirus replication. The pathological and clinical manifestations of parvovirus infections reflect their dependence on replicating cells. The single-stranded genome must be converted to a duplex molecule before amplification and RNA transcription of the viral genes can occur. Parvoviruses of veterinary importance are presented in Table 45.1.

Parvovirus virions are highly resistant and may remain stable in the environment for several months. They are stable in the presence of lipid solvents, at a range of pH values from 3–9 and at 56°C for at least 60 minutes. Viral inactivation can be achieved using formalin, beta-propriolactone, sodium hypochlorite and oxidizing agents. The parvoviruses of vertebrates with the exceptions of Aleutian mink disease virus (AMDV) and goose parvovirus (GPV) have the ability to agglutinate erythrocytes. Haemagglutination inhibition (HAI) by specific antisera is a widely used and convenient method for the identification of these viruses. Specific monoclonal antibodies must be employed to distinguish the closely related feline panleukopenia virus (FPV), mink enteritis virus (MEV), canine parvovirus (CPV) and racoon parvovirus (RPV). It is considered that MEV, CPV and RPV are host range mutants of FPV and are classified as strains of FPV.

FELINE PANLEUKOPENIA

Feline panleukopenia (FP), also referred to as feline distemper or feline infectious enteritis, is a highly contagious, generalized, viral disease of domestic and wild cats. It is caused by feline panleukopenia virus (FPV), which has a worldwide distribution. Infection is common but frequently subclinical. Disease typically occurs in young kittens as maternally derived antibody levels decline and is characterized by a sudden onset of severe depression, anorexia and fever followed by vomiting, sometimes accompanied by diarrhoea, within one to two days. The immunity conferred following natural infection or vaccination is strong and long-lived. Vaccination has successfully controlled the disease in pet cat populations.

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Pathogenesis

The incubation period is typically four to five days but can vary from two to 10 days. Following ingestion or inhalation, primary infection occurs in the lymphoid tissues of the oropharynx. The regional lymph nodes then become infected. Within 24 hours the subsequent viraemia results in the infection of mitotically active cells throughout the body, particularly the lymphopoietic cells of the bone marrow, thymus and spleen and the crypt cells of the intestinal villi. The rapid destruction of these target tissues results in a panleukopenia and the loss of regenerative intestinal epithelium. Depending on the stage of gestation, transplacental infection may result in a range of foetal effects from death of the foetus to cerebellar hypoplasia and retinal dysplasia.

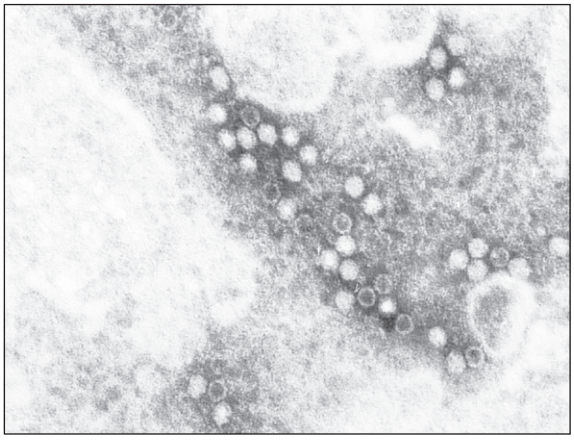

Figure 45.1 Electron micrograph of parvovirus.

Diagnosis

A presumptive diagnosis of FP may be possible on the basis of history, particularly vaccination status, and the presenting clinical signs.

- Suitable specimens for laboratory confirmation include oropharyngeal swabs, faeces, spleen, mesenteric lymph node and ileum.
- Affected animals in the acute stages of the disease usually have a white cell count of less than 7×10^9/L. In severe cases counts of less than 2×10^9/L are common. Neutropenia is more common than lymphopenia. If the cat survives, white cell counts recover after a few days.

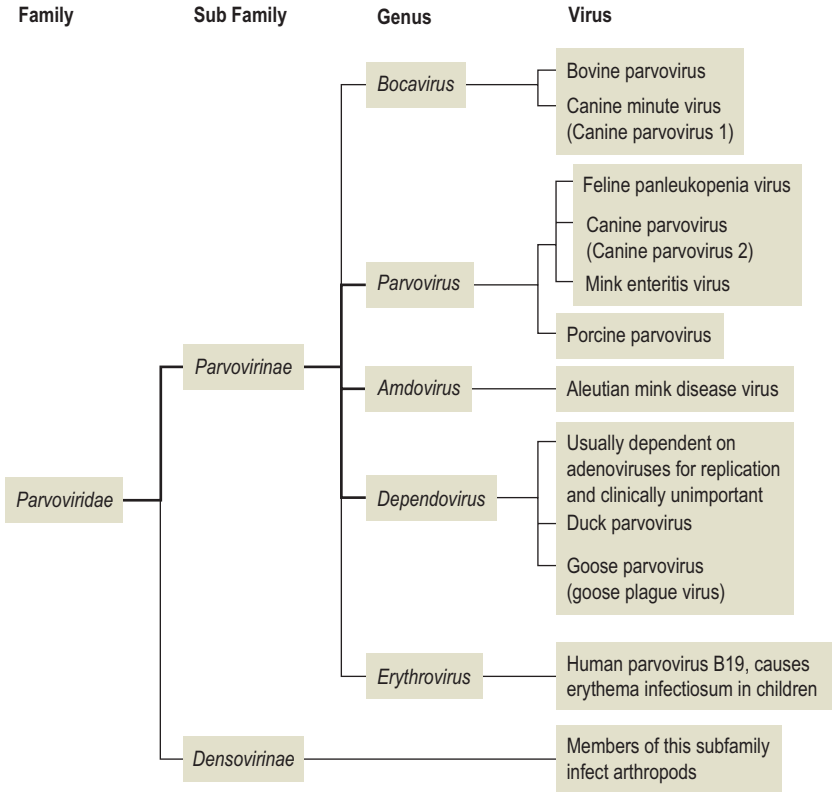

Figure 45.2 Classification of parvoviruses of vertebrates.

Table 45.1 Parvoviruses of animals

Virus	Host species	Disease
Feline panleukopenia virus	Domestic and wild cats	Highly contagious systemic disease of cats characterized by fever, depression, vomiting and diarrhoea in weaned kittens. Abortion or cerebellar ataxia in neonates following intrauterine infection
Canine parvovirus	Domestic and wild dogs	Highly contagious systemic disease characterized by depression, vomiting, dysentery
Porcine parvovirus	Pigs	Important cause of stillbirths, mummification, embryonic deaths and infertility (SMEDI syndrome), particularly in gilts
Mink enteritis virus	Mink	Disease of young mink similar to feline panleukopenia
Aleutian mink disease virus	Mink	Immune complex disease, most severe in mink homozygous for pale coat colour. Characterized by persistent viraemia, plasmacytosis and hypergammaglobulinaemia
Goose parvovirus	Geese	Fatal disease of goslings (Derzsy's disease) characterized by hepatitis, myositis and myocarditis
Canine minute virus	Dogs	Widespread virus, uncertain role in disease
Bovine parvovirus	Cattle	Uncertain role in disease

- Virus isolation using thinly seeded cultures of primary or secondary feline cells or an established feline cell line such as Crandell feline kidney may be attempted. Cytopathic effect may be difficult to detect and staining for intranuclear inclusions or fluorescent antibody techniques may be required.
- As high levels of virus are shed in the faeces during the acute stages of FP, virus may be detected using electron microscopy.
- Latex agglutination, ELISA or haemagglutination (HA) with pig or rhesus monkey red cells may be used to demonstrate viral antigen in faeces. A commercial immunochromatographic test strip for canine parvovirus has been used to detect feline parvovirus in intestinal tract contents from kittens (Addie et al. 1998).
- The haemagglutination inhibition (HAI), ELISA, indirect immunofluorescence and virus neutralization (VN) tests may be used to detect a rising antibody titre in acute and convalescent sera. The HAI test is less sensitive than the VN test and non-specific inhibitors of HA may be present in some sera.
- Samples of several regions of small intestine, mesenteric lymph node and spleen should be submitted in formal saline for histopathological examination. Histopathological changes are most prominent in the ileum and jejunum where the crypts of Lieberkuhn are usually dilated and filled with sloughed, necrotic epithelial cells. The intestinal villi are blunted and may be fused. Intranuclear inclusions may be present in crypt cells. In kittens with ataxia, microscopic CNS lesions in the cerebellum include a marked reduction in the numbers of Purkinje and granular cells.
- Rapid and sensitive diagnostic methods utilizing the polymerase chain reaction method have been developed for the detection of parvovirus in faeces (Schunck et al. 1995) or tissues (Meurs et al. 2000).

CANINE PARVOVIRUS INFECTION

A severe viral enteritis of acute onset affecting dogs and characterized by vomiting and bloody diarrhoea emerged in the late 1970s. The disease has a worldwide distribution and is caused by canine parvovirus (CPV), formerly known as canine parvovirus type 2. It is believed that CPV evolved from FPV in a cat or FPV-like virus in a wild carnivore by mutations in the capsid protein gene and then adapted to the canine host (Steinel et al. 2001). Within a few years CPV underwent further antigenic variation resulting in the emergence of new antigenic types designated CPV-2a, CPV-2b and 'CPV-2c'. These newer antigenic types have replaced the original type and have also regained the ability to replicate in cats (Truyen 2006). Infection or vaccination with one subtype will generally protect against infection with another.

The pattern of disease has changed since the virus first appeared. Early outbreaks were panzootic in nature, with both high morbidity and high mortality. The majority of adult dogs are now immune as a result of vaccination or natural infection and parvoviral infection principally

presents as an enteric disease of young dogs between weaning and six months of age. A second clinical form of acute or subacute heart failure occurs in puppies infected in utero or in the perinatal period but is now rare. Most breeding bitches are immune and confer protection on their offspring during the critical early neonatal period.

Pathogenesis

The incubation period usually lasts between four and seven days. Following ingestion CPV appears to initially replicate in pharyngeal lymphoid tissue and Peyer's patches. The main target tissues are the myocardium, intestinal epithelium and lymphoid tissues. During the first two weeks of life there is rapid cardiac myocyte multiplication while the turnover of intestinal epithelial cells is low. This situation reverses in older puppies. The virus infects the actively dividing epithelial cells of the crypts of the small intestinal villi resulting in an acute or subacute haemorrhagic enteritis. Excretion of virus in the faeces usually begins about day three postinfection (PI). The concentration of faecal virus reaches a maximum at about day five or six PI, when there may be 10^9 TCID/g of faeces. The rate of virus excretion falls off rapidly and it is frequently not possible to detect virus after day 12 PI. Initial signs include the sudden onset of vomiting, anorexia, depression and possibly fever. Diarrhoea follows a day or two later. The faeces are frequently streaked with blood or in severe cases are frankly haemorrhagic with a fetid smell. Dehydration and weight loss are marked and rapid.

Diagnosis

Sudden death in puppies and characteristic histopathological changes in the myocardium are highly suggestive of the myocardial form of disease. Parvoviral enteritis could be confused with intestinal obstruction by foreign bodies or other enteric infections such as coronavirus, *Salmonella* spp. or *Campylobacter jejuni*.

- Suitable specimens for laboratory confirmation include faeces, blood and tissues, particularly affected portions of intestine and myocardium.
- Leukopaenia (usually a lymphopaenia) may be detected, particularly in severely affected animals.
- The nature and site of the gross and microscopic intestinal lesions are strongly suggestive. Immunocytochemical staining can be used to positively identify viral antigen in tissue samples.
- Definitive diagnosis usually depends on the demonstration of virus or viral antigen. Samples should be taken early in the course of the infection when there is a high titre of virus in the faeces. Electron microscopy, ELISA or haemagglutination may be used. Canine parvovirus will haemagglutinate pig or rhesus monkey red blood cells. Virus isolation can be carried out in thinly seeded cell cultures of a number of susceptible primary or established canine and feline cell lines including the A72 canine fibroma-derived cell line and Crandell feline kidney cells. Cytopathic effect may be difficult to detect and immunofluorescent staining may be required to demonstrate viral antigen in the nuclei of infected cells. A number of commercial assays are also available (Addie et al. 1998, Lacheretz et al. 2003).
- Rapid and sensitive diagnostic methods utilizing the polymerase chain reaction method have been developed for the detection of canine parvovirus in faeces (Mochizuki et al. 1993, Uwatoko et al. 1995) and paraffin-embedded tissues (Truyen et al. 1994).
- Serological tests including haemagglutination inhibition, ELISA, virus neutralization and indirect immunofluorescence may confirm the diagnosis by demonstrating a high antibody titre or by detecting IgM antibody.

PORCINE PARVOVIRUS

Porcine parvovirus (PPV) has a worldwide distribution and is an important cause of reproductive failure in pigs. Infection is enzootic in many conventional pig herds and as a result most sows are immune. Piglets receive passive immunity through colostral antibodies. The titres of these antibodies decline over several months. During this period the maternally derived antibodies interfere with the development of active immunity and consequently groups of gilts may be seronegative and susceptible to infection as they approach breeding age.

Pathogenesis

Following infection the virus replicates extensively and is disseminated throughout the body as a result of a viraemia. The virus has a predilection for the mitotically active cells of foetal tissues. Transplacental infection occurs about 10–14 days after the dam has been exposed. Disease only follows exposure of susceptible dams during the first half of gestation. Embryos infected during the first few weeks of gestation die and are resorbed. If the number of viable embryos is reduced below four the entire pregnancy is lost and the dam generally returns to oestrus. Foetuses infected before day 70 of gestation also die but the foetal skeleton precludes resorption and the dead foetuses become mummified. Infection after day 70 results in a protective immune response being mounted by the foetus.

Diagnosis

Several mummified foetuses should be submitted for laboratory diagnosis.

- The detection of viral antigen in cryostat sections of foetal tissues, particularly lung tissue, by immunofluorescence is a reliable and sensitive diagnostic procedure. It is also possible to detect viral haemagglutinin in foetal tissue homogenates using guinea pig erythrocytes.
- Standard and nested PCR assays have been developed for the detection of viral DNA in foetal tissues (Gradil et al. 1994).
- Virus isolation can be attempted using semi-confluent monolayers of swine kidney cells. However, viral infectivity is progressively lost in foetal tissues following the death of the foetus and isolation from mummified tissues may be unsuccessful. The cytopathic effect is characterized by rounding up and lysis of cells. Primary and secondary cell lines should be monitored by immunofluorescence for endogenous infection with PPV.
- Serological tests may be used to detect antibodies in the sera and body fluids of older foetuses or stillborn pigs because there is no placental transfer of maternal antibodies. Suitable tests include HAI and VN. Testing of the dam's serum is generally of little value unless infection has only recently been introduced into the herd or to exclude PPV infection.

REFERENCES

Addie, D.D., Toth, S., Thompson, H., et al., 1998. Detection of feline parvovirus in dying pedigree kittens. Veterinary Record 142, 353–356.

Gradil, C.M., Harding, M.J., Lewis, K., 1994. Use of polymerase chain reaction to detect porcine parvovirus associated with swine embryos. American Journal of Veterinary Research 55, 344–347.

Lacheretz, A., Laperrousaz, C., Kodjo, A., et al., 2003. Diagnosis of canine parvovirus by rapid immunomigration on a membrane. Veterinary Record 152, 48–50.

Meurs, K.M., Fox, P.R., Magnon, A.L., et al., 2000. Molecular screening by polymerase chain reaction detects panleukopenia virus DNA in formalin-fixed hearts from cats with idiopathic cardiomyopathy and myocarditis. Cardiovascular Pathology 9, 119–126.

Mochizuki, M., San Gabriel, M.C., Nakatani, H., et al., 1993. Comparison of polymerase chain reaction with virus isolation and haemagglutination assays for the detection of canine parvovirus in faecal specimens. Research in Veterinary Science 55, 60–63.

Schunck, B., Kraft, W., Truyen, U., 1995. A simple touch-down polymerase chain-reaction for the detection of canine parvovirus and feline panleukopenia virus in faeces. Journal of Virological Methods 55, 427–433.

Steinel, A., Parrish, C.R., Bloom, M.E., et al., 2001. Parvovirus infections in wild carnivores. Journal of Wildlife Diseases 37, 594–607.

Truyen, U., 2006. Evolution of canine parvovirus – A need for new vaccines? Veterinary Microbiology 117, 9–13.

Truyen, U., Platzer, G., Parrish, C.R., et al., 1994. Detection of canine parvovirus DNA in paraffin-embedded tissues by polymerase chain reaction. Journal of Veterinary Medicine Series B 41, 148–152.

Uwatoko, K., Sunairi, M., Nakajima, M., et al., 1995. Rapid method utilizing the polymerase chain-reaction for detection of canine parvovirus in faeces of diarrhoeic dogs. Veterinary Microbiology 43, 315–323.

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Chapter 46

Circoviridae

This family is relatively new and comprises viruses of vertebrates. Circoviruses are non-enveloped, icosahedral in structure and generally range from 20–26 nm in diameter. A distinctive capsid surface structure is visible in electron micrographs of circoviruses. Chicken anaemia virus is larger and has a more distinct surface structure than porcine circovirus or beak and feather disease virus (Fig. 46.1). The genome consists of a single molecule of circular negative-sense or ambisense single-stranded DNA and replication occurs in the nucleus of dividing cells by means of a circular, double-stranded replicative form of the viral genome. Genetic sequencing studies (Niagro et al. 1998) demonstrated the existence of three distinct groups within the family and this has led to classification changes including the creation of two new genera, *Circovirus* and *Gyrovirus* (Fig. 46.2). The third grouping, the plant circoviruses, has now been assigned to the family *Nanoviridae*. The viruses are stable in the environment at pH 3 to 9 and resistant to heat at 60°C for 30 minutes.

Circoviruses are host-specific and have a worldwide distribution (Table 46.1). They are associated with infection of cells of the haemolymphatic system.

CHICKEN ANAEMIA VIRUS INFECTION

Chicken anaemia virus infection in young birds is characterized by aplastic anaemia and generalized lymphoid atrophy with accompanying immunosuppression. The virus only infects chickens and is present in poultry flocks worldwide. All field isolates belong to a single serotype and appear to be equally pathogenic.

Horizontal and vertical transmission occur. Infection by the faecal–oral route results from direct contact or via contaminated fomites. Vertical transmission via the egg occurs in laying hens during the one- to three-week viraemic period following infection. Once infection is established in a breeder flock most birds become infected and develop antibody before they begin to lay. Maternally derived antibody protects from disease but does not prevent chicks becoming infected and shedding virus. An age resistance to disease but not to infection develops at one to two weeks of age. Subclinical infection is common in immune flocks with birds acquiring infection soon after maternal antibody has waned at about three weeks of age. However, the protective effects of maternal antibody and age resistance can be overcome by dual infections involving other immunosuppressive viruses such as infectious bursal disease virus or gallid herpesvirus 2.

Pathogenesis

Following infection of susceptible day-old chicks viraemia develops and virus can be recovered from most organs and from rectal contents for a period of three to four weeks. The principal target cells are precursor T cells in the cortex of the thymus and haemocytoblasts in the bone marrow. Destruction of these cells gives rise to immunosuppression and anaemia. The mortality rate is usually about 10% but may be up to 50%. Surviving birds gradually recover. Subclinical infection in broiler progeny of immune breeder flocks has been associated with significant economic loss associated with poor feed conversion and reduced average weights.

Diagnosis

A presumptive diagnosis may be based on clinical signs and gross pathological lesions including atrophy of thymus and bursa, pale bone marrow and haemorrhages

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Table 46.1 Circoviruses of animals

Virus	Genus	Host species	Disease significance
Chicken anaemia virus	*Gyrovirus*	Chicken	Worldwide; widespread infection of chickens. Cause of anaemia and immunosuppression in chicks infected vertically via egg or horizontally via contact within days of hatching
Beak and feather disease virus	*Circovirus*	Psittacine species	Debilitating, immunosuppressive disease of young psittacine birds, particularly cockatoos
Pig circovirus 1	*Circovirus*	Pig	Isolated from pig cell line. Probably non-pathogenic
Pig circovirus 2	*Circovirus*	Pig	Associated with postweaning multisystemic wasting syndrome (PMWS)

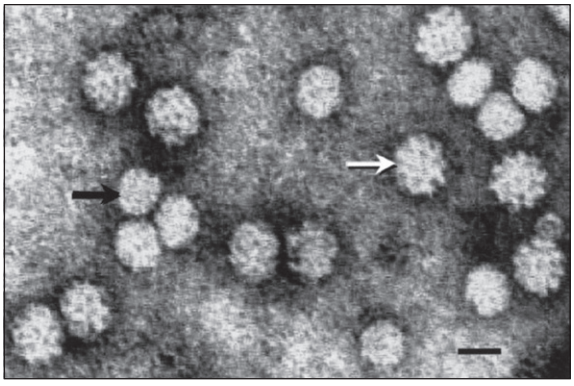

Figure 46.1 Negative contrast electron microscopy of particles of an isolate of chicken anaemia virus (white arrow) and beak and feather disease virus (black arrow) stained with uranyl acetate. The bar represents 20 nm. Reprinted with permission: Fauquet CM (ed) et al. 2005 Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press.

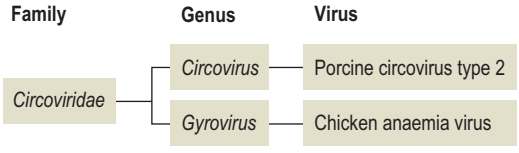

Figure 46.2 Classification of *Circoviridae* of veterinary importance.

under the skin and in skeletal muscle. Laboratory confirmation is based on detection of viral antigen by immunocytochemical techniques or of viral DNA by in situ hybridization, dot blot hybridization and PCR in bone marrow and thymus (Noteborn et al. 1992, Tham & Stanislawek 1992, Todd et al. 1992). Virus isolation is possible but rather specialized, slow and expensive. Serum antibodies can be detected by virus neutralization, indirect immunofluorescence and ELISA (Todd et al. 1999). A commercial ELISA is available and can be used to screen breeder flocks at point of lay.

PORCINE CIRCOVIRUS INFECTION

Porcine circovirus was first described as a picornavirus-like contaminant of the continuous pig kidney cell line PK/15. Experimental infections suggest that this virus, termed porcine circovirus 1, is not particularly pathogenic. An antigenically and genomically distinct circovirus, porcine circovirus 2 (PCV-2) was isolated from piglets affected by a wasting disease subsequently. Isolates can be divided by genetic analysis into several clusters contained in three subgroups PCV-2a, PCV-2b and PCV-2c (Olvera et al. 2007, Segales et al. 2008). Seroepidemiological studies suggest that infection with porcine circovirus is widespread in pig populations worldwide. Postweaning multisystemic wasting syndrome (PMWS), a progressive wasting condition associated with lesions in several organ systems, which was first described in Canada in 1991 in high health, specific pathogen-free (SPF) herds, is believed to be associated with PCV-2 infection (Allan & Ellis 2000). Affected animals are usually about six weeks of age and present with signs of weight loss, dyspnoea and enlarged lymph nodes. Moderate to severe lymphoid depletion and/or granulomatous lymphadenopathy are characteristic histopathological lesions present in affected pigs. In some animals basophilic intracytoplasmic inclusions in histiocytes and multinucleated giant cells may be demonstrable. The exact mechanisms by which PCV-2 induces the granulomatous inflammation is unclear at present. The disease has a low morbidity but a relatively high mortality, which may reach 20% on affected farms in acute outbreaks. Environmental factors and/or infection with other infectious agents such as porcine parvovirus or porcine respiratory and reproductive syndrome virus appear to be necessary for the production of clinical disease although disease has also been induced experimentally in germ-free pigs in the absence of known co-factors (Lager et al. 2007).

The diagnosis of PMWS is based on clinical signs and pathological examination. In order to confirm a diagnosis it is necessary to link the presence of PCV-2 to the characteristic tissue lesions because PCV-2 can be detected in normal healthy pigs. The demonstration of antibodies to PCV-2 using indirect immunofluorescence, immunoperoxidase monolayer assay or ELISA is suggestive but not indicative of a disease problem. Virus isolation in pig cell lines is also suggestive rather than confirmatory. Definitive diagnosis requires demonstration of PCV-2 antigen or nucleic acid in association with characteristic lesions in diseased animals. This can be achieved by the use of immunohistochemistry and in situ hybridization (Kim & Chae 2004).

REFERENCES

Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. Journal of Veterinary Diagnostic Investigation 12, 3–14.

Kim, J., Chae, C., 2004. A comparison of virus isolation, polymerase chain reaction, immunohistochemistry, and in situ hybridization for the detection of porcine circovirus 2 and porcine parvovirus in experimentally and naturally coinfected pigs. Journal of Veterinary Diagnostic Investigation 16, 45–50.

Lager, K.M., Gauger, P.C., Vincent, A.L., et al., 2007. Mortality in pigs given porcine circovirus type 2 subgroup 1 and 2 viruses derived from DNA clones. Veterinary Record 161, 428–429.

Niagro, F.D., Forsthoefel, A.N., Lawther, R.P., et al., 1998. Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminviruses and plant circoviruses. Archives of Virology 143, 1723–1744.

Noteborn, M.H.M., Verschueren, C.A.J., Vanroozelaar, D.J., et al., 1992. Detection of chicken anaemia virus by DNA hybridization and polymerase chain reaction. Avian Pathology 21, 107–118.

Olvera, A., Cortey, M., Segales, J., 2007. Molecular evolution of porcine circovirus type 2 genomes phylogeny and clonality. Virology 357, 175–185.

Segales, J., Olvera, A., Grau-Roma, L., et al., 2008. PCV-2 genotype definition and nomenclature. Veterinary Record 162, 867–868.

Tham, K.M., Stanislawek, W.L., 1992. Detection of chicken anaemia agent DNA sequences by the polymerase chain reaction. Archives of Virology 127, 245–255.

Todd, D., Mawhinney, K., McNulty, M.S., 1992. Detection and differentiation of chicken anaemia virus isolates by using the polymerase chain reaction. Journal of Clinical Microbiology 30, 1661–1666.

Todd, D., Mawhinney, K.A., Graham, D.A., 1999. Development of a blocking enzyme-linked immunosorbent assay for the serological diagnosis of chicken anaemia virus. Journal of Virological Methods 82, 177–184.

FURTHER READING

Chae, C., 2004. Post weaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Veterinary Journal 168, 41–49.

Segales, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Animal Health Research Reviews 6, 119–142.

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Chapter 47

Papillomaviridae

Members of the family *Papillomaviridae* are non-enveloped with icosahedral capsids (Fig. 47.1). Virions contain a single molecule of circular double-stranded DNA. The name is derived from the Latin *papilla* meaning nipple and the Greek suffix *-oma* used to form the names of tumours. The family originally contained a single genus, *Papillomavirus*, but now contains 16 genera (de Villiers et al. 2004; Fig. 47.2). There are proposals to increase the number of genera to 29 (Bernard et al. 2010). Papillomaviruses are 55 nm in diameter and replicate in the nucleus of host cells. The viruses are resistant to lipid solvents, acid and heating to 60°C for 30 minutes. Infections are typically persistent and are usually established early in life. In man several viral species and more than 80 genotypes are recognized while in cattle three species and 10 genotypes are known to exist. Individual types share less than 50% sequence homology and display differences when tested using reciprocal serological assays.

Papillomaviruses are epitheliotropic, causing proliferative lesions termed papillomas or warts in epidermal or mucosal epithelia often at specific sites on the body. Papillomavirus infections occur in several animal species but only those occurring in cattle, horses and dogs are of significant veterinary importance (Table 47.1). Lesions are most commonly observed in young animals and usually regress spontaneously after a period of several weeks or months, with multiple warts regressing simultaneously. Regression is thought to be due to the development of cell-mediated immunity. In certain cases, in association with co-factors, the benign tumours induced by these viruses may progress to malignant neoplasms. Cultivation of papillomaviruses in cell culture is problematical but molecular biology techniques have made it possible to sequence several members and to detect their presence in lesions with great sensitivity. In infected cells the viral DNA usually remains episomal within the host cell nucleus. Papillomaviruses have been used in genetic engineering to insert foreign DNA into cultured cells.

Pathogenesis

Papillomaviruses gain entry to and infect the basal layer cells of the epithelium via abrasions and microlesions or where such cells become naturally exposed such as at the junction between different types of epithelia. The infected cells are stimulated to excessive growth by three viral encoded oncoproteins; E5, E6 and E7. Viral gene expression is restricted during this proliferative phase. Once differentiation of the cells begins the expression of late viral genes can proceed resulting in the synthesis of viral capsids within the stratum spinosum and stratum granulosum. New virions can only be visualized by electron microscopy in the nuclei of the terminally differentiated keratinized cells. The release of virus occurs during desquamation from the surface of lesions. Two main types of papilloma (wart) are described histologically, papillomas and fibropapillomas. The latter contain a fibrous core of connective tissue. The progression of these benign tumours of squamous epithelium to malignancy in association with certain co-factors has been documented in humans, cattle and rabbits.

Diagnosis

The clinical appearance of warts is distinctive and frequently precludes the necessity of laboratory confirmation.

- Histopathology carried out on a biopsy from the lesion will indicate the histological type of papilloma or tumour present. In addition, it will determine if the lesion is benign or otherwise.

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- Electron microscopic examination of homogenates of affected epidermis may reveal the characteristic viral particles.
- Hybridization assays and PCR methods are available for the detection of papillomavirus DNA (Bredal et al. 1996, Teifke et al. 1998), but are not in routine diagnostic use. The typing of isolates can be carried out by DNA extraction and restriction endonuclease analysis or by Southern blotting (Le Net et al. 1997).

Bovine Papillomatosis

At least 10 genotypes of bovine papillomavirus (BPV) are described. On the basis of the type of lesion induced and size of genome, types 1 to 6 may be grouped into Group A (types 1, 2 and 5) which infect both epithelial cells and subepithelial fibroblasts and Group B (types 3, 4 and 6) which infect epithelial cells only. Three bovine papillomaviruses are taxonomically recognized: *bovine papillomavirus 1* (includes bovine papillomavirus genotypes 1 and 2), *bovine papillomavirus 3* (includes bovine papillomavirus genotypes 3, 4 and 6) and *bovine papillomavirus 5*.

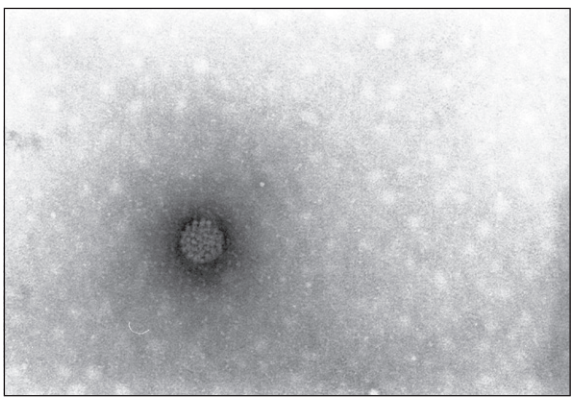

Figure 47.1 Electron micrograph of a papillomavirus.

Bovine papillomavirus types 1 and 2 cause cutaneous fibropapillomas which are commonly seen on the skin of cattle. The condition is worldwide, infectious and self-limiting although the duration of the condition may vary considerably from individual to individual. All ages of cattle may be affected but the condition is most common in young animals less than two years of age. The condition is more common in housed animals. The virus is quite resistant and transmission may occur directly or indirectly following contact with contaminated fence posts, grooming equipment, halters and other objects. The incubation period is about 30 days. The lesions appear as raised, verrucous masses, often with a cauliflower-like appearance, ranging in size from a few millimetres to several centimetres. The most common sites are the neck, head and shoulders. Lesions may occur on the genitalia but are now uncommon due to the widespread use of artificial insemination. Regression may not occur for one to 12 months. Prolonged persistence of extensive lesions is sometimes described and is thought to be due to some subtle deficit in the immune system. Infection of urinary bladder epithelium with bovine papillomavirus type 2 results in a non-productive, abortive replication cycle. The ingestion of bracken fern, which contains a number of toxic factors, is believed to contribute to transformation of infected cells to a squamous cell carcinoma (Campo et al. 1992). Haemorrhage from malignant tumours in the urinary bladder is responsible for the clinical condition known as enzootic haematuria. Squamous cell carcinomas of this type are most commonly reported from particular regions in Scotland, Brazil and Colombia where bracken is abundant.

Bovine papillomavirus type 3 causes a cutaneous papilloma which is usually flat with a broad base and has a tendency to persist.

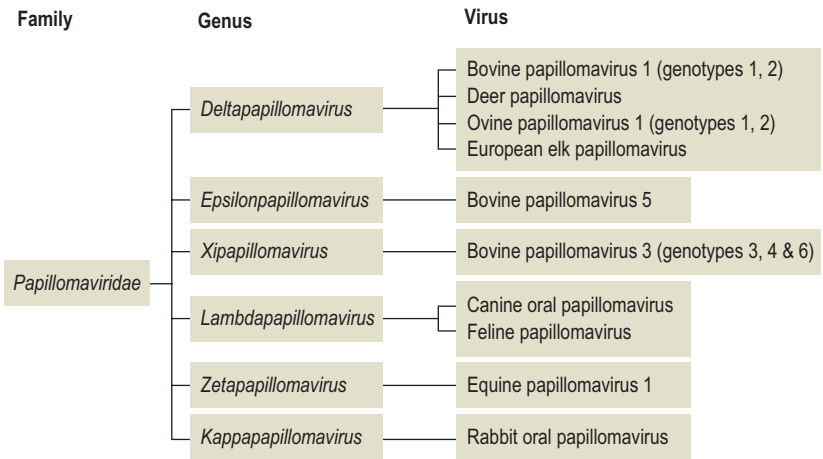

Figure 47.2 Classification of papillomaviruses of veterinary significance.

Table 47.1 Papillomaviruses of animals

Virus	Host species	Disease
Bovine papillomavirus types 1, 2	Cattle	Fibropapillomas of the head and neck of young cattle, sometimes affecting penis or teats. Implicated in the aetiology of sarcoids in horses. Type 2 has been implicated in neoplasms of bladder wall and enzootic haematuria
type 3		Persistent cutaneous papillomas
type 4		Upper alimentary tract papillomas capable of progression to malignancy in association with bracken fern ingestion
type 5		Teat fibropapilloma
type 6		Teat papilloma
type 7		Papillomas on skin and teats
type 8		Papillomas on teats and skin
types 9, 10		Papillomas on teats
Equine papillomavirus	Horse, donkey, mule	Papillomas of young horses
Canine oral papillomavirus	Dogs	Papillomas in oral cavity
Ovine papillomavirus types 1, 2	Sheep	Fibropapillomas
Cottontail rabbit papillomavirus (Shope's papillomavirus)	Cottontail rabbits	Lesions appear as localized keratinized horns
Rabbit oral papillomavirus	Rabbit	Benign papillomas of oral cavity

Infection with BPV type 4 causes papillomas of the oesophagus, rumen and reticulum. Ingestion of bracken fern is believed to be responsible for the transformation of these papillomas to squamous cell carcinomas.

Infection of the teats and udders of cows may be caused by BPV type 1, type 5 or type 6. 'Rice grain' fibropapillomas of the teats of cows associated with BPV-5 have been described. Generally these lesions are of little consequence unless large enough to interfere with milking. Bovine papillomavirus type 6 causes 'frond' papillomas on the teats of cows and may also affect the udder. Several new genotypes have been described associated with the teats of cows (Ogawa et al. 2004).

Equine Papillomatosis

Papillomas are common in horses between one and three years of age. DNA studies have indicated two types of equine papillomavirus: type 1, associated with papillomas on the muzzle and legs; type 2, associated with papillomas of the genital tract. Lesions usually regress spontaneously after a variable period of one to nine months and the animals are immune to re-infection.

Equine Sarcoids

The equine sarcoid is the most common neoplasm of horses, donkeys and mules. It is a locally invasive fibroblastic skin tumour caused by bovine papillomavirus types 1 and 2 or closely related viruses (Otten et al. 1993, Goodrich et al. 1998). Sarcoids are usually observed in horses between three and six years of age. Multiple cases can occur in families or groups of horses kept together. Sarcoids can occur on any part of the body, singly or in clusters. The most commonly affected sites are the head, ventral abdomen and limbs.

Canine Oral Papillomatosis

Multiple, transmissible papillomas in the oropharyngeal region of dogs are reasonably common. The condition is most common in young dogs and is caused by canine oral papillomavirus (COPV). Spontaneous regression occurs within months of the onset of the condition. Papillomas affecting other sites of the body have also been associated with papillomavirus infection (Narama et al. 1992, Nagata et al. 1995, Le Net et al. 1997). It is considered that different papillomaviruses are responsible for the multiplicity of lesions described in the dog (Nicholls & Stanley 1999).

REFERENCES

Bernard, H.U., Burk, R.D., Chen, Z., et al., 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 401, 70–79.

Bredal, W.P., Thoresen, S.I., Rimstad, E., et al., 1996. Diagnosis and clinical course of canine oral papillomavirus infection. Journal of Small Animal Practice 37, 138–142.

Campo, M.S., Jarett, W.F.H., Farron, R., et al., 1992. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. Cancer Research 52, 6898–6904.

de Villiers, E.M., Fauquet, C., Broker, T.R., et al., 2004. Classification of papillomaviruses. Virology 20, 17–27.

Goodrich, L., Gerber, H., Marti, E., et al., 1998. Equine sarcoids. Veterinary Clinics of North America: Equine Practice 14, 607–623.

Le Net, J., Orth, G., Sundberg, J.P., et al., 1997. Multiple pigmented cutaneous papules associated with a novel canine papillomavirus in an immunosuppressed dog. Veterinary Pathology 34, 8–14.

Nagata, M., Nanko, H., Moriyama, A., et al., 1995. Pigmented plaques associated with papillomavirus infection in dogs: is this epidermodysplasia verruciformis? Veterinary Dermatology 6, 179–186.

Narama, I., Ozaki, K., Maeda, H., et al., 1992. Cutaneous papilloma with viral replication in an old dog. Journal of Veterinary Medical Science 54, 387–389.

Nicholls, P.K., Stanley, M.A., 1999. Canine papillomavirus – a centenary review. Journal of Comparative Pathology 120, 219–233.

Ogawa, T., Tomita, Y., Okada, M., et al., 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. Journal of General Virology 85, 2191–2197.

Otten, N., von Tscharner, C., Lazary, S., et al., 1993. DNA of bovine papillomavirus Type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Archives of Virology 132, 121–131.

Teifke, J.P., Lohr, C.V., et al., 1998. Detection of canine oral papillomavirus-DNA in canine oral squamous cell carcinomas and p53 over-expressing skin papillomas of the dog using the polymerase chain reaction and non-radioactive in situ hybridisation. Veterinary Microbiology 60, 119–130.

FURTHER READING

Nasir, L., Campo, M.S., 2008. Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. Veterinary Dermatology 19, 243–254.

Chapter 48

Adenoviridae

Adenoviruses (from the Greek word *adenos* meaning gland) were first isolated from explant cultures of human adenoids. They are non-enveloped, icosahedral viruses, 70–90 nm in diameter, containing a single linear molecule of double-stranded DNA (Fig. 48.1). One to two protein fibres protrude from each of the 12 vertices of the capsid. The family *Adenoviridae* contains five genera designated *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and a genus of fish adenoviruses, *Ichtadenovirus*. Mammalian adenoviruses, assigned to the genus *Mastadenovirus*, share a common antigen and are serologically distinct from those that infect birds, genus *Aviadenovirus*. Many adenoviruses agglutinate rat or monkey erythrocytes. The fibre protein is responsible for this property and contains type-specific determinants. The haemagglutination inhibition test is used to confirm serospecificity. Adenoviruses are moderately resistant, surviving in the environment for days or weeks under suitable conditions. Adenoviruses can withstand freezing, mild acids and lipid solvents. Infectivity is lost following heating at 56°C for more than 10 minutes. The adenoviruses of veterinary importance are shown in Figure 48.2 and Table 48.1.

Adenoviruses replicate in the nucleus of the cell. Newly assembled virions form crystalline aggregates which appear as intra-nuclear basophilic inclusions when viewed using light microscopy. Infection with adenoviruses is common in many animal species and man. They are generally host species-specific having a natural host range confined to a single species or closely related animal species. More than 50 human serotypes, grouped into seven virus species, have been identified to date. However, the vast majority of infections appear to be subclinical or mild with more severe disease signs confined to immunodeficient individuals. A similar situation pertains in the case of many animal adenovriuses. In contrast, canine adenovirus type 1 causes a generalized disease affecting the liver and vascular system of dogs.

AVIAN ADENOVIRUSES

Adenoviruses have been isolated from fowl, turkeys, pheasants, pigeons, ducks, quail, geese, guinea fowl and budgerigars. Some avian adenoviruses exhibit a marked species specificity while others have a broad host species range. The distribution of infection is worldwide and extremely common in poultry flocks. Faeces are an important source of infection. Egg transmission also occurs. Maternal antibodies control infections in young birds. As these antibodies are lost, the birds may undergo infection. The majority of fowl adenovirus infections are subclinical or only associated with mild disease. A number of fowl adenoviruses are associated with a specific clinical syndrome such as inclusion body hepatitis.

Inclusion Body Hepatitis

Inclusion body hepatitis (IBH) occurs chiefly in broilers but can also be seen in rearing pullets. Several fowl adenoviruses have been associated with IBH but the exact aetiology and pathogenesis requires further clarification. There is a sudden increase in mortality in affected flocks. Mortality averages 10% but may be as high as 30% where there is immunosuppression due to infectious bursal disease or chicken anaemia virus infection. Lesions include an enlarged, friable liver with haemorrhages on the surface, intramuscular haemorrhages and anaemia. Histologically there is hepatic necrosis and intranuclear inclusions in hepatocytes. Diagnosis is usually based on clinical signs

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and post mortem examination because apparently healthy birds can also excrete fowl adenoviruses and possess antibodies. Control measures are general in nature because vaccines are not routinely available and the aetiology and pathogenesis of IBH is not fully understood.

Egg Drop Syndrome

Egg drop syndrome was first described in Northern Ireland in 1976. Infection with duck adenovirus A is widespread in ducks and is believed to have been introduced into chickens through the use of a contaminated vaccine. The disease is characterized by a drop in egg production, by the laying of abnormal eggs or by a failure to peak. The condition is usually seen in hens between the start of lay and 36 weeks of age. Lesions are found in the oviduct, particularly the pouch shell gland, of affected birds. Intranuclear inclusions are commonly seen in the epithelial cells of the pouch shell gland. Suitable specimens for isolation of the virus in avian cell lines, particularly duck kidney or fibroblast cells, include samples of oviduct and material from the pouch shell gland. Detection of the virus is possible using immunofluorescence, ELISA or PCR. The virus agglutinates avian red cells and the HAI test is the test of choice for the detection of antibodies.

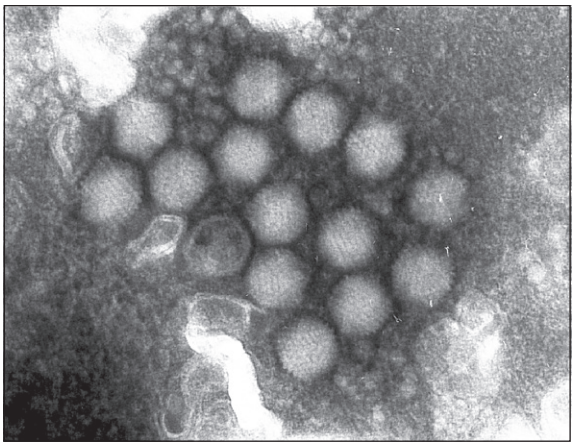

Figure 48.1 Electron micrograph of adenoviruses.

CANINE ADENOVIRUS INFECTION

Two closely related serotypes of canine adenovirus, canine adenovirus type 1 (CAV-1) and canine adenovirus type 2 (CAV-2), are described in dogs. Both serotypes can induce mild respiratory disease following experimental aerosol infection. However, under natural conditions CAV-1 is associated with a generalized infection, infectious canine hepatitis (see below), while CAV-2 is more commonly associated with respiratory disease. Canine adenovirus type 1 has been isolated on occasion from cases of infectious canine tracheobronchitis but simultaneous

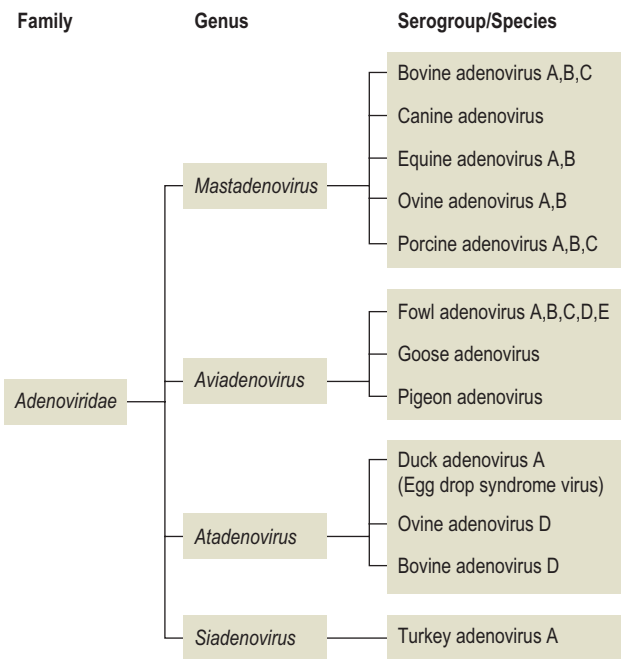

Figure 48.2 Classification of adenoviruses of veterinary significance.

Table 48.1 Adenoviruses of animals

Virus	Host species	Disease
Canine adenovirus serotype 1	Dog, fox	Cause of infectious canine hepatitis, a generalized infection affecting liver and blood vascular system
Canine adenovirus serotype 2	Dog	One of the causes of infectious tracheobronchitis (kennel cough), a highly contagious respiratory disease complex
Equine adenovirus A	Horse	Generally subclinical or mild respiratory infection but also associated with fatal pneumonia in Arabian foals affected by combined immunodeficiency disease
Bovine adenoviruses	Cattle	Occasional outbreaks of enteric and respiratory disease
Ovine adenoviruses	Sheep	Occasional outbreaks of enteric and respiratory disease
Porcine adenoviruses	Pig	Occasional cases of diarrhoea, usually subclinical
Fowl adenoviruses	Fowl, quail	Numerous serotypes. Frequently isolated from healthy birds or following mild respiratory disease. Associated with inclusion body hepatitis and quail bronchitis
Duck adenovirus A	Fowl, duck	Cause of egg drop syndrome in laying hens
Turkey adenovirus A	Turkey, pheasant	Cause of turkey haemorrhagic enteritis which affects four- to 12-week-old turkey poults and of marble spleen disease which affects two- to eight-month-old pheasants

presentation of the respiratory and the systemic forms of disease in the one animal does not appear to take place.

Canine adenovirus type 2 is readily transmitted by aerosol, replicating in both the upper and lower respiratory tract. Clinical signs are typically mild or inapparent. Affected dogs present with signs typical of canine infectious tracheobronchitis ('kennel cough'). Most dogs recover uneventfully and are immune to subsequent challenge. Occasional cases of bronchopneumonia develop as a result of secondary bacterial infection. Virus is shed for about eight or nine days post infection and can be isolated from nasal or oropharyngeal swabs. Diagnosis can be confirmed by virus isolation in susceptible cells such as Madin–Darby canine kidney cells, in situ hybridization and PCR (Benetka et al. 2006). Vaccines containing CAV-2 also confer protection against CAV-1 infection.

INFECTIOUS CANINE HEPATITIS

Infectious canine hepatitis (ICH) or Rubarth's disease is a worldwide, generalized viral disease of dogs that principally affects the liver and blood vascular system. However, ICH is a relatively rare disease because of a low morbidity rate and the widespread use of effective commercial vaccines. It is caused by canine adenovirus 1 (CAV-1). Urine is an important source of virus for the transmission of infection as virus can be shed in urine for six months or more.

Pathogenesis

The incubation period is four to seven days. Following ingestion CAV-1 localizes in the tonsils and Peyer's patches. A viraemia develops and CAV-1 replicates in vascular endothelium, resulting in the rapid distribution of virus to virtually every tissue of the body. Virus replication also occurs in the parenchymal cells of the liver and lymphatic tissues. Lesions at this stage are due to the cytotoxic effects of the virus. Subclinical infection is common. Clinically affected dogs may present with peracute or acute illness. The acute form of the disease is accompanied by fever, depression, anorexia, increased thirst, vomiting and diarrhoea. Abdominal palpation may elicit pain and reveal hepatomegaly. Tonsillitis, pharyngitis and cervical lymphadenopathy are frequently evident. Jaundice is not a common presentation.

The production of neutralizing antibodies commences from about day seven post infection. Most dogs recover at this stage. Immune mediated lesions of glomerulonephritis, corneal oedema ('blue eye') and anterior uveitis, associated with the deposition of antigen–antibody complexes, are not uncommon in recovering animals.

Diagnosis

A history of fever, sudden collapse and abdominal pain in a young, unvaccinated dog is suggestive.

- Haematological examination is helpful. There is a marked reduction in the numbers of neutrophils and lymphocytes during the febrile stage. Prolonged blood clotting time is also suggestive.
- Histopathology: the presence of intranuclear inclusion bodies in hepatocytes and Kupffer cells is diagnostic for ICH. Viral antigen may be demonstrated in cryostat sections of liver using immunofluorescence.
- Use of PCR for detection of the DNA of CAV-1 in clinical specimens and for differentiating CAV-1 from CAV-2 has been described (Chouinard et al. 1998, Hu et al. 2001, Maxson et al. 2001).
- Virus isolation may be carried out in canine kidney cells. Suitable specimens include oropharyngeal swabs, blood, urine and faeces from the live animal, during the febrile stage of the disease. Suitable post mortem samples include spleen, lymph nodes and kidney. Liver samples are not suitable due to high levels of arginase which have an inherent inhibitory effect on viral replication in cell culture.
- Serology: demonstration of a rising antibody titre, using virus neutralization or haemagglutination inhibition tests, provides good evidence of CAV-1 involvement.

REFERENCES

Benetka, V., Weissenbock, H., Kudielka, I., et al., 2006. Canine adenovirus type 2 infection in four puppies with neurological signs. Veterinary Record 158, 91–94.

Chouinard, L., Martineau, D., Forget, C., et al., 1998. Use of polymerase chain reaction and immunohistochemistry for detection of canine adenovirus type 1 in formalin-fixed, paraffin-embedded liver of dogs with chronic hepatitis or cirrhosis. Journal of Veterinary Diagnostic Investigation 10, 320–325.

Hu, R.L., Huang, G., Qiu, W., et al., 2001. Detection and differentiation of CAV-1 and CAV-2 by polymerase chain reaction. Veterinary Research Communications 25, 77–84.

Maxson, T.R., Meurs, K.M., Lehmkuhl, L.B., et al., 2001. Polymerase chain reaction analysis for viruses in paraffin-embedded myocardium from dogs with dilated cardiomyopathy or myocarditis. American Journal of Veterinary Research 62, 130–135.

Chapter 49

Herpesviridae

The family *Herpesviridae* includes more than 100 viruses infecting fish, amphibians, reptiles, birds and mammals including man. Their ubiquitous occurrence, evolutionary diversity and involvement in a range of important medical and veterinary diseases make this group of viruses one of the most important. The name is derived from the Greek word 'herpein' meaning 'creeping' in reference to the recurrence of vesicles in people infected with herpes simplex virus. Herpesviruses are enveloped and range in size from 200–250 nm in diameter (Fig. 49.1). Virions contain linear, double-stranded DNA within an icosahedral capsid, approximately 125 nm in diameter. Between the envelope and the capsid there is a layer of amorphous material containing several proteins, termed the tegument. Herpesviruses enter cells by fusion with the plasma membrane. The capsid then moves to the nucleus where transcription, viral DNA replication and capsid assembly take place. The envelope is probably derived from the nuclear membrane of the host cell, although this is currently a matter of some debate. It is modified such that it contains several different viral-encoded glycoproteins. The enveloped virions accumulate in the endoplasmic reticulum prior to final processing of glycoproteins in the Golgi apparatus and release by exocytosis. Productive infection results in cell death. Intranuclear inclusions are characteristic of herpesvirus infection. Infected cells may fuse to form syncytia permitting virus to infect neighbouring cells without being exposed to antibody. Host protective antibody responses are usually directed against the envelope glycoproteins. Herpesvirus virions are fragile, sensitive to detergents and lipid solvents, and do not survive well in the environment.

The family is divided into three subfamilies (Fig. 49.2) containing twelve genera; *Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae*. A previously unassigned genus *Ictalurivirus* containing ictalurid herpesvirus 1, a virus of channel catfish, has now been assigned to a separate family *Alloherpesviridae* within the order *Herpesvirales*. The subfamily classification was originally based solely on biological properties but nucleotide sequence and phylogenetic analyses have resulted in some recent changes, most noticeably Marek's disease virus which was originally classified as a gammaherpesvirus has now been placed in its own genus in the alphaherpesvirus subfamily. Alphaherpesviruses replicate and spread rapidly, destroying host cells and often establishing latent infections in sensory nerve ganglia. Betaherpesviruses replicate slowly, spread slowly and cause infected cells to become enlarged, hence the common name cytomegalovirus. They may become latent in cells of the monocyte series. Gammaherpesviruses are specific for T or B lymphocytes and can establish latent infections in these cells. However, infection of lymphocytes is frequently arrested with minimal expression of the viral genome. A number of gammaherpesviruses are associated with lymphoproliferative disorders such as human herpesvirus 4 (Epstein–Barr virus) which is associated with Burkitt's lymphoma in children and human herpesvirus 8 which is associated with Kaposi's sarcoma.

A feature common to all herpesviruses is the ability to establish life-long latent infections which may be reactivated to cause further bouts of clinical disease (Table 49.1). Shedding of virus may be periodic, coinciding with recrudescence, or continuous. During latency the episomal viral genome becomes closed and circular with only a small subset of genes expressed. Reactivation of infection is associated with various stressors including transportation, overcrowding, intercurrent infection and adverse weather. In nature herpesviruses are usually restricted to a single host species. The viruses are highly adapted to their natural hosts as a result of co-evolution and infections are

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usually inapparent or trivial. In some circumstances, particularly in the very young, where there is immunosuppression or following infection of an alternative host, infections can be life-threatening. A number of herpesviruses, such as gallid herpesvirus 2 (Marek's disease virus), have been implicated in neoplastic transformation.

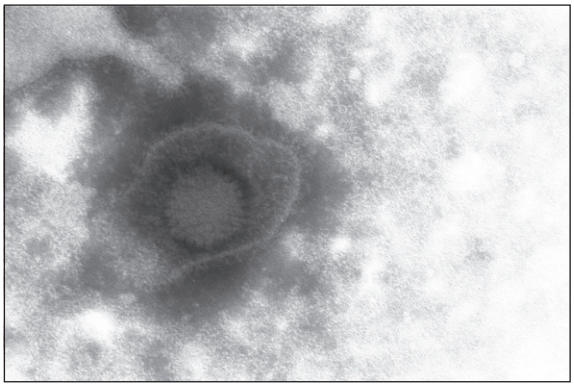

Figure 49.1 Electron micrograph of a herpesvirus.

INFECTIOUS BOVINE RHINOTRACHEITIS/INFECTIOUS PUSTULAR VULVOVAGINITIS

Bovine herpesvirus 1 (BoHV-1) is one of the most important viruses of cattle, causing significant losses to domestic and wild cattle around the world. It is associated with several clinical conditions: infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, abortion, balanoposthitis, conjunctivitis and fatal generalized disease of newborn calves. Successful eradication programmes have been carried out in Austria, Denmark, Finland, Norway, Sweden and Switzerland. Only a single antigenic type of BoHV-1 occurs but three subtypes, 1.1, 1.2a and 1.2b, have been recognized on the basis of restriction enzyme analysis. Subtype 1.1 is associated with respiratory disease and most vaccines are based on this subtype. Subtypes 1.2a and 1.2b are associated with mild respiratory disease and the genital form of infection, infectious balanoposthitis/infectious pustular vulvovaginitis (IBP/IPV). Both subtypes 1.1 and 1.2a are capable of causing abortions while the less virulent subtype 1.2b is not. Many infections are

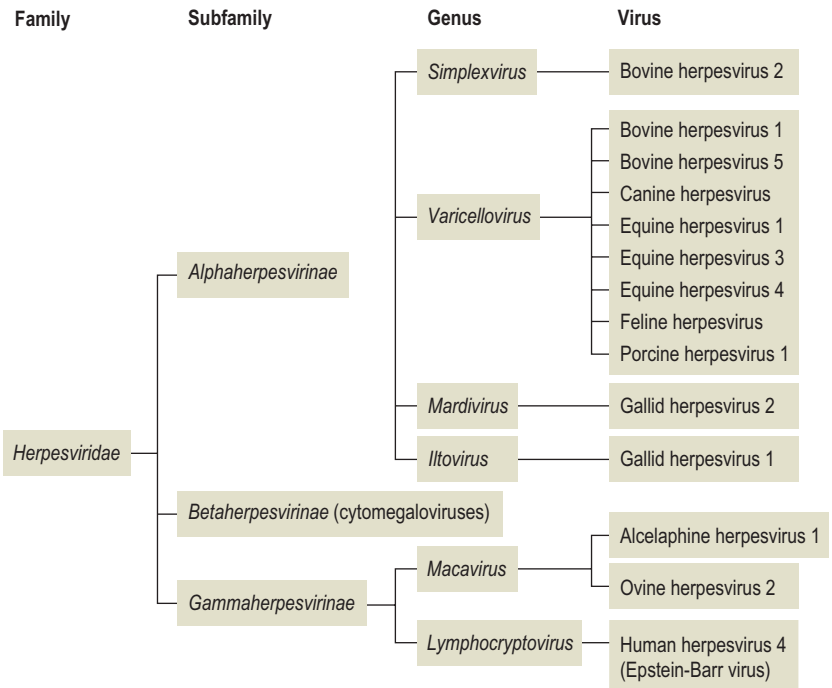

Figure 49.2 Classification of herpesviruses of veterinary importance.

Table 49.1 Herpesviruses of veterinary significance

Virus	Host	Significance of infection
Bovine herpesvirus 1	Cattle	Worldwide, important viral infection of cattle. Separate respiratory (infectious bovine rhinotracheitis) and genital (infectious pustular vulvovaginitis) forms of disease. Abortion occurs in pregnant cows following respiratory disease outbreaks
Bovine herpesvirus 2	Cattle	Infection in temperate regions associated with ulcerative mammillitis. Mild, generalized skin infection, pseudo-lumpy skin disease, described in tropical and subtropical regions
Bovine herpesvirus 4	Cattle	Frequently subclinical but has been associated with mastitis and endometritis
Bovine herpesvirus 5	Cattle	Associated with outbreaks of non-suppurative encephalitis in calves in USA, Argentina, Hungary and Australia
Alcelaphine herpesvirus 1	Wildebeest, cattle, deer	Subclinical infection in wildebeest. Associated with malignant catarrhal fever in cattle and deer. Distribution confined to Africa and zoological collections
Ovine herpesvirus 2	Sheep, goats, cattle, deer	Subclinical infection in sheep and goats. Associated with malignant catarrhal fever in cattle and deer. Worldwide distribution
Equine herpesvirus 1	Horses	Infection associated with abortion, respiratory disease, generalized neonatal infection and nervous disease. Common, worldwide infection
Equine herpesvirus 3	Horses	Mild, venereal infection characterized by lesions on the external genitalia of mares and stallions
Equine herpesvirus 4	Horses	Cause of equine rhinopneumonitis in young horses and sporadic cause of abortion. Common, worldwide infection
Porcine herpesvirus 1	Pigs	Cause of Aujeszky's disease in pigs, characterized by high mortality in piglets, weight loss and respiratory disease in fatteners and reproductive disorders in sows and boars. Transmission to other farm species may occur, associated with a fatal neurological disease (pseudorabies)
Porcine herpesvirus 2 (pig cytomegalovirus)	Pigs	Associated with upper respiratory tract infection in young pigs (inclusion body rhinitis) and generalized infection of foetuses. Widespread distribution but infection inapparent in herds where enzootic
Canine herpesvirus	Dogs	Uncommon cause of disease, associated with fatal, generalized infection in susceptible neonates
Feline herpesvirus	Cats	Common infection with worldwide distribution. Cause of feline viral rhinotracheitis, an acute, upper respiratory tract disease of young cats
Anatid herpesvirus 1	Ducks (duck plague), geese, swans	Highly contagious, acute disease characterized by ocular and nasal discharge, diarrhoea and high mortality. Worldwide distribution
Gallid herpesvirus 1	Chickens	Cause of infectious laryngotracheitis, an acute, upper respiratory tract disease. Worldwide distribution. Significant mortality associated with high-virulence strains of virus
Gallid herpesvirus 2 (Marek's disease virus)	Chickens	Marek's disease is an economically important, lymphoproliferative disease of chickens characterized by leg and wing paralysis in 12–24-week-old birds. Infection is common and worldwide
Pigeon herpesvirus 1	Pigeons	Conjunctivitis and upper respiratory tract infection of young birds. Asymptomatic, latent infection in adult birds. Worldwide distribution
Psittacid herpesvirus 1	Psittacine species	Cause of Pacheco's disease. Fatal, generalized infection associated with stress

subclinical. A single clinical form, either respiratory or genital, usually predominates in an outbreak because the virus tends to remain localized at the site of infection following transmission. Conjunctivitis is commonly seen together with the respiratory form.

Transmission of virus usually occurs via respiratory or genital secretions and is facilitated by close grouping of animals. Aerosol transmission probably only occurs over short distances. The semen of infected bulls may contain virus and genital infection may follow natural service or artificial insemination. Aborted foetuses are also an important source of infection. Reactivation of latent infections can occur following a stressful incident such as parturition or transport resulting in virus being synthesized at the site(s) of latency and then transported intra-axonally to the original portal of entry. Such animals usually show no clinical signs when the infection is reactivated but shed virus into the environment and are an important means of perpetuating infection in herds.

Pathogenesis

The incubation period is two to four days for both the respiratory and genital forms. In the case of infectious bovine rhinotracheitis, infection usually occurs by aerosol, entering the animal via the nares and replicating in the mucous membranes of the upper respiratory tract. Large quantities of virus are produced and excreted. The virus also enters the axons of local nerve cells and migrates by intra-axonal transport to the trigeminal ganglion where latency is established. In most cases the infection is brought under control within one to two weeks by a pronounced immune response. However, the tissue damage caused can facilitate secondary bacterial infection resulting in severe damage and possibly death. Wider dissemination of virus can occur following viraemia and may produce foetal infection and abortion in pregnant cows. Following genital infection, the virus replicates in the mucous membranes of the vagina or prepuce. Latent infection is established in the sacral ganglia. Viraemia does not appear to occur in these cases and pregnant cows rarely abort.

The primary lesion, resulting from the cytopathic effects of the virus, is a focal necrosis of upper respiratory or genital mucosa. Focal lesions of pustular necrosis may coalesce to form larger areas of ulcerated mucosa, covered by a diphtheritic membrane. There is an intense inflammatory reaction. Secondary bacterial infection may give rise to pneumonia or endometritis. Necrotic foci are found in various organs, particularly the liver, in aborted foetuses.

Clinical signs in the respiratory form vary from mild to severe. Animals recover after about a week in uncomplicated cases. Where bacterial infection becomes established dyspnoea, coughing, open-mouth breathing and death may be observed. In severe outbreaks such as occur in feedlots morbidity may approach 100% with mortality rates as high as 10%. Abortions may occur at the same time or up to three months after the respiratory disease. Cows with IPV show signs of frequent urination, elevated tailhead and vaginal discharge.

Severe, generalized disease characterized by fever, nasal and ocular discharges, respiratory signs, diarrhoea, incoordination, convulsions and death has been described in young calves but is more commonly associated with bovine herpesvirus 5 than BoHV-1.

Diagnosis

- Ocular swabs and nasal or genital swabs rubbed vigorously against the mucosal surface and collected from several affected animals in the early acute phase of the disease are suitable for virus isolation (Nettleton et al. 1983). The virus is reasonably fragile and specimens should be submitted in viral transport medium at 4°C. Mucous membranes of the respiratory tract as well as lung and bronchial lymph node samples can be collected during post mortem examination of animals that have died due to the respiratory disease. Suitable foetal samples include liver, lung, spleen and kidney. A wide range of bovine cell lines are susceptible to infection including primary, secondary and continuous cell lines such as Madin-Darby bovine kidney cell line. The virus produces a rapid cytopathic effect (CPE) in bovine cell lines, usually within three days of inoculation, characterized by holes in the monolayer surrounded by grape-like clusters of rounded cells. Raw semen is toxic to cells and must be prediluted.
- For a more rapid diagnosis it is possible to make smears from ocular, nasal or genital swabs or to cut frozen sections from the tissues of aborted foetuses in order to demonstrate the presence of viral antigen by immunofluorescence. Nasal samples should be obtained from a number of animals in the early stages of the respiratory disease in order to increase the chances of detection. Viral antigen can also be detected using ELISA (Edwards & Gitao 1987). These techniques are not as sensitive as virus isolation.
- The presence of characteristic gross and microscopic lesions, particularly intranuclear inclusions, in aborted foetuses is highly suggestive and helpful in establishing a diagnosis of BoHV-1.
- The polymerase chain reaction has been adapted for detection of BoHV-1 viral DNA in clinical samples (Moore et al. 2000) and is the method of choice for the detection of virus in semen (Smits et al. 2000). It is more sensitive and rapid than virus isolation and can also be used to detect latent infection in sensory ganglia. Assays based on detection of gE sequences can be used to discriminate between wild-type virus and gE-deleted vaccine strains (Fuchs et al. 1999). Real time PCR protocols have also been described (Kramps 2008).

- The collection of paired sera and demonstration of a rising antibody titre by virus neutralization or ELISA (Kramps et al. 1993) is indicative of infection. Several ELISA kits are available commercially as well as gE-ELISAs that can be used in conjunction with marker vaccines to distinguish infected cattle from vaccinates (Wellenberg et al. 1998). The blocking ELISA format, particularly gB-specific ELISAs, is considered to be more sensitive than the indirect ELISA format (Perrin et al. 1993, Kramps et al. 2004). Serological testing to demonstrate a rising titre is less useful in abortion cases due to the delay between infection and time of abortion such that animals often have high titres at the time of abortion. The ELISA has been adapted for the testing of bulk milk samples for surveillance purposes (Nylin et al. 2000). Infected herds where less than 20% of the animals tested have antibodies to BoHV-1 will give a negative bulk milk test. Animals infected in early life when maternally derived antibodies are present may become latently infected but not develop an active antibody response (Lemaire et al. 2000). The failure to detect these seronegative latent carriers (SNLC) serologically, presents a difficulty in the implementation of control programmes.

MALIGNANT CATARRHAL FEVER

Malignant catarrhal fever (MCF) is a sporadic, acute, frequently fatal disease of cattle, deer and some wild ruminants. It is caused by two related but distinct gammaherpesviruses, alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OHV-2). The natural hosts for AlHV-1 and OHV-2 are wildebeest and sheep, respectively. Infection in the natural host is common and subclinical. The corresponding diseases caused by these viruses in cattle and farmed deer is referred to as wildebeest-associated MCF (WA-MCF) and sheep-associated MCF (SA-MCF). The distribution of AlHV-1 is largely confined to Africa and zoological gardens while OHV-2 occurs in sheep and goats worldwide.

In endemically infected wildebeest populations alcelaphine herpesvirus 1 is transmitted both vertically and horizontally. Latent infection is thought to occur in lymphoid cells. In most cases infection is acquired by calves shortly after being born from their mothers or infected comrades via nasal secretions. Transplacental transmission occurs in some instances. A persistent viraemia occurs in young wildebeest for the first few months of life and it is at this time that large quantities of cell-free, infectious virus is shed in nasal and ocular secretions. Cattle in contact with wildebeest at this time may become infected. The situation in sheep is thought to be similar to that in wildebeest with the greatest risk of transmission to cattle associated with lambing time and contact with young lambs. However, a number of studies indicate that young sheep at six to nine months of age are the highest risk group for transmission (Li et al. 2004). Cattle and deer are considered to be 'end-hosts' as virus does not appear to be transmitted by infected animals. Both seroconversion and the presence of OHV-2 DNA have been reported in clinically normal cattle suggesting that cattle may become infected without developing clinical signs (Powers et al. 2005). The disease has been shown to also occur in pigs (Loken et al. 1998).

Pathogenesis

The pathogenesis of MCF is not well understood. The presumed site of entry to the body is the upper respiratory tract. A cell-associated viraemia occurs but several studies have found little evidence of viral antigen in affected organs. For many years the cause of lesions had been ascribed to an autoimmune phenomenon characterized by a necrotizing process and destruction of normal host cells. However, recent evidence suggests that lesions are caused by the activities of virus-infected, dysregulated cytotoxic T cells (Russell et al. 2009). The incubation period is highly variable but typically lasts about three to four weeks. Four clinical forms have been described; peracute, 'head-and-eye', intestinal and mild. The 'head-and-eye' form is the most common. It is characterized by sudden onset, fever, ocular and nasal discharges, enlarged lymph nodes, conjunctivitis with corneal opacity and erosive mucosal lesions in the upper respiratory tract leading to profuse mucopurulent nasal discharge and encrustation of the muzzle. Some animals display central nervous signs including muscular tremors, incoordination and head pressing. Diarrhoea or dysentery may be a feature of the disease. The course of the disease is usually three to seven days, typically ending in death of the animal. However, chronic cases lasting several weeks or months as well as recovery have been described (O'Toole et al. 1997). In peracute cases, particularly in deer, there may be sudden death without premonitory signs.

Diagnosis

- Diagnosis is generally based on clinical signs and characteristic histopathological changes including disseminated fibrinoid vasculitis, widespread lymphoid infiltration and ulceration of surface epithelia.
- In the clinically affected live animal detection of OHV-2 DNA by PCR is the test of choice and can be used with peripheral blood leukocytes, fresh tissues and paraffin-embedded tissues (Baxter et al. 1993, Muller-Doblies et al. 1998, Crawford et al. 1999).
- Antibody to OHV-2 can be detected in 70 to 80% of clinically affected cattle by indirect

immunofluoresence or the immunoperoxidase test using AlHV-1-infected cells. A competitive inhibition ELISA has been developed for the detection of serum antibodies to AlHV-1 and OHV-2 (Li et al. 1994, 2001). It is not as sensitive a test as PCR or histopathology for the diagnosis of MCF in clinically affected animals but is extremely useful in epidemiological studies.

- Isolation of AlHV-1 in bovine thyroid cells is possible from the buffy coat and lymph nodes of animals with WA-MCF. Evidence of CPE may not be visible for up to 21 days postinoculation. Ovine herpesvirus-2 has not been isolated to date.

BOVINE HERPES MAMMILLITIS AND PSEUDO-LUMPY SKIN DISEASE

Infection with bovine herpesvirus 2 (BoHV-2) causes outbreaks of a severe, ulcerative condition of the teats of dairy cows. The condition is worldwide in occurrence. In tropical and subtropical regions bovine herpesvirus 2 infection is associated with a generalized, mild skin infection termed pseudo-lumpy skin disease, to distinguish it from the more serious lumpy skin disease caused by a poxvirus.

In temperate regions outbreaks of herpes mammillitis are sporadic in nature, usually occurring in the autumn or early winter. Latent infection and subsequent reactivation facilitates the spread and perpetuation of infection within and between herds. Outbreaks usually begin with a few affected animals, typically first-calvers, a few days after calving. Serous exudate from lesions contains large amounts of virus. Transmission to other cows in the herd occurs via direct and indirect contact principally during milking. A wide range of wild animal species are thought to act as subclinical reservoirs of infection in Africa. Insect involvement in transmission is likely to be much more important in warmer climates and may account for the occurrence of the generalized skin form of the disease.

Pathogenesis

The incubation period is three to eight days. Viral replication is optimal at a temperature somewhat lower than normal body temperature. Following intradermal or subcutaneous inoculation BoHV-2 replicates locally. Dissemination to other sites does not occur. In contrast, the intravenous inoculation of BoHV-2 has been shown to produce generalized infection with the development of widespread skin nodules.

Subclinical infection is not uncommon and the number of animals displaying clinical signs during outbreaks is highly variable. Lesions appear as thickened plaques on one or more teats. The surface sloughs leaving an ulcerated area that becomes covered in a dark scab. The lesions are painful and there is a significant reduction in milk yield due to difficulties in milking and predisposition to mastitis. Circular ulcers may be seen on the lips and muzzle of calves suckling affected cows. In cases of pseudo-lumpy skin disease a variable number of circular and hard skin nodules with a depressed centre appear over the neck, shoulders, back and perineum. Healing occurs without a scar within a couple of weeks.

Diagnosis

Diagnosis is based on clinical signs and demonstration of virus in scrapings or vesicular fluid by direct electron microscopy or alternatively using virus isolation in primary bovine cell lines. The temperature of incubation for inoculated cell cultures should be reduced to 32°C. A PCR assay has been published that is suitable for the detection of BoHV-2 in skin lesions (d'Offay et al. 2003). Paired sera can be used to try to demonstrate a rise in antibody titre but a high titre of antibody is frequently already present by the time the first sample is obtained. Suitable serological tests include virus neutralization, complement fixation, agar gel immunodiffusion and indirect immunofluorescence.

EQUINE RHINOPNEUMONITIS AND EQUINE HERPESVIRUS ABORTION

Equine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4) are closely related alphaherpesviruses endemic in horse populations worldwide. They are responsible for annual outbreaks of respiratory disease among young horses and for outbreaks of abortion among pregnant mares. Infection with EHV-1 is associated with respiratory disease, abortion, fatal generalized neonatal disease and myeloencephalopathy. Infection with EHV-4 is primarily associated with respiratory disease but sporadic abortions have also been reported. Close contact facilitates the transmission of these rather fragile viruses. Transmission usually occurs by the respiratory route following direct or indirect contact with nasal secretions or with aborted foetuses, fluids and placentae. Serological studies have shown that the prevalence of antibody to EHV-4 approaches 100% in all ages whereas the prevalence of antibody to EHV-1 is about 30% in adults and somewhat lower in foals (Gilkerson et al. 1999b). It is thought that regular subclinical episodes of reactivation of latent EHV-4 infection occur in adult horses resulting in transmission to and respiratory disease in the annual foal crop. In contrast, foals become infected with EHV-1 from their dams or from other lactating mares in the group. Foal-to-foal spread then occurs both before and after weaning (Gilkerson et al. 1999a). Not all females infected with EHV-1 subsequently abort but all infected mares are potential

shedders of virus. The fact that not all mares are exposed to EHV-1 means that when reactivation of infection in a latent carrier mare occurs it may result in infection in a large number of non-immune, in-contact pregnant mares leading to abortion storms.

Pathogenesis

Respiratory disease caused by EHV-4 and characterized by signs of fever, pharyngitis and profuse serous nasal discharge follows an incubation period of two to eight days. Primary replication occurs in the upper respiratory tract and local lymph nodes with spread in some cases to the lower respiratory tract and lungs. Secondary bacterial infection is common, giving rise to a mucopurulent nasal discharge, coughing and in some cases bronchopneumonia. In uncomplicated cases recovery is usual within one to two weeks. Respiratory disease associated with EHV-1 is clinically indistinguishable. Latent infection with both viruses is believed to occur in the trigeminal ganglia. In the case of EHV-4 infection appears to be restricted to the respiratory tract and viraemia is rare. In contrast, local replication by EHV-1 may be succeeded by a lymphocyte-associated viraemia which may result in abortion or nervous disease. Both latency and reactivation have been demonstrated for EHV-1 in lymphocytes. The virus is capable of spreading directly from an infected leukocyte to contiguous cells without the requirement for an extracellular phase, thus avoiding inactivation by circulating antibody. Transplacental infection of the foetus results in widespread viral damage. Equine herpesvirus 1 has a tropism for vascular endothelium. Viral vasculitis and thrombosis in the pregnant uterus along with infection of the foetus results in abortion. Aborting mares infected with EHV-1 rarely show any preceding signs. Abortion typically occurs several weeks or months after exposure, usually during the last four months of gestation. Mares appear to abort from EHV-1 only once and subsequent fertility is not impaired. Infection close to term may result in the birth of an infected foal. Such foals usually die due to interstitial pneumonia and viral damage to various tissues, often complicated by secondary bacterial infection.

Cases of nervous disease associated with EHV-1 infection, referred to as equine herpes myeloencephalopathy or equine herpesvirus-associated neurological disease, are characterized by vasculitis and thrombosis of arterioles in the CNS, particularly the spinal cord, resulting in hypoxic degeneration of adjacent neural tissue (Borchers et al. 2006). Both viral damage and the immune response to the infection are involved in the pathogenesis of the condition. There is evidence that only certain neuropathogenic strains of EHV-1 are associated with nervous disease (Nugent et al. 2006). Nervous disease associated with EHV-1 infection is relatively uncommon and can occur in a single horse or in several horses usually in association with an outbreak of abortion and/or respiratory disease. Pregnant and lactating mares appear to be at somewhat greater risk of developing the nervous form of disease. Signs may vary from mild incoordination to severe paralysis, recumbency and death. The signs in mildly affected animals usually stabilize in a day or two with gradual recovery over a period of several days or weeks.

Immunity to re-infection following primary respiratory tract infection only lasts a few months and is only protective against infection with the same virus species. However, infection does result in an immunity that prevents or reduces the severity of respiratory disease and multiple infections will result in significant cross-protection against the heterologous herpesvirus.

Diagnosis

- Virus isolation and identification are routinely used for laboratory confirmation of herpesviral disease in horses. Nasopharyngeal swabs should be collected from affected animals during the early stages of the respiratory disease and rapidly despatched in viral transport medium to the laboratory. Specimens of lung, liver, kidney and spleen are suitable from aborted foetuses. Suitable cells for both viruses include primary equine kidney, equine dermal fibroblasts and equine lung fibroblasts. A wider range of cell lines will support the growth of EHV-1 including rabbit kidney (RK-13), baby hamster kidney (BHK-21) and Madin–Darby bovine kidney (MDBK). A characteristic herpesvirus CPE of focal rounding, increase in refractility and detachment of cells is usually seen within a couple of days.
- Viral antigen may be demonstrated in cryostat sections of tissues such as lung, liver and spleen collected from aborted foetuses using immunofluorescence. Immunohistochemical methods have been applied successfully to the detection of viral antigen in paraffin-embedded tissues from aborted foetuses (Schultheiss et al. 1993) and from neurologically affected horses (Whitwell et al. 1992).
- The presence of characteristic gross and microscopic lesions, particularly intranuclear inclusions in cells at the edge of areas of hepatic necrosis, may be sufficient for a diagnosis in cases of herpesviral abortion. Diagnosis of herpesviral myeloencephalopathy is usually dependent on the demonstration of characteristic lesions of a degenerative thrombotic vasculitis in the CNS. Virus isolation from tissues or the demonstration of antibodies in cerebrospinal fluid is frequently unsuccessful in these cases. Virus isolation from peripheral blood leukocytes may be successful in the early stages of the paralytic disease.

- The polymerase chain reaction has been adapted for the detection of viral DNA in clinical specimens (Sharma et al. 1992) and in paraffin-embedded tissue (O'Keefe et al. 1994). Protocols are available for the simultaneous detection and differentiation of both viruses (Wagner et al. 1992, Varrasso et al. 2001). The interpretation of positive PCR results in adult horses is complicated by the high prevalence of latent infection.
- Serological testing of paired serum samples and demonstration of a fourfold rise in antibody titre is useful in confirming a diagnosis retrospectively. Suitable serological tests include virus neutralization, complement fixation test and ELISA (Dutta et al. 1983). Complement fixing antibody titres become negative within a few months of recovery from infection. Most serological tests are unable to distinguish between infection with EHV-1 from infection with EHV-4 due to antigenic cross-reactivity between the two viruses. However, virus-specific ELISAs based on discriminating monoclonal antibody or on recombinant glycoprotein G antigens are capable of distinguishing between the two infections (Crabb et al. 1995).

EQUINE COITAL EXANTHEMA

Equine coital exanthema is a benign, venereal disease of horses caused by equine herpesvirus 3 (EHV-3). The infection is considered to have a worldwide distribution. Serological surveys suggest a prevalence of about 50% in sexually active horses. However, the reported incidence of clinical disease is much lower and many infections are subclinical or so mild as to go unnoticed. The principal mode of transmission is venereal but may also occur via human intervention and contaminated instruments.

Pathogenesis

The incubation period ranges from two to 10 days. It is thought that latent infection with EHV-3 probably occurs in sacral ganglia and that outbreaks of disease result from the reactivation of latent infection. The virus has a tropism for keratinized epithelium and is temperature-sensitive such that replication is restricted at core body temperature. Viraemia and abortion do not occur.

Lesions appear on the external genitalia, initially as red papules then as vesicles and pustules. The pustules rupture leaving ulcers that may coalesce into larger areas. Secondary bacterial infection is common, resulting in a mucopurulent discharge. In uncomplicated cases the lesions usually heal within two weeks. On pigmented skin the site of healed lesions is marked for life by white spots. The infection has no effect on fertility other than affected stallions may be reluctant to copulate during the acute disease.

Diagnosis

A clinical diagnosis may be made based on the presence of the characteristic lesions. Confirmation can be achieved by electron microscopy of lesion scrapings or by virus isolation in equine cell lines at 33–35°C. A cytopathic effect is usually evident after one to two days. PCR protocols for detection of viral DNA in skin lesions are available (Kleiboeker & Chapman 2004, Dynon et al. 2005). Virus neutralization and ELISA are suitable assays for demonstrating a rising antibody titre to EHV-3 in paired serum samples. In some cases the antibody response may be minimal and failure to demonstrate a fourfold rise is not necessarily evidence of absence of infection.

AUJESZKY'S DISEASE

Aujeszky's disease (AD), also referred to as pseudorabies, is caused by Aujeszky's disease virus (porcine or suid herpesvirus 1). Only a single serotype of the virus is recognized. The pig is the natural host, with subclinical and latent infections occurring in this species. A number of other farm animal species can also be infected including cattle, sheep, goats, dogs and cats. Infection in these secondary hosts is fatal.

Infection is endemic in the pig populations of many countries worldwide. The disease has been eradicated from Denmark and Great Britain. In naive herds the outbreaks of disease can be quite devastating with rapid spread to all ages within a week. The virus is shed in nasal and oral secretions, milk and semen. Transmission usually occurs via nose-to-nose contact or via aerosolized virus. Transplacental transmission occurs in pregnant sows and aborted foetuses are a source of virus. The virus is not particularly stable in the environment but may remain infectious for a few days under suitable conditions. Wind-borne transmission over distances of a few kilometres has been recorded. Sheep are highly susceptible and may acquire infection following contact with pigs or by sharing the same air space. Scavenging animals, particularly cats, may become infected following consumption of tissues from dead pigs. The incubation and clinical course in secondary hosts is short and as a result the opportunities for further dissemination of virus are limited.

Pathogenesis

The incubation period may be as short as 36 hours in neonates or as long as five days in fatteners. Following oronasal infection the virus replicates in the epithelium of the nasopharynx and tonsils. The virus spreads from these

primary sites to regional lymph nodes via the lymphatics and to the CNS via the axons of cranial nerves. Higher-virulence strains of ADV produce a brief viraemia and are widely distributed around the body, particularly to the respiratory tract for which the virus has a predilection. The virus replicates in alveolar macrophages, inhibiting their function. Transplacental infection in pregnant sows results in generalized foetal infection. Infected animals continuously excrete virus for two to three weeks following exposure. Infection results in a high percentage of latently infected animals with virus persisting in trigeminal ganglia and tonsils. Clinical signs vary according to age and virulence of the infecting virus strain. Younger pigs are the most severely affected and in suckling piglets neurological signs predominate, mortality may approach 100%. In susceptible fatteners fever, weight loss and respiratory signs of sneezing, coughing, nasal discharge and dyspnoea are seen. Nervous signs are uncommon in animals of this age and affected animals typically recover in about a week. Infection of pregnant females in the first trimester may result in resorption of foetuses and return to oestrus while infection later in pregnancy frequently results in abortion or birth of stillborn and weak piglets. Following the primary episode a herd returns to normal productivity and signs of disease are greatly reduced despite the persistence of infection in the herd due to the presence of latently infected animals. Neonates are protected by the presence of maternal antibody.

Disease in other farm species occurs sporadically and is characterized by an acute nervous disorder which has been likened clinically to rabies. Intense pruritis and self-mutilation or 'mad itch' is a feature of the disease, particularly in ruminants. The clinical course is short and most affected animals die within a few days.

Diagnosis

The history, clinical signs and characteristic pathological lesions may be sufficient to make a diagnosis. Laboratory confirmation is based on detection of the virus and demonstration of a serological response.

- Brain, tonsil, spleen and lung from acutely affected animals are the most suitable tissues for virus isolation. In live pigs, nasal swabs or tonsil biopsies can be used. Samples should be kept at fridge temperature and promptly inoculated onto susceptible tissue culture cells. Numerous cell lines are suitable including the porcine kidney cell line PK-15, primary pig and rabbit kidney cells and chick embryo fibroblasts. Cytopathic effect with syncytia and large balloon-like cells is usually evident within 72 hours of inoculation.
- Cryostat sections of tonsil or brain are suitable for immunofluorescent detection of viral antigen.
- Several PCR assays have been described for the detection of ADV genomes in secretions or tissue samples in acutely (Belak et al. 1989, Jestin et al. 1990) and latently infected pigs (Wheeler & Osorio 1991). Real-time assays have also been described (McKillen et al. 2007).
- Suitable serological tests for the detection of ADV antibodies include virus neutralization, ELISA (Banks 1983) and latex agglutination (Yong et al. 2005). Kits for the latex agglutination test (LAT) and ELISA are commercially available. The LAT is commonly used for screening for antibodies. The ELISA can be used to detect antibodies in meat juice as well as serum and has also been adapted to test filter paper discs moistened with a small quantity of blood followed by air-drying before transport to the laboratory (Banks 1985). In young pigs maternal antibodies may be present for up to four months. Differential ELISAs (Eloit et al. 1989) have been developed to detect antibodies to specific surface glycoproteins, currently available tests detect antibodies to gC, gE and gG. These assays are designed to identify animals infected with field virus in herds being vaccinated with vaccines based on gene deletion mutant viruses which lack one of these non-essential surface glycoproteins.

CANINE HERPESVIRUS INFECTION

Infection with canine herpesvirus 1 (CHV-1) is common and occurs worldwide in domestic and wild *Canidae* species. The occurrence of disease is uncommon and is characterized by high mortality following generalized infection in neonates. Prevalence rates of 88% and 42% based on serological surveys of dogs have been reported in England and the Netherlands, respectively (Reading & Field 1998, Rijsewijk et al. 1999). Infection occurs via the oronasal route following direct contact between infected and susceptible animals. Latent infections occur with reactivation and shedding of virus following periods of stress. Sensory ganglia act as sites of latency (Burr et al. 1996, Miyoshi et al. 1999). Virus is shed in oral, nasal and vaginal secretions. Newborn pups may acquire infection during parturition and transmit the infection horizontally to their littermates. In utero infection may also occur.

Pathogenesis

Puppies infected at birth or soon afterwards develop clinical signs from three to eight days later. Following infection virus replication occurs in the nasal mucosa, pharynx and tonsils. Canine herpesvirus 1 replicates most efficiently at temperatures a little below normal adult body temperature, confining infection in most animals to the

upper respiratory tract or external genitalia. However, in puppies under four weeks of age the hypothalamic thermoregulatory centre is not fully operational such that neonates are very dependent on ambient temperature and maternal contact for normal body heat. In addition, very young puppies do not demonstrate a full pyrexial response. A cell-associated viraemia and widespread viral replication in visceral organs occurs in infected neonates that become chilled. Onset is sudden with puppies ceasing to nurse, displaying signs of abdominal pain and crying persistently. The course of the disease is acute and rapid with affected pups dying within one to two days. The morbidity and mortality rates in affected litters are high. The bitches of affected litters generally produce healthy litters subsequently. Infection in older puppies and adult dogs is usually asymptomatic. Occasionally vesicular lesions on external genitalia and mild vaginitis or balanoposthitis may be observed.

Diagnosis

Characteristic focal areas of necrosis and haemorrhage, particularly in the kidneys, in puppies that have died as well as the presence of intranuclear inclusions are generally sufficient for a diagnosis. Virus isolation can be carried out in canine cell lines from fresh specimens of liver, kidney, lung and spleen. In situ hybridization and PCR protocols for the detection of CHV-1 in tissues have been published (Burr et al. 1996, Schulze & Baumgartner 1998).

FELINE VIRAL RHINOTRACHEITIS

Feline viral rhinotracheitis is an acute upper respiratory tract infection of young cats caused by feline herpesvirus 1 (FHV-1). The virus has a worldwide distribution and accounts for about 40% of respiratory infections in cats. Both domestic and exotic species of *Felidae* are susceptible. Close contact is required for efficient transmission. The prevalence of infection is higher in colony cats than in individual household cats. Virus is shed in oral, nasal and ocular secretions. Indirect transmission via fomites is possible but only for short periods due to the relative fragility of the virus. It is thought that virtually all recovered cats remain latently infected. Reactivation and virus shedding may occur at any time but is particularly associated with periods of stress such as parturition, lactation or change of housing. There is an interval of several days between the stressful episode and the shedding of virus. The kittens of carrier queens may become infected subclinically under the protection of maternal antibody, going on to become carriers themselves and perpetuating the cycle of infection. The trigeminal ganglia are an important site of latency.

Pathogenesis

The incubation period is short, usually about two days but occasionally as long as six days. Following infection, FHV-1 replicates locally in oral, nasal and conjunctival tissues. Spread occurs throughout the upper respiratory epithelium with signs of fever, sneezing, inappetence, hypersalivation, conjunctivitis and oculonasal discharge. Viraemia and generalized infection do not appear to occur except in the very young, the very old or immunocompromised individuals. Secondary bacterial infection is common and increases the severity of the clinical signs. The discharges become mucopurulent, forming crusts around the eyes and sometimes sticking the eyelids together. In more severe cases evidence of pneumonia or ulcerative keratitis may be present. The mortality rate is low except in very young or immunosuppressed animals. On rare occasions cats may present with facial and nasal dermatitis, this has been associated with reactivation of latent infection (Hargis & Ginn 1999). Abortion may occur on occasion in pregnant queens and is considered to be due to debilitation rather than to any direct viral effect.

Diagnosis

Clinical differentiation of feline viral rhinotracheitis from feline calicivirus infection is usually not possible.

- Virus isolation from oropharyngeal or conjunctival swabs is the method of choice for diagnosis and can be carried out in feline cell lines. Virus isolation results need to be interpreted with care as FHV-1 may be detected in clinically normal cats (Maggs et al. 1999). Samples should be collected before the application of fluorescein or Rose Bengal stain to the eye, which can inactivate the virus (Brooks et al. 1994).
- Specific viral antigen can be demonstrated in acetone-fixed nasal and conjunctival smears using immunofluorescence.
- Detection of viral DNA in conjunctival and nasal swabs by PCR has been described (Hara et al. 1996, Sykes et al. 2001). Several PCR protocols have been compared for sensitivity (Maggs & Clarke 2005). Typically the primers are based on the thymidine kinase gene, which is highly conserved. In the absence of characteristic clinical signs, a positive result may simply be an incidental finding as all latently infected cats may shed virus intermittently. The use of real time PCR to provide a quantitative assessment of viral load in clinical samples may be more informative (Vogtlin et al. 2002).
- Seroprevalence is high in cats as a result of natural infection and vaccination. Paired serum samples tested by virus neutralization or ELISA to demonstrate a rising titre can be useful in confirming an active infection. Neutralizing antibodies do not appear till 20 to 30 days following infection.

INFECTIOUS LARYNGOTRACHEITIS

Infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens. It is caused by gallid herpesvirus 1 (GaHV-1) which has a worldwide distribution. Strains of GaHV-1 vary widely in virulence but are antigenically homogeneous. The disease is well controlled in areas of intensive poultry production by vaccination and biosecurity measures. However, the virus tends to persist as a result of enzootic infections in backyard and fancier chicken flocks. Natural infection occurs in chickens and sometimes in pheasants. Transmission occurs via the upper respiratory and ocular routes. It is most efficient when birds are confined and infectious respiratory exudates are aerosolized or expectorated. The virus can become latent in the trigeminal ganglia of infected birds and these carrier birds shed virus intermittently throughout their lives particularly after periods of stress such as onset of lay or mixing with unfamiliar birds. Indirect spread via contaminated fomites may be responsible for spread from one production site to another.

Pathogenesis

The incubation period is six to 12 days. Following infection the virus replicates locally in the upper respiratory tract. The principal lesion is tracheitis. Viraemia is not thought to occur but neural migration along sensory nerves results in spread to the trigeminal ganglia. The epizootic form of the disease associated with virulent strains of the virus is characterized by coughing, gasping, moist rales, nasal and ocular discharge, expectoration of blood-stained mucus and head shaking. The mortality rate may be as high as 70%, usually due to occlusion of the trachea as a result of the severe haemorrhagic laryngotracheitis and the formation of diphtheritic membranes. Low-virulence strains of GaHV-1 are associated with mild respiratory signs, conjunctivitis and lowered egg production.

Diagnosis

In severe outbreaks the clinical signs and pathological lesions may be sufficiently characteristic for a diagnosis to be made. Laboratory confirmation is required in cases of the mild form of ILT.

- Virus isolation can be carried out on the chorioallantoic membrane of embryonated hens' eggs or in avian cell cultures such as chicken embryo liver cells.
- Rapid methods of diagnosis include examination of tracheal exudate by electron microscopy for herpesvirus particles, detection of viral antigen in smears or frozen sections by immunofluorescence and detection of viral antigen in tracheal samples by ELISA (York & Fahey 1988) or AGID.
- PCR is considered to be more sensitive than virus isolation for detection of GaHV-1 (Williams et al. 1994, Alexander & Nagy 1997). Both conventional and real time PCR assays have been published (Callison et al. 2007). It is possible to differentiate between field strains and vaccine strains of the virus using restriction fragment length polymorphism of PCR products (Chang et al. 1997).
- Antibodies to GaHV-1 can be demonstrated by virus neutralization, indirect immunofluorescence, ELISA and AGID (Adair et al. 1985). A commercial ELISA kit is available.

MAREK'S DISEASE

Marek's disease (MD) is a contagious, lymphoproliferative disease of chickens caused by a highly cell-associated, oncogenic herpesvirus, gallid herpesvirus 2 (Marek's disease virus). It has a ubiquitous, worldwide distribution and is of major economic significance in the poultry industry. Herpesviruses of chickens and turkeys, members of the genus *Mardivirus,* can be divided immunologically into 3 serotypes. Serotype 1 (gallid herpesvirus 2) includes all pathogenic strains and attenuated variants derived from these strains, serotype 2 (gallid herpesvirus 3) contains the naturally avirulent and non-oncogenic strains while serotype 3 is represented by the related, avirulent herpesvirus of turkeys, meleagrid herpesvirus 1. Serotype 1 strains vary markedly in pathogenicity and may be divided into mildly virulent, virulent and highly virulent pathotypes.

Productive replication with release of infective virus occurs only in the feather follicle epithelium. Cell-free virus is released with feather follicle desquamated cells and this dander can remain infective for several months in poultry-house dust and litter. Infected birds remain carriers for life. The chicks of infected birds are protected by maternal antibody but acquire infection within a few weeks of hatching, usually by the respiratory route. Several factors besides the virulence of the infecting virus strain affect the severity of disease including the genetic makeup, sex and age at infection of the host bird. Two genetic loci have been identified as important, one associated with a major histocompatibility complex allele which influences the immune response to MD virus and a second locus which influences the susceptibility of T lymphocytes to transformation. Female birds are more susceptible to the disease than males but the basis for this difference is not known. Resistance to the development of disease increases as the age at which infection occurs increases. The main environmental factor associated with an increased incidence of disease is stress due to changes such as movement, vaccination, handling and beak trimming.

Pathogenesis

Following inhalation the virus replicates locally before being transported to lymphoid organs, probably within macrophages. The virus causes an acute cytolytic infection, primarily of B cells within the bursa, thymus and spleen, resulting in suppression of humoral immunity. Adjacent T cells, activated as a result of this cytotoxic infection, become susceptible to infection and latent infection is established in these cells. A persistent, cell-associated viraemia follows with widespread dissemination of the virus throughout the body. A secondary cytolytic infection occurs in feather follicle epithelium, about two weeks post infection, resulting in the shedding of infectious cell-free virus into the environment. In non-resistant chickens likely to develop tumours, permanent immunosuppression occurs at this stage associated with apoptosis of T cells and thymocytes and downregulation of CD8 molecules. Gross lymphomatous lesions may appear as early as two to three weeks post infection or as much as several months after infection. Transformation of T cells is believed to be due to the presence of oncogenes in the genome of oncogenic serotype 1 strains. Multiple copies of the virus genome, both integrated into the host cell DNA and as episomal DNA, are found in transformed cells. The oncogene considered most likely to be responsible for transformation is *meq*, a basic-leucine zipper gene (Calnek 1998). Immune surveillance mechanisms, compromised by the immunosuppression associated with the infection, may fail to prevent a transformed cell from multiplying and forming characteristic lymphoid neoplasms. In classical Marek's disease the peripheral nerves are commonly involved and may be affected by proliferative, inflammatory or minor infiltrative changes, termed type A, B and C lesions respectively. Demyelination occurs in nerves displaying type A and B lesions and results in paralysis. Birds aged between 12 and 24 weeks of age are most commonly affected and present with partial or complete assymmetrical paralysis of the legs and wings. The mortality rate rarely exceeds 10 to 15% with deaths occurring over a number of weeks or over several months. The acute form of MD is characterized by widespread, diffuse, lymphomatous involvement of multiple internal organs. Birds are severely depressed before death or may die without any prior signs. The mortality rate of this form is 10 to 30% but outbreaks with mortality as high as 70% have been reported. A surface antigen present on transformed lymphocytes, Marek's disease tumour-associated surface antigen (MATSA), was once thought to be tumour specific but is now considered to be a host marker for activated T cells.

Diagnosis

The diagnosis of MD is based on clinical signs and gross or microscopic pathology. Paralysis of legs and wings in conjunction with enlargement of peripheral nerves is pathognomonic for MD. However, nerve involvement is sometimes not seen, particularly in adult birds, and differentiation of MD from lymphoid leukosis is important. Differentiation is usually possible based on age of birds affected, incidence of disease and histopathological findings. Lymphomas caused by GaHV-2 consist of various types of lymphoid cells, T cell in type, while in lymphoid leukosis the lymphomatous infiltrations are composed of uniform lymphoblasts, B cell in type. Demonstration of the presence of infection with gallid herpesvirus 2 does not confirm the presence of MD in a flock as persistent infection can occur in the absence of clinical disease.

- The virus can be isolated from the buffy coat from heparinized blood samples or suspensions of spleen or lymphoma cells from infected birds. The virus is highly cell associated and samples must contain viable cells. Rapid transportation of samples to the laboratory under chilled conditions is required. Isolation can be carried out in monolayers of chicken kidney cells or duck embryo fibroblasts at 38.5°C. Characteristic focal areas of CPE termed plaques usually appear in three to five days in positive cultures.
- Viral antigen can be detected in preparations of skin or of feather tips using a radial precipitin test or ELISA (Davidson et al. 1988).
- Primers capable of detecting and distinguishing attenuated and wild-type strains have been developed for PCR assays (Becker et al. 1992, Handberg et al. 2001). Suitable sources of viral DNA include blood (Davidson et al. 1995) and feather tips (Davidson & Borenshtain 2002). Quantitative PCR protocols have been published (Reddy et al. 2000).
- Serum antibodies to GaHV-2 may be detected using AGID, ELISA (Cheng et al. 1984), indirect immunofluorescence and virus neutralization.

REFERENCES

Adair, B.M., Todd, D., McKillop, E.R., et al., 1985. Comparison of serological tests for the detection of antibodies to infectious laryngotracheitis virus. Avian Pathology 14, 461–469.

Alexander, H.S., Nagy, E., 1997. Polymerase chain reaction to detect infectious laryngotracheitis virus in conjunctival swabs from experimentally infected chickens. Avian Diseases 41, 646–653.

Banks, M., 1983. Rapid ELISA for Aujeszky's disease eradication. Veterinary Record 113, 94–95.

Banks, M., 1985. Detection of antibodies to Aujeszky's disease virus in whole blood by ELISA-disc. Journal of Virological Methods 12, 41–45.

Baxter, S.I.F., Pow, I., Bridgen, A., et al., 1993. PCR detection of the sheep-associated agent of malignant catarrhal fever. Archives of Virology 132, 145–159.

Becker, Y., Asher, Y., Tabor, E., et al., 1992. Polymerase chain reaction for differentiation between pathogenic and non-pathogenic serotype 1 Marek's disease virus (MDV) and vaccine viruses of MDV-serotypes 2 and 3. Journal of Virological Methods 40, 307–322.

Belak, S., Ballagi-Pordany, A., Flensburg, J., et al., 1989. Detection of pseudorabies virus DNA sequences by the polymerase chain reaction. Archives of Virology 108, 279–286.

Borchers, K., Thein, P., Steiner-Kock, A., 2006. Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation. Equine Veterinary Journal 38, 283–287.

Brooks, S.E., Kaza, V., Nakamura, T., et al., 1994. Photo-inactivation of herpes simplex virus by Rose Bengal and fluorescein: in vitro and in vivo studies. Cornea 13, 43–50.

Burr, P.D., Campbell, M.E.M., Nicolson, L., et al., 1996. Detection of canine herpesvirus 1 in a wide range of tissues using the polymerase chain reaction. Veterinary Microbiology 53, 227–237.

Callison, S.A., Riblet, S.M., Oldoni, I., et al., 2007. Development and validation of a real-time TaqMan PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of Virological Methods 139, 31–38.

Calnek, B.W., 1998. Lymphomagenesis in Marek's disease. Avian Pathology 27, S54–S64.

Chang, P.C., Lee, Y.L., Shien, J.H., et al., 1997. Rapid differentiation of vaccine strains and field isolates of infectious laryngotracheitis virus by restriction fragment length polymorphism of PCR products. Journal of Virological Methods 66, 179–186.

Cheng, Y.-Q., Lee, L.F., Smith, E.J., et al., 1984. An enzyme-linked immunosorbent assay for the detection of antibodies to Marek's disease virus. Avian Diseases 28, 900–911.

Crabb, B.S., MacPherson, C.M., Reubel, G.H., et al., 1995. A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. Archives of Virology 140, 245–258.

Crawford, T.B., Li, H., O'Toole, D., 1999. Diagnosis of malignant catarrhal fever by PCR using formalin-fixed, paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation, 11, 111–116.

Davidson, I., Borenshtain, R., 2002. The feather tips of commercial chickens are a favourable source of DNA for the amplification of MDV and ALV-J. Avian Pathology 31, 237–240.

Davidson, I., Borovskaya, A., Perl, S., et al., 1995. Use of the polymerase chain-reaction for the diagnosis of natural infection of chickens and turkeys with Mareks-disease virus and reticuloendotheliosis virus. Avian Pathology 24, 69–94.

Davidson, I., Malkinson, M., Strenger, C., et al., 1988. An improved ELISA method, using a streptavidin-biotin complex, for detecting Marek's disease virus antigens in feather-tips of infected chickens. Journal of Virological Methods 14, 237–241.

d'Offay, J.M., Floyd, J.G., Eberle, R., et al., 2003. Use of a polymerase chain reaction assay to detect bovine herpesvirus type 2 DNA in skin lesions from cattle suspected to have pseudo-lumpy skin disease. Journal of the American Veterinary Medical Association 222, 1404–1407.

Dutta, S.K., Talbot, N.C., Myrup, A.C., 1983. Detection of equine herpesvirus-1 antigen and the specific antibody by enzyme-linked immunosorbent assay. American Journal of Veterinary Research 44, 1930–1934.

Dynon, K., Varrasso, A., Ficorilli, N., et al., 2005. Identification of equine herpesvirus 3 (equine coital exanthema virus), equine gammaherpesviruses 2 and 5, equine adenoviruses 1 and 2, equine arteritis virus and equine rhinitis A virus by polymerase chain reaction. Australian Veterinary Journal 79, 695–702.

Edwards, S., Gitao, G.C., 1987. Highly sensitive antigen detection procedures for the diagnosis of infectious bovine rhinotracheitis: amplified ELISA and reverse passive haemagglutination. Veterinary Micorobiology 13, 135–141.

Eloit, M., Fargeaud, D., Vannier, P., et al., 1989. Development of an ELISA to differentiate between animals either vaccinated with or infected by Aujeszky's disease virus. Veterinary Record 124, 91–94.

Fuchs, M., Hubert, P., Detterer, J., et al., 1999. Detection of bovine herpesvirus type 1 in blood from naturally infected cattle by using a sensitive PCR that discriminates between wild-type virus and virus lacking glycoprotein. European Journal of Clinical Microbiology & Infectious Diseases 37, 2498–2507.

Hara, M., Fukuyama, M., Suzuki, Y., et al., 1996. Detection of feline herpesvirus 1 DNA by the nested polymerase chain reaction. Veterinary Microbiology 48, 345–352.

Gilkerson, J.R., Whalley, J.M., Drummer, H.E., et al., 1999a. Epidemiological studies of equine herpesvirus 1 (EHV-1) in thoroughbred foals: a review of studies conducted in the Hunter Valley of New South Wales between 1995 and 1997. Veterinary Microbiology 68, 15–25.

Gilkerson, J.R., Whalley, J.M., Drummer, H.E., et al., 1999b. Epidemiology of EHV-1 and EHV-2 in the mare and foal populations on a Hunter Valley study farm: are mares the source of EHV-1 for unweaned foals. Veterinary Microbiology 68, 27–34.

Handberg, K.J., Nielson, O.L., Jorgensen, P.H., 2001. The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. Avian Pathology 30, 243–249.

Hargis, A.M., Ginn, P.E., 1999. Feline herpesvirus 1-associated facial and nasal dermatitis and stomatitis in domestic cats. Veterinary Clinics of North America: Small Animal Practice 29, 1281–1290.

Jestin, A., Foulon, T., Pertuiset, B., et al., 1990. Rapid detection of pseudorabies virus genomic sequences in biological samples from infected pigs using polymerase chain reaction DNA amplification. Veterinary Microbiology 23, 317–328.

Kleiboeker, S.B., Chapman, R.K., 2004. Detection of equine herpesvirus 3 in equine skin lesions by polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 16, 74–79.

Kramps, J.A., 2008. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis. In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, Paris. Chapter 2.4.13. pp. 752–767.

Kramps, J.A., Quak, S., Weerdmeester, K., et al., 1993. Comparative study on sixteen enzyme-linked immunosorbent assays for the detection of antibodies to bovine herpesvirus 1 in cattle. Veterinary Microbiology 35, 11–21.

Kramps, J.A., Banks, M., Beer, M., et al., 2004. Evaluation of tests for antibodies against bovine herpesvirus 1 performed in national reference laboratories in Europe. Veterinary Microbiology 102, 169–181.

Lemaire, M., Weynants, V., Godfroid, J., et al., 2000. Effects of bovine herpesvirus type 1 infection in calves with maternal antibodies on immune response and virus latency. Journal of Clinical Microbiology 38, 1885–1894.

Li, H., Shen, D.T., Knowles, D.P., et al., 1994. Competitive inhibition enzyme-linked immunosorbent assay for antibody in sheep and other ruminants to a conserved epitope of malignant catarrhal fever virus. Journal of Clinical Microbiology 32, 1674–1679.

Li, H., Taus, N.S., Lewis, G.S., et al., 2004. Shedding of ovine herpesvirus 2 in sheep nasal secretions: the predominant mode for transmission. Journal of Clinical Microbiology 42, 5558–5564.

Loken, T., Aleksandersen, M., Reid, H., et al., 1998. Malignant catarrhal fever caused by ovine herpesvirus-2 in pigs in Norway. Veterinary Record 143, 464–467.

Maggs, D.J., Clarke, H.E., 2005. Relative sensitivity of polymerase chain reaction assays used for detection of feline herpesvirus type 1 DNA in clinical samples and commercial vaccines. American Journal of Veterinary Research 66, 1550–1555.

Maggs, D.J., Lappin, M.R., Reif, J.S., et al., 1999. Evaluation of serologic and viral detection methods for diagnosing feline herpesvirus-1 infection in cats with acute respiratory tract or chronic ocular disease. Journal of the American Veterinary Medical Association 214, 502–507.

McKillen, J., Hjertner, B., Millar, A., et al., 2007. Molecular beacon real-time PCR detection of swine viruses. Journal of Virological Methods 140, 155–165.

Miyoshi, M., Ishii, Y., Takiguchi, M., et al., 1999. Detection of canine herpesvirus DNA in the ganglionic neurons and the lymph node lymphocytes of latently infected dogs. Journal of Veterinary Medical Science 61, 375–379.

Moore, S., Gunn, M., Walls, D., 2000. A rapid and sensitive PCR-based diagnostic assay to detect bovine herpesvirus 1 in routine diagnostic submissions. Veterinary Microbiology 75, 145–153.

Muller-Doblies, U.U., Li, H., Hauser, B., et al., 1998. Field validation of laboratory tests for clinical diagnosis of sheep-associated malignant catarrhal fever. Journal of Clinical Microbiology 36, 2970–2972.

Nettleton, P.F., Herring, J.A., Herring, A.J., 1983. Evaluation of an immunofluorescent test for the rapid diagnosis of field infections of infectious bovine rhinotracheitis. Veterinary Record 112, 298–300.

Nugent, J., Birch-Machin, I., Smith, K.C., et al., 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus non-neuropathogenic disease outbreaks. Journal of Virology 80, 4047–4060.

Nylin, B., Stroger, U., Ronsholt, L., 2000. A retrospective evaluation of a bovine herpesvirus-1 (BHV-1) antibody ELISA on bulk-tank milk samples for classification of the BHV-1 status of Danish dairy herds. Preventive Veterinary Medicine 47, 91–105

O'Keefe, J.S., Julian, A., Moriarty, K., et al., 1994. A comparison of the polymerase chain reaction with standard laboratory methods for the detection of EHV-1 and EHV-4 in archival tissue samples. New Zealand Veterinary Journal 42, 93–96.

O'Toole, D., Li, H., Miller, D., et al., 1997. Chronic and recovered cases of sheep-associated malignant catarrhal fever in cattle. Veterinary Record 140, 519–524.

Perrin, B., Bitsch, V., Cordioli, P., et al., 1993. A European comparative study of serological methods for the diagnosis of infectious bovine rhinotracheitis. Revue scientifique et technique (International Office of Epizootics) 12, 969–984.

Powers, J.G., VanMetre, D.C., Collins, J.K., et al., 2005. Evaluation of ovine herpesvirus type 2 infections, as detected by competitive inhibition ELISA and polymerase reaction assay in dairy cattle without clinical signs of malignant catarrhal fever. Journal of the American Veterinary Medical Association 227, 606–611.

Reading, M.J., Field, H.J., 1998. A serological survey of canine herpesvirus 1 infection in the English dog population. Archives of Virology 143, 1477–1488.

Reddy, S.M., Witter, R.L., Gimeno, I.M., 2000. Development of a quantitative-competitive polymerase chain reaction assay for serotype 1

Marek's disease virus. Avian Diseases 44, 770–775.

Rijsewijk, F.A.M., Luiten, E.J., Daus, F.J., et al., 1999. Prevalence of antibodies against canine herpesvirus 1 in dogs in The Netherlands in 1997–1998. Veterinary Microbiology 65, 1–7.

Russell, G.C., Stewart, J.P., Haig, D.M., 2009. Malignant catarrhal fever: a review. Veterinary Journal 179, 324–335.

Schultheiss, P.C., Collins, J.K., Carman, J., 1993. Use of an immunoperoxidase technique to detect equine herpesvirus-1 antigen in formalin-fixed paraffin-embedded equine fetal tissues. Journal of Veterinary Diagnostic Investigation 5, 12–15.

Schulze, C., Baumgartner, W., 1998. Nested polymerase chain reaction and in situ hybridization for diagnosis of canine herpesvirus infection in puppies. Veterinary Pathology 35, 209–217.

Sharma, P.C., Cullinane, A.A., Onions, D.E., et al., 1992. Diagnosis of equid herpesviruses-1 and -4 by polymerase chain reaction. Equine Veterinary Journal 24, 20-25.

Smits, C.B., Van Maanen, C., Glas, R.D., et al., 2000. Comparison of three polymerase chain reaction methods for routine detection of bovine herpesvirus 1 DNA in fresh bull semen. Journal of Virological Methods 85, 65–73.

Sykes, J.E., Allen, J.L., Studdert, V.P., et al., 2001. Detection of feline calicivirus, feline herpesvirus 1 and *Chlamydia psittaci* mucosal swabs by multiplex RT-PCR/PCR. Veterinary Microbiology 81, 95–108.

Varrasso, A., Dynon, K., Ficorilli, N., et al., 2001. Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. Australian. Veterinary Journal 79, 563–569.

Vogtlin, A., Fraefel, C., Albini, S., et al., 2002. Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR. Journal of Clinical Microbiology 40, 519–523.

Wagner, W.N., Bogdan, J., Haines, D., et al., 1992. Detection of equine herpesvirus and differentiation of equine herpesvirus type 1 from type 4 by the polymerase chain reaction. Canadian Journal of Microbiology 38, 1193–1196.

Wellenberg, G.J., Verstraten, E.R.A.M., Mars, M.H., et al., 1998. Detection of bovine herpesvirus 1 glycoprotein E antibodies in individual milk samples by enzyme-linked immunosorbent assays. Journal of Clinical Microbiology 36, 409–413.

Wheeler, J.G., Osorio, F.A., 1991. Investigation of sites of pseudorabies virus latency, using polymerase chain reaction. American Journal of Veterinary Research 52, 1799–1803.

Whitwell, K.E., Gower, S.M., Smith, K.C., 1992. An immunoperoxidase method applied to the diagnosis of equine herpesvirus abortion, using conventional and rapid microwave techniques. Equine Veterinary Journal 24, 10–12.

Williams, R.A., Savage, C.E., Jones, R.C., 1994. A comparison of direct electron microscopy, virus isolation and a DNA amplification method for the detection of avian infectious laryngotracheitis virus in field material. Avian Pathology 23, 709–720.

Yong, T., Chen, H.C., Xiao, S.B., et al., 2005. Development of a latex agglutination test using the major epitope domain of glycoprotein E of pseudorabies virus expressed in *E. coli* to differentiate between immune responses in pigs naturally infected or vaccinated with pseudorabies virus. Veterinary Research Communications 29, 487–497.

York, J.J., Fahey, K.J., 1988. Diagnosis of infectious laryngotracheitis using a monoclonal antibody ELISA. Avian Pathology 17, 173–182.

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Chapter 50

Asfarviridae

African swine fever virus was formerly assigned to the family *Iridoviridae*. Subsequently, the virus was listed as the only member of a floating genus termed 'African swine fever-like viruses'. Taxonomic changes have resulted in the creation of a new family *Asfarviridae* with a single genus *Asfivirus*. African swine fever virus is the type species of this genus. The virus shares a number of similarities in genome structure and replication strategy with poxviruses but differs substantially in morphology and other properties. Virions are 175–215 nm in diameter. They consist of a membrane-bound nucleoprotein core within an icosahedral capsid and surrounded by an outer lipid-containing envelope (Fig. 50.1). They are complex viruses containing over 50 proteins including a large number of structural proteins and several virus-encoded enzymes required for transcription and post-translational modification of mRNA. The genome is a single molecule of linear, double-stranded DNA. Replication occurs in the cytoplasm of host cells with release by budding through the plasma membrane or by cell destruction. African swine fever virus is stable in the environment at 20 °C or 4 °C and over a wide pH range, permitting the virus to persist for weeks or months in meat. Infectivity is destroyed by heating, lipid solvents and certain disinfectants such as the para-phenylphenolic disinfectants.

AFRICAN SWINE FEVER

African swine fever is an economically important viral haemorrhagic disease of pigs characterized by fever, haemorrhages in the reticuloendothelial system and a high mortality rate. It occurs over large areas of Africa and in Sardinia and Madagascar. Outbreaks have occurred in Belgium, Italy, Malta, Brazil and the West Indies. The disease was successfully cleared from the Iberian Peninsula in 1995, almost 30 years after its introduction. Sequencing of the gene encoding the major capsid protein p72 has led to the identification of 22 genotypes. Genotyping has proven useful in determining the source of an outbreak in Georgia in 2007 (Rowlands et al. 2008).

Pigs are the only domesticated species susceptible to infection. Wild boars are also susceptible. Isolates vary in virulence, producing clinical disease ranging from peracute to chronic and apparently healthy carriers. In Africa ASFV is maintained in a sylvatic cycle involving inapparent infection of warthogs, bush pigs and soft ticks of the genus *Ornithodoros*. Following infection young warthogs develop viraemia sufficient to infect feeding ticks. Older warthogs are rarely viraemic despite being persistently infected. Replication of the virus occurs in the ticks with both transovarial and transstadial transmission occurring. Soft ticks feed quickly on their host before returning to cracks in the ground, in walls and in burrows. The presence of infection in ticks in a given region renders the eradication of ASF much more difficult. The principal tick species involved are *O. porcinus porcinus* (*O. moubata)* in Africa and *O. erraticus* in Spain and Portugal (Kleiboeker et al. 1998). Ingestion of uncooked meat from infected pigs or warthogs can result in transmission. The feeding of scraps to pigs is an important mechanism of international spread of the infection with outbreaks arising close to airports or harbours. Once established in domesticated pigs transmission can occur by direct contact usually via oral or nasal secretions.

Pathogenesis

The incubation period varies from four to 19 days but is typically five to seven days in acute cases. Infection in domestic pigs is usually acquired via the oronasal route.

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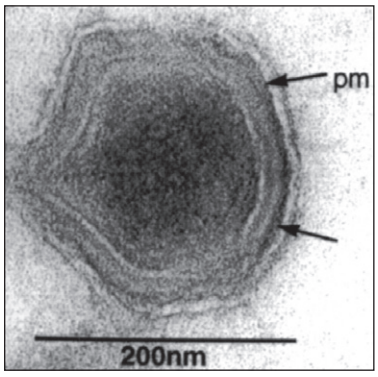

Figure 50.1 Cryosection negative contrast electron micrograph of African swine fever virus particle. The arrows indicate the membrane components of the virus; pm = plasma membrane. *Reprinted with permission: Fauquet, C.M., et al. (Eds.), 2005. Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, p. 135.*

The virus replicates initially in the pharyngeal mucous membrane and tonsils before spreading to the draining lymph nodes. Infection then extends via the bloodstream to the target organs which include lymph nodes, bone marrow, spleen, lung, liver and kidney. These are the main sites of secondary replication, which gives rise to a prolonged viraemia. The virus replicates primarily in the cells of the lymphoreticular system, particularly cells of the mononuclear phagocyte system, but also infects megakaryocytes, endothelial cells, kidney cells and hepatocytes. Lesions are widespread in the body and include splenic enlargement, swollen and haemorrhagic gastrohepatic and renal lymph nodes, subcapsular petechiae in the kidneys, petechial and ecchymotic haemorrhages in the heart walls and serosal surfaces, oedema of the lungs, hydrothorax and haemorrhages of the pleura. The pathogenesis of the haemorrhages appears to be largely related to the development of disseminated intravascular coagulation and the destruction of megakaryocytes (Rodriquez et al. 1996). There is a marked leukopenia in acute cases. The virus does not appear to replicate in T and B lymphocytes and the associated lymphopenia is thought to be due to apoptosis of lymphocytes and necrosis of lymphoid organs (Carrasco et al. 1996). In chronic cases of ASF, lesions are largely confined to the respiratory tract and include pneumonia, fibrinous pleuritis and pericarditis, pleural adhesions and hyperplasia of lymphoreticular tissues.

The immune mechanisms responsible for recovery and protection from ASFV are not well understood. Cell-mediated immunity is considered to be an important part of the protective response. It is not possible to demonstrate antibodies capable of fully neutralizing the virus in the sera of recovered animals.

Diagnosis

Laboratory confirmation of ASFV is required on account of the similarity of the clinical signs and lesions to a number of other pig diseases such as classical swine fever, erysipelas and septicaemic salmonellosis. Suitable samples include blood, serum, tonsil, spleen, kidney and lymph nodes.

- The preferred tests for detection of ASFV are direct immunofluorescence and haemadsorption (Sanchez-Vizcaino 1999). Direct immunofluorescence is fast and economical and can be carried out on impression smears or cryostat sections. In the case of subacute and chronic forms of ASF the sensitivity of the test is only 40% due to the blocking action of antigen–antibody complexes in the tissues of infected pigs. The haemadsorption (HA) test can be carried out by inoculating primary pig leukocyte cultures with blood or tissue specimens from suspect cases or more conveniently by the collection and separation of peripheral leukocytes from the blood of suspect pigs. Pig erythrocytes will adhere to and form a characteristic rosette on the surface of ASFV-infected monocyte or macrophage cells. Only a few field strains of ASFV have been isolated that do not induce haemadsorption.
- A challenge experiment involving the inoculation of suspect material into pigs vaccinated against classical swine fever and unvaccinated pigs may be used as an additional procedure to aid differentiation between these two clinically similar diseases. Alternative laboratory tests are now available making expensive challenge experiments unnecessary.
- The detection of ASFV DNA by polymerase chain reaction is particularly useful where tissues are unsuitable for virus isolation or antigen detection due to virus inactivation and putrefaction respectively (Aguero et al. 2003, King et al. 2003).
- Following infection antibodies persist for long periods in recovered animals. Several techniques have been successfully applied to the detection of antibodies to ASFV in serum or tissue extracts including ELISA (Wardley et al. 1979), immunoblotting, indirect immunofluorescence, complement fixation and radioimmunoassay. Serological testing may be the only means of detecting animals infected with low-virulence isolates.

REFERENCES

Aguero, M., Fernandez, J., Romero, L., et al., 2003. Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. Journal of Clinical Microbiology 41, 4431–4434.

Carrasco, L., Chacon, M.-L., Martin de las Mulas, J., et al., 1996. Apoptosis in lymph nodes in acute African swine fever. Journal of Comparative Pathology 115, 415–428.

King, D.P., Reid, S.M., Hutchings, G.H., et al., 2003. Development of a TaqMan® PCR assay with internal amplification control for the detection of African swine fever virus. Journal of Virological Methods 107, 53–61.

Kleiboeker, S.B., Burrage, T.G., Scoles, G.A., et al., 1998. African swine fever virus infection in the Argasid host, *Ornithodoros porcinus porcinus*. Journal of Virology 72, 1711–1724.

Rodriquez, F., Fernandez, A., Perez, J., et al., 1996. African swine fever: morphopathology of a viral haemorrhagic disease. Veterinary Record 139, 249–254.

Rowlands, R.J., Michaud, V., Heath, L., et al., 2008. African swine fever isolate, Georgia, 2007. Emerging Infectious Diseases 14, 1870–1874.

Sanchez-Vizcaino, J.M., 1999. African swine fever. In: Straw, B.E., D'Allaire, S., Mengeling, W.L. (Eds.), Diseases of Swine, eighth ed, Blackwell Science, Oxford, pp. 93–102.

Wardley, R.C., Abu Elzein, E.M.E., Crowther, J.R., et al., 1979. A solid-phase enzyme-linked immunosorbent assay for the detection of African swine fever antigen and antibody. Journal of Hygiene 83, 363–369.

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Chapter 51

Poxviridae

The name pox is derived from an old English word 'poc' which means a vesicular skin lesion. Poxviral diseases typically affect the skin. Poxviruses are among the largest and most complex viruses with more than 100 proteins and several virus-specified enzymes. Members of this family infect many vertebrate and invertebrate hosts. Virions are large (220–450 nm × 140–260 nm) and usually brick-shaped with the external surface membrane containing lipid and displaying tubular or globular protein structures (Fig. 51.1); in contrast, parapoxviruses are ovoid with the surface membrane displaying a regular spiral filament (Fig. 51.2). Poxvirus symmetry is described as complex. There is a biconcave core or nucleoid that contains the linear, double-stranded DNA genome. One or two lateral bodies are present in the concave region between the core wall and the surface membrane. An additional cell-derived envelope may enclose the virus and these are termed extracellular enveloped virions (EEV).

The family is divided into two subfamilies (Fig. 51.3), *Chordopoxvirinae* (poxviruses of vertebrates) and *Entomopoxvirinae* (poxviruses of insects). The subfamily *Chordopoxvirinae* is comprised of nine genera, *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Cervidpoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus* and *Yatapoxvirus*. Genetic recombination and extensive serological cross-reaction and cross-protection occurs within genera. Three closely related parapoxviruses, pseudocowpox, bovine papular stomatitis and orf virus, infect cattle, sheep and goats. These viruses are transmissible to man producing clinically indistinguishable lesions. The three viruses are morphologically identical and identification of the causal species relies on genetic studies. Capripoxviruses are economically important viruses producing generalized infections in domestic ruminants with significant mortality. The three members of the genus, sheeppox, goatpox and lumpy skin diesase virus, are closely related and share a group-specific structural protein named P32, making it possible to protect sheep, goats and cattle with a single vaccine. Squirrelpox virus was formerly assigned to the genus *Parapoxvirus* but has been removed and is presently unassigned.

Poxviral infections are generally characterized by skin lesions (Table 51.1). Lesions may be localized to the teats and mouth or be multiple and distributed generally. Several virus-specified proteins are secreted from infected cells including a homologue of epidermal growth factor. This protein stimulates the proliferation of epidermal cells. Orthopoxviruses and swinepox virus produce lesions that begin as macules and progress through papules, vesicles and pustules to scabs that finally separate leaving a scar. The lesions produced by parapox, capripox and fowlpox viruses tend to be proliferative in nature with a granulomatous or nodular appearance. Infection with myxomatosis virus, a leporipoxvirus, is characterized by tumours.

Replication occurs in the host cell cytoplasm in circumscribed areas termed 'viral factories'. Release can occur by budding, by exocytosis or by cell disruption resulting in enveloped and non-enveloped viruses respectively. Both forms are infectious. Enveloped viruses possess host cell lipids and additional virus-specified proteins including, in the case of orthopoxviruses, a virus haemagglutinin protein. Virions are stable at room temperature under dry conditions but sensitive to heat, detergents, formaldehyde and oxidizing agents. Ether-sensitivity varies between genera.

COWPOX VIRUS

Cowpox virus is endemic in Europe. Although infection and disease have been described in cattle, cats, man and

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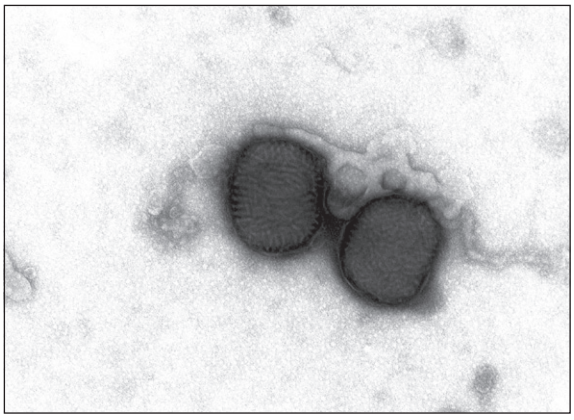

Figure 51.1 Electron micrograph of othopoxvirus.

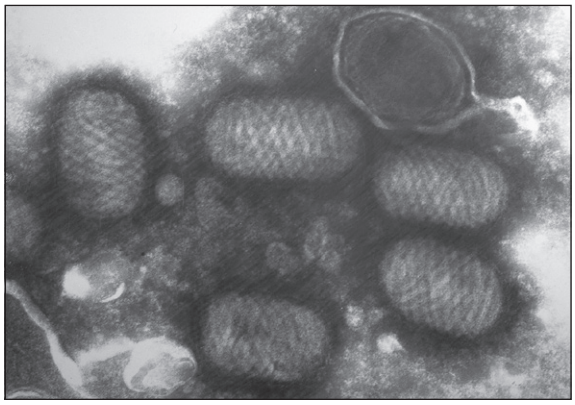

Figure 51.2 Electron micrograph of parapoxvirus

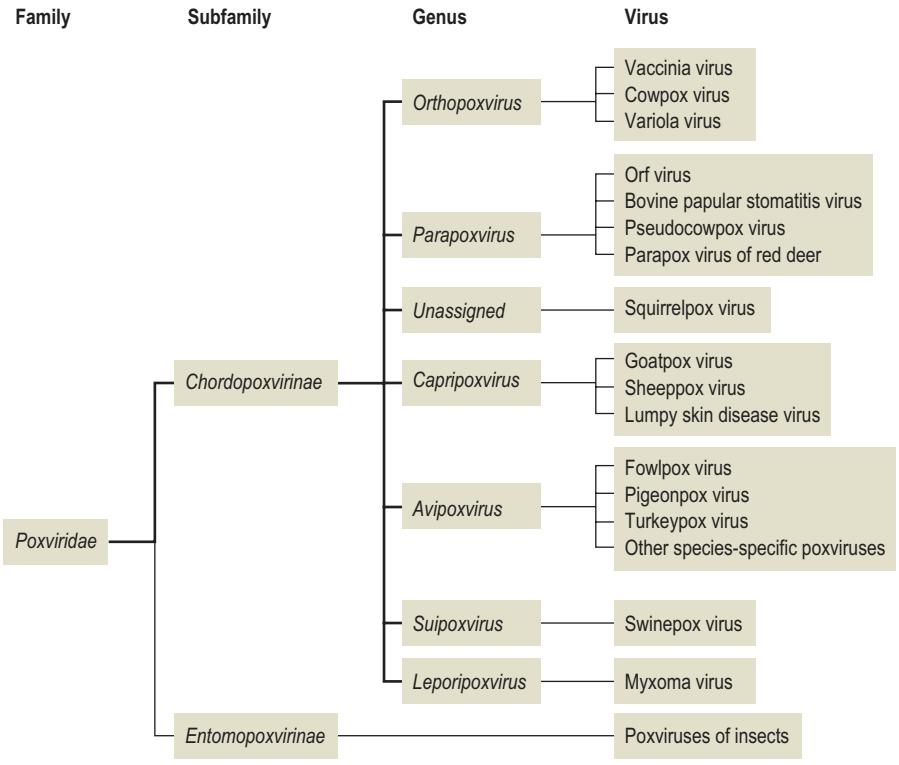

Figure 51.3 Classification of poxviruses of veterinary importance.

a range of captive mammals in zoological collections, these species are probably incidental hosts and the reservoir hosts are believed to be wild rodents (Chantrey et al. 1999). Genetic studies indicate that cowpox virus is probably a composite of up to five strains and support the creation of as many as four new species within the traditional 'cowpox' group (Carroll et al. 2011). Voles and woodmice are the principal reservoir hosts in western Europe. Clinical disease in cattle is rare and affects the teats of milking cows. Affected domestic cats usually

Table 51.1 Poxviruses of animals

Virus	Genus	Host species	Disease
Vaccinia virus (buffalopox and rabbitpox viruses are subspecies)	*Orthopoxvirus*	Wide host range including humans	Mild infections. Used as recombinant virus vector vaccine for rabies
Cowpox virus	*Orthopoxvirus*	Rodents, cats, cattle	Small rodents are the natural hosts, while cats are principal incidental host (skin lesions). Rare cause of bovine teat lesions. Transmissible to humans
Camelpox virus	*Orthopoxvirus*	Camel	Systemic infection with typical pox exanthema, most severe in young animals
Pseudocowpox virus	*Parapoxvirus*	Cattle	Common cause of teat lesions. Transmissible to humans (milker's nodule)
Bovine papular stomatitis virus	*Parapoxvirus*	Cattle	Mild, papular lesions affecting the muzzle and buccal cavity. Transmissible to humans
Orf virus	*Parapoxvirus*	Sheep, goats	Proliferative lesions involving muzzle and lips, most severe in young lambs. Transmissible to humans
Lumpy skin disease	*Capripoxvirus*	Cattle	Generalized infection with severe skin lesions. Endemic in Africa
Sheeppox/ Goatpox virus	*Capripoxvirus*	Sheep, goats	Generalized infection with characteristic skin lesions. Endemic in Africa, Middle East and India
Fowlpox virus	*Avipoxvirus*	Chickens	Cutaneous (dry) and diphtheritic (wet) forms. Transmitted by biting arthropods
Swinepox virus	*Suipoxvirus*	Pigs	Mild skin disease transmitted by pig louse (*Haematopinus suis*)
Myxoma virus	*Leporipoxvirus*	Rabbits	Mild disease in natural host (cottontail rabbits) but severe disease in European rabbits. Used for biological control of rabbits in several countries
Squirrelpox virus	Unassigned	Red and grey squirrels	Thought to be a significant factor in the demise of red squirrels in Great Britain and Ireland

come from rural areas and are described as good hunters. Infections in cats tend to peak in the Autumn when rodent populations are at their highest. There is often a history of a single bite-like wound on the head or a forelimb followed a few days or weeks later by widespread secondary skin lesions. Although cat-to-cat transmission can occur it is rare. These lesions begin as small papules but ulcerate over a period of two to three days. Scab formation follows with complete recovery usually in about six weeks. The diagnosis can be confirmed by histopathology, PCR, electron microscopy of unfixed scab or biopsy material and by virus isolation. Human infections with cowpox virus are uncommon and frequently associated with contact with infected cats. There is usually only a single lesion but affected individuals may be systemically ill. More severe disease and even fatal cases have been described in immunocompromised individuals.

PSEUDOCOWPOX VIRUS

Pseudocowpox virus, also referred to as milker's nodule or paravaccinia virus, is a parapoxvirus that infects cattle worldwide. It is a common, mild infection of the teats of lactating cows. The infection spreads slowly through milking herds with a proportion of animals showing lesions at any one time. Re-infection may occur at subsequent lactations. The lesions appear as small red papules on the teats or udder that quickly scab over and heal from the centre, producing a characteristic ring or horseshoe-shaped scab. Typical parapoxvirus particles can be visualized in scab material using electron microscopy. Infection is frequently acquired by people through contact with infected animals, appearing as spherical reddish-blue lesions on the hand, forearm or face.

BOVINE PAPULAR STOMATITIS VIRUS

Bovine papular stomatitis is a mild viral infection of cattle that is transmissible to humans. Infection is believed to be common and worldwide. The virus typically produces a subclinical infection. Older animals are thought to serve as a reservoir of infection for successive generations of calves. Lesions are most commonly seen in calves on the mucous membranes of the buccal cavity and muzzle. They are characterized by hyperaemic foci that develop into papules with concentric zones of inflammation. Affected animals typically recover within three weeks. A more severe chronic form has been described occasionally and may be associated with concurrent infections or other immunosuppressive factors (Yeruham et al. 1994). The condition can be confirmed by electron microscopic detection of the typical parapoxvirus particles in lesion scrapings.

ORF VIRUS

Orf virus, also called contagious pustular dermatitis or contagious ecthyma virus, infects sheep, goats, camels and man. It is an important, common infection with a worldwide distribution. The name is thought to be derived from an Old English word meaning rough or scabby. The disease primarily affects young animals and may be so mild as to go unnoticed or so severe as to result in significant mortality. Lesions are most commonly seen on the commissures of the lips and muzzle but may also appear on the feet, genitalia and teats. Severely affected lambs with lesions in the buccal cavity often fail to eat and lose condition. Outbreaks last six to eight weeks and usually do not reappear till a new crop of susceptible lambs becomes available.

Pathogenesis

The incubation period is about four to seven days. The virus is highly epitheliotropic producing proliferative wart-like lesions in affected animals following entry into the host through abrasions of the skin. The virus replicates in regenerating epidermal keratinocytes. Orf virus codes for a vascular endothelial growth factor which is thought to be important in stimulating vascular endothelial cell proliferation (Savory et al. 2000) and possibly providing the virus with further target cells to infect (Haig & Mercer 1998). Lesions progress through a series of characteristic phases. Initially papules develop but rapidly give way to a vesicular and then pustular stage. Scabs form within a few days while proliferation of the underlying dermis produces a verrucose mass. The lesions usually heal within four weeks leaving no scar. Secondary bacterial infection may prolong the course. The virus is readily transmissible to humans.

Diagnosis

A diagnosis of orf is normally possible on the basis of the characteristic clinical presentation. If necessary, electron microscopy can be performed on active scab material to confirm the diagnosis. Primers have been designed and used for the detection of parapoxvirus infections of ruminants by the polymerase chain reaction (Inoshima et al. 2000, Torfason & Guonadottir 2002, Gallina et al. 2006, Kottaridi et al. 2006).

LUMPY SKIN DISEASE VIRUS

Lumpy skin disease is endemic in sub-Saharan Africa and Madagascar with temporary incursions into the Middle East. It is an acute disease caused by a distinct species of capripoxvirus, lumpy skin disease virus (Neethling virus). It is classed by the OIE as a listed disease.

Pathogenesis

Following inoculation into the skin by insects, the virus multiplies locally in the dermis producing a primary nodule. A viraemia follows accompanied by fever and the appearance of widespread skin nodules which involve both the dermis and epidermis. The nodules arise from damage to endothelial cells, vasculitis, thrombosis and infarction leading to coagulative necrosis. Nodules on the mucous membranes of the mouth and nares quickly ulcerate. Some skin lesions may develop into 'sit-fasts' where a central plug of necrotic tissue develops over a number of weeks before sloughing off to reveal a deep granulating pock involving all layers of the skin. Secondary infection and myiasis are common complications in untreated cases. Recovery is slow and animals are frequently debilitated for several months. Lesions may also occur in the rumen, abomasum, trachea and lungs.

Diagnosis

- Clinical signs of a generalized nodular skin disease are highly suggestive. Biopsy or post mortem material from skin nodules are suitable diagnostic samples. However, material for virus isolation or antigen detection should be collected early in the clinical course before the development of neutralizing antibodies.
- The histopathological changes in lesions are characteristic and include vasculitis of dermal vessels, perivascular cuffing and thrombotic infarction.

Intracytoplasmic inclusions may be seen in early cases.
- Electron microscopy and demonstration of capripoxvirus particles in biopsy material or desiccated crusts provides a rapid means of confirming lumpy skin disease.
- The virus can be isolated in lamb testis cells where it produces a characteristic cytopathic effect ('moth-eaten' appearance to the monolayer) and intracytoplasmic inclusion bodies.
- An antigen trapping ELISA has been developed for the detection of the highly antigenic capripoxvirus P32 structural protein (Carn 1995). Expressed recombinant P32 antigen is available for the production of diagnostic reagents.
- Capripox virus-specific primers for the viral attachment protein gene and for the viral fusion protein gene can be used to detect viral DNA in biopsy or tissue culture material by PCR (Ireland & Binepal 1998, Tuppurainen et al. 2005).
- Several serological assays including virus neutralization, Western blot analysis, indirect fluorescent antibody test (Gari et al. 2008) and indirect ELISA (Carn et al. 1994) are suitable for the detection of antibodies to capripoxviruses.

SHEEPPOX AND GOATPOX VIRUSES

Sheeppox and goatpox are currently ascribed to two separate viruses. It has been suggested that the two diseases should be referred to as capripox on account of the many features common to the two diseases and their causal viruses. Genetic recombination occurs between strains and a wide spectrum of capripoxvirus strains have been isolated from sheep and goats (Gershon et al. 1989). Although most strains cause more severe disease in either sheep or goats, some strains are equally pathogenic in both animal species. However, genetic studies suggest that sheeppox virus and goatpox virus are phylogentically distinct viruses (Tulman et al. 2002). Capripox is endemic in central and north Africa, the Middle East and India. It is classified by the OIE as a listed disease and is notifiable in many countries.

Pathogenesis

The virus replicates locally in the skin or lungs depending on the route of infection. Spread to the regional lymph nodes is followed by a primary viraemia and replication in various internal organs. Skin lesions appear at about seven days post infection as macules. Within 24 hours the macules become papules which persist for about one week before becoming necrotic. Scabs form during the following few days. The scabs fall off leaving a permanent depressed scar.

Diagnosis

A diagnosis can often be made based on clinical signs and post mortem appearance. Papules may be present at many sites internally including tongue, oesophagus, rumen, abomasum and large intestine. Lesions in the lungs often coalesce into areas of consolidation and haemorrhage. Skin biopsies from live animals or necropsy specimens can be submitted for laboratory confirmation. Material intended for virus isolation or antigen detection should be collected early in the clinical course prior to the development of neutralizing antibodies.

- Histopathological examination of acute-stage skin lesions typically reveals a large cellular infiltration, vasculitis, oedema and the presence of eosinophilic intracytoplasmic inclusions in cells in the dermis.
- Electron microscopy can be used to rapidly identify poxvirus particles in lesion material. The morphology of capripoxviruses is easily distinguishable from parapoxviruses.
- Virus isolation is possible in lamb testis or kidney cells and results in a characteristic cytopathic effect and intracytoplasmic inclusion bodies.
- An antigen trapping ELISA has been developed for the detection of the highly antigenic P32 capripoxvirus structural protein (Carn 1995).
- Capripoxvirus-specific primers have been designed for the detection of viral DNA in biopsy or tissue culture samples using PCR (Ireland & Binepal 1998, Heine et al. 1999, Balinsky et al. 2008). It is possible to differentiate sheeppox and goatpox viruses on the basis of restriction enzyme analysis of the PCR-amplicons of the P32 gene, which encodes a major antigen (Hosamani et al. 2004).
- A number of serological methods including virus neutralization, indirect fluorescent antibody test, Western blot analysis (Chand et al. 1994) and indirect ELISA (Carn et al. 1994, Heine et al. 1999) are available for the detection of antibodies to capripoxviruses.

FOWL POX

Fowlpox is a term used to describe the disease associated with poxvirus infection of domestic poultry, particularly chickens and turkeys. Fowlpox, pigeonpox and turkeypox viruses are closely related viruses that are not strictly host-specific. Infection spreads slowly through contact and on the mouthparts of arthropods such as mosquitoes. Disease is characterized by proliferative cutaneous lesions (dry

pox) and diphtheritic lesions (wet pox) in the upper digestive and respiratory tracts.

Pathogenesis

The virus multiplies at the site of entry resulting in the formation of primary lesions. Infection with less pathogenic strains may be limited to the site of inoculation. In other cases viraemia occurs with spread to internal organs; secondary viraemia and development of secondary lesions follows. It is thought that inhalation or ingestion of virus-contaminated materials may be important in the development of diphtheritic lesions. Contributing factors such as malnutrition, debilitation and stress may also be necessary.

Diagnosis

- The identification of large, intracytoplasmic inclusion bodies (Bollinger bodies) which contain smaller elementary bodies (Borrel bodies) in histological sections of lesions is confirmatory. Immunofluorescent and immunoperoxidase techniques can be used to specifically stain the intracytoplasmic inclusions (Tripathy et al. 1973).
- Electron microscopy can be used to detect and identify the typical poxvirus particles in lesion material.
- Virus isolation can be attempted on the chorioallantoic membrane of nine- to 12-day-old embryonated hens' eggs. After five to seven days' incubation the CAM is examined for focal white pock lesions or generalized thickening.
- Nucleic acid probes have been used successfully for detection by hybridization with viral DNA extracted from lesion material (Fatunmbi et al. 1995). Genomic DNA sequences can be amplified by the polymerase chain reaction using specific primers (Lee & Lee 1997, Fallavena et al. 2002).
- Suitable serological tests for the detection of antibodies include ELISA (Buscaglia et al. 1985), virus neutralization (Morita 1973), agar gel precipitation and passive haemagglutination (Tripathy et al. 1970).

SWINEPOX

Swinepox virus infection occurs worldwide but is mild and often overlooked or undiagnosed. It is the only member of the genus *Suipoxvirus*. The virus can be transmitted mechanically by the pig louse, *Haematopinus suis*. Infected animals display a slight fever and rash after an incubation period of about one week. This is followed by the development of papules and subsequently pustules within the space of a few days. The lesions crust over and appear umbilicated. Healing is complete after three to four weeks. The clinical signs are generally sufficient for a diagnosis. Direct electron microscopy of lesion material can be used to confirm the condition.

MYXOMATOSIS

Myxomatosis is a severe generalized disease of European rabbits. The causal agent, myxoma virus, is the type species of the genus *Leporipoxvirus*. The natural hosts of myoma virus are New World species of rabbit, *Sylvilagus brasiliensis* in South America and *S. bachmani* in California. Infection has long been endemic in South America and western North America. In the natural hosts myxoma virus is associated with a benign cutaneous fibroma. However, the virus is associated with a severe, lethal infection in *Oryctolagus cuniculus*, the European rabbit. In the 1950s South American isolates of myxoma virus were deliberately introduced into Europe, Chile and Australia to control European rabbit populations and infection is now endemic in these regions.

Pathogenesis

Initial replication occurs at the site of skin inoculation followed by replication in the draining lymph node. The virus produces a generalized infection with primary and secondary viraemias. The viraemia is mainly cell-associated with most virus circulating within lymphocytes. Secondary lesions appear in the skin about a week after infection.

The first characteristic sign is blepharoconjunctivitis which becomes more marked and is accompanied by an opalescent ocular discharge. Subcutaneous gelatinous swellings develop all over the body. The ears droop and there is a swollen appearance to the head and anogenital region.

Diagnosis

The clinical signs in severe cases are sufficiently characteristic for a clinical diagnosis to be made. Laboratory confirmation can be achieved by isolation of the virus in primary or established rabbit cell lines from disrupted lesion material. A cytopathic effect typically develops after 24 to 48 hours. Alternatively, poxvirus particles may be detected in exudate or lesion material by electron microscopy. Complement fixation (Saurat et al. 1980, Chantal et al. 1993), indirect fluorescent antibody (Gilbert et al. 1989) and ELISA (Gelfi et al. 1999) are the most appropriate serological tests. A simple agar gel immunodiffusion test has been described for the detection of antibodies and antigen (Sobey et al. 1966).

REFERENCES

Balinsky, C.A., Delhon, G., Smoliga, G., et al., 2008. Rapid preclinical detection of sheeppox virus by a real-time PCR assay. Journal of Clinical Microbiology 46, 438–442.

Buscaglia, C., Bankowski, R.A., Miers, L., 1985. Cell-culture virus neutralization test and enzyme-linked immunosorbent assay for evaluation of immunity in chickens against fowlpox. Avian Diseases 29, 672–680.

Carn, V.M., 1995. An antigen trapping ELISA for the detection of capripoxvirus in tissue culture supernatant and biopsy samples. Journal of Virological Methods 51, 95–102.

Carn, V.M., Kitching, R.P., Hammond, J.M., et al., 1994. Use of a recombinant antigen in an indirect ELISA for detecting bovine antibody to capripoxvirus. Journal of Virological Methods 49, 285–294.

Carroll, D.S., Emerson, GL., Li, Y., et al., 2011. Chasing Jenner's vaccine: revisiting cowpox virus classification. PLoS One 6, e23086.

Chand, P., Kitching, R.P., Black, D.N., 1994. Western blot analysis of virus-specific antibody responses to capripoxvirus and contagious pustular dermatitis infections in sheep. Epidemiology & Infection 113, 377–385.

Chantal, J., Boucraut-Baralon, C., Ganiere, J.P., et al., 1993. Réaction de fixation du complément en plaques de microtitration: application à la sérologie de la myxomatose. Etude comparative des résultats avec la réaction d'immunofluorescence indirecte. Revue scientifique et technique (International Office of Epizootics) 12, 895–907.

Chantrey, J., Meyer, H., Baxby, D., et al., 1999. Cowpox: reservoir hosts and geographic range. Epidemiology & Infection 122, 455–460.

Fallavena, L.C., Canal, C.W., Salle, C.T., et al., 2002. Presence of avipoxvirus DNA in avian dermal squamous cell carcinoma. Avian Pathology 31, 241–246.

Fatunmbi, OO., Reed, W.M., Schwartz, D.I., et al., 1995. Dual infection of chickens with pox and infectious laryngotracheitis (ILT) confirmed with specific pox and ILT DNA dot-blot hybridization assays. Avian Diseases 39, 925–930.

Gallina, L., Dal Pozzo, F., Mc Innes, C.J., et al., 2006. A real time PCR assay for the detection and quantification of orf virus. Journal of Virological Methods 134, 140–145.

Gari, G., Biteau-Coroller, F., Legoff, C., et al., 2008. Evaluation of indirect fluorescent antibody test (IFAT) for the diagnosis and screening of lumpy skin disease using Bayesian method. Veterinary Microbiology 129, 269–280.

Gelfi, J., Chantal, J., Phong, T.T., et al., 1999. Development of an ELISA for detection of myxoma virus-specific rabbit antibodies; test evaluation for diagnostic applications on vaccinated and wild rabbit sera. Journal of Veterinary Diagnostic Investigation 11, 240–245.

Gilbert, Y., Picavet, D.P., Chantal, J., 1989. Diagnostic de la myxomatose: mise au point d'une technique d'immunofluorescence indirecte. Utilisation de prélèvements sanguins sur papier buvard pour la recherche d'anticorps. Revue scientifique et technique (International Office of Epizootics) 8, 209–220.

Gershon, P.D., Kitching, R.P., Hammond, J.M., et al., 1989. Poxvirus genetic recombination during natural virus transmission. Journal of General Virology 70, 485-489.

Haig, D.M., Mercer, A.A., 1998. Orf. Veterinary Research 29, 311–326.

Heine, H.G., Stevens, M.P., Foord, A.J., et al., 1999. A capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, the homolog of the vaccinia virus H3L gene. Journal of Immunological Methods 227, 187–196.

Hosamani, M., Mondal, B., Tembhurne, P.A., et al., 2004. Differentiation of sheep pox and goat poxviruses by sequence analysis and PCR-RFLP of P32 gene. Virus Genes 29, 73–80.

Inoshima, Y., Morooka, A., Sentsui, H., 2000. Detection and diagnosis of parapoxvirus by the polymerase chain reaction. Journal of Virological Method 84, 201–208.

Ireland, D.C., Binepal, Y.S., 1998. Improved detection of capripoxvirus in biopsy samples by PCR. Journal of Virological Methods 74, 1–7.

Kottaridi, C., Nomikou, K., Lelli, R., et al., 2006. Laboratory diagnosis of contagious ecthyma: comparison of different PCR protocols with virus isolation in cell culture. Journal of Virological Methods 134, 119–124.

Lee, L.H., Lee, K.H., 1997. Application of the polymerase chain reaction for the diagnosis of fowlpoxvirus infection. Journal of Virological Methods 63, 113–119.

Morita, C., 1973. Studies on fowlpox viruses. II. Plaque neutralization test. Avian Diseases 17, 93–98.

Saurat, P., Chantal, J., Ganiere, J.P., et al., 1980. La réponse immunitaire dans la myxomatose. Etude de la réponse humorale. Bulletin mensuel de l'Office National de la Chasse 12, 297–309.

Savory, L.J., Stacker, S.A., Fleming, S.B., et al., 2000. Viral vascular endothelial growth factor plays a critical role in orf virus infection. Journal of Virology 74, 10699–10706.

Sobey, W.R., Conolly, D., Adams, K.M., 1966. Myxomatosis: a simple method of sampling blood and testing for circulating soluble antigens or antibodies to them. Australian Journal of Science 28 (No. 9), 354.

Torfason, E.G., Gunadóttir, S., 2002. Polymerase chain reaction for laboratory diagnosis of orf virus infections. Journal of Clinical Virology 24, 79–84.

Tripathy, D.N., Hanson, L.E., Myers, W.L., 1970. Passive hemagglutination test with fowlpox virus. Avian Diseases 14, 29–38.

Tripathy, D.N., Hanson, L.E., Killinger, A.H., 1973. Immunoperoxidase technique for detection of fowlpox antigen. Avian Diseases 17, 274–278.

Tulman, E.R., Afonso, C.L., Lu, Z., et al., 2002. The genomes of sheepox and

goatpox viruses. Journal of Virology 76, 6054–6061.

Tuppurainen, E.S., Venter, E.H., Coetzer, J.A., 2005. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. Onderspoort Journal of Veterinary Research 72, 153–164.

Yeruham, I., Abraham, A., Nyska, A., 1994. Clinical and pathological description of a chronic form of bovine papular stomatitis. Journal of Comparative Pathology 111, 279–286.

FURTHER READING

Babiuk, S., Bowden, T.R., Boyle, D.B., et al., 2008. Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle. Transboundary and Emerging Diseases 55, 263–272.

Bhanuprakash, V., Indrani, B.K., Hosamani, M., et al., 2006. The current status of sheep pox disease. Comparative Immunology, Microbiology and Infectious Diseases 29, 27–60.

Chapter 52

Picornaviridae

Picornaviruses get their name from the prefix 'pico' meaning very small and the abbreviation for ribonucleic acid. In keeping with their name these viruses are only 22–30 nm in diameter and contain a single molecule of infectious, positive-sense, single-stranded RNA. Individual virions are icosahedral and non-enveloped (Fig. 52.1). The capsid is composed of 60 protein subunits. Each subunit consists of four proteins VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A). The protein VP4 is located on the inner surface of the capsid. Viral replication occurs in the cytoplasm of cells in membrane-associated complexes. Infection is usually cytolytic. The family is part of the order *Picornavirales* and is divided into 12 genera (Fig. 52.2): *Enterovirus, Teschovirus, Cardiovirus, Aphthovirus, Hepatovirus, Erbovirus, Kobuvirus*, *Parechovirus, Tremovirus, Sapelovirus*, *Avihepatovirus* and *Senecavirus*. The human rhinoviruses, a major cause of the common cold in man, have been moved to the genus *Enterovirus* and the genus *Rhinovirus* removed completely. Significant re-classification of the porcine enteroviruses has occurred with many being transferred to the newly created genus *Teschovirus*. Many former avian enteroviruses have been re-named and re-assigned to another genus or left unassigned. Avian nephritis virus 1, 2 have been re-classified in the family *Astroviridae*. The genera *Hepatovirus* and *Parechovirus* contain viruses of human importance including hepatitis A virus. Seneca Valley virus has a marked affinity for human tumour cell lines and is being evaluated as a treatment for human metastatic neuroendocrine cancers. Enteroviruses are responsible for the important human disease poliomyelitis. Equine rhinovirus 1 has been assigned to the genus *Aphthovirus* and renamed equine rhinitis A virus.

Picornaviruses are generally stable and resistant to ether, chloroform and non-ionic detergents. Thermal and pH stability varies within the genus. Aphthoviruses are unstable below pH 6.5 while rhinoviruses are unstable below pH 5. The enteroviruses, cardioviruses, hepatoviruses and parechoviruses are generally stable at acid pH.

In general, picornaviruses infect only one or a very small number of host species. However, foot-and-mouth disease virus and encephalomyocarditis virus infect a very wide host range. Horizontal transmission occurs, usually by faecal–oral, fomite or airborne routes. Persistent infections occur with a number of species, notably foot-and-mouth disease virus (Bergmann et al. 1996, Mezencio et al. 1999) and swine vesicular disease virus (Lin et al. 1998). The mechanism of persistence is unclear but may be due to antigenic variation (Woodbury 1995). Genetic recombination, complementation and phenotypic mixing have been described. Mixed infections in individual animals, particularly the African buffalo, with different serotypes of foot-and-mouth disease virus are known to occur. Infections with porcine teschoviruses (PTVs) are widespread in pig herds but usually subclinical. Certain PTV serotypes (PTV-1, PTV-2, PTV-3, PTV5, PTV-6) are associated with a range of clinical conditions including SMEDI, encephalomyelitis, diarrhoea, pneumonia and heart disease. Picornaviruses of animals are summarized in Table 52.1.

FOOT-AND-MOUTH DISEASE

Foot-and-mouth disease (FMD) is a highly contagious disease of even-toed ungulates, characterized by fever and the formation of vesicles. It is a listed disease of major international, economic importance on account of its speed of spread and the severe losses of production in infected animals. Seven major serotypes are recognized FMDV-A, FMDV-Asia 1, FMDV-O, FMDV-C, FMDV-Southern African Territories (SAT) 1, FMDV-SAT 2 and FMDV-SAT 3. Infection with one serotype does not

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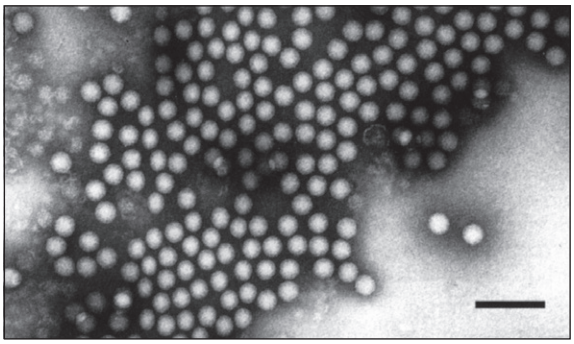

Figure 52.1 Negative contrast electron micrograph of poliovirus particles. The bar represents 100 nm. *Reprinted with permission: Fauquet, C.M., et al. (Eds.), 2005. Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, pp. 757.*

confer immunity against another serotype, while a large number of strains are recognized within each serotype.

Cattle, sheep, goats, pigs and buffalo are susceptible to FMD. In addition several wildlife species including African buffalo, elephants, hedgehogs, deer and antelope are susceptible. Large amounts of virus are shed by infected animals in all secretions and excretions. Virus shedding begins during the incubation period, usually beginning about 24 hours before the appearance of clinical signs, and infectivity of animals is much reduced by four to five days after the lesions develop. Transmission occurs by direct contact, by animal products including meat, offal, milk, semen and embryos, by the airborne route or by mechanical carriage by people, vehicles and fomites. Infected groups of animals, particularly pigs, shed large quantities of virus in exhaled air as an aerosol. Under suitable conditions of low temperature, high humidity and

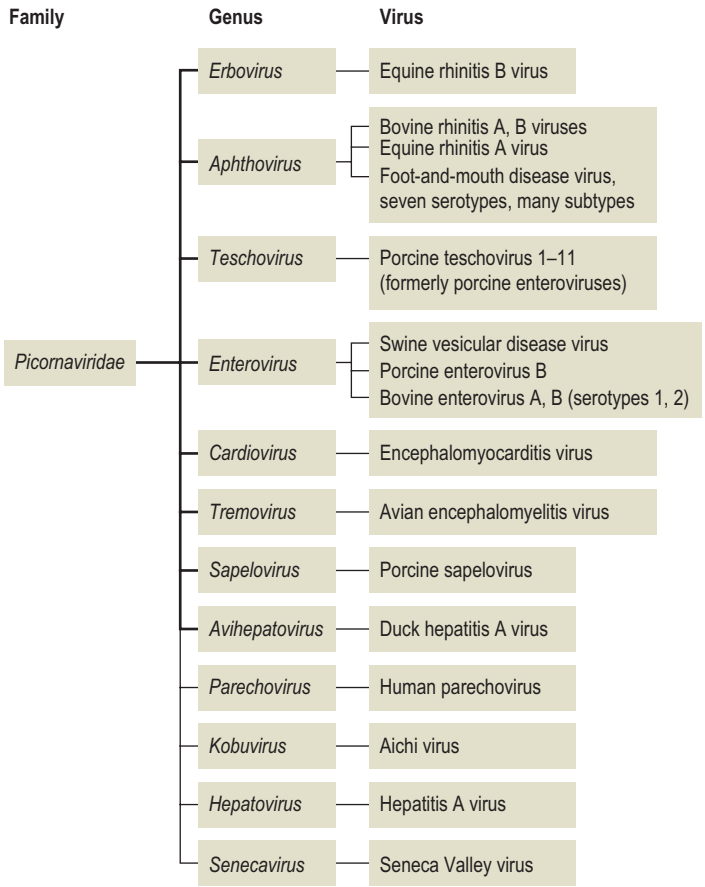

Figure 52.2 Classification of picornaviruses of importance.

Table 52.1 Picornaviruses of animals

Virus	Genus	Host species	Significance of infection
Foot-and-mouth disease viruses	*Aphthovirus*	Cloven hoofed animals	Seven serotypes: O, Asia 1, A, C, SAT1, SAT 2, SAT 3. Many subtypes and numerous distinct antigenic strains recognized. Listed disease, highly contagious disease of international importance
Swine vesicular disease	*Enterovirus*	Pig	Mild vesicular disease, clinically indistinguishable from foot-and-mouth disease
Porcine sapelovirus (porcine enterovirus A, porcine enterovirus 8)	*Sapelovirus*	Pig	Infection is frequently asymptomatic. Associated with SMEDI
Porcine teschovirus 1	*Teschovirus*	Pig	Varying virulence PTV-1 strains associated with encephalomyelitis. Highly virulent strains of PTV-1 (Teschen disease) confined to Eastern Europe and Madagascar. Worldwide distribution of mild PTV-1 strains (Talfan disease)
Porcine teschoviruses 2–11	*Teschovirus*	Pig	Infection is frequently asymptomatic. Associated with encephalomyelitis, SMEDI, pneumonia and diarrhoea
Avian encephalomyelitis virus	*Tremovirus*	Chicken	Associated with avian encephalomyelitis in young chickens
Bovine enterovirus A, B	*Enterovirus*	Wide range of ruminant species	Widespread distribution. Isolated from normal cattle and from cattle with enteric, respiratory and reproductive disease problems
Equine rhinitis virus A	*Aphthovirus*	Horse	Common, systemic infection. Usually subclinical infection but also associated with respiratory disease
Encephalomyocarditis virus	*Cardiovirus*	Wide host range, rodents are natural hosts	Infection is often subclinical in pigs but associated with sudden death in young pigs and reproductive failure in sows
Bovine rhinitis B virus	*Aphthovirus*	Cattle	Widespread distribution. Isolated from normal cattle and from animals with respiratory disease, usually in association with other bovine respiratory viruses
Equine rhinitis B virus	*Erbovirus*	Horse	Widely distributed. Considered to be minor respiratory pathogen

moderate winds such aerosols may spread the virus over long distances. Typically spread over land is within 10 km of the source. However, turbulence is generally less over water than over land and in 1981 the disease spread over 200 km across the English Channel from France to the south coast of England. Cattle are most susceptible to infection because of their large respiratory volume and the low infective dose required. The virus is moderately resistant but is sensitive to pH outside the range 6.0 to 9.0. Virus can remain infective on soil for three days in the summer and for 28 days in the winter. Following death and rigor mortis, the production of lactic acid in muscle inactivates the pH-labile virus but virus may persist in offal and bone marrow. Foot-and-mouth disease virus can persist in the pharyngeal region of animals that have recovered from FMD or in vaccinated animals following contact with live virus. Persistence can last up to three years in cattle, several months in sheep and up to five years in African Cape buffalo. It is unclear if persistence occurs in pigs (Bergmann et al. 1996, Mezencio et al. 1999). The transmission of infection from carrier to susceptible animals has only been demonstrated satisfactorily for African Cape buffalo.

Pathogenesis

The principal route of infection is by inhalation, although infection can also occur by ingestion, insemination, inoculation or contact with abraded skin. Following inhalation viral replication occurs in the pharynx followed by spread

to the bloodstream and distribution to predilection sites including the epithelium of the mouth, muzzle, feet and teats. Vesicle formation results from swelling and rupture of infected keratinocytes in the stratified squamous epithelium at these sites.

Following an incubation period of two to eight days, infected cattle demonstrate fever, loss of appetite, and a marked drop in milk production. There is profuse salivation as vesicles appear in the mouth and rapidly rupture leaving raw, painful ulcers. Vesicles also appear in the interdigital cleft and on the coronary band giving rise to lameness and the shifting of weight from one foot to another. Vesicles may appear on the teats and udder of lactating cows. The lesions heal rapidly but may become secondarily infected giving rise to mastitis and underrunning of the sole. Infected animals show a marked loss in condition and growth rate but mortality in adult cattle is rare. Death may occur in calves due to acute myocarditis. Although the virus does not cross the placenta, abortion may occur, probably as a result of the fever. In pigs feet lesions are more pronounced and the hooves may slough. Lameness is marked and the most prominent clinical sign in pigs. The disease in sheep, goats and wild ruminants is generally milder, presenting as a rapidly spreading lameness accompanied by fever.

Infection in man has been described on a few occasions, usually in laboratory personnel working with the virus or in people handling infected animals. The disease is mild and characterized by fever and vesiculation of the skin and mucous membranes.

Diagnosis

Laboratory confirmation is required due to the clinical similarities between foot-and-mouth disease and other vesicular diseases of domestic animals; vesicular stomatitis in cattle and pigs, swine vesicular disease and vesicular exanthema in pigs. Handling of virus and the performance of diagnostic tests must be carried out in virus-secure laboratories. The sample of choice is epithelium collected from an unruptured or recently ruptured vesicle. In convalescent, persistent or subclinical infections samples of oesophageal-pharyngeal fluid can be obtained by means of a probang (sputum) cup. Samples should be placed in a transport medium composed of equal parts of PBS and glycerol at pH 7.2 to 7.6 with added antibiotics and antifungal agents.

- Diagnosis is based on the demonstration of FMD viral antigen in samples of tissue or fluid. Samples may be tested using ELISA or CFT. The indirect sandwich ELISA (Roeder & Le Blanc Smith 1987) is the preferred test as it is more sensitive and specific and is available in kit form.
- The reverse transcription polymerase chain reaction (RT-PCR) is suitable for the amplification of genome fragments of FMDV in diagnostic material (Amarel-Doel et al. 1993, Bastos 1998, Reid et al. 2000). Sensitive real time RT-PCR protocols are also available (Reid et al. 2001, Alexandersen et al. 2002, Reid et al. 2002, King et al. 2006). Efforts are being made to adapt the methodology for use in the field (King et al. 2008). Nucleotide sequencing of the VP1 gene, which encodes a capsid protein, of an isolate from an outbreak of FMD is used to provide a means of comparison with other isolates of the same serotype in order to determine the possible origin of the outbreak. In addition, the study of VP1-based phylogenies has revealed that different genotypes within the O and SAT types evolve in discrete geographical regions, the resulting variants are known as topotypes.
- Virus isolation can be carried out in sensitive cell lines such as primary bovine thyroid or kidney cells. Established cell lines such as IB-RS-2 and BHK-2 may be used but are less sensitive than primary cells. The epithelium sample should be blotted dry to remove most of the glycerol, which is toxic to cells, and homogenized to form a suspension in a small volume of tissue culture medium. Following clarification the suspension is inoculated onto cell culture and examined over 48 hours for evidence of cytopathic effect (CPE). In the event of failure to detect CPE a second passage should be carried out and examined for a further 48 hours.
- Demonstration of specific antibody can also be used to confirm a diagnosis but generally requires absence of vaccination. Interpretation of antibody titres in enzootic areas may be difficult due to the possibility of previous infection. Suitable serological tests include virus neutralization and liquid-phase blocking or competitive ELISA (Hamblin et al. 1986, Van Maanen 1990). These assays detect antibodies to structural proteins and are therefore serotype-specific. The detection of antibodies to non-structural proteins (NSPs) by ELISA or immunoblotting has been shown to be useful in detecting animals currently or previously infected regardless of whether the animal has been vaccinated (De Diego et al., 1997, Bergmann et al. 2000, Paton et al. 2006). The polyproteins 3AB or 3ABC are generally considered the most useful NSP test antigens and are produced by recombinant techniques (Mackay et al. 1997, Sorensen et al. 1998). It is important that the FMD vaccines used do not contain even trace amounts of NSPs. Some infected animals may not be detected using NSP antibody assays (Mackay 1998) and therefore these assays are probably best used on a herd rather than an individual animal basis. The NSPs are highly conserved and are not serotype-specific.

SWINE VESICULAR DISEASE

Swine vesicular disease (SVD) is a mild vesicular disease of pigs which occurs sporadically in parts of Europe and Asia. The importance of the disease is due to the fact that it is clinically indistinguishable from foot-and-mouth disease.

Swine vesicular disease virus is an enterovirus and considered a porcine variant of human coxsackievirus B5, which is a subtype of human enterovirus B. The pig is the natural host but the virus is also capable of infecting laboratory workers handling infected material. The infection in humans is generally characterized by a mild febrile illness. Transmission can occur by direct or indirect contact, the virus is stable for long periods in the presence of organic matter in the environment. The spread of disease from farm to farm is dependent on the movement of infected pigs or contaminated materials. The tissues of infected pigs contain large quantities of virus. The infectivity of the virus is retained despite the pH changes that occur during rigor mortis and the virus can persist indefinitely in refrigerated pork.

Pathogenesis

The incubation period is two to seven days. Subclinical disease is common. Following the entry of virus through damaged skin or ingestion, there is local replication followed by spread via the lymphatics to the bloodstream. During the two- to three-day viraemia many organs and tissues become infected. Shedding of virus can start before clinical signs are evident and is heaviest in the first week following infection. The faeces and lesion material of infected pigs may contain infective virus for many weeks or months (Lin et al. 1998).

The clinical disease is characterized by a transient fever followed by the development of vesicular lesions on the feet, particularly on the coronary band, and less commonly on the lips, tongue and snout. Lameness, dullness and inappetence occur but are not constant signs. The animals do not lose condition and the lesions heal rapidly over the course of a few weeks. The severity of the clinical signs is influenced by the strain of SVDV, the route and dose of infection and the husbandry conditions.

Diagnosis

Laboratory diagnosis is essential to differentiate SVD from the other major vesicular diseases of pigs, foot-and-mouth disease and vesicular stomatitis. Samples from suspect animals should be handled as if they contain foot-and-mouth disease virus and be transported in phosphate buffered saline (PBS) with glycerol (1/1) and antimicrobial agents at PH 7.2 to 7.6.

- The indirect sandwich ELISA is the method of choice for the rapid detection of viral antigen in vesicular fluid or epithelium (10% suspension of lesion material in PBS).
- Samples should also be inoculated onto monolayers of susceptible porcine cells such as IB-RS-2 cells. The virus produces a cytopathic effect (CPE). Negative cultures should be blind-passaged every 48 or 72 hours for two to three passages. The supernatant fluid from cultures displaying CPE is harvested and tested for viral antigen using the ELISA.
- An RT-PCR protocol has been described for the detection of SVD viral genome in clinical samples using primers to highly conserved regions of genes coding for major structural proteins (Lin et al. 1997). Alternative protocols are available (Nunez et al. 1998). Real time RT-PCR assays have also been evaluated (Reid et al. 2004). Amplification and sequencing of the *1D* gene, which codes for the major structural protein VP1, has been used for epidemiological studies of strains of SVDV (Brocchi et al. 1997).
- On account of the mild nature of the disease, the first indication of infection may often be obtained following serological screening for antibodies to SVDV. Several test procedures are suitable, of which ELISA (Brocchi et al. 1995) and virus neutralization are the most frequently used. The VN test is the accepted standard test but requires tissue culture procedures and takes longer than the ELISA to perform. A small proportion of animals may produce false-positive results and repeat samples should be collected from the individual animal and from cohorts.

TESCHEN/TALFAN DISEASE

Also referred to as enterovirus encephalomyelitis this condition varies widely in severity depending on the strain of porcine teschovirus involved. The disease was first described in Teschen in 1929 in the Czech Republic and has caused significant losses in several European countries. Severe clinical disease is now rare and largely confined to Eastern Europe and Madagascar.

Originally porcine enteroviruses (PEV) were divided into 13 serotypes (PEV 1 to 13) grouped into three groups; I, II and III, on the basis of replication in different cell lines, CPE produced, and serological assays. Group II contains porcine sapelovirus (formerly PEV 8, subsequently referred to as porcine enterovirus A). Group III contains PEV 9 and 10, now referred to as porcine enterovirus B. As a result of nucleotide sequencing and phylogenetic analysis group I serotypes (PEV 1 to 7 and PEV 11 to 13) have been placed in their own genus *Teschovirus*. The most

important neurotropic strains belong to porcine teschovirus 1 (PEV 1) which includes both highly virulent isolates (Teschen disease) as well as many less virulent but more widely distributed strains (Talfan disease or endemic posterior paresis). Other porcine 'enterovirus' serotypes associated with encephalomyelitis include porcine teschovirus 2 (PEV 2), porcine teschovirus 3 (PEV 3) and porcine teschovirus 5 (PEV 5).

Transmission is usually by the faecal–oral route and can occur by direct or indirect contact with infected pigs. Clinical disease is most severe in young pigs and usually follows the introduction of the virus or a new serotype to which the herd has not been previously exposed. In endemically infected herds, infection tends to occur at the time of weaning as a result of a decline in passive maternal immunity and the mixing of pigs. Sporadic clinical cases usually occur at about this time.

Pathogenesis

The incubation period is about 14 days. Initial signs include fever, depression and listlessness followed by the onset of nervous signs at about one week post infection. Following ingestion the virus replicates in the tonsils, intestines and associated lymph nodes. Viraemia may follow, particularly in the case of virulent strains, permitting invasion of the CNS. Faecal excretion of virus continues for several weeks. The mortality rate is high in Teschen disease with death occurring within three to four days of the onset of nervous signs. Mild cases are characterized by ataxia and paresis, usually followed by recovery.

Diagnosis

Appropriate samples include brain and spinal cord for histopathological examination and detection of virus. Paired serum samples should be collected for the detection of specific antibodies.

- Histopathological examination of the brain and spinal cord reveals a non-suppurative encephalomyelitis with perivascular lymphocyte infiltration. The extent of the lesions varies with the severity of the condition. The presence of viral antigen can be confirmed by immunohistochemistry.
- The virus can be isolated from samples of brain and spinal cord from piglets showing early nervous signs in porcine kidney cell lines or other cell lines derived from porcine tissue. The virus produces a characteristic cytopathic effect with foci of rounded refractile cells. The identity of the virus can be confirmed by virus neutralization or immunofluorescence using specific antisera or monoclonal antibodies.
- RT-PCR provides a rapid means of detection of the viral RNA (Palmquist et al. 2002) and specific primer sets have been designed for the differentiation of groups I, II and III viruses by nested and real time RT-PCR (Zell et al. 2000, Krumbholz et al. 2003).
- Several serological methods are available for the detection of antibodies to porcine enterovirus type 1. Virus neutralization and ELISA (Hubschle et al. 1983) are the most frequently used tests. On account of the wide distribution of porcine teschovirus 1 strains in pig populations, it is necessary to demonstrate a fourfold rise in titre between acute and convalescent sera.

PORCINE ENTEROVIRAL REPRODUCTIVE DISORDERS

Several former enterovirus serotypes, including PEV 1, 3 and 6 (porcine teschoviruses 1, 3 and 6) and 8 (porcine sapelovirus, porcine enterovirus A) are associated with SMEDI (stillbirths, mummification, embryonic death and infertility) in pigs. These SMEDI-enteroviruses are widely distributed in commercial pig herds but are only pathogenic to embryos and foetuses. Clinical disease only follows infection of naive, pregnant animals. Serotypes are not cross-protective. Transmission is by the faecal–oral route or by indirect contact with fomites. Infection of the enteric tract is followed by viraemia and transplacental spread to some of the developing foetuses. The clinical effects of infection vary with the stage of gestation; infection during early to mid-gestation results in embryonic death and mummification whereas infection during late pregnancy may result in stillbirth or the birth of live piglets. Infections typically result in a mixture of mummified, stillborn and live piglets, reflecting the slow rate of intrauterine spread. The clinical signs are indistinguishable from porcine parvovirus infection, a more common cause of reproductive failure involving mummification. Laboratory confirmation of diagnosis can be achieved by isolation of the virus from the lung tissue of stillborn piglets or demonstration of antibody in serum from stillborn piglets or newborn piglets prior to the feeding of colostrum. Mummified foetuses carried to term rarely contain live virus but may provide samples suitable for viral antigen detection by immunofluorescence.

AVIAN ENCEPHALOMYELITIS

Avian encephalomyelitis is a viral disease of young birds that has been recorded in chickens, pheasants, quail and turkeys. It is of considerable economic importance in chickens. Avian encephalomyelitis virus (AEV),

formerly considered an enterovirus, was thought to be a hepatovirus and had been assigned to the genus *Hepatovirus* (Todd et al. 1999). It has now been placed in its own genus *Tremovirus*. Both horizontal and vertical transmission occur. Following infection of the enteric tract the virus is shed in the faeces. In addition a proportion of the eggs laid by infected hens will be infected. Congenitally infected chicks generally hatch normally but shed virus and infect their hatch-mates. Clinical disease ensues in congenitally infected chicks and in susceptible chicks exposed at an early age. Infection of the enteric tract is followed by viraemia and infection may then establish in the CNS. Clinical signs usually become evident at one to two weeks of age and include ataxia, poor muscle coordination and fine tremors of the head and neck. Except for a temporary drop in egg production, clinical signs do not occur in older birds following the development of immunological competence. Avian encephalomyelitis can be diagnosed on the basis of an absence of gross lesions and the presence of characteristic histopathological lesions in the CNS and viscera. Lesions in the CNS are characteristic of non-suppurative encephalomyelitis while those in the viscera consist of lymphocytic accumulations. Confirmation can be obtained by demonstration of viral antigen in tissues by immunofluorescence or by virus isolation from brain or pancreas in embryonated eggs. An RT-PCR assay has been described (Xie et al. 2005). Serological testing of paired sera may also be helpful.

EQUINE RHINITIS VIRUSES

Formerly three distinct equine rhinoviruses, unassigned to a genus, were recognized. Genetic and other studies have revealed that equine rhinovirus 1 is closely related to foot-and-mouth disease virus. As a result it has been re-named equine rhinitis A virus and placed in the genus *Aphthovirus*. Equine rhinitis B virus (equine rhinovirus 2, 3), of which there are three serotypes recognized, is a member of the genus *Erbovirus*. Infection with these viruses appears to be widespread with most horses exposed early in life. Both equine rhinitis A virus and equine rhinitis B virus have been associated with acute respiratory disease (Carman et al. 1997, Klaey et al. 1998, Dynon et al. 2007) but are generally thought of as minor respiratory pathogens. These viruses may contribute to the development of disease as a result of a mixed viral or bacterial infection or following surgery or strenuous exercise. Viraemia and prolonged viral shedding in urine occurs following infection with equine rhinitis A virus.

ENCEPHALOMYOCARDITIS VIRUS

The natural hosts of encephalomyocarditis virus (EMCV) are rats and mice, which excrete the virus in faeces and urine. However, the virus has a wide host range with infections occurring in man, monkeys, squirrels, elephants and pigs. Infection in pigs is usually subclinical but sporadic deaths and minor outbreaks occur. Infection is usually acquired by ingestion of contaminated feed. The virus is quite stable in the environment. It is thought that pig-to-pig transmission can also occur (Koenen et al. 1999). Outbreaks on farms are typically restricted to one particular age category. It appears that virus isolates associated with myocardial disease differ from isolates responsible for reproductive disease (Koenen et al. 1999).

Pathogenesis

Following ingestion, virus can be found in the bloodstream within a few days. The highest titres of virus occur in heart muscle and in spleen and mesenteric lymph nodes. Transplacental infection may occur in pregnant sows. The severity of disease varies with the strain of virus and the age of pigs involved. Infection in young pigs is characterized by sudden death as a result of acute heart failure whereas in older pigs infection is generally subclinical. Infection of pregnant sows is characterized by abortion, mummified foetuses, stillbirths and weak piglets.

Diagnosis

Sudden deaths or reproductive failure in conjunction with high pre-weaning mortality is suggestive of EMCV infection in a pig herd. Gross and microscopic myocardial lesions are significant. Laboratory confirmation can be achieved by virus isolation and identification. Virus isolation can be carried out in mice or in susceptible cell lines such as BHK-21. The virus produces a rapid and complete cytopathic effect in cell monolayers. An RT-PCR protocol has been described for the rapid detection of EMCV RNA in clinical specimens (Vanderhallen & Koenen 1997). Virus neutralization, ELISA and haemagglutination tests can be used to detect serum antibodies. Determination of the significance of serological titres in sows can be difficult.

REFERENCES

Alexandersen, S., Zhang, Z., Reid, S.M., et al., 2002. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. Journal of General Virology 83, 1915–1923.

Amarel-Doel, C.M.F., Owen, N.E., Ferris, N.P., et al., 1993. Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction. Vaccine 11, 415–421.

Bastos, A.D.S., 1998. Detection and characterization of foot-and-mouth disease virus in sub-Saharan Africa. Onderstepoort Journal of Veterinary Research 65, 37–47.

Bergmann, I.E., Malirat, V., de Mello, P.A., et al., 1996. Detection of foot-and-mouth viral sequences in various fluids and tissues during persistence of the virus in cattle. American Journal of Veterinary Research 57, 134–137.

Bergmann, I.E., Malirat, V., Neitzert, E., et al., 2000. Improvement of serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot. Archives of Virology 145, 473–489.

Brocchi, E., Berlinzani, A., Gamba, D., et al., 1995. Development of two novel monoclonal antibody-based ELISAs for the detection of antibodies and the identification of swine isotypes against swine vesicular disease virus. Journal of Virological Methods 52, 155–167.

Brocchi, E., Zhang, G., Knowles, N.J., et al., 1997. Molecular epidemiology of recent outbreaks of swine vesicular disease: two genetically and antigenically distinct variants in Europe, 1987–1994. Epidemiology & Infection 118, 51–61.

Carman, S., Rosendal, S., Huber, L., et al., 1997. Infectious agents in acute respiratory disease in horses in Ontario. Journal of Veterinary Diagnostic Investigation 9, 17–23.

De Diego, M., Brocchi, E., Mackay, D., et al., 1997. The use of the non-structural polyprotein 3ABC of FMD virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Archives of Virology 142, 2021–2033.

Dynon, K., Black, W.D., Ficorilli, N., et al., 2007. Detection of viruses in nasal swab samples from horses with acute, febrile, respiratory disease using virus isolation, polymerase chain reaction and serology. Australian Veterinary Journal 85, 46–50.

Hamblin, C., Barnett, I.T.R., Hedger, R.S., 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus I Development and method of ELISA. Journal of Immunological Methods 93, 115–121.

Hubschle, O.J.B., Rajoanarison, J., Koko, M., et al., 1983. ELISA for detection of Teschen virus antibodies in swine serum samples. Deutsche Tierarztliche Wochenschrift 90, 86–88.

King, D.P., Ferris, N.P., Shaw, A.E., et al., 2006. Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time RT-PCR assays. Journal of Veterinary Diagnostic Investigation 18, 92–96.

King, D.P., Dukes, J.P., Reid, S.M., et al., 2008. Prospects for rapid diagnosis of foot-and-mouth disease in the field using reverse transcriptase-PCR. Veterinary Record 162, 315–316.

Klaey, M., Sanchez-Higgins, M., Leadon, D.P., et al., 1998. Field case study of equine rhinovirus 1 infection: clinical signs and clinicopathology. Equine Veterinary Journal 30, 267–269.

Koenen, F., Vanderhallen, H., Castryck, F., et al., 1999. Epidemiologic, pathogenic and molecular analysis of recent encephalomyocarditis outbreaks in Belgium. Journal of Veterinary Medicine, Series B 46, 217–231.

Krumbholz, A., Wurm, R., Scheck, O., et al., 2003. Detection of porcine teschoviruses and enteroviruses by LightCycler real-time PCR. Journal of Virological Methods 113, 51–63.

Lin, F., Mackay, D.K.J., Knowles, N.J., 1997. Detection of swine vesicular disease virus RNA by reverse transcription-polymerase chain reaction. Journal of Virological Methods 65, 111–121.

Lin, F., MacKay, D.K.J., Knowles, N.J., 1998. The persistence of swine vesicular disease virus infection in pigs. Epidemiology & Infection 121, 459–472.

Mackay, D.K., 1998. Differentiating infection from vaccination in foot-and-mouth disease. Veterinary Quarterly 20 (Suppl 2), 2–5.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., et al., 1997. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine 16, 446–459.

Mezencio, J.M.S., Babcock, G.D., Kramer, E., et al., 1999. Evidence for the persistence of foot-and-mouth disease virus in pigs. Veterinary Journal 157, 213–217.

Nunez, J.I., Blanco, E., Hernandez, T., et al., 1998. A RT-PCR assay for the differential diagnosis of vesicular viral diseases of swine. Journal of Virological Methods 72, 227–235.

Palmquist, J., Munir, S., Taku, A., et al., 2002. Detection of porcine teschovirus and enterovirus type II by reverse transcription-polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 14, 476–480.

Paton, D.J., de Clercq, K., Greiner, M., et al., 2006. Application of non-structural protein antibody tests in substantiating freedom from foot-and-mouth disease virus infection after emergency vaccination of cattle. Vaccine 24, 6503–6512.

Reid, S., Ferris, N.P., Hutchings, G.H., et al., 2000. Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. Journal of Virological Methods 89, 167–176.

Reid, S., Ferris NP, Hutchings, G.H., et al., 2001. Diagnosis of foot-and-mouth disease by real-time fluorogenic PCR assay. Veterinary Record 149, 621–623.

Reid, S., Ferris NP, Hutchings, G.H., et al., 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time fluorogenic reverse transcription polymerase chain reaction assay. Journal of Virological Methods 105, 67–80.

Reid, S.M., Ferris, N.P., Hutchings, G.H., et al., 2004. Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. Journal of Virological Methods 116, 169–176.

Roeder, P.L., Le Blanc Smith, P.M., 1987. The detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. Research in Veterinary Science 43, 225–232.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., et al., 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Archives of Virology 143, 1461–1476.

Todd, D., Weston, J.H., Mawhinney, K.A., et al., 1999. Characterization of the genome of avian encephalomyelitis virus with cloned cDNA fragments. Avian Diseases 43, 219–226.

Vanderhallen, H., Koenen, F., 1997. Rapid diagnosis of encephalomyocarditis virus infections in pigs using a reverse transcription-polymerase chain reaction. Journal of Virological Methods 66, 83–89.

Van Maanen, C., 1990. A complex-trapping-blocking (CTB) ELISA, using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types A, O and C 1 Method and characteristics. Veterinary Microbiology 24, 171–178.

Woodbury, E.L., 1995. A review of the possible mechanisms for the persistence of foot-and-mouth disease virus. Epidemiology & Infection 114, 1–13.

Xie, Z., Khan, M.I., Girshick, T., et al., 2005. Reverse transcriptase-polymerase chain reaction to detect avian encephalomyelitis virus. Avian Diseases 49, 227–230.

Zell, R., Krumbholz, A., Henke, A., et al., 2000. Detection of porcine enteroviruses by nRT-PCR: differentiation of CPE groups I–III with specific primer sets. Journal of Virological Methods 88, 205–218.

FURTHER READING

Sutmoller, P., Bartelling, S.S., Olascoaga, R.C., et al., 2003. Control and eradication of foot-and-mouth disease. Virus Research 91, 101–144.

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Chapter 53

Caliciviridae

Caliciviruses derive their name from the Latin word *calix* meaning cup, which refers to the cup-shaped depressions visible on the surface of the virions in negative-contrast electron micrographs. The virions are icosahedral, non-enveloped and 27–40 nm in diameter with a genome that consists of a single molecule of linear, positive-sense, single-stranded RNA (Fig. 53.1). Replication occurs in the cytoplasm of infected cells with release by cell lysis. Several caliciviruses have not yet been cultivated in vitro. The virions are resistant to ether, chloroform, mild acids and mild detergents.

Caliciviruses (Table 53.1) are most closely related to picornaviruses and formerly were classified as the genus *Calicivirus* within the *Picornaviridae*. The family *Caliciviridae* is divided into five genera (Fig. 53.2); *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus* and *Nebovirus*. Important veterinary viruses in the genus *Vesivirus* include the prototype virus of the family, vesicular exanthema of swine virus, and feline calicivirus. The *Lagovirus* genus contains two viruses of lagomorphs, rabbit haemorrhagic disease virus and European brown hare syndrome virus. The human caliciviruses, Norwalk virus and Sapporo virus, are important causes of gastroenteritis. Viruses in the genus *Norovirus* are also referred to as small, round structured viruses (SRSV). Norwalk virus strains are divided into five genogroups (GI to GV). Bovine noroviruses (GIII) such as Newbury-2 virus as well as porcine noroviruses (GII) and porcine sapoviruses have been described. Other bovine enteric calicivirus isolates have been shown to be distinct and the genus *Nebovirus* has been created with type species Newbury-1 virus. Hepatitis E virus of man has been removed from the family and placed in a new family *Hepeviridae*.

Caliciviruses have been recovered from a wide range of species including man, cattle, dogs, cats, pigs, marine mammals, rabbits, hares, reptiles, amphibians and insects. Calicivirus infections are associated with a diverse range of diseases including respiratory disease, vesicular lesions, gastroenteritis and necrotizing hepatitis. Caliciviruses frequently cause persistent infections with outcomes ranging from inapparent to acute disease. Transmission occurs by direct and indirect means.

VESICULAR EXANTHEMA OF SWINE

Vesicular exanthema of swine (VES) is an acute, highly contagious vesicular disease of pigs. It was first reported in southern California in 1932 and became widespread throughout the USA during the 1950s. The disease was confined to the USA. A vigorous eradication campaign, including the implementation of garbage cooking laws, was successful. The last case was recorded in 1956 and the USA was declared free of the disease in 1959. However, a reservoir of the virus exists in marine mammals. A closely related virus, San Miguel sea lion virus (SMSV), was isolated from Californian sea lions showing signs of disease including vesicles on the flippers and premature parturition. Other related caliciviruses have since been isolated from a range of marine mammals and the opal eye fish. It is likely that the original outbreak of VES arose through feeding meat from infected marine mammals to pigs in uncooked swill. The virus then spread between pigs by direct and indirect contact. Vesicular exanthema of swine virus isolates show significant antigenic heterogenicity and SMSV is classified as a strain of VESV while the whole group of related viruses is referred to as marine caliciviruses.

The incubation period for VES is about 24–72 hours and the course of the disease approximately one to two weeks. Vesicles occur in the oral cavity, on the tongue,

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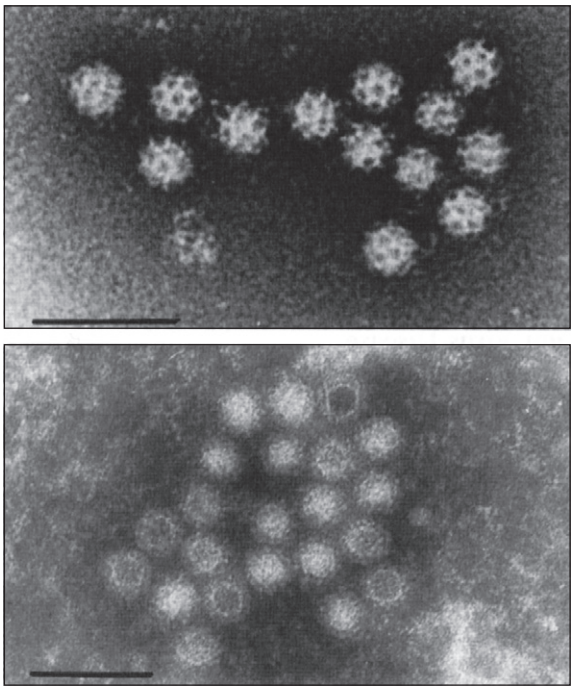

Figure 53.1 (Top) Vesicular exanthema of swine virus showing the cup-shaped depressions that are characteristic of many of the caliciviruses. (Bottom) Enteric calicivirus showing the lack of surface detail that is characteristic of most of these viruses as seen in diagnostic faecal specimens. Negative stain electron microscopy. Bars: 100 nm. *Reprinted with permission: Murphy et al., Veterinary Virology Third Edition (1999). Academic Press.*

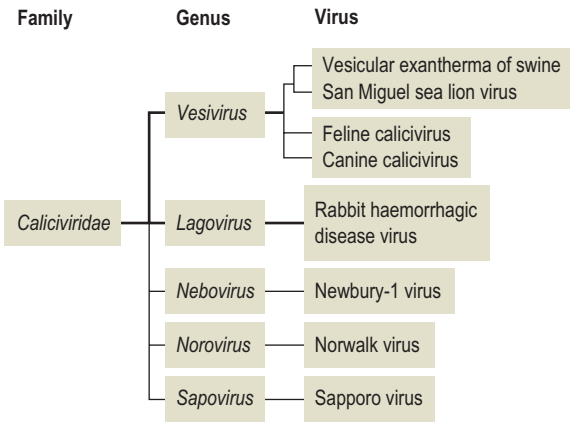

Figure 53.2 Classification of caliciviruses of note.

Table 53.1 Caliciviruses of animals

Virus	Host species	Disease significance
Vesicular exanthema of swine (VES) virus	Pigs	Natural disease not seen since 1956, confined to USA. Acute, contagious, vesicular disease, clinically similar to foot-and-mouth disease. Believed to have resulted from feeding of SMSV-infected sea lion and seal carcasses in swill
San Miguel sea lion virus (SMSV)	Marine mammals, opal eye fish	Causes VES when inoculated into pigs. Cause of cutaneous vesicles and premature parturition in pinnipeds
Feline calicivirus	Domestic and large cats	Upper respiratory tract disease in cats, occurs worldwide. Outbreaks with severe systemic form occasionally described
Rabbit haemorrhagic disease virus	European wild and domestic rabbits	Acute, fatal disease of European rabbits. Physiological resistance in rabbits less than two months of age
European brown hare syndrome virus	European brown hare	Related but distinct from RHDV. Similar disease to RHD, hepatic necrosis and diffuse generalized haemorrhaging. High mortality rate
Canine calicivirus	Dogs	Associated with diarrhoea on occasion

lips, snout, interdigital spaces and coronary band. Affected pigs are pyrexic and acutely lame. The morbidity is high but mortality is low. The disease is clinically indistinguishable from the other vesicular diseases of pigs, namely foot-and-mouth disease, vesicular stomatitis and swine vesicular disease. The principal importance of VES lies in its similarity to foot-and-mouth disease, the weight loss in fattening pigs and deaths which can occur in neonatal pigs.

Samples rich in virus include vesicular fluid and the overlying flap of epithelium. Diagnostic techniques

include ELISA and CFT for antigen detection, immunoelectron microscopy and virus isolation in pig kidney cell lines, with identification by virus neutralization. Primers suitable for both VESV and SMSV have been described for use in RT-PCR (Neill & Seal 1995). A real time assay has also been described (Reid et al. 2007).

FELINE CALICIVIRUS INFECTION

Feline calicivirus (FCV) is responsible for about 40% of all cases of upper respiratory tract infections in cats worldwide. The majority of FCV isolates belong to a single genotype, a second genotype has been described in Japan (Sato et al. 2002). A high degree of antigenic heterogeneity exists among FCV isolates. Genome sequence analysis studies indicate that individual isolates of FCV exist as quasispecies, which rapidly evolve and undergo antigenic drift. The alterations in the antigenic profile of sequential virus isolates from carrier cats are driven by immune selection and play an important part in viral persistence (Radford et al. 1998). All species of *Felidae* appear to be susceptible to FCV infection but natural disease occurs in domestic cats and cheetahs. Cats of all ages are susceptible to infection by FCV but acute disease is most commonly seen in kittens two to six months of age, following the waning of maternally derived antibody. A proportion of cats remain persistently infected following recovery from the acute infection or following subclinical infection while protected by maternally derived antibody or by vaccination. The maintenance of infection in a particular cat population is largely due to these carriers which shed virus continually from the oropharynx for several months, occasionally years (Coyne et al. 2007).

Pathogenesis

The incubation period is two to five days. Virus replication occurs primarily in the oropharynx. This is followed by rapid spread throughout the upper respiratory tract and to the conjunctivae. A transient viraemia occurs. Strains of FCV differ considerably in virulence and infections vary from subclinical to severe. Morbidity rates can be high but mortality is generally low. The virus has been recovered from the joints of cats showing signs of lameness. An association between chronic gingivitis/stomatitis and FCV infection has been suggested. Co-infection with feline immunodeficiency virus appears to be an important element of such cases. Virulent strains of FCV are capable of causing interstitial pneumonia in young kittens, while a few strains have been associated with a virulent systemic disease (VSD) form characterized by high mortality despite vaccination (Hurley et al. 2004).

Diagnosis

- Upper respiratory signs, particularly ulcers in the oral cavity, are suggestive of FCV infection but definitive differentiation from feline herpesvirus 1 infection requires laboratory confirmation.
- Oropharyngeal swabs or lung tissue are suitable samples for isolation of feline calicivirus in feline cells lines. However, isolation of FCV alone may not indicate aetiological significance on account of the large number of carrier animals.
- Assays for the detection of viral RNA in clinical specimens have been described using RT-PCR (Sykes et al. 1998) and real-time RT-PCR (Helps et al. 2002, Scansen et al. 2004, Wilhelm & Truyen 2006) protocols. Due to the variability of the viral genome, the sensitivity of these assays can vary depending on the primers used.
- Demonstration of a rising antibody titre by virus neutralization or ELISA (Lappin et al. 2002) is required on account of the large number of cats that have been previously infected or vaccinated.

RABBIT HAEMORRHAGIC DISEASE

Rabbit haemorrhagic disease is a highly contagious, acute, often fatal disease of European rabbits (*Oryctolagus cuniculus*) over two months of age. It was first reported in China in 1984 and has since spread to many parts of the world. The virus has been used for the biological control of rabbits in Australia and New Zealand. The virus escaped from a research facility in Australia in 1995 and was illegally introduced into New Zealand in 1997. Outbreaks of disease have been more severe in some commercial rabbitries than others in Europe and this has led to the suggestion that rabbit haemorrhagic disease virus (RHDV) is a mutant form of a non-pathogenic virus, rabbit calicivirus (RCV), that has been enzootic in commercial and wild rabbits in Europe for a long time (Capucci et al. 1996). An alternative hypothesis is that RHDV may be propagated through both pathogenic and non-pathogenic modes of transmission (White et al. 2004). Non-pathogenic strains of RHDV have been identified in wild rabbits (Forrester et al. 2007).

A single serotype is recognized with two major subtypes: RHDV and the antigenic variant RHDVa. Virus is shed in all excretions and secretions by infected rabbits. The principal mode of transmission among rabbits in close contact is the faecal–oral route. Spread of the virus between rabbitries and between countries is due to the movement of infected rabbits, rabbit meat, insects or fomites.

Pathogenesis

The incubation period is one to three days. The most consistent pathological lesion in affected rabbits is severe necrosis of the liver. In addition there is evidence of disseminated intravascular coagulation (DIC). It is unclear whether the DIC is triggered by the liver damage or if another pathogenic mechanism is involved. Cells of the mononuclear phagocyte lineage may be the major target of the virus (Ramiro-Ibanez et al. 1999). The disease is characterized by high morbidity and high mortality (40–90%). Rabbits less than four weeks of age can become infected and develop immunity but do not display clinical signs or pathological lesions. This resistance decreases rapidly and is not present in rabbits over two months. The basis for the resistance to clinical disease is physiological and may be related to changes in liver function which occur after weaning at about five weeks of age.

Diagnosis

Rabbit haemorrhagic disease is characterized by high mortality and the presence of characteristic gross lesions including acute hepatitis, swollen spleen and congested and haemorrhagic lungs. Cultivation of RHDV in vitro has proven to be extremely difficult. However, high concentrations of virus are present in liver from affected rabbits and confirmation is based on detection of virus or viral antigen using electron microscopy, ELISA, immunostaining, western blotting or haemagglutination using human Group O erythrocytes. Reverse transcription-PCR has been developed for detection of RHDV nucleic acid (Guittre et al. 1995, Gould et al. 1997, Moss et al. 2002). A real time assay is also available (Gall et al. 2007). Serological tests suitable for the detection of specific antibodies to the virus include haemagglutination inhibition and ELISA (Cooke et al. 2000).

REFERENCES

Capucci, L., Fusi, P., Lavazza, A., et al., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but non-pathogenic. Journal of Virology 70, 8614–8623.

Cooke, B.D., Robinson, A.J., Merchant, J.C., et al., 2000. Use of ELISAs in field studies of rabbit haemorrhagic disease RHD in Australia. Epidemiology & Infection 124, 563–576.

Coyne, K.P., Gaskell, R.M., Dawson, S., et al., 2007. Evolutionary mechanisms of persistence and diversification of a calicivirus within endemically infected natural host populations. Journal of Virology 81, 1961–1971.

Forrester, N.L., Trout, R.C., Gould, E.A., 2007. Benign circulation of rabbit haemorrhagic disease virus on Lambay Island. Eire Virology, 358, 18–22.

Gall, A., Hoffmann, B., Teifke, J.P., et al., 2007. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Veterinary Microbiology 120, 17–32.

Gould, A.R., Kattenbelt, J.A., Lenghaus, C., et al., 1997. The complete nucleotide sequence of rabbit haemorrhagic disease virus Czech strain V351: use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. Virus Research 47, 7–17.

Guittre, C., Baginski, I., Le Gall, G., et al., 1995. Detection of rabbit haemorrhagic disease virus isolates and sequence comparison of the N-terminus of the capsid protein gene by the polymerise chain reaction. Research in Veterinary Science 58, 128–132.

Helps, C., Lait, P., Tasker, S., et al., 2002. Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR. Journal of Virological Methods 106, 241–244.

Hurley, K.F., Pesavento, P.A., Pedersen, N.C., et al., 2004. An outbreak of virulent systemic feline calicivirus disease. Journal of the American Veterinary Medical Association 224, 241–249.

Lappin, M.R., Andrews, J., Simpson, D., et al., 2002. Use of serologic tests to predict resistance to feline herpesvirus 1, feline calicivirus, and feline parvovirus infection in cats. Journal of the American Veterinary Medical Association 220, 38–42.

Moss, S.R., Turner, S.L., Trout, R.C., et al., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. Journal of General Virology 83, 2461–2467.

Neill, J.D., Seal, B.S., 1995. Amplification of two distinct regions of the genomes of San Miguel sea lion and vesicular exanthema of swine viruses. Molecular and Cellular Probes 9, 33–37.

Radford, A.D., Turner, P.C., Bennett, M., et al., 1998. Quasispecies evolution of a hypervariable region of the feline calicivirus capsid gene in cell culture and in persistently infected cats. Journal of General Virology 79, 1–10.

Ramiro-Ibanez, F., Martin-Alonso, J.M., Garcia-Palencia, P., et al., 1999. Macrophage tropism of rabbit haemorrhagic disease virus is associated with vascular pathology. Virus Research 60, 21–28.

Reid, S.M., King, D.P., Shaw, A.E., et al., 2007. Development of a real-time reverse transcription polymerase chain reaction assay for detection of marine caliciviruses genus *Vesivirus*. Journal of Virological Methods 140, 166–173.

Sato, Y., Ohe, K., Murakami, M., et al., 2002. Phylogenetic analysis of field isolates of feline calcivirus FCV in Japan by sequencing part of its capsid gene. Veterinary Research Communications 26, 205–219.

Scansen, B.A., Wise, A.G., Kruger, J.M., et al., 2004. Evaluation of a p30

gene-based real-time reverse transcriptase polymerase chain reaction assay for detection of feline caliciviruses. Journal of Veterinary Internal Medicine 18, 135–138.

Sykes, J.E., Studdert, V.P., Browning, G.F., 1998. Detection and strain differentiation of feline calicivirus in conjunctival swabs by RT-PCR of the hypervariable region of the capsid protein gene. Archives of Virology 143, 1321–1334.

White, P.J., Trout, R.C., Moss, S.R., et al., 2004. Epidemiology of rabbit haemorrhagic disease virus in the United Kingdom: evidence for seasonal transmission by both virulent and avirulent modes of infection. Epidemiology & Infection 132, 555–567.

Wilhelm, S., Truyen, U., 2006. Real-time reverse transcription polymerase chain reaction assay to detect a broad range of feline calicivirus isolates. Journal of Virological Methods 133, 105–108.

FURTHER READING

Radford, A.D., Coyne, K.P., Dawson, S., et al., 2007. Feline calicivirus. Veterinary Research 38, 319–335.

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Chapter 54

Astroviridae

The name of the family is derived from the Greek word *astron* meaning star and referring to the star-like surface structure of member virions. Astroviruses are non-enveloped with icosahedral symmetry, 28–30 nm in diameter (Fig. 54.1). The genome consists of a single molecule of linear, positive-sense, single-stranded RNA. The viruses are resistant to pH 3, various detergents and heat (60°C for 5 minutes). Replication is cytolytic and occurs in the cytoplasm of host cells. The presence of trypsin is required for cultivation of these viruses in cell culture. There are two genera, *Mamastrovirus* and *Avastrovirus* representing mammalian and avian astroviruses (Fig. 54.2) with type species *Human astrovirus* and *Turkey astrovirus* respectively. Species within these genera are defined on the basis of the animal species they infect. Isolates from different host species are antigenically unrelated and are species-specific. Eight serotypes of human astrovirus have been described, two serotypes of bovine astrovirus and three of turkey astrovirus.

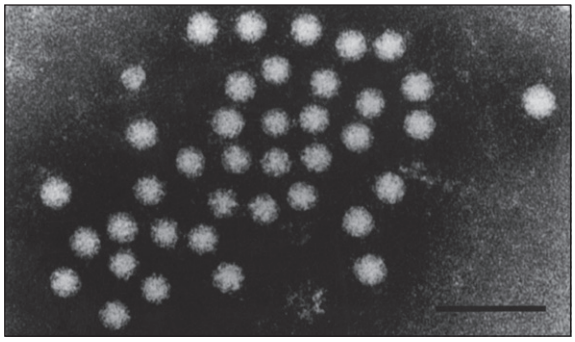

Figure 54.1 Negative contrast electron micrograph of virions of human astrovirus from a stool specimen. The bar represents 100 nm. *Reprinted with permission: Fauquet CM (ed) et al. 2005 Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, p. 859.*

CLINICAL INFECTIONS

Astroviruses have a worldwide distribution and are associated with acute, self-limiting gastroenteritis in animals and man. They have been detected in the faeces of humans, cattle, pigs, sheep, dogs, cats, deer, mice, ducks, chickens and turkeys. Originally classified as a picornavirus, avian nephritis virus has been shown to be an astrovirus and renamed chicken astrovirus. An astrovirus has been associated with poult enteritis mortality syndrome (PEMS) in turkeys. Transmission of astroviruses occurs by the faecal–oral route. Following an incubation period of one to four days, a watery diarrhoea may be seen but the majority of infections are probably subclinical. Severe disease and

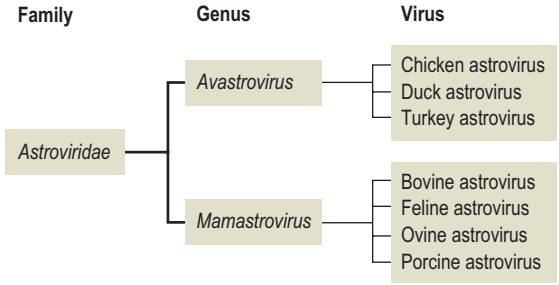

Figure 54.2 Classification of astroviruses of veterinary interest.

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death are rare except in ducks in which astrovirus infection is associated with a severe hepatitis of young ducklings.

Diagnosis

Diagnosis is based on detection of astroviruses in faeces using electron microscopy, immunoelectron microscopy or ELISA. Only a proportion of viral particles may exhibit the characteristic five- or six-pointed star-like morphology making it difficult to identify the particles as astroviruses by electron microscopy. Virus isolation in embryonated hens' eggs or primary cell lines is possible. Detection of viral RNA using reverse transcriptase PCR has been demonstrated (Koci et al. 2000, Day et al. 2007).

REFERENCES

Day, J.M., Spackman, E., Pantin-Jackwood, M., 2007. A multiplex RT-PCR test for the differential identification of turkey astrovirus type 1, turkey astrovirus type 2, chicken astrovirus, avian nephritis virus, and avian rotavirus. Avian Diseases 51, 681–684.

Koci, M.D., Seal, B.S., Schultz-Cherry, S., 2000. Development of an RT-PCR diagnostic test for an avian astrovirus. Journal of Virological Methods 90, 79–83.

FURTHER READING

Koci, M.D., Schultz-Cherry, S., 2002. Avian astroviruses. Avian Pathology 31, 213–227.

Chapter 55

Reoviridae

The term reovirus is an acronym derived from respiratory, enteric and orphan viruses. Original isolations of these viruses were from respiratory and enteric samples but without associated clinical disease, hence the term orphan. Members of the *Reoviridae* are icosahedral in structure, 60–80 nm in diameter, non-enveloped and possess a one-, two- or three-layered capsid. Two morphological forms are recognized, reflected in the division of the family into two subfamilies: *Spinareovirinae* (Fig. 55.1) and *Sedoreovirinae* (Fig. 55.2). Viruses in the subfamily *Spinareovirinae* have spikes or turrets at the vertices of the icosahedral virus while members of the subfamily *Sedoreovirinae* lack such projections. The development of infectivity by some reoviruses requires the action of a protease on the outer capsid to produce infectious or intermediate subviral particles (ISVPs). The genome of the virion comprises 10–12 segments of linear, double-stranded RNA. Genetic reassortment occurs readily between members of the same genus. Replication occurs in the cytoplasm of host cells and frequently gives rise to intracytoplasmic inclusion bodies. The family contains 15 genera, of which members of the genera *Orthoreovirus, Rotavirus* and *Orbivirus* infect animals and man (Fig. 55.3). Members of the genus *Coltivirus* are associated with infections of arthropods, rodents and man while viruses belonging to the genus *Aquareovirus* infect fish. The genera *Fijivirus, Phytoreovirus, Seadornavirus, Idnoreovirus, Cypovirus, Dinovernavirus, Cardoreovirus,* and *Oryzavirus* contain viruses of arthropods and plants, while the genera *Mycoreovirus* and *Mimoreovirus* contain viruses of fungi and algae respectively. The viruses are moderately resistant to heat, organic solvents and non-ionic detergents. Reoviruses and rotaviruses are stable over a wide pH range while orbiviruses lose infectivity at low pH.

Orthoreoviruses are widespread in nature and have been isolated from many mammalian and avian species (Table 55.1). Infections are generally considered non-pathogenic except in poultry and rodents. Mammalian and avian orthoreoviruses have distinct group antigens. Orthreoviruses may also be divided into subgroups on the basis of their ability to induce cell–cell fusion from within, termed fusogenic and non-fusogenic orthoreoviruses. Typically, avian isolates are fusogenic while mammalian isolates are non-fusogenic. Atypical, fusogenic orthoreoviruses have been isolated from baboons, flying foxes and snakes. Phylogenetic analysis based on the nucleotide sequences of the three S-class genome segments encoding the major sigma class core, outer capsid, and non-structural proteins indicates at least four genogroups or species of orthoreoviruses (Duncan 1999), five species are currently recognized. Rotaviruses are a significant worldwide cause of human infantile gastroenteritis and of acute diarrhoea in intensively reared farm animals. Transmission of orthoreoviruses and rotaviruses is by direct and indirect contact with contaminated faeces. A number of important animal diseases are caused by orbiviruses. Twenty-two serogroups or species of orbiviruses are currently recognized, as well as a number of unclassified viruses. The groups include serotypes and antigenic complexes. Both African horse sickness and bluetongue are categorized as listed diseases by the Office International des Epizooties (OIE). Epizootic haemorrhagic disease virus and Ibaraki virus of deer and cattle respectively are similar to bluetongue virus in infection and disease patterns. Infection with equine encephalosis virus has only been recognized in southern Africa and Israel. Serological evidence suggests widespread infection with this virus but acute disease is sporadic in occurrence. Orbivirus transmission involves arthropod vectors, primarily *Culicoides* species.

AVIAN ORTHOREOVIRUSES

Reoviruses are frequently recovered from the gastrointestinal tract of clinically normal birds. However, under certain

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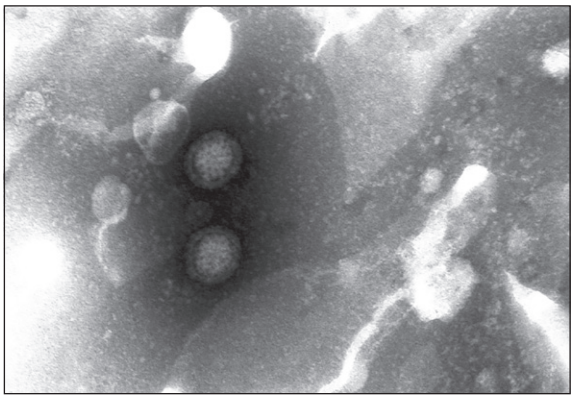

Figure 55.1 Electron micrograph of orthoreoviruses.

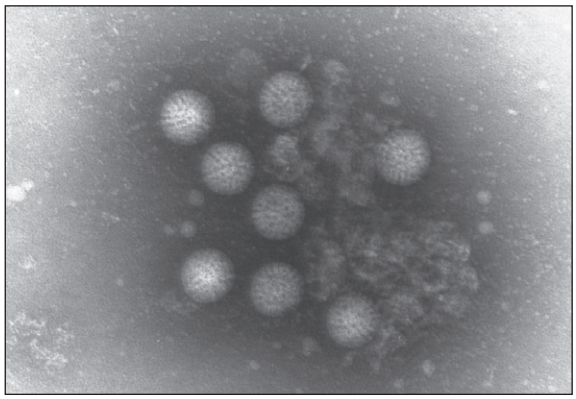

Figure 55.2 Electron micrograph of rotaviruses.

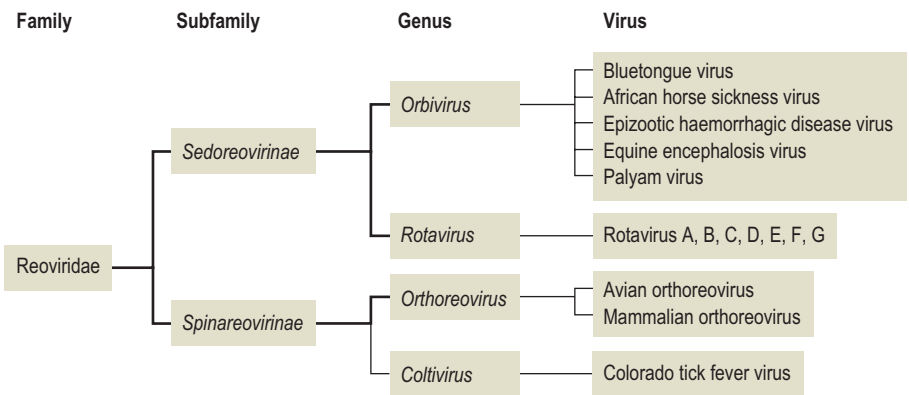

Figure 55.3 Classification of reoviruses of veterinary significance.

circumstances they may give rise to disease directly or enhance the pathogenicity of other infectious agents. Avian othoreoviruses have been linked with a diverse range of conditions including arthritis, tenosynovitis, chronic respiratory disease and enteritis. More than nine serotypes can be distinguished using serum neutralization tests. Transmission is mainly horizontal by the faecal–oral route but vertical transmission through the embryonated egg is also possible. Reoviruses are an important cause worldwide of viral arthritis/tenosynovitis in meat-type chickens between four and 16 weeks of age. Morbidity is typically less than 10%. The condition principally presents as lameness. Rupture of the gastrocnemius tendon may occur such that the bird is unable to bear weight on the affected leg. Affected birds lose weight and may die of starvation as a result of difficulties in reaching feed and water. The lesions are not pathognomonic as similar lesions can be induced by *Mycoplasma synoviae* and *Staphylococcus aureus*. Diagnosis of reovirus involvement is best carried out by virus isolation. The specimens of choice are hock articular cartilage and affected tendons. Synovial fluid from the hock joint is also suitable but gives lower rates of virus recovery. Suspensions of suspect material can be inoculated into the yolk sac of embryonated hens' eggs or onto monolayers of chick embryo liver cells. Viral antigen may be detectable by immunofluorescence in cryostat sections of affected tendons. Reverse transcription-PCR protocols have been described for the detection and differentiation of avian orthoreoviruses (Liu et al. 1999, Caterina et al. 2004, Liu et al. 2004). Serological testing is not particularly useful due to the high prevalence of subclinical infections. It may be useful on a flock basis to determine the immune status of parent flocks and young chicks.

Table 55.1 Members of *Reoviridae* of veterinary significance

Virus	Natural host	Significance of infection
Mammalian orthoreovirus	Wide range of species	Four serotypes. Associated with mild enteric and respiratory disease. Significance often dependent on presence of secondary infectious agents
Avian orthoreovirus	Chickens, turkeys and other avian species	Multiple serotypes. Important cause of viral arthritis/tenosynovitis in chickens and possibly turkeys
Rotavirus A to G	Neonates of many intensively reared domestic species including calves, lambs, piglets, foals and poultry	Mild to severe diarrhoea dependent on age, colostral intake, accommodation and concurrent infections. Rotavirus E is associated with pigs while rotaviruses D, F and G are associated with birds
African horse sickness virus	Equidae	Non-contagious arthropod-borne infection. Endemic in Africa south of the Sahara. Mortality rate often high in susceptible horses. Listed disease
Bluetongue virus	Sheep, cattle, goats, deer, antelopes	Arthropod-borne infection of ruminants. Clinical disease highly variable in severity. Severe disease seen in fine wool and mutton breeds of sheep and in certain deer species. Teratogenic virus. Clinical disease is uncommon in cattle. Listed disease
Epizootic haemorrhagic disease virus (EHDV)	Deer, cattle, buffalo	Arthropod-borne, principally *Culicoides* species. Important disease of deer in North America. Clinically similar to bluetongue. Infection of cattle is usually subclinical or mild. Seven serotypes
Ibaraki virus	Cattle	Acute febrile disease similar to bluetongue. Described in southeast Asia. Member of the EHDV group (EHDV-2)
Equine encephalosis virus	Horse	Seven serotypes. Only reported in South Africa and Israel. Majority of infections are subclinical with sporadic cases of an acute fatal, disease characterized by brain oedema, fatty liver degeneration and enteritis
Colorado tick fever virus	Arthropod-borne, primarily ticks but also mosquitoes. Prolonged viraemia in rodent species (reservoir host)	Humans and other mammals may be dead-end hosts. May cause encephalitis in children
Palyam virus	Cattle	Large serogroup of viruses. Arthropod-borne. Associated with abortions and teratogenicity. Recorded in southern Africa, southeast Asia and Australia

ROTAVIRUSES

Rotaviruses are an important cause of diarrhoea in intensively reared farm animals worldwide. Isolates are divided into seven antigenically distinct species (serogroups), rotaviruses A–G, on the basis of reactions with the highly conserved, major capsid protein VP6. The majority of isolates belong to group A, the 'conventional' rotaviruses. Some 14 serotypes (G1-G15) are recognized within rotavirus A on the basis of the antigenicity of the glycoprotein VP7, the main constituent of the outer capsid, which is highly immunogenic and induces type-specific neutralizing antibodies. This antigenic classification has been largely superseded by a system based on the sequencing of the VP7 gene and 27 G genotypes have been described to date. Rotavirus A isolates are also frequently classified into P serotypes or P genotypes on the basis of differences in VP4.

Although rotavirus isolates from one host species can successfully infect another species under experimental conditions, cross-species infection does not appear to be

important under field conditions and rotaviruses are considered largely species-specific. Very high titres of virus (10^9 virions per gram of faeces) are excreted in the faeces of scouring animals. Horizontal transmission occurs by ingestion of infected faeces. The virions can survive for a number of months in the environment, leading to significant build-up of infection in rearing pens or other areas where animals congregate. As a consequence intensively reared animals are most commonly affected.

Pathogenesis

The severity of infection varies greatly and is influenced by several factors including age, viral virulence, colostral immunity, overcrowding and the presence of other enteric pathogens. The incubation period is usually less than a day. Following oral ingestion, virus passes largely unaffected through the acidic medium of the stomach and infects the columnar enterocyctes lining the apices of the villi of the small intestine. In young animals the rate of replacement of enterocytes is relatively slow and affected villi become shortened and covered by cuboidal replacement cells. These changes are most pronounced in the proximal small intestine. These immature cells have reduced levels of disaccharidases and impaired glucose-coupled sodium transport. In addition a non-structural protein of the virus NSP4 has been shown to act as an enterotoxin affecting the co-transport of sodium ions and galactose/glucose (Lorrot & Vasseur 2007). Undigested lactose in milk forms a substrate for bacterial proliferation and exerts an osmotic effect. As a result, increased amounts of fluid are retained in the lumen of the gut and diarrhoea ensues. Affected animals are anorexic, depressed and produce light-coloured semi-liquid or pasty faeces. In uncomplicated cases animals frequently recover within three to four days without treatment. However, concurrent infection with other enteric pathogens such as *Escherichia coli*, *Salmonella* species and *Cryptosporidium* species may give rise to severe diarrhoea and significant mortality.

Diagnosis

Suitable samples include faeces and intestinal contents.

- Negative-contrast electron microscopy is rapid and capable of detecting combined virus infections. However, it requires a large concentration of virus particles (10^6 per gram of faeces) and specialized equipment. The sensitivity of the procedure can be improved by using immunoelectron microscopy.
- Viral antigen can be detected in faeces by ELISA or latex agglutination. The antisera employed in these tests is usually reactive with group A rotaviruses only. These assays are available commercially. Immunofluorescence can also be used to detect viral antigen in smears or cryostat sections of affected small intestine.
- Sodium dodecyl sulphate-polyacrylamide gel electrophoesis (SDS-PAGE) has been used to detect the RNA segments of rotaviruses in clinical samples (Herring et al. 1982). The sensitivity of this procedure is similar to that obtained using electron microscopy. Differentiation of the different rotavirus serogroups is possible using the electrophoretic patterns obtained.
- Rotaviruses are difficult to isolate in vitro. However, the presence of low concentrations of trypsin in the growth medium will facilitate viral uncoating and improve viral replication.
- Reverse transcription PCR protocols are available for the detection (Gouvea et al. 1990) and genotyping of rotaviruses (Gentsch et al. 1992, Gouvea et al. 1994, Iturriza-Gómara et al. 2000).

AFRICAN HORSE SICKNESS

African horse sickness is a non-contagious, listed disease of horses, zebras, mules and donkeys caused by African horse sickness virus (AHSV). Nine serotypes of the virus are distinguishable using neutralization tests and form the African horse sickness virus serogroup. The disease is endemic in subtropical and tropical Africa south of the Sahara. Serious outbreaks have occurred in the Middle East, India and Pakistan but the disease has not persisted. Outbreaks have also occurred in Spain (1987–1990), Portugal (1989) and Morocco (1989–1991).

The virus is transmitted through blood via haematophagous insects. The principal vector is *Culicoides imicola* which is a true biological vector and infected for life. There is evidence that *C. bolitinos* may also be an important vector (Meiswinkel & Paweska 2003). *Culicoides imicola* is an Afro-Asian midge that prefers a warm climate and is susceptible to frost. Below 10°C *C. imicola* will aestivate and virus replication ceases (Mellor et al. 1998). The virus cycles silently in maintenance hosts such as zebra and African donkey. Enzootic disease only occurs where adult *C. imicola* are continuously present throughout the year. African house sickness is periodically reported beyond its enzootic regions as a result of movement of infected midges on the wing (up to 25 km) or on wind currents (up to 700 km). Outbreaks of disease tend to be seasonal, typically occurring in late summer.

Pathogenesis

The incubation period is about five to seven days. Following inoculation the primary sites of replication are believed to be regional lymph nodes, spleen and lungs. The onset of viraemia corresponds with the appearance of fever and persists throughout the febrile period. Endothelial cells appear to be an important site of secondary replication.

Vascular endothelium is affected resulting in increased vesicular permeability, oedema, haemorrhage and intravascular coagulation due to thrombocytopenia. Four forms of disease are described, all four forms can occur in an outbreak:

- Peracute pulmonary form: the incubation period is short at three to five days and the mortality rate may be close to 100%.
- Subacute cardiac form: the incubation period is seven to 14 days with a mortality rate of about 50%.
- Mixed/acute cardio pulmonary form: the incubation period varies from five to seven days and the mortality rate is more than 80%.
- Horse sickness fever is a mild to subclinical disease that is usually associated with infection in partially immune animals or in resistant species such as zebras and donkeys. Affected horses recover.

Diagnosis

Clinical signs may be sufficiently characteristic, such as oedema of the supraorbital fossa, to allow a clinical diagnosis. Post mortem changes including excess pericardial and pleural effusions may also be suggestive. Suitable samples for laboratory confirmation include unclotted whole blood collected during the early febrile stage of the disease and samples of spleen, lymph node and lung from animals that have died. Samples should be transported and stored at 4°C.

- Sample suspensions may be inoculated into newborn mice (intracerebral inoculation), embryonated hens' eggs (intravenous inoculation) or onto cell culture cells. Susceptible cell lines include insect cell cultures and mammalian cells such as BHK-21 and Vero. A cytopathic effect may be observed in the mammalian derived cell lines two to eight days post inoculation. Three blind passages are required before a sample is considered negative. Identification of the virus can be carried out by immunofluorescence. The serotyping of virus isolates is achieved by neutralization tests with monovalent antisera, permitting selection of the correct vaccine. Although there is no evidence of humans becoming infected with field strains of AHSV, infections in laboratory workers with a neurotropic vaccine strain have been described (Meyden et al. 1992).
- The presence of viral antigen can be detected in samples by sandwich ELISA using either polyclonal (Hamblin et al. 1991) or monoclonal antibodies (Laviada et al. 1992a).
- Viral RNA detection using RT-PCR (Stone-Marschat et al. 1994, Zientara et al. 1994) has been shown to be as sensitive as virus isolation (Zientara et al. 1998). Results can be available within 24 hours compared to a period of several days for virus isolation. An RT-PCR assay for the discrimination of the nine AHSV serotypes has been described (Sailleau et al. 2000).
- Serological assays such as CFT, AGID, ELISA and serum neutralization tests are all suitable. Both indirect and competitive ELISAs (Hamblin et al. 1990, Laviada et al. 1992b) using either soluble AHSV antigen or a recombinant protein VP7 have been recognized by the European Union. Animals develop antibodies at about two weeks post infection. Outside of enzootic areas infected animals may die before antibodies are produced. Donkeys generally do not develop severe AHS and are often used as sentinel animals based on regular serological monitoring and detection of seroconversion. An indirect ELISA using serotype 4 non-structural protein NS3 as antigen has been described for the differentiation of infected or vaccinated with live vaccine animals from those vaccinated with a purified inactivated vaccine or recombinant structural protein subunit vaccine (Laviada et al. 1995).

BLUETONGUE

Bluetongue is an insect-borne disease of sheep and other domestic and wild ruminants. The disease is of most significance in sheep and deer. It is classified as a listed disease by the OIE. Isolates of bluetongue virus (BTV) belong to a distinct serogroup within the *Orbivirus* genus. Twenty-six serotypes of BTV have been described (Maan et al. 2011) with one of the most recent serotypes (BTV-25) detected in clinically healthy goats in Switzerland, Toggenburg orbivirus. The virions are composed of three protein layers. The outer layer contains two proteins, VP2 and VP5. Of these, VP2 is the determinant of serotype specificity and the major neutralizing antigen responsible for haemagglutination and binding to mammalian cells. The icosahedral core is composed of two major proteins, VP3 and VP7, as well a number of minor proteins. VP7 is the principal determinant of serogroup specificity.

Infections vary from subclinical to severe depending on the serotype of virus, breed of sheep and local conditions. All breeds of sheep are susceptible to BTV infection but the clinical presentation is highly variable from subclinical infection to severe disease with high mortality. Severe disease is generally confined to the Merino and European mutton breeds. Nutritional status, exposure to sunlight and age also appear to play a role.

Bluetongue viruses have a worldwide distribution between latitudes 53°N and 30°S reflecting the distribution of the biological vectors, species of *Culicoides* midge. In recent years the disease has extended its range northwards into areas of Europe resulting in significant losses of sheep (Mellor & Wittmann 2002). Molecular

epidemiology studies support the concept of distinct, reasonably stable ecosystems in different regions of the world where strains of BTV and their specific *Culicoides* vectors co-evolved over long periods (Zhang et al. 1999). In Africa and the Middle East *C. imicola* is the principal vector while in Australia *C. fulvus, C. wadai* and *C. brevitarsis* are involved. Other *Culicoides* species are competent to transmit the virus and may play a lesser role. In North America the most important vector is *C. variipennis* var. *sonorensis* while in Central and South America it is *C. insignis*. Indigenous European *Culicoides* species including *C. dewulfi* and *C. obsoletus* complex are considered to be responsible for the large epizootic and subsequent establishment of BTV-8 in northern Europe (Saegerman et al. 2008). Female insects taking a blood meal from viraemic animals become infected. Temperatures above 12 °C are necessary for viral replication within the vector. Once infected a *Culicoides* species begins transmitting the virus in saliva within seven to 10 days and remains infected for life. The optimum conditions for activity of these insects are temperatures of 18–29 °C and high humidity. As a result the disease has a seasonal occurrence in many parts of the world, generally occurring in late summer or autumn in temperate regions. *Culicoides* species are crepuscular with most activity at dawn and dusk. Four potential routes may be responsible for the overwintering of virus in ruminant populations, permitting viral recrudescence in spring: vertical transmission in ruminants (dam to offspring), prolonged subclinical viraemia in certain infected individual animals, vertical transmission in the vector with survival of infected offspring through the winter, survival of infected adult midges (Wilson et al. 2008).

Within enzootic regions localized 'hot spots' of BTV activity occur where susceptible animals are present and conditions are particularly suitable for vector breeding (damp areas with large amounts of animal dung). Spread to neighbouring areas occurs through the movement of viraemic animals or infected vectors. Although the flight range of *Culicoides* species is short, infected insects may be passively transported over long distances on the wind resulting in the introduction of BTV to susceptible ruminant populations in areas outside normal enzootic regions. Such introductions frequently result in dramatic epizootics but are usually self-limiting unless the climate is suitable for vector activity throughout the year. In enzootic areas infection of cattle is common and usually inapparent. However, BTV infection of cattle is characterized by prolonged viraemia that commonly lasts several weeks and provides extended opportunity for transmission to the insect vector. As a result cattle are considered important amplifiers and reservoirs of virus (Barratt-Boyes & MacLachlan 1995). Clinical disease in cattle has been a feature of the BTV-8 epizootic in northern Europe. Other routes of transmission besides insect borne such as venereal or transplacental are possible but uncommon (Menzies et al. 2008).

Pathogenesis

The incubation period in sheep is five to 10 days. Following intradermal inoculation the virus multiplies in the local draining lymph nodes. The virus is then carried in blood or lymph to other lymphoid tissues where additional replication occurs before general release of virus into the bloodstream. The virus multiplies in the vascular endothelium of small blood vessels resulting in vascular occlusion, stasis and exudation. This in turn produces hypoxia of overlying tissues with secondary lesion development in epithelium. The severity of the lesions is strongly influenced by mechanical abrasion and secondary bacterial infection. Bluetongue virus is concentrated in the small blood vessels underlying stratified squamous epithelium, particularly in the oral cavity, around the mouth and coronet of the hoof. In the bloodstream the virus is highly cell associated, particularly with erythrocytes. It has been suggested that this association may protect the virus from antibody. Clinical disease is uncommon in cattle but sporadic cases are described. It is thought that clinical disease in cattle is due to an IgE-mediated hypersensitivity reaction as a result of previous exposure to BTV or related orbiviruses.

Diagnosis

Bluetongue is a listed disease and diagnosis of the presence of the virus invokes significant international trade sanctions. Australia has suffered disruption of trade in live animals, semen and embryos following the discovery of a number of serotypes of BTV in the Northern Territory despite a lack of evidence of clinical disease in the field in sheep or cattle (Muller 1995). A presumptive diagnosis of BT may be made based on clinical and pathological findings. A distinctive haemorrhage is present at the base of the pulmonary artery in the majority of cases. Confirmation of the diagnosis is based on identification of the virus or by demonstration of BTV-specific antibodies.

- Suitable samples for virus isolation include blood in anticoagulant from febrile animals or fresh spleen and lymph nodes from animals that have died. Isolation can be carried out by intravenous inoculation of nine- to 12-day-old embryonated eggs. Infected embryos typically die between two and seven days post inoculation and often have a haemorrhagic appearance. Cell culture is not as sensitive for viral isolation as embryonated eggs. Susceptible cell lines include mouse L, BHK-21, Vero and *Aedes albopictus* cells. Isolated viruses may be identified and serogrouped using serogroup-specific monoclonal antibodies by immunofluorescence or antigen capture ELISA (Hawkes et al. 2000). Serotyping is usually carried out by plaque assay or virus neutralization using serotype specific antisera.

- Several highly sensitive RT-PCR assays have been developed for the rapid detection of BTV nucleic acid in clinical samples (Dangler et al. 1990, Wade-Evans et al. 1990, Billinis et al. 2001, Aradaib et al. 2003). These techniques can provide information on virus serogroup and serotype (Johnson et al. 2000). Bluetongue virus nucleic acid can be detected in blood by PCR methods for at least 30 days and possibly over 90 days beyond the period during which virus can be isolated. However, attempts to infect the vector with PCR-positive, isolation-negative blood were unsuccessful. The presence of viral nucleic acid does not necessarily indicate the presence of infectious virus. Protocols for differentiating viral strains (Mertens et al. 2007) and for quantifying viral load (Toussaint et al. 2007. Shaw et al. 2007) have been published.
- Antigen detection ELISAs have been described (Stanislawek et al. 1996, Hamblin et al. 1998).
- A number of serological tests are available for the detection of antibodies to the BTV serogroup including CFT, AGID, indirect immunofluorescence and competitive ELISA. The competitive or blocking ELISA based on use of a BTV serogroup-reactive monoclonal antibody was developed to ensure the specificity of the test, in particular avoiding cross-reactivity between members of the BTV and epizootic haemorrhagic disease virus serogroups (Afshar et al. 1989). The assay is available commercially. Neutralization or HAI assays are required for the demonstration of type-specific antibodies. In enzootic regions paired serum samples are required for the demonstration of a rising titre.

REFERENCES

Afshar, A., Thomas, F.C., Wright, P.F., et al., 1989. Comparison of competitive ELISA, indirect ELISA and standard AGID tests for detecting bluetongue virus antibodies in cattle and sheep. Veterinary Record 124, 136–141.

Aradaib, I.E., Smith, W.L., Osburn, B.I., et al., 2003. A multiplex PCR for simultaneous detection and differentiation of North American serotypes of bluetongue and epizootic hemorrhagic disease viruses. Comparative Immunology, Microbiology and Infectious Diseases 26, 77–87.

Barratt-Boyes, S.M., MacLachlan, N.J., 1995. Pathogenesis of bluetongue virus infection of cattle. Journal of the American Veterinary Medicine Association 206, 1322–1329.

Billinis, C., Koumbati, M., Spyrou, V., et al., 2001. Bluetongue virus diagnosis of clinical cases by a duplex reverse transcription–PCR: a comparison with conventional methods. Journal of Virological Methods 98, 77–89.

Caterina, K.M., Frasca, S., Girshick, T., et al., 2004. Development of a multiplex PCR for detection of avian adenovirus, avian reovirus, infectious bursal disease virus, and chicken anemia virus. Molecular and Cellular Probes 18, 293–298.

Dangler, C.A., De Mattos, C.A., De Mattos, C.C., et al., 1990. Identifying bluetongue virus ribonucleic acid sequences by the polymerase chain reaction. Journal of Virological Methods 28, 281–292.

Duncan, R., 1999. Extensive sequence divergence and phylogenetic relationships between the fusogenic and non-fusogenic orthoreoviruses: A species proposal. Virology 260, 316–328.

Gentsch, J.R., Glass, R.I., Woods, P., et al., 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. Journal of Clinical Microbiology 30, 1365–1373.

Gouvea, V., Glass, R.I., Woods, P., et al., 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. Journal of Clinical Microbiology 28, 276–282.

Gouvea, V., Santos, N., Timenetsky, M.C., 1994. Identification of bovine and porcine rotavirus G types by PCR. Journal of Clinical Microbiology 32, 1338–1340.

Hamblin, C., Graham, S.D., Anderson, E.C., et al., 1990. A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus. Epidemiology & Infection 104, 303–312.

Hamblin, C., Mertens, P.P., Mellor, P.S., et al., 1991. A serogroup specific enzyme-linked immunosorbent assay for the detection and identification of African horse sickness viruses. Journal of Virological Methods 31, 285–292.

Hamblin, C., Salt, J.S., Graham, S.D., et al., 1998. Bluetongue virus serotypes 1 and 3 infection in Poll Dorset sheep. Australian Veterinary Journal 76, 622–629.

Hawkes, R.A., Kirkland, P.D., Sanders, D.A., et al., 2000. Laboratory and field studies of an antigen capture ELISA for bluetongue virus. Journal of Virological Methods 85, 137–149.

Herring, A.J., Inglis, N.F., Ojeh, C.K., et al., 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. Journal of Clinical Microbiology 16, 473–477.

Iturriza-Gómara, M., Green, J., Brown, D.W., et al., 2000. Diversity within the VP4 gene of rotavirus P[8] strains: implications for reverse transcription-PCR genotyping. Journal of Clinical Microbiology 38, 898–901.

Johnson, D.J., Wilson, W.C., Paul, P.S., 2000. Validation of a reverse transcriptase multiplex PCR test for the serotype determination of US isolates of bluetongue virus. Veterinary Microbiology 76, 105–115.

Laviada, M.D., Arias, M., Dominguea, J., et al., 1992a. Detection of African horse sickness virus in infected

spleen by sandwich ELISA using two monoclonal antibodies specific for VP7. Journal of Virological Methods 38, 229–242.

Laviada, M.D., Roy, P., Sanchez-Vizcaino, J.M., 1992b. Adaptation and evaluation of an indirect ELISA and immunoblotting test for African horse sickness antibody detection. In: Walton, T.E., Osburn, B.I. (Eds.), Bluetongue, African Horse Sickness and Related Orbiviruses Proceedings of the Second International Symposium, CRC Press, Boca Raton, Florida, USA, pp. 646–650.

Laviada, M.D., Roy, P., Sanchez-Vizcaino, J.M., et al., 1995. The use of African horse sickness virus NS3 protein, expressed in bacteria, as a marker to differentiate infected from vaccinated horses. Virus Research 38, 205–218.

Liu, H.J., Liao, M.H., Chang, C.D., et al., 1999. Comparison of two molecular techniques for the detection of avian reoviruses in formalin-fixed, paraffin-embedded chicken tissues. Journal of Virological Methods 80, 197–201.

Liu, H.J., Lee, L.H., Shih, W.L., et al., 2004. Rapid characterization of avian reoviruses using phylogenetic analysis, reverse transcription-polymerase chain reaction and restriction enzyme fragment length polymorphism. Avian Pathology 33, 171–180.

Lorrot, M., Vasseur, M., 2007. How do the rotavirus NSP4 and bacterial enterotoxins lead differently to diarrhoea? Virology Journal 4, 31.

Maan, S., Maan, N.S., Nomikou, K., et al., 2011. Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait. PLoS One 6, e26147.

Meiswinkel, R., Paweska, J.T., 2003. Evidence for a new field *Culicoides* vector of African horse sickness in South Africa. Preventive Veterinary Medicine 60, 243–253.

Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998–2001. Veterinary Journal 164, 20–37.

Mellor, P.S., Rawlings, P., Baylis, M., et al., 1998. Effect of temperature on African horse sickness virus infection in *Culicoides*. In: Mellor, P.S., Baylis, M., Hamblin, C., et al. (Eds.), African Horse Sickness, Springer-Verlag, Wien, pp. 156–163.

Menzies, F.D., McCullough, S.J., McKeown, I.M., et al., 2008. Evidence for transplacental and contact transmission of bluetongue virus in cattle. Veterinary Record 163, 203–209.

Mertens, P.P.C., Maan, N.S., Prasad, G., et al., 2007. Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. Journal of General Virology 88, 2811–2823.

Meyden, C.H., Van Der Erasmus, B., Swanepoel, R., et al., 1992. Encephalitis and chorioretinitis associated with neurotropic African horse sickness virus infection in laboratory workers. South African Medical Journal 81, 451–454.

Muller, M.J., 1995. Veterinary arbovirus vectors in Australia – a retrospective. Veterinary Microbiology 46, 101–116.

Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue epidemiology in the European Union. Emerging Infectious Diseases 14, 539–544.

Sailleau, C., Hamblin, C., Paweska, J., et al., 2000. Identification and differentiation of nine African horse sickness virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. Journal of General Virology 81, 831–837.

Shaw, A.E., Monaghan, P., Alpar, H.O., et al., 2007. Development and validation of a real-time RT-PCR assay to detect genome bluetongue virus segment 1. Journal of Virological Methods 145, 115–126.

Stanislawek, W.L., Lunt, R.A., Blacksell, S.D., et al., 1996. Detection by ELISA of bluetongue antigen directly in the blood of experimentally infected sheep. Veterinary Microbiology 52, 1–12.

Stone-Marschat, M., Carville, A., Skowronek, A., et al., 1994. Detection of African horse sickness virus by reverse transcription PCR. Journal of Clinical Microbiology 32, 697–700.

Toussaint, J.F., Sailleau, C., Breard, E., et al., 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. Journal of Virological Methods 140, 115–123.

Wade-Evans, A.M., Mertens, P.P.C., Bostock, C.J., 1990. Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. Journal of Virological Methods 30, 15–24.

Wilson, A., Darpel, K., Mellor, P., 2008. Where does bluetongue virus sleep in winter? Public Library of Science Biology 6, 1612–1617.

Zientara, S., Sailleau, C., Moulay, S., et al., 1994. Diagnosis of the African horse sickness virus serotype 4 by a one-tube, one manipulation RT-PCR reaction from infected organs. Journal of Virological Methods 46, 179–188.

Zientara, S., Sailleau, C., Moulay, S., et al., 1998. Use of reverse transcriptase-polymerase chain reaction RT-PCR and dot-blot hybridization for the detection and identification of African horse sickness virus nucleic acids. In: Mellor, P.S., Baylis, M., Hamblin, C., et al. (Eds.), African Horse Sickness, Springer-Verlag, Wien, pp. 317–327.

Zhang, N., MacLachlan, N.J., Bonneau, K.R., et al., 1999. Identification of seven serotypes of bluetongue virus from the People's Republic of China. Veterinary Record 145, 427–429.

FURTHER READING

Lundgren, O., Svensson, L., 2001. Pathogenesis of Rotavirus diarrhea. Microbes and Infection 3, 1145–1156.

Savini, G., Afonso, A., Mellor, P., et al., 2011. Epizootic haemorrhagic disease. Research in Veterinary Science 91, 1–17.

Chapter 56

Birnaviridae

Birnaviruses derive their name from the two segments of linear, double-stranded RNA that make up their genome. The virions are non-enveloped, about 65 nm in diameter with icosahedral symmetry (Fig 56.1). Five polypeptides, VP1, VP2, VP3, VP4 and VP5 have been identified. The major capsid protein VP2 contains the virus neutralizing epitopes and is the type-specific antigen. Replication occurs in the cytoplasm of host cells and involves a virion-associated RNA-dependent RNA polymerase (VP1). The family is divided into four genera (Fig. 56.2); *Aquabirnavirus* and *Blosnavirus* contain viruses of fish while *Avibirnavirus* and *Entomobirnavirus* contain viruses of chickens and insects respectively. The virions are resistant to ether and chloroform, and stable at pH 3 to 9 and 60°C for one hour.

CLINICAL INFECTIONS

Birnaviruses are responsible for two economically important diseases: infectious pancreatic necrosis (IPN) of salmonids and infectious bursal disease of chickens. These diseases occur worldwide and cause considerable losses in intensive aquaculture and poultry units respectively.

INFECTIOUS BURSAL DISEASE

Infectious bursal disease (IBD) is a highly contagious viral disease of young chickens that occurs worldwide. The causal virus, IBD virus, was first isolated in Gumboro, Delaware giving rise to the original name of the disease, Gumboro disease. Infection occurs in chickens, turkeys, ostriches and ducks but clinical disease only occurs in chickens. Transmission occurs by the oro-faecal route. Severe, acute disease with high mortality typically occurs in three- to six-week-old birds, while a mild or subacute disease is common in birds younger than three weeks. However, lymphoid depletion of the bursa of Fabricius due to IBDV infection occurring in the first two weeks of life may result in significant depression of the humoral immune response.

Isolates of IBDV can be divided into serotype 1 and serotype 2 on the basis of serum neutralization tests. Serotype 2 isolates are not associated with clinical disease. A wide range of antigenic diversity and variation in virulence exists among isolates within serotype 1. Three subgroups are recognized:

1. Classical strains.
2. Variant serotype 1 isolates were first recognized in the United States in the mid-1980s in flocks that had been vaccinated with classical strain vaccines. These antigenic variants are highly immunosuppressive in young chicks, causing rapid bursal atrophy without clinical signs of disease.
3. Very virulent (vv) serotype 1 strains were first reported in Europe and Asia in the late 1980s. These vvIBDV strains are similar antigenically to classical serotype 1 strains but have caused disease even in the presence of maternal antibody induced by classical vaccine strains.

Pathogenesis

The severity of clinical signs varies with the virulence of the virus, age of infection, breed and level of maternal antibody. Infection usually occurs when maternal

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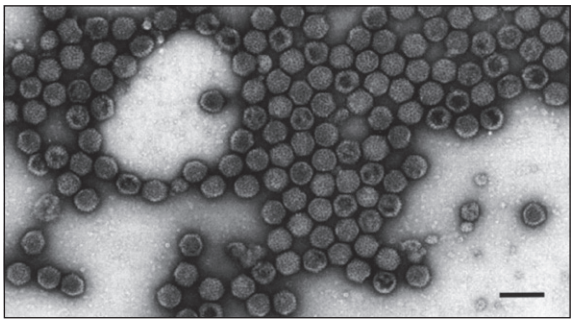

Figure 56.1 Negative contrast electron micrograph of IBDV virions. The bar represents 100 nm. *Reprinted with permission: Fauquet, C.M., et al. (Ed.), 2005. Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press.*

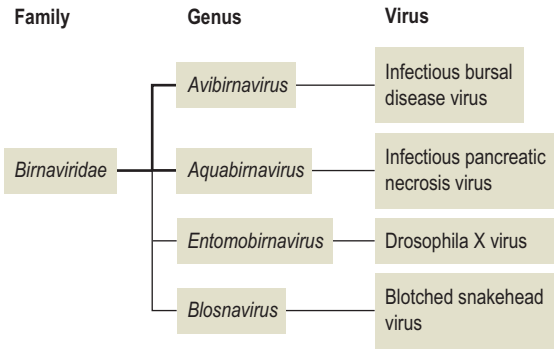

Figure 56.2 Classification of *Birnaviridae*.

antibody levels are waning at two to three weeks of age and is generally acquired by the oral route. The bursa of Fabricius reaches maximum development at this age and the acute form of the disease occurs following an incubation of two to three days. Within a few hours of ingestion of the virus it is possible to detect viral antigen in macrophages and lymphoid cells in the caeca, duodenum and jejunum. The virus reaches the liver via the portal circulation, infecting Kuppfer cells. The bursa of Fabricius is also reached via the bloodstream resulting in massive replication, a pronounced secondary viraemia and dissemination to other tissues. B lymphocytes and their precursors in the bursa are the main target cells.

Infection tends to be subclinical in birds less than three weeks of age on account of the smaller population of susceptible B lymphoblast cells and the less severe systemic effects caused by the virus. However, B lymphocyte destruction is often more severe in these birds because all the susceptible cells are still in the bursa. Bursal damage occurring after about two weeks of age tends to have a much less severe effect on immune competence as immunocompetent cells have already migrated out into the peripheral lymphoid tissues. Depletion of B lymphocytes in early life can result in impaired immune responses, lowered resistance to infectious diseases and suboptimal responses to vaccines.

Diagnosis

- Clinical signs and the gross appearance of a swollen, oedematous bursa are frequently sufficient for a clinical diagnosis of IBD in acute cases. Confirmation or identification of subclinical infection requires laboratory testing.
- Viral antigen can be detected using immunofluorescence of smears or frozen sections of the bursa. Macerated bursae are suitable for detection of viral antigen by ELISA (Kwang et al. 1987) or in a gel diffusion test.
- The virus is difficult to isolate and this is usually not attempted routinely. Specimens of bursa, spleen or faeces are suitable for isolation of the virus. Most strains will grow on the chorio-allantoic membrane of nine- to 11-day-old embryonated hens' eggs from flocks serologically negative for IBDV. Chicken embryo fibroblast cultures may be used but are usually not suitable for the isolation of the more pathogenic strains of the virus.
- Reverse transcription-PCR is frequently used for the diagnosis of IBD (Wu et al. 1992, Liu et al. 2000). Most protocols utilize primers directed against the VP2 gene (Eterradossi et al. 1998) although some primers have been described based on the VP1 gene (Tiwari et al. 2003). Restriction enzyme analysis (Jackwood & Sommer 1999) and nucleotide sequencing (Liu et al. 2002) of PCR products is widely used for the characterization of IBDV strains, particularly for the identification of vvIBDV (Zierenberg et al. 2001). Real-time RT-PCR protocols have been published (Moody et al. 2000).
- Recovered birds develop high antibody titres as mature peripheral B lymphocytes are still functional. Suitable serological assays include agar gel immunodiffusion, ELISAs, which are commercially available, and virus neutralization.

REFERENCES

Eterradossi, N., Arnauld, C., Toquin, D., et al., 1998. Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. Archives of Virology 143, 1627–1636.

Jackwood, D.J., Sommer, S.E., 1999. Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses from outside the United States. Avian Diseases 43, 310–314.

Kwang, M.J., Lu, Y.S., Lee, L.H., et al., 1987. Detection of infectious bursal disease viral antigen prepared from the cloacal bursa by ELISA. Journal of the Chinese Society of Veterinary Science 13, 265–269.

Liu, J., Zhou, J., Kwang, J., 2002. Antigenic and molecular characterization of recent infectious bursal disease virus isolates in China. Virus Genes 24, 135–147.

Liu, X., Giambrone, J.J., Hoerr, F.J., 2000. In situ hybridization, immunohistochemistry and in situ reverse transcription-polymerase chain reaction for detection of infectious bursal disease virus. Avian Diseases 44, 161–169.

Moody, A., Sellers, S., Bumstead, N., 2000. Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. Journal of Virological Methods 85, 55–64.

Tiwari, A.K., Kataria, R.S., Prasad, N., et al., 2003. Differentiation of infectious bursal disease viruses by restriction enzyme analysis of RT-PCR amplified VP1 gene sequence. Comparative Immunology, Microbiology & Infectious Diseases 26, 47–53.

Wu, C.C., Lin, T.L., Zhang, H.G., et al., 1992. Molecular detection of infectious bursal disease virus by polymerase chain reaction. Avian Diseases 36, 221–226.

Zierenberg, K., Raue, R., Muller, H., 2001. Rapid identification of 'very virulent' strains of infectious bursal disease virus by reverse transcription-polymerase chain reaction combined with restriction enzyme analysis. Avian Pathology 30, 55–62.

FURTHER READING

Muller, H., Islam, M.R., Raue, R., 2003. Research on infectious bursal disease – the past, the present and the future. Veterinary Microbiology 97, 153–165.

Nagarajan, M.M., Kibenge, F.S.B., 1997. Infectious bursal disease virus: a review of molecular basis for variations in antigenicity and virulence. Canadian Journal of Veterinary Research 61, 81–88.

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Chapter 57

Flaviviridae

The name of the family *Flaviviridae* is derived from the Latin word *flavus* meaning yellow and referring to the virus of yellow fever, which is a type species within the family. The viruses are spherical, 40–60 nm in diameter with an icosahedral capsid and a tightly adherent envelope containing two or three virus-encoded proteins depending on the genus (Fig. 57.1). The family is made up of three genera containing over 60 species: *Flavivirus, Pestivirus* and *Hepacivirus* (Fig. 57.2). The genera are antigenically unrelated but serological cross-reactivity occurs between members within the genera *Flavivirus* and *Pestivirus*. The *Flavivirus* genus is the largest containing about 50 species arranged into several serologically defined groups. A fourth genus, *Pegivirus*, has been proposed for a group of viruses isolated from primates (*Pegivirus A*, also known as hepatitis G virus) and from fruit bats (*Pegivirus B*).

Replication occurs in the cytoplasm of infected cells in cytoplasmic replication complexes that are associated with perinuclear membranes. Assembly and envelopment of virions occurs at intracellular membranes with transport in cytoplasmic vesicles through the secretory pathway and release from the cell by exocytosis. Replication is often accompanied by a characteristic proliferation of intracellular membranes. The nucleic acid is positive-sense single-stranded RNA and contains a single long, open reading frame from which a single polyprotein of more than 3000 amino acids is translated. Cleavage by viral and cellular proteinases results in three to four structural proteins and a number of non-structural proteins. The mature virions are generally labile, being sensitive to heat, detergents and organic solvents. Members of the *Flavivirus* genus agglutinate goose red cells. Pestiviruses are composed of four structural proteins, the capsid protein and three envelope glycoproteins designated E^{rns} (soluble RNase), E1 and E2. E2 (gp55) is the major glycoprotein and the main target of neutralizing antibodies.

The majority of members of the genus *Flavivirus* are arboviruses requiring either a mosquito or a tick vector (Table 57.1). Two major groups of mosquito-borne flaviviruses are distinguished on the basis of ecology and disease presentation in man. The encephalitic group includes Japanese encephalitis virus, West Nile virus, Murray Valley encephalitis virus and St Louis encephalitis virus, which infect birds as the natural vertebrate hosts and *Culex* species of mosquitoes as the primary vectors. The viscerotropic group includes yellow fever virus and dengue fever virus, which infect lower primates as the vertebrate hosts and *Aedes* species of mosquitoes as the principal vectors. About 30 members of the *Flavivirus* genus are known to be associated with disease in man including yellow fever, dengue, Japanese encephalitis, St Louis encephalitis, West Nile encephalitis and tick-borne encephalitis. Japanese encephalitis virus, St Louis encephalitis virus and West Nile virus are members of the same serogroup (Japanese encephalitis virus group). A number of members of the genus *Flavivirus*, including louping ill, Japanese encephalitis, West Nile and Wesselsbron viruses, cause disease in domestic animals.

The four members of the *Pestivirus* genus, bovine viral diarrhoea virus (BVDV) 1 and 2, border disease virus (BDV) and classical swine fever (hog cholera) virus (CSFV), infect ruminants and pigs. They are antigenically diverse, antigenically cross-reactive and display an overlapping host spectrum. Bovine viral diarrhoea virus and border disease virus can cross-infect other ruminants and swine. Genetic studies based on comparison of the immunodominant major envelope glycoprotein E2 (gp55) indicate six genotypes within the genus (van Rijn et al. 1997);

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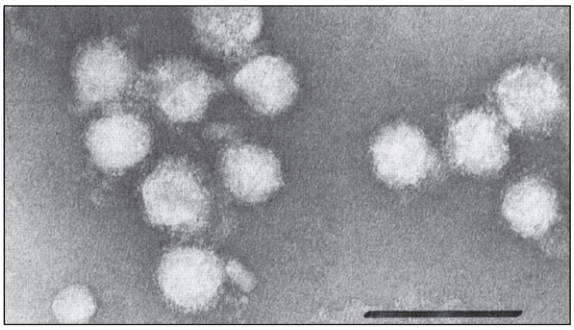

Figure 57.1 Negative stain electron micrograph of central European tick-borne encephalitis virus. The bar represents 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 558.*

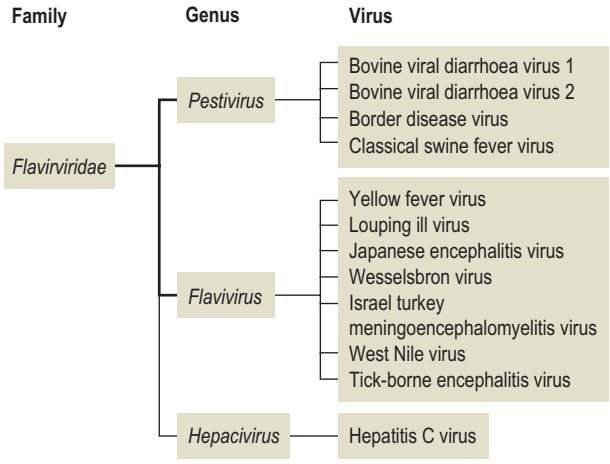

Figure 57.2 Classification of members of the family *Flaviviridae* with emphasis on species which affect domestic animals.

Table 57.1 Flaviviruses and pestiviruses of animals

Virus	Host species	Disease
Bovine viral diarrhoea virus 1 and 2	Cattle (sheep, pigs)	Causes inapparent infection, acute disease (bovine viral diarrhoea) and sporadic fatal infection (mucosal disease). Infection of pregnant animals may result in abortion, congenital defects or persistent infection (immunotolerance)
Border disease virus	Sheep	Important infection of pregnant ewes and cause of abortion or congenital abnormalities (hairy shaker lambs)
Classical swine fever (hog cholera) virus	Pigs	Economically significant disease. Highly contagious, generalized infection that is frequently fatal. Nervous signs, abortion and congenital tremor are features of the disease
Louping ill virus	Red grouse, sheep, cattle, horses and humans	Present in specific regions of Europe. Transmitted by the tick *Ixodes ricinus*. Produces encephalitis in sheep
Japanese encephalitis	Water birds, pigs, horses and humans	Widespread distribution in Asia. Transmitted by mosquitoes. Water birds are reservoir host. Infection in pigs is associated with abortion and neonatal mortality. May cause nervous disease in horses
West Nile virus	Birds, humans and horses	Transmitted by mosquitoes; birds are the natural hosts. Sporadic cause of serious CNS disease in humans and horses
Wesselsbron virus	Sheep	Occurs in parts of sub-Saharan Africa. Causes generalized infection, hepatitis and abortion. Transmitted by mosquitoes
Israel turkey meningoencephalitis virus	Turkeys	Outbreaks of progressive paresis and paralysis in turkeys in Israel and South Africa. Mosquito-borne virus

classical swine fever virus isolates, border disease virus isolates, bovine viral diarrhoea virus isolates predominantly from cattle (classical BVDV isolates), bovine viral diarrhoea virus isolates from sheep, swine and cattle (atypical BVDV) isolates, deer pestivirus isolate and giraffe pestivirus isolate. A novel pestivirus genotype has been identified in pronghorn antelope (Vilcek et al. 2005). The analysis of pestivirus genetic sequences suggests three main branches in the pestivirus 'family tree'; the first branch includes BVDV 1 and 2, the second branch includes

CSFV, BDV and pestiviruses from giraffe and reindeer, the third branch has a single member from a pronghorn antelope (Ridpath 2003). Liu et al. (2009) have proposed nine species of pestivirus. Pestivirus infections may be inapparent, acute or persistent and are important economically worldwide. Hepatitis C virus is currently the only member of the *Hepacigenus* and is an important cause of hepatitis in humans.

BOVINE VIRAL DIARRHOEA

Infection with bovine viral diarrhoea virus (BVDV) is common in cattle populations worldwide. Successful eradication schemes have been carried out in Scandinavia. The virus is responsible for acute disease (bovine viral diarrhoea) and a chronic syndrome associated with persistent infection (mucosal disease). Isolates of BVDV can be segregated into two genotypes now considered separate species, BVDV 1 (classical BVDV isolates) and BVDV 2 (atypical BVDV isolates), on the basis of comparison of the conserved 5′ untranslated region of the viral genome. Isolates of the two viruses can be further subdivided into a and b subgenotypes (Ridpath 2003) and in the case of BVDV 1 several additional subgenotypes (Vilcek et al. 2001). Both BVDV 1 and BVDV 2 can exist as one of two biotypes based on their activity in cell cultures: cytopathic or non-cytopthic. Non-cytopathic isolates circulate widely in cattle populations. Cytopathic isolates arise from non-cytopathic BVDV as a result of recombination events that include the insertion of host RNA, the duplication of viral RNA sequences or mutations in the NS2-3 gene (Meyers et al. 1996, Kummerer et al. 2000). In some cases the cytopathic mutant results from recombination between the resident non-cytopathic virus and a superinfecting heterotypic cytopathic virus, such as has occurred in vaccine-associated outbreaks.

Bovine viral diarrhoea virus 1 and BVDV 2 produce similar clinical syndromes in cattle. However, only non-cytopathic BVDV 2 isolates have been associated with thrombocytopenia and a haemorrhagic syndrome, first described in North America and now referred to as severe acute BVD (Rebuhn et al. 1989). The BVDV isolates used in vaccines and diagnostic tests have traditionally been BVDV 1.

Animals exposed to BVDV for the first time transiently shed the virus in the early stages of infection and may transmit infection to other animals. Chronic shedding of virus in semen has been reported (Voges et al. 1998). Of far greater importance is the role of persistently infected animals, which shed the virus in all excretions and secretions and are efficient transmitters of the infection. Persistent infections are produced following foetal infection before day 120 gestation with non-cytopathic strains. Approximately 1% of animals in an infected population are persistently infected and viraemic while from 60% to 85% are antibody-positive (Houe 1999). Persistently infected cows may breed and will always transmit the virus to the calf. The familial occurrence of persistent infection is reasonably common. The presence of persistently infected animals in a herd results in ongoing exposure to the virus and a high level of herd immunity. Typically more than 80% of animals in infected herds are serologically positive.

Cattle are the primary host but the virus is capable of infecting most even-toed ungulates. The inter-species spread of bovine and ovine pestiviruses has been demonstrated under natural conditions but its importance in the transmission of infection is largely dependent on husbandry practices.

Pathogenesis

Acute BVDV infections are characterized by a transient viraemia and a rise in specific antibody post infection. The virus is typically acquired by the oronasal route and initial replication occurs in the oronasal mucosa. Viraemia follows and the virus spreads systemically, free in serum or associated with leukocytes. There is a transient decrease in B and T lymphocyte numbers following infection. The virus has an immunosuppressive effect and as a result may play an important part in the calf respiratory disease complex, increasing the severity of clinical disease.

Transplacental spread to the foetus will occur in susceptible pregnant animals. The outcome of foetal infection is highly dependent on the age of the foetus. During the first 30 days of gestation early embryonic death and infertility may follow infection. Infection during the first and second trimesters may result in abortion, mummification or congenital abnormalities such as cerebellar hypoplasia. Foetuses infected during the last trimester are able to mount an active immune response and are usually normal at birth. Foetal infection occurring up to about day 120 may result in the birth of a persistently infected calf. The developing immune system of the foetus does not recognize the virus as foreign but comes to regard viral antigens as self-antigens. As a result of this immunotolerance, specific antibody is not produced and the virus is able to persist for the lifetime of the animal. At some stage after birth, usually betwen six to 24 months of age, an antigenically homologous cytopathic biotype of virus arises as a result of a genetic alteration in the virus. This event is responsible for the subsequent development of mucosal disease. Cytopathic isolates demonstrate a pronounced tropism for gut-associated lymphoid tissue and continually produce an 80-kDa non-structural protein known as NS3 (p80), due to cleavage of the NS2-3 gene product. The role of NS3 in the pathogenesis of mucosal disease is unclear but is correlated with the development of disease. Non-structural 3 protein is present in leukocytes and lymphoid tissue in cattle persistently infected with non-cytopathic virus

(Kameyama et al. 2008) and may account for signs such as poor development and reduced immunocompetence reported in some persistently infected animals. The virus has a necrotizing effect on epithelial tissues of the gastrointestinal tract, integument and respiratory tract.

Mucosal disease is usually a sporadic disease and typically affects persistently infected animals between six months and two years of age. The clinical picture is characterized by depression, fever, mucopurulent nasal discharge, salivation, profuse watery diarrhoea and lameness. Erosive lesions occur throughout the intestinal tract and in the interdigital clefts. The case fatality rate is 100% with death usually occurring about one week after the onset of signs. A small number of animals become chronically affected and may survive for several months before dying from severe debilitation. This form of disease has been referred to as chronic mucosal disease.

Diagnosis

A tentative diagnosis may be possible based on the clinical presentation. Pathological examination is frequently sufficient to confirm classical cases of mucosal disease. However, in many cases the clinical signs are vague and require laboratory confirmation involving demonstration of antibody, viral antigen or viral RNA. The detection of seroconversion and persistently infected (PI) animals are required to confirm on-going infection in a herd.

- Virus isolation is possible in a wide range of cell cultures; primary bovine kidney, turbinate and testis cells are highly sensitive. The harvesting of the buffy coat from whole blood provides a useful specimen in the live animal. Suitable specimens from post mortem cases include spleen, liver, kidney, lymph node and sections of the gastro-intestinal tract containing lesions. Two samples taken three weeks apart should be analysed to confirm persistent infection in an animal. Cell lines and foetal calf serum, used as a cell culture medium supplement, must be screened for the presence of BVDV before attempting isolation of the virus (Bolin et al. 1991). The foetal calf serum should also be free from BVDV-specific antibodies. Cytopathic BVDV isolates will produce foci of lysed cells in the cell monolayer while non-cytopathic virus can be detected using fluorochrome or enzyme-labelled BVDV-specific antibodies. A microtitre virus isolation method using sera, the immunoperoxidase monolayer assay (IPMA), for whole herd screening for PI cattle has been developed (Saliki et al. 1997). It has been reported that some PI cattle can be IPMA-negative on serum but virus-isolation-positive on buffy coat (Grooms et al. 2001).
- Viral antigen can be detected directly by immunofluorescence on frozen sections or buffy coat smears. Immunohistochemical staining of skin sections ('ear notch' test) has been shown to be reliable and to correlate well with findings from blood testing (Thür et al. 1996, Njaa et al. 2000). Several antigen capture ELISAs have been developed for rapid detection of BVDV antigens extracted from tissues or blood leukocytes (Fenton et al. 1991, Shannon et al. 1991) and commercial kit sets are available. These assays are usually based on the use of monoclonal antibodies that recognize the antigenically conserved non-structural protein NS2-3 and therefore should detect most if not all BVDV strains.
- Dot blot, in situ hybridization and RT-PCR techniques for the detection of viral RNA have been described (Roberts et al. 1991, Sandvik et al. 1997, McGoldrick et al. 1999, Letellier & Kerkhofs 2003) as well as a multiplex PCR for the detection and differentiation of BVDV 1 and BVDV 2 (Gilbert et al. 1999). Reverse transcription-PCR is sufficiently sensitive for the detection of viral nucleic acid in pooled samples such as bulk milk (Radwan et al. 1995, Drew et al. 1999) and serum (Weinstock et al. 2001). This is a particularly useful approach where the herd in question has been vaccinated and antibody testing is not appropriate. It is possible to discriminate between BVDV-1 and -2 using separate sets of primers and probes (Letellier & Kerkhofs 2003).
- Virus neutralization and ELISA are the preferred methods for the detection of antibodies to BVDV (Edwards 1990). The virus neutralization test detects antibodies to the viral glycoproteins, particularly E2 (gp55). Although these antibodies are capable of cross-reacting with several strains of BVDV, it is important to use a challenge virus antigenically similar to the field virus circulating in the test population. The tissue culture cells used can also influence the apparent titre of a test serum (Saliki & Dubovi 2004). ELISA kits for detection of BVDV-specific antibodies are available commercially. The collection of paired serum samples and the detection of a fourfold increase in antibody titre are necessary to detect recent infection. The systematic serological testing of bulk milk or pooled blood samples from herds is an important part of identifying herds with PI animals and as such is also a key step in national eradication programmes. In order to confirm the removal of all PI animals from a herd, unvaccinated animals between eight to 12 months should be tested for antibodies. These animals are free of maternal antibody and have been at risk of infection for some time. The use of antibody testing as a screening test for PI animals is not recommended where BVDV vaccines are being used as PI cattle can have neutralizing antibody titres to the vaccine strain of the virus. The NS3 protein, alone or in combination with other BVDV proteins, is frequently

used as the basis of commercial ELISAs because it is highly conserved among the pestiviruses. Recent studies have shown that NS3-specific antibody levels in serum and milk are low or undetectable following vaccination with an inactivated BVDV vaccine. As a result it is possible to monitor infection in vaccinated herds using a suitable combination of inactivated vaccine and NS3 antibody test (Makoschey et al. 2007, Kuijk et al. 2008).

BORDER DISEASE

Border disease (hairy shaker disease) is a congenital disorder of sheep that occurs worldwide. It is associated with foetal infection by a non-cytopathic biotype of border disease virus (BDV). The condition was first reported from the border area between England and Wales. Border disease virus is closely related to BVDV. Pestivirus isolates from sheep are capable of infecting pigs and other ruminant species including cattle and goats. Conversely, pestivirus isolates from other species, particularly cattle, can cause border disease in sheep. Recent sequencing studies of ovine pestivirus isolates indicate at least three distinct genotypes within the species BDV with classical BDV isolates being termed BDV-1 (Becher et al. 2003).

Persistently infected animals constitute the main virus reservoir, shedding virus continuously into the environment through all excretions and secretions. Although persistently infected animals tend to have a low survival rate under field conditions they may live for several years without obvious clinical signs.

Pathogenesis

Virus is usually acquired via the oronasal route. Infection of healthy sheep is transient and generally subclinical. However, in pregnant susceptible ewes the virus crosses the placenta within a few days of infection. The immune response of the ewe quickly eliminates the virus from maternal tissues but has no effect on virus infecting the foetus. The most important factor governing the outcome of foetal infection is the stage of gestation at which it occurs. The foetus acquires the ability to respond immunologically to BDV between 60 and 85 days gestation. Infection after this period elicits an immune response capable of eliminating the infection and resulting in the birth of a clinically healthy animal. Infection of the foetus before day 60 may result in death and resorption, abortion or mummification. In other cases the foetus survives, develops immunotolerance for the infecting virus and remains persistently infected for life. These animals may be clinically normal at birth or may display tremors and increased hairiness due to a number of congenital defects arising from viral interference with organogenesis and the ensuing growth retardation. The principal defects include hypomyelination or dysmyelination of the CNS, skeletal defects and enlargement of primary hair follicles with a concurrent reduction in the number of secondary follicles. The effect of foetal infection during midgestation when the immune system is beginning to develop ranges from an absence of clinical signs to widespread CNS inflammation leading to cerebral cavitation and cerebellar dysplasia. An immune-mediated mechanism has been suggested to explain the severity of these lesions. Surviving, persistently infected lambs may subsequently suffer from a condition analogous to mucosal disease (Monies et al. 2004).

Diagnosis

- A clinical diagnosis may be possible on the basis of the characteristic clinical signs but only a proportion of affected animals may show obvious signs.
- Histopathology of the brain usually reveals characteristic lesions including dysmyelination, perivascular infiltration of mononuclear cells and focal hypercellularity in the white matter. The presence of viral antigen in the brain may be detectable using immunocytochemical staining.
- Virus isolation can be carried out in susceptible bovine or ovine cell lines to demonstrate the presence of virus in affected lambs. However, immunocytochemical staining will be required to detect the presence of non-cytopathic virus. Suitable samples include whole blood and tissues. Pre-colostral blood is preferable as the presence of colostral antibody may mask the presence of virus for several weeks.
- Viral antigen can be detected in frozen or fixed sections using immunofluorescence or immunoperoxidase techniques respectively. The detection of viral antigen in blood may be carried out by ELISA.
- Reverse transcription-PCR techniques have been described for BDV (Fulton et al. 1999, Vilcek & Paton 2000, Willoughby et al. 2006). These assays have principally been used for typing of BDV strains and differentiation from BVDV strains.
- Serological testing for specific antibody is useful in determining the presence and extent of infection in a flock. Suitable methods include serum neutralization and ELISA.

CLASSICAL SWINE FEVER (HOG CHOLERA)

Classical swine fever is a very contagious and frequently fatal disease of pigs. It is present in many countries but has been eradicated from North America, Australia and many

European countries. It is a disease that is listed (formerly List A) by the Office International des Epizooties (OIE). Significant outbreaks have occurred in recent years in Italy, Belgium, Germany and the Netherlands. Although probably the least variable member of the *Pestivirus* genus, CSFV isolates can be divided into three major groupings on the basis of nucleotide sequence data (Lowings et al. 1996, Moennig et al. 2003), particularly data relating to E2 (gp55). Recent European isolates (Group 2) are quite distinct from those responsible for outbreaks in the 1940s and 1950s (Group 1) and those currently circulating in Asia (Group 3). Isolates are of a single major antigenic type and are generally non-cytopathic but vary considerably in virulence. Congenital infection with BVDV can give rise to clinical signs resembling CSF (Terpstra & Wensvoort 1988).

Pigs and wild boar are the only natural hosts of CSFV and direct contact between infected and susceptible animals is the main means of transmission. In enzootic areas the main means of disease spread is by movement of infected pigs. Congenital infections with low-virulence strains may result in the birth of persistently infected piglets. The virus is reasonably fragile and does not persist for long in the environment. However, CSFV can survive for long periods in protein-rich media such as meat or body fluids, especially if the material is chilled or frozen.

Pathogenesis

The incubation period is five to 10 days. Pigs are usually infected by the oronasal route and the tonsil is the primary site for viral multiplication. The virus spreads to the draining regional lymph nodes. Following multiplication there the virus enters the bloodstream. The virus has an affinity for vascular endothelium and reticuloendothelial cells. The virus also replicates in visceral lymph nodes, lymphoid tissue lining the intestine and in bone marrow. Late in the viraemic phase, the virus invades parenchymatous organs. Degeneration of endothelial cells in conjunction with a severe thrombocytopenia is responsible for the multiple haemorrhages seen in acute CSF. A non-suppurative encephalitis is present in most CSFV-infected pigs. The principal CNS lesion is perivascular cuffing. A chronic form of disease may occur with viral strains of reduced virulence.

In pregnant sows the virus may cross the placenta and the outcome will be largely determined by the age of the foetus and the virulence of the strain of virus. The earlier in gestation that infection occurs the greater the foetal damage that is likely to occur. Infection early in gestation typically results in resorption or abortion. Infection late in gestation may result in a protective immune response and the birth of healthy piglets. Congenital infection may also result in stillbirths, the birth of weak piglets with congenital tremors or occasionally clinically normal piglets. Possible congenital abnormalities include deformities of the head and limbs, cerebellar hypoplasia and hypomyelinogenesis. Surviving piglets have a specific immune tolerance to the virus and are persistently infected, excreting virus continuously. They may subsequently develop late-onset disease. The factors triggering the appearance of disease in such cases are unclear.

Diagnosis

The history and clinical signs may be sufficiently characteristic for a provisional diagnosis to be made, but laboratory confirmation is essential, particularly in the case of infections with low-virulence strains.

- In acute cases, haemorrhages in many organs and on serosal surfaces are characteristically seen. Petechiae on the surface of the kidneys and in the lymph nodes are the most consistent findings. Splenic infarction, if present, is considered highly characteristic of acute swine fever. Button ulcers in the intestinal mucosa near the ileocaecal valve may be present in chronic or late-onset disease.
- Rapid confirmation can be achieved using direct immunofluorescence on frozen sections. Specific cytoplasmic fluorescence is most frequently obtained using tonsillar tissue although kidney, spleen, distal ileum and lymph nodes may also be used. As pigs can become infected with BVDV it is necessary to employ monoclonal antibodies specific to CSFV in order to confirm positive test results. An antigen-capture ELISA has been described for the detection of viral antigens in blood (Depner et al. 1995). Commercial antigen capture ELISAs are available and can be used to detect viral antigen in blood and organ suspensions (Hergarten et al. 2001). As such assays are less sensitive than virus isolation, they are best used on a herd rather than on an individual animal basis.
- Virus isolation can be attempted in porcine cell lines, such as the pig kidney cell line PK-15, using homogenates of spleen and tonsil. The majority of isolates are non-cytopathic and immunostaining is required to confirm the presence of virus after 24 to 72 hours.
- Reverse transcription-PCR has been shown to be a rapid and more sensitive alternative to antigen-capture ELISA and viral isolation (McGoldrick et al. 1998, Dewulf et al. 2004, Hoffmann et al. 2005). Amplification of viral RNA followed by sequencing can be used to compare genetic differences between virus isolates and for genetic typing studies (Paton et al. 2000).
- Serological testing is useful on farms infected with low-virulence strains of virus and for large-scale monitoring of infection. The most widely used tests are virus neutralization and ELISA. The definitive test for differentiation of CSFV-specific antibodies from cross-reactive antibodies against ruminant

pestiviruses is the comparative neutralization test which compares the titres obtained using a range of challenge pestiviruses. As CSFV is non-cytopathic, immunostaining must be used to detect non-neutralized virus. Microtitre plate formats are commonly used such as the fluorescent antibody virus neutralization test and the neutralizing peroxidase-linked assay, which are both accepted tests for international trade purposes. A complex-trapping-blocking ELISA has been developed for distinguishing between CSFV and BVDV infections (Wensvoort et al. 1988, Colijn et al. 1997). Commercial ELISA kits are also available. A number of marker vaccines are now available for CSFV. These vaccines are subunit vaccines that employ the E2 glycoprotein as immunogen. A companion ELISA that detects antibodies to the E^{rns} protein is capable of distinguishing between vaccinated and naturally infected animals (Langedijk et al. 2001).

LOUPING ILL

Louping ill virus belongs to a serologically related group of viruses known collectively as the mammalian tick-borne virus group or complex. The members of this group are distributed in northern temperate latitudes and are regarded essentially as human pathogens. However, louping ill is primarily a disease of sheep and although the virus is fully pathogenic for man, human infection is rare. The disease is largely confined to Britain and Ireland but has also been described in Norway, Spain, Bulgaria and Turkey. Isolates from cases in Spain and Turkey have been shown to be distinct from each other and from those isolates recovered in Britain, Ireland and Norway (Marin et al. 1995). Irish, British, Spanish and Turkish subtypes are currently recognized.

The virus is transmitted by the tick *Ixodes ricinus*. As a result the timing and distribution of the disease are closely associated with periods of tick activity and suitable habitats, namely rough upland grazing or areas of woodland. The host range of the ticks is wide and infection can occur in several vertebrate species including sheep, cattle, horses, deer, man and red grouse. However, sufficient viraemia to pass virus on to feeding ticks has only been demonstrated in sheep and red grouse. The maintenance of louping ill virus in enzootic areas is considered to be dependent on a sheep–tick cycle. However, it has been suggested that the mountain hare may be an important host reservoir for the virus (Jones et al. 1997, Laurenson et al. 2003). It has also been demonstrated that the virus may be transmitted from infected to uninfected ticks feeding in close proximity on the same vertebrate host in the absence of viraemia. Transstadial but not transovarial transmission of the virus occurs in the tick. On farms where the infection is enzootic, losses mainly occur in animals under two years of age. Lambs are protected by colostral antibody while adult animals are usually immune.

Pathogenesis

Initial viral replication occurs in the lymph node draining the site of inoculation. Viraemia follows with dissemination of the virus to the brain and lymphatic organs. The speed of onset and vigour of the subsequent immune response are important factors in determining the extent of spread of the virus in the brain. The severity of the disease is highly variable ranging from ataxia to the rapid onset of coma and death. The name of the disease is derived from a Scottish word meaning to leap, which refers to the gait of some affected animals. Immunosuppression associated with tick-borne fever infections is believed to be responsible for the increased mortality observed in animals with dual infections.

The disease is zoonotic, transmitted to man by biting ticks, contact with infected sheep tissues or by ingestion of goat's milk from infected lactating does. Initial signs are influenza-like followed by mild nervous signs.

Diagnosis

A history of nervous signs or sudden death in areas of tick activity is highly suggestive. On account of the wide range of clinical signs, laboratory confirmation is usually required.

- Lesions of non-suppurative encephalomyelitis may be detected by histopathology. The lesions are usually most pronounced in the brain stem while the forebrain is relatively unaffected. A specific diagnosis may be possible following detection of viral antigen using immunohistochemical staining.
- Isolation of the virus is possible using specimens of brain collected aseptically into 50% glycerol saline and inoculated into tissue culture (pig kidney cells) or intracerebrally into mice.
- A RT-PCR protocol for detection of louping ill virus has been described (Gaunt et al. 1997, Marriott et al. 2006).
- Antibody titres to the virus can be determined using CFT, gel diffusion, HI and IFAT. The virus will haemagglutinate gander red cells. The detection of IgM is a useful indicator of recent infection.

JAPANESE ENCEPHALITIS

Japanese encephalitis is essentially a human disease. It has a wide distribution over large parts of Asia. The virus is transmitted by mosquitoes (*Culex* species, in particular *Cx.*

tritaeniorhynchus) and is maintained by a mosquito–aquatic bird (principally egrets, herons) cycle. Infection of a number of animal species can occur, particularly horses and pigs. The disease in horses is of less importance than formerly due to declining numbers and effective vaccines. Pigs are an important amplifying host reared close to human populations. The virus can be transmitted in the semen of boars and infection may result in various forms of reproductive failure in pregnant sows. The litters of infected pregnant sows may contain mummified foetuses, stillborns, weak piglets with nervous signs and apparently normal piglets. Virus diagnosis is based on virus isolation, detection of viral RNA by RT-PCR (Lian et al. 2002) or the demonstration of a rising antibody titre. Antibody titres may be demonstrated by haemagglutination, virus neutralization, ELISA or latex agglutination (Jia et al. 2002).

WESSELSBRON DISEASE

Wesselsbron virus is transmitted by mosquitoes and has a wide host range including domestic species, wild mammals and man. However, disease is generally only seen in sheep and goats, while infections in other species tend to be mild or subclinical. Human infections present as a febrile influenza-like disease. Infection is widespread over much of sub-Saharan Africa. The disease in sheep is similar but less severe than Rift Valley fever with signs of abortion, congenital abnormalities such as hydranencephaly or arthrogryposis and neonatal mortality. The disease is most severe in newborn lambs, with signs of fever, depression, general weakness and increased respiration. Characteristic lesions include hepatomegaly, icterus and necrosis of the liver parenchyma. Virus diagnosis is based on virus isolation by intracerebral inoculation of mice, demonstration of viral antigens in tissues by immunohistochemical staining and the demonstration of antibody (Williams et al. 1997).

WEST NILE VIRUS

Prior to the emergence of West Nile virus (WNV) in New York in 1999, WNV was generally considered to be a relatively unimportant virus, endemic in the Nile river basin and other parts of Africa. A number of limited outbreaks had been described in Europe and Israel since 1994. In particular, significant mortality was observed in domestic geese in Israel between 1998 to 2000. Isolates of the virus represent a continuum of closely related viruses that display varying pathogenicity for birds and other vertebrates and are continuing to evolve. Strains of WNV can be divided into five distinct lineages. Lineage 1 viral isolates are further divided into two clades: clade 1a viruses are responsible for significant disease in birds, humans and horses, clade 1b includes Kunjin virus which is present in Australasia. Lineage 2 viral isolates are generally found in sub-Saharan Africa and are considered relatively non-pathogenic but have recently been associated with avian mortality in central Europe. Lineages 3 and 4 have been identified in Russia. Lineage 5 (formerly classified as 1c) has been found in India.

The virus is transmitted by mosquitoes to birds which in turn develop a viraemia sufficient to infect subsequent biting mosquitoes. The magnitude and duration of viraemia appear to be highest in crows, magpies, house sparrows, house finches and other passerine species. Man, horses and other mammals do not develop sufficient viraemia to transmit to mosquitoes and are dead-end hosts. Since 1999 WNV has spread across most of the United States, southern Canada and into Mexico and the Caribbean. This rapid spread is probably due to the movement of infected birds. In addition, the virus has been responsible for outbreaks of disease in birds, humans and horses and these outbreaks have recurred year after year. The seasonal re-emergence of WNV in the United States and Europe each spring is believed to be due to both migrating birds and over-wintering of the virus in mosquitoes. Deaths in infected birds usually occur from mid-summer onwards with human and equine cases beginning a few weeks later. The virus is highly infectious for horses but only a small percentage ultimately develop clinical signs of myeloencephalitis.

Diagnosis

West Nile virus infection should be suspected in birds displaying neurological signs during the warm months of the year. Sick birds, particularly crows and raptor species, are often viraemic and virus can be detected using RT-PCR in saliva, blood and droppings. Post mortem examination and diagnostic procedures should only be carried out where the appropriate biosecurity level 3 facilities are available. Infection with WNV can be confirmed by detection of viral antigen by immunohistochemistry or by immunoassay. Commercial antigen detection assays are available for humans. Infection can also be confirmed by detection of viral RNA by RT-PCR (Johnson et al. 2001, Komar et al. 2002, Tewari et al. 2004). The virus is present in a range of tissues in birds including brain, heart, lung, liver and spleen. However, brain tissue is the tissue of choice to collect in horses (Kleiboeker et al. 2004) and also appears to be the best tissue for viral isolation in Vero cells (Panella et al. 2001). Serological testing is the primary method of diagnosis of WNV infection. Suitable serological tests for the detection of WNV antibodies include haemagglutination inhibition, plaque reduction neutralization assay and ELISA (Blitvich et al. 2003, Weingartl et al. 2003). All of these assays are influenced by the presence of antibodies to other flaviviruses, the plaque reduction assay is the most specific assay.

REFERENCES

Becher, P., Ramirez, R.A., Orlich, M., et al., 2003. Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. Virology 311, 96–104.

Blitvich, B.J., Bowen, R.A., Marlenee, N.L., et al., 2003. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. Journal of Clinical Microbiology 41, 2676–2679.

Bolin, S.R., Matthews, P.J., Ridpath, R., 1991. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhoea virus and antibodies against bovine viral diarrhoea virus. Journal of Veterinary Diagnostic Investigation 3, 199–203.

Colijn, E.O., Bloemraad, M., Wensvoort, G., 1997. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. Veterinary Microbiology 59, 15–25.

Depner, K., Paton, D.J., Cruciere, C., et al., 1995. Evaluation of the enzyme-linked immunosorbent assay for the rapid screening and detection of classical swine fever virus antigens in the blood of pigs. Revue Scientifique et Technique, Office International des Epizooties 14, 677–689.

Dewulf, J., Koenen, F., Mintiens, K., et al., 2004. Analytical performance of several classical swine fever laboratory diagnostic techniques on live animals for detection of infection. Journal of Virological Methods 119, 137–143.

Drew, T.W., Yapp, F., Paton, D.J., 1999. The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single tube RT-PCR. Veterinary Microbiology 64, 143–152.

Edwards, S., 1990. The diagnosis of bovine virus diarrhoea – mucosal disease in cattle. Revue Scientifique et Technique, Office International des Epizooties 9, 115–130.

Fenton, A., Nettleton, P.F., Entrican, G., et al., 1991. Identification of cattle infected with bovine virus diarrhoea virus using a monoclonal antibody capture ELISA. Archives of Virology 3, 169–174.

Fulton, R.W., d'Offay, J.M., Saliki, J.T., et al., 1999. Nested reverse transcriptase-polymerase chain reaction RT-PCR for typing ruminant pestiviruses: bovine viral diarrhea viruses and border disease virus. Canadian Journal of Veterinary Research 63, 276–281.

Gaunt, M.W., Jones, L.D., Laurenson, K., et al., 1997. Definitive identification of louping ill virus by RT-PCR and sequencing in field populations of *Ixodes ricinus* on the Lochindorb Estate. Archives of Virology 142, 1181–1191.

Gilbert, S.A., Burton, K.M., Prins, S.E., et al., 1999. Typing of bovine viral diarrhoea viruses directly from blood of persistently infected cattle by multiplex PCR. Journal of Clinical Microbiology 37, 2020–2023.

Grooms, D.L., Kaiser, L., Walz, P.H., et al., 2001. Study of cattle persistently infected with bovine viral diarrhoea virus that lack detectable virus in serum. Journal of the American Veterinary Medical Association 219, 629–631.

Hergarten, G., Hurter, K.P., Hess, R.G., 2001. Diagnosis of classical swine fever infection in wild boar: a comparison of different laboratory methods. Deutsche Tierarztliche Wochenschrift 108, 51–54.

Hoffmann, B., Beer, M., Schelp, C., et al., 2005. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. Journal of Virological Methods 130, 36–44.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus BVDV infections. Veterinary Microbiology 64, 89–107.

Jia, X.L., Chen, H.C., He, Q.G., et al., 2002. The development and application of the latex agglutination test to detect serum antibodies against Japanese encephalitis virus. Veterinary Research Communications 26, 495–503.

Johnson, D.J., Ostlund, E.N., Pedersen, D.D., et al., 2001. Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. Emerging Infectious Diseases 7, 739–741.

Jones, L.D., Gaunt, M., Hails, R.S., et al., 1997. Transmission of louping ill virus between infected and uninfected ticks co-feeding on mountain hares. Medical and Veterinary Entomolgy 11, 172–176.

Kameyama, K., Sakoda, Y., Matsuno, K., et al., 2008. Cleavage of the NS2-3 protein in the cells of cattle persistently infected with non-cytopathogenic bovine virus diarrhoea virus. Microbiology and Immunology 52, 277–282.

Kleiboeker, S.B., Loiacono, C.M., Rottinghaus, A., et al., 2004. Diagnosis of West Nile virus infection in horses. Journal of Veterinary Diagnostic Investigation 16, 2–10.

Komar, N., Lanciotti, R., Bowen, R., et al., 2002. Detection of West Nile virus in oral and cloacal swabs collected from bird carcasses. emerging infectious Diseases 8, 741–742.

Kuijk, H., Franken, P., Mars, M.H., et al., 2008. Monitoring of BVDV in a vaccinated herd by testing milk for antibodies to NS3 protein. Veterinary Record 163, 482–484.

Kummerer, B.M., Tautz, N., Becher, P., et al., 2000. The genetic basis for cytopathogenicity of pestiviruses. Veterinary Microbiology 77, 117–128.

Langedijk, J.P., Middel, W.G., Meloen, R.H., et al., 2001. Enzyme-linked immunosorbent assay using a virus type-specific peptide based on a subdomain of envelope protein E(rns) for serologic diagnosis of pestivirus infections in swine. Journal of Clinical Microbiology 39, 906–912.

Laurenson, M.K., Norman, R.A., Gilbert, L., et al., 2003. Identifying disease reservoirs in complex systems: mountain hares as reservoirs of ticks and louping-ill virus, pathogens of red grouse. Journal of Animal Ecology 72, 177–185.

Letellier, C., Kerkhofs, P., 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. Journal of Virological Methods 114, 21–27.

Lian, W.C., Liau, M.Y., Mao, C.L., 2002. Diagnosis and genetic analysis of Japanese encephalitis virus infected in horses. Journal of Veterinary Medicine series B – Infectious Diseases and Veterinary Public Health 49, 361–365.

Liu, L., Xia, H., Wahlberg, N., et al., 2009. Phylogeny, classification and evolutionary insights into pestiviruses. Virology 385, 351–357.

Lowings, P., Ibata, G., Needham, J., et al., 1996. Classical swine fever virus diversity and evolution. Journal of General Virology 77, 1311–1321.

Makoschey, B., Sonnemans, D., Munoz Bielsa, J., et al., 2007. Evaluation of the induction of NS3 specific BVDV antibodies using a commercial inactivated BVDV vaccine in immunization and challenge trials. Vaccine 25, 6140–6145.

Marin, M.S., McKenzie, J., Gao, G.F., et al., 1995. The virus causing encephalomyelitis in sheep in Spain: a new member of the tick-borne encephalitis group. Research in Veterinary Science 58, 11–13.

Marriott, L., Willoughby, K., Chianini, F., et al., 2006. Detection of louping ill virus in clinical specimens from mammals and birds using TaqMan RT-PCR. Journal of Virological Methods 137, 21–28.

McGoldrick, A., Lowings, J.P., Ibata, G., et al., 1998. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe Taq Man. Journal of Virological Methods 72, 125–135.

McGoldrick, A., Bensaude, E., Ibata, G., et al., 1999. Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. Journal of Virological Methods 79, 85–95.

Meyers, G., Tautz, N., Dubovi, E.J., et al., 1996. Origin and diversity of cytopathogenic pestiviruses. In: International Symposium Bovine Viral Diarrhoea Virus: A 50 year Review Cornell University, New York, pp. 24–34.

Moennig, V., Floegel-Niesmann, G., Greiser-Wilke, I., 2003. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. Veterinary Journal 165, 11–20.

Monies, R.J., Paton, D.J., Vilcek, S., 2004. Mucosal disease-like lesions in sheep infected with border disease virus. Veterinary Record 155, 765–769.

Njaa, B.L., Clark, E.G., Janzen, E., et al., 2000. Diagnosis of persistent bovine viral diarrhoea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. Journal of Veterinary Diagnostic Investigation 12, 393–399.

Panella, N.A., Kerst, A.J., Lanciotti, R.S., et al., 2001. Comparative West Nile virus detection in organs of naturally infected American crows *Corvus brachyrhynchos*. Emerging Infectious Diseases 7, 754–755.

Paton, D.J., McGoldrick, A., Greiser-Wilke, I., et al., 2000. Genetic typing of classical swine fever. Veterinary Microbiology 73, 137–157.

Radwan, G.S., Brock, K.V., Hogan, J.S., et al., 1995. Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhoea virus. Veterinary Microbiology 44, 77–92.

Rebuhn, W.C., French, T.W., Perdrizet, J.A., et al., 1989. Thrombocytopenia associated with acute bovine virus diarrhoea infection in cattle. Journal of Veterinary Internal Medicine 3, 42–46.

Ridpath, J.F., 2003. BVDV genotypes and biotypes: practical implications for diagnosis and control. Biologicals 31, 127–131.

Roberts, K.L., Collins, J.K., Carman, J., et al., 1991. Detection of cattle infected with bovine viral diarrhoea virus using nucleic acid hybridisation. Journal of Veterinary Diagnostic Investigation 3, 10–15.

Saliki, J.T., Dubovi, E.J., 2004. Laboratory diagnosis of bovine viral diarrhoea virus infections. Veterinary Clinics of North America Food Animal Practice 20, 69–83.

Saliki, J.T., Fulton, R.W., Hull, S.R., Dubovi, E.J., 1997. Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhoea virus in cattle serum. Journal of Clinical Microbiology 35, 803–807.

Sandvik, T., Paton, D.J., Lowings, P.J., 1997. Detection and identification of ruminant and porcine pestiviruses by nested amplification of 5′ untranslated cDNA regions. Journal of Virological Methods 64, 43–56.

Shannon, A.D., Richards, S.G., Kirkland, P.D., et al., 1991. An antigen-capture ELISA detects pestivirus antigens in blood and tissues of immunotolerant carrier cattle. Journal of Virological Methods 34, 1–12.

Terpstra, C., Wensvoort, G., 1988. Natural infections of pigs with bovine viral diarrhoea virus associated with signs resembling swine fever. Research in Veterinary Science 45, 137–142.

Tewari, D., Kim, H., Feria, W., et al., 2004. Detection of West Nile virus using formalin fixed paraffin embedded tissues in crows and horses: quantification of viral transcripts by real-time RT-PCR. Journal of Clinical Virology 30, 320–325.

Thür, B., Zlinszky, K., Ehrensperger, F., 1996. Immunohistochemical detection of bovine viral diarrhoea virus in skin biopsies: a reliable and fast diagnostic tool. Journal of Veterinary Medicine Series B 43, 163–166.

van Rijn, P.A., Gennip, H.G.P., Leendertse, C.H., et al., 1997. Subdivision of the *Pestivirus* genus based on envelope glycoprotein E2. Virology 237, 337–348.

Vilcek, S., Paton, D.J., 2000. A RT-PCR assay for the rapid recognition of border disease virus. Veterinary Research 31, 437–445.

Vilcek, S., Paton, D.J., Durkovic, B., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Archives of Virology 146, 99–115.

Vilcek, S., Ridpath, J.F., Van Campen, H., et al., 2005. Characterization of a novel pestivirus originating from a pronghorn antelope. Virus Research 108, 187–193.

Voges, J., Horner, G.W., Rowe, S., et al., 1998. Persistent bovine pestivirus infection localized in the testes of

immunocompetent, non-viraemic bull. Veterinary Microbiology 61, 165–175.

Weingartl, H.M., Drebot, M.A., Hubalek, Z., et al., 2003. Comparison of assays for the detection of West Nile virus antibodies in chicken serum. Canadian Journal of Veterinary Research 67, 128–132.

Weinstock, D., Bhudevi, B., Castro, A.E., 2001. Single-tube single-enzyme reverse transcriptase PCR assay for detection of bovine viral diarrhoea virus in pooled serum. Journal of Clinical Microbiology 39, 343–346.

Wensvoort, G., Bloemraad, M., Terpestra, C., 1988. An enzyme immunoassay employing monoclonal antibodies and detecting specifically antibodies to classical swine fever virus. Veterinary Microbiology 17, 129–140.

Williams, R., Schoeman, M., Van Wyk, A., et al., 1997. Comparison of ELISA and HI for detection of antibodies against Wesselsbron disease virus. Onderstepoort. Journal of Veterinary Research 64, 245–250.

Willoughby, K., Valdazo-González, B., Maley, M., et al., 2006. Development of a real time RT-PCR to detect and type ovine pestiviruses. Journal of Virological Methods 132, 187–194.

FURTHER READING

Blitvich, B.J., 2008. Transmission dynamics and changing epidemiology of West Nile virus. Animal Health Research Reviews 9, 71–86.

Blome, S., Meindl-Bohmer, A., Loeffen, W., et al., 2006. Assessment of classical swine fever diagnostics and vaccine performance. Revue scientifique et technique. Office Internationale des Epizooties 25, 1025–1038.

Dauphin, G., Zientara, S., 2007. West Nile virus: recent trends in diagnosis and vaccine development. Vaccine 25, 5563–5576.

de Smit, A.J., 2000. Laboratory diagnosis, epizootiology, and efficacy of marker vaccines in classical swine fever: a review. Veterinary Quarterly 22, 182–188.

Ellis, P.M., Daniels, P.W., Banks, D.J., 2000. Japanese encephalitis. Veterinary Clinics of North America – Equine Practice 16, 565–575.

Graham, D.A., Beggs, N., Mawhinney, K., 2009. Comparative evaluation of diagnostic techniques for bovine viral diarrhoea virus in aborted and stillborn fetuses. Veterinary Record 164, 56–58.

Nettleton, P.F., Gilray, J.A., Russo, P., et al., 1998. Border disease of sheep and goats. Veterinary Resarch 29, 327–340.

Phalen, D.N., Dahlhausen, B., 2004. West Nile virus. Seminars in Avian and Exotic Pet. Medicine 13, 67–78.

Sandvik, T., 1999. Laboratory diagnostic investigations for bovine viral diarrhoea virus infections in cattle. Veterinary Microbiology 64, 123–134.

Sandvik, T., 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. Preventive Veterinary Medicine 72, 3–16.

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Chapter 58

Arteriviridae

Arteriviruses were formerly classified as members of *Togaviridae* on the basis of morphological similarities. However, their genome organization and replication strategy is similar to members of *Coronaviridae*. A new family *Arteriviridae* was created by the International Committee on Taxonomy of Viruses in 1996. It is part of the Order *Nidovirales,* which also includes the family *Coronaviridae*. The name of the family is derived from equine arteritis which is the disease caused by the type virus species. Arteriviruses are medium-sized, spherical viruses, 50–74 nm in diameter (Fig. 58.1). They have an envelope which possesses a surface pattern of small, indistinct projections. The isometric nucleocapsid contains a single molecule of linear, positive-sense, single-stranded RNA. As well as the nucleocapsid protein (N) there are two major (GP_5, M) and four minor (GP_2, GP_3, GP_4, E) envelope proteins. Twelve non-structural proteins (nsp1–12) have been described. Replication occurs in the cytoplasm of infected cells. Nucleocapsids bud into the lumen of smooth endoplasmic reticulum and are released from infected cells by exocytosis. Arteriviruses are quite labile and are sensitive to heat, UV irradiation, low pH, lipid solvents, detergents and many disinfectants.

There is a single genus *Arterivirus* (Fig. 58.2). Members of the genus infect horses, pigs, mice and monkeys (Table 58.1). They are not antigenically related and are host-specific. Spread of infection is horizontal and includes aerosol, biting and venereal transmission. The primary target cells are macrophages and persistent infections are frequently established.

EQUINE VIRAL ARTERITIS (EVA)

Infection with equine arteritis virus (EAV) is common in horses worldwide. However, the number of clinical outbreaks of disease is comparatively rare. Upper respiratory tract disease, ventral oedema and abortion are prominent features in disease outbreaks. Isolates of EAV display biological and genomic differences but antigenic variation is limited and only one serotype has been recognized to date. Some isolates are associated with more severe disease. Horses, donkeys, mules and possibly zebras are susceptible to infection with EAV.

The percentage of seropositive horses varies according to breed and country. In general seroprevalence is high among standardbreds but low among thoroughbreds. It is unclear whether this difference reflects differences in susceptibility or differences in risk of exposure due to management practice differences. Equine arteritis virus is spread during the acute phase of infection primarily by aerosols from respiratory secretions. Close contact is required for the efficient spread of the infection. Mares and geldings eliminate the virus within one to two months of infection but about 35% of infected stallions become persistently infected. Carrier stallions are asymptomatic but shed the virus continuously in their semen and can venereally transmit the infection to 85–100% of mares covered by them. Persistent infection does not impair the fertility of stallions and appears to be testosterone-dependent (McCollum et al. 1994). Mares infected venereally may return to the home farm and spread the infection horizontally to in-contact susceptible animals. Infected pregnant mares can transmit the virus transplacentally to their foetus resulting in abortion or the birth of an infected foal.

PATHOGENESIS

The incubation period ranges from three to 14 days. Following aerosol infection, initial replication occurs in

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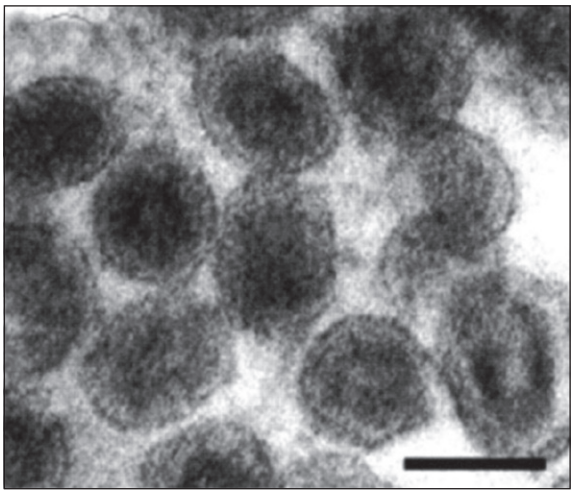

Figure 58.1 Electron micrograph of negatively stained particles of porcine reproductive and respiratory syndrome virus particles. Bar is 50 nm. *Reprinted with permission: Fauquet, C.M., et al. (Ed.), 2005. Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, pp. 965.*

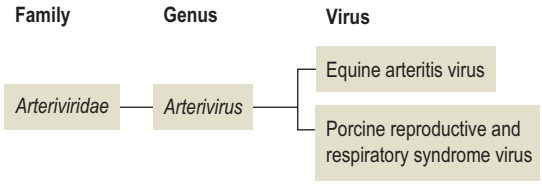

Figure 58.2 Classification of arteriviruses of veterinary significance.

Table 58.1 Overview of disease significance of arteriviruses

Virus	Host species	Disease
Equine arteritis virus	Horse	Rhinitis, conjunctivitis and oedema of the legs and trunk. Abortion is common in pregnant mares
Porcine reproductive and respiratory syndrome virus	Pig	Reproductive failure, pneumonia in piglets and increased pre-weaning mortality
Lactate dehydrogenase-elevating virus	Mouse	Life-long persistent viraemia with no clinical disease usually. Elevated enzyme levels in blood
Simian haemorrhagic fever virus	Macaque monkey	Haemorrhagic fever in macaques, other monkey species may carry the virus

pulmonary macrophages. Infection spreads to the bronchial lymph nodes and subsequently throughout the body via the bloodstream. The virus infects macrophages and endothelial cells primarily. Gross lesions include oedema, congestion and haemorrhage in many tissues as a direct consequence of a generalized vascular necrosis. Aborted foetuses are usually expelled partly autolysed and rarely display specific lesions. It is thought that abortions occur due to a combination of impairment of uterine and placental blood supply and direct viral damage to the foetus.

Diagnosis

Equine arteritis clinically resembles a number of other infectious diseases and definitive diagnosis requires laboratory confirmation. Internationally accepted testing procedures have been published (Timoney 2008).

- Virus isolation can be carried out in permissive cell lines such as rabbit or equine kidney. Early passage RK-13 cells are the cell system of choice. Appropriate samples include nasopharyngeal swabs, conjunctival swabs or placenta, foetal tissues and fluids. Heparinized blood is not suitable because of the inhibitory effects of heparin. Visible CPE is usually evident within two to six days.
- The characteristic vascular lesions found in affected adults are not a feature of EVA-related abortions but viral antigens can be detected in placental and foetal tissues by immunohistochemistry.
- Viral RNA can be detected in semen and other specimens using reverse transcription-polymerase chain reaction. Single, nested and real-time RT-PCR protocols have been described (Belak et al. 1995, Gilbert et al. 1997b, Belasuriya et al. 2002). A universal primer set has not been agreed upon yet and it is recommended that RT-PCR should be used in conjunction with virus isolation (Timoney 2008).
- Acute and convalescent blood samples should be collected for serological testing. Several serological tests including virus neutralization, complement fixation, indirect fluorescent antibody, agar gel immunodiffusion and ELISA are suitable. The virus neutralization test (Senne et al. 1985) is considered to be sensitive and highly specific. It is the most widely adopted test and is typically carried out in microtitre plates with the addition of complement to enhance sensitivity. The first ELISAs developed tended to identify a high number of false-positives

due to the presence of antibodies to tissue culture antigens in horses vaccinated with tissue culture-derived vaccines (Cook et al. 1989). Improved performance has been achieved by the use of more specific reagents such as recombinant major envelope GP_5 (previously known as G_L) protein as antigen (Hedges et al. 1998, Nugent et al. 2000) or monoclonal antibody to the major envelope GP_5 protein in a blocking ELISA (Cho et al. 2000).

- In the case of carrier stallions, semen should be collected for virus isolation from animals with positive titres. The sperm-rich fraction of a semen ejaculate is suitable for virus isolation or RT-PCR. An alternative strategy is to mate such animals to seronegative mares that are then monitored for seroconversion.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

Porcine reproductive and respiratory syndrome is an economically important condition characterized by late-term reproductive failure and pneumonia in young pigs. The syndrome was initially described in the USA in 1987 and known as mystery swine disease. Following an epidemic phase and despite early attempts to control its spread the disease is now endemic in many countries worldwide. The virus responsible was first isolated in the Netherlands and originally termed Lelystad virus (Wensvoort et al. 1991). The virus was characterized as an arterivirus and renamed porcine reproductive and respiratory syndrome virus (PRRSV). Significant antigenic and genomic differences are demonstrable between American and European isolates. As a result two major antigenic subtypes or genotypes are recognized, type I (European) and type II (American). However, distinction between the two genotypes may be less useful in the future as evidence of transfer between continents and recombination has been reported. A more virulent variant associated with high fever and high mortality rates appeared in China in 2006 and has spread throughout southeast Asia.

Infection occurs in wild boars and pigs. Typically, infection is introduced onto farms by infected pigs or by infected semen. On endemically infected farms infection tends to persist and to circulate either continuously or in consecutive waves. Several factors are important in maintaining farm infections (Albina 1997). Persistent infection in experimentally infected pigs has been demonstrated for up to 157 days after challenge (Wills et al. 1997). The introduction of susceptible replacement pigs provides a new pool of animals to support infection. Infection may spread in a slow and uneven manner on farms following its introduction resulting in subpopulations of susceptible animals within a large herd.

Pathogenesis

Following transmission, the resident macrophages within the exposed mucosal surface become infected. Infection most frequently occurs by the respiratory route. The virus has a tropism for pulmonary alveolar macrophages and the lungs are the principal target organ (Van Reeth 1997). Antibody produced early in the course of infection is not effective at clearing virus. Rather antibody-dependent enhancement of infection of pulmonary alveolar macrophages has been documented. The virus is transported in lymph to regional lymph nodes and is subsequently distributed systemically to mononuclear cells and tissue macrophages throughout the body. Transplacental infection of foetuses occurs in pregnant animals. Reproductive failure is more difficult to reproduce experimentally in early gestation than in late gestation (Kranker et al. 1998). The reason for this is unknown. Foetal and placental abnormalities are not always present and the mechanism of foetal death and reproductive failure remains unclear. Porcine reproductive and respiratory syndrome virus predisposes to other infections such as *Streptococcus suis*, porcine respiratory coronavirus and *Haemophilus parasuis* infections. However, the virus does not appear to have a systemic immunosuppressive effect (Albina et al. 1998). Where infection is endemic the clinical signs vary widely among infected herds. Subclinical infection is common. Factors increasing the severity of clinical disease include large numbers and high density of pigs, slatted floors and strain of virus. Some herds report sporadic respiratory or reproductive disease while a few experience severe chronic disease problems (Zimmerman et al. 1997). More severe outbreaks with average mortality rates of 20% across all ages have been described in China (Tian et al. 2007).

Diagnosis

Initial diagnosis is based on the recognition of clinical signs suggestive of the syndrome. On account of the variable nature of clinical signs, particularly in endemically infected herds, laboratory confirmation is required.

- Serology is the most widely used diagnostic method due to its ease of use and high sensitivity and specificity when applied on a herd basis. However, it will not distinguish carriers or vaccinated animals. Several serological tests are suitable including ELISA (Sorensen et al. 1998), virus neutralization (Jusa et al. 1996), indirect fluorescent antibody (Yoon et al. 1992) and immunoperoxidase monolayer assay (Houben et al. 1995). Commercial ELISAs are available (Drew 1995, Okinga et al. 2009).
- The presence of PRRSV may be demonstrated by several methods. Suitable samples include serum (due to the prolonged viraemia), foetal fluids and lung. Virus isolation is considered difficult, requiring the use of porcine alveolar macrophages harvested

from young SPF pigs. Immunohistochemical detection of PRRSV antigen in tissues has been described (Halbur et al. 1994), while in situ hybridization has been used to detect and differentiate North American and European PRRSV genotypes in formalin-fixed tissues (Larochelle & Magar 1997). Highly sensitive RT-PCR (Mardassi et al. 1994), nested PCR (Kono et al. 1996) and real time quantitative RT-PCR (Chung et al. 2005, Kleiboeker et al. 2005) protocols for the detection of viral RNA have been reported. A multiplex PCR has been described that differentiates European and North American PRRSV isolates (Gilbert et al. 1997a).

REFERENCES

Albina, E., 1997. Epidemiology of porcine reproductive and respiratory syndrome PRRS: an overview. Veterinary Microbiology 55, 309–316.

Albina, E., Piriou, L., Hutet, E., et al., 1998. Immune response in pigs infected with porcine reproductive and respiratory syndrome virus PRRSV. Veterinary Immunology and Immunopathology 61, 49–66.

Belak, S., Ballagi-Pordany, A., Timoney, P., et al., 1995. Evaluation of a nested PCR assay for the detection of equine arteritis virus infection. Proceedings of the 7th International Conference on Equine Infectious Diseases, Tokyo, Japan, 1994, 33–38.

Belasuriya, U.B.R., Leutenegger, C.M., Topol, J.B., et al., 2002. Detection of equine arteritis virus by real-time TaqMan® reverse transcription-PCR assay. Journal of Virological Methods 101, 21–28.

Cho, H.J., Entz, S.C., Deregt, D., et al., 2000. Detection of antibodies to equine arteritis virus by a monoclonal antibody-based blocking ELISA. Canadian Journal of Veterinary Research 64, 38–43.

Chung, W.B., Chan, W.H., Chaung, H.C., et al., 2005. Real-time PCR for quantitation of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 in naturally-infected and challenged pigs. Journal of Virological Methods 124, 11–19.

Cook, R.F., Gann, S.J., Mumford, J.A., 1989. The effects of vaccination with tissue culture-derived viral vaccines on detection of antibodies to equine arteritis virus by enzyme-linked immunosorbent assay ELISA. Veterinary Microbiology 20, 181–189.

Drew, T.W., 1995. Comparative serology of porcine reproductive and respiratory syndrome in eight European laboratories, using immunoperoxidase monolayer assay and enzyme-linked immunosorbent assay. Revue Scientifique et Technique, Office International des Epizooties 14, 761–775.

Gilbert, S.A., Larochelle, R., Magar, R., et al., 1997a. Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. Journal of Clinical Microbiology 35, 264–267.

Gilbert, S.A., Timoney, P.J., McCollum, W.H., et al., 1997b. Detection of equine arteritis virus in the semen of carrier stallions using a sensitive nested PCR assay. Journal of Clinical Microbiology 35, 2181–2183.

Halbur, P.G., Andrews, J.J., Huffman, E.L., et al., 1994. Development of a streptavidin-biotin immunoperoxidase procedure for the detection of porcine reproductive and respiratory syndrome virus antigen in porcine lung. Journal of Veterinary Diagnostic Investigation 6, 254–257.

Hedges, J.F., Belasuriya, U.B.R., Shabbir, A., et al., 1998. Detection of antibodies to equine arteritis virus by enzyme linked immunosorbent assays utilizing G_L, M and N proteins expressed from recombinant baculoviruses. Journal of Virological Methods 76, 127–137.

Houben, S., Callebaut, P., Pensaert, M.B., 1995. Comparative study of a blocking enzyme-linked immunosorbent assay and immunoperoxidase monolayer assay for the detection of antibodies to the porcine reproductive and respiratory syndrome virus in pigs. Journal of Virological Methods 51, 125–128.

Jusa, E.R., Inaba, Y., Kouno, M., et al., 1996. Slow-reacting and complement-requiring neutralizing antibody in swine infected with porcine reproductive and respiratory syndrome PRRSV virus. Journal of Veterinary Medical Science 58, 749–753.

Kleiboeker, S.B., Schommer, S.K., Lee, S.M., et al., 2005. Simultaneous detection of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative reverse transcriptase-PCR. Journal of Veterinary Diagnostic Investigation 17, 165–170.

Kono, Y., Kanno, T., Shimzu, M., et al., 1996. Nested PCR for the detection and typing of porcine reproductive and respiratory syndrome PRRSV virus in pigs. Journal of Veterinary Medical Science 58, 941–946.

Kranker, S., Nielsen, J., Bille-Hansen, V., et al., 1998. Experimental inoculation of swine at various stages of gestation with a Danish isolate of porcine reproductive and respiratory syndrome virus PRRSV. Veterinary Microbiology. 61, 21–31.

Larochelle, R., Magar, R., 1997. Differentiation of North American and European porcine reproductive and respiratory syndrome virus genotypes by in situ hybridization. Journal of Virological Methods 68, 161–168.

Mardassi, H., Wilson, L., Mounir, S., et al., 1994. Detection of porcine reproductive and respiratory syndrome virus and efficient differentiation between Canadian and European strains by reverse transcription and PCR amplification. Journal of Clinical Microbiology 32, 2197–2203.

McCollum, W.H., Little, T.V., Timoney, P.J., et al., 1994. Resistance of castrated male horses to attempted establishment of the carrier state with equine arteritis virus. Journal of

Comparative Pathology 111, 383–388.

Nugent, J., Sinclair, R., deVries, A.A.F., et al., 2000. Development and evaluation of ELISA procedures to detect antibodies against the major envelope protein (G_L) of equine arteritis virus. Journal of Virological Methods 90, 167–183.

Okinga, T., Yamagishi, T., Yoshii, M., et al., 2009. Evaluation of unexpected positive results from a commercial ELISA for antibodies to PRRSV. Veterinary Record 164, 455–459.

Senne, D.A., Pearson, J.E., Cabrey, E.A., 1985. Equine viral arteritis: a standard procedure for the virus neutralization test and comparison of results of a proficiency test performed at five laboratories. Proceedings of the United States Animal Health Association 89, 29–34.

Sorensen, K.J., Strandbygaard, B., Botner, A., et al., 1998. Blocking ELISAs for the distinction between antibodies against European and American strains of porcine reproductive and respiratory syndrome virus. Veterinary Microbiology 60, 169–177.

Tian, K., Yu, X., Zhao, T., et al., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS ONE 2, e526.

Timoney, P.J., 2008. Equine viral arteritis. In: Manual of Standards for Diagnostic Tests and Vaccines, sixth ed. Office International des Epizooties, Paris, Chapter 2.5.10.

Van Reeth, K., 1997. Pathogenesis and clinical aspects of a repiratory porcine reproductive and respiratory syndrome virus infection. Veterinary Microbiology 55, 223–230.

Wensvoort, G., Terpstra, C., Pol, T.J.M., et al., 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Veterinary Quarterly 13, 121–130.

Wills, R.W., Zimmerman, J.J., Yoon, K.-J., et al., 1997. Porcine reproductive and respiratory syndrome virus: a persistent infection. Veterinary Microbiology 55, 231–240.

Yoon, I.J., Joo, H.S., Christianson, W.T., et al., 1992. An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. Journal of Veterinary Diagnostic Investigation 4, 144–147.

Zimmerman, J.J., Yoon, K.-J., Wills, R.W., et al., 1997. General overview of PRRSV: a perspective from the United States. Veterinary Microbiology 55, 187–196.

FURTHER READING

Choo, J.G., Dee, S.A., 2006. Porcine reproductive and respiratory syndrome virus. Theriolgenology 66, 655–662.

Glaser, A.L., Chirnside, E.D., Horzinek, M.C., et al., 1997. Equine arteritis virus. Theriogenology 47, 1275–1295.

Holyoak, G.R., Balasuriya, U.B.R., Broaddus, C.C., et al., 2008. Equine viral arteritis: current status and prevention. Theriogenology 70, 403–414.

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Chapter 59

Togaviridae

The family name is taken from the Latin word *toga* meaning cloak or mantle and is a reference to the viral envelope. Members of the family are 60–70 nm in diameter. The envelope contains glycoprotein spikes and is tightly adherent around an icosahedral capsid (Fig. 59.1). The family is composed of two genera: *Alphavirus* and *Rubivirus* (Fig. 59.2). The genus *Alphavirus* contains more than 25 species of which a number are important veterinary pathogens. Alphaviruses are thought to have arisen from an insect-borne plant virus, probably in the New World. They can be divided into several groupings or complexes on the basis of antigenic and genetic studies; Venezuelan equine encephalitis complex, Eastern equine encephalitis complex, Semliki Forest complex and Western equine encephalitis/Sindbis complex (Powers et al. 2001). Western equine encephalitis virus is believed to have arisen by recombination between Eastern equine encephalitis and Sindbis-like viruses (Strauss & Strauss 1994, Weaver et al. 1997). Relatively recent discoveries include an alphavirus of salmonids, the cause of salmon pancreas disease and sleeping disease of rainbow trout, as well as southern elephant seal virus. These alphaviruses are unusual in that they do not seem to require an invertebrate vector. Rubella virus, which causes German measles in humans, does not require a vector and is the sole member of the genus *Rubivirus*.

Replication occurs in the cytoplasm of infected cells. The nucleic acid is positive-sense single-stranded RNA. The viral nucleocapsids are assembled in the cytosol. Alphavirus infection of vertebrate cells involves the shutdown of macromolecular synthesis and is cytolytic. Release from infected cells is effected by budding through the virus protein-modified plasma membrane with the consequent acquisition of a lipid envelope with glycoprotein spikes. In invertebrate cells the infection is usually non-cytolytic with host cells surviving and becoming persistently infected. In this case, the assembled nucleocapsids bud into cytoplasmic vesicles. The mature virions are not very stable in the environment, being sensitive to pH, heat, detergents and disinfectants. Togaviruses agglutinate goose and chick erythrocytes.

Almost all members of the genus *Alphavirus* are arboviruses (arthropod-borne). Arboviruses are defined as viruses maintained in nature through biological transmission between vertebrate hosts by haematophagous arthropods. The viruses multiply in the tissues of the arthropod vector. The term arbovirus has no taxonomic status, being a heterogeneous group of viruses belonging to several viral families including *Togaviridae, Flaviviridae, Reoviridae, Rhabdoviridae, Arenaviridae* and *Bunyaviridae*. Most arboviruses are mantained in complex sylvatic life cycles involving a primary vertebrate host and a primary arthropod host. Such cycles usually remain undetected unless domestic animals and humans encroach or the virus escapes its primary cycle by means of a secondary vector or vertebrate host due to ecological change thus bringing the virus into the peridomestic environment. Domestic animals and humans are generally 'dead-end' hosts as they do not develop sufficient viraemia to contribute to the transmission of the virus. Almost all arbovirus infections are zoonotic. The majority of arboviruses are found in tropical developing countries and have a distinct geographical distribution. Ecological factors limiting the distribution of particular arboviruses include temperature, rainfall and distribution of both vertebrate reservoir host and of the arthropod vector. The most important arthropod vectors are mosquitoes, ticks, sandflies and midges. The vector remains infected for life.

A number of important equine diseases are associated with infections by members of the genus *Alphavirus*

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(Table 59.1). The three equine encephalitis viruses (Venezuelan, Eastern and Western) are confined to the western hemisphere and are transmitted by mosquitoes. Getah virus is confined to southeast Asia and Australia. A number of outbreaks of disease have occurred in Japan.

EQUINE ENCEPHALITIS VIRUSES

Venezuelan, Eastern and Western equine encephalitis viruses are important causes of nervous disease in horses in the New World. The three viruses produce similar clinical signs although infections with Western equine encephalitis virus tend to be milder.

Several epidemiological features are common to the three diseases. The peak of disease incidence coincides with the time of maximum vector numbers in late summer or following the rains. Clinical cases dramatically cease once vector numbers diminish due to cold or drought. The distribution of the viruses is intimately associated with the distribution of the mosquito vectors.

The Venezuelan equine encephalitis (VEE) complex comprises several viral species, divided into six subtypes I to VI (Powers et al. 2001). Five antigenic variants or serotypes (AB to F) are recognized within subtype I, Venezuelan equine encephalitis virus (VEEV). The epizootic form of VEE is associated with two highly virulent subtype I serotypes of the virus known as I-AB and I-C. All other subtypes and serotypes are considered non-pathogenic or of low pathogenicity for horses, although I-E isolates from some recent outbreaks in horses in Mexico produced neurological disease but were incapable of generating a high-titre viraemia in horses. Subtype II viruses are also referred to as Everglades virus, subtype III as Mucambo virus and

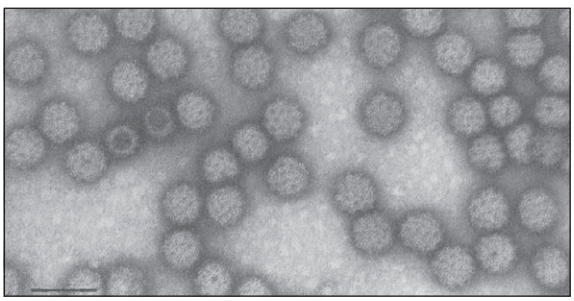

Figure 59.1 Electron micrograph of negatively stained particles of Eastern equine encephalitis virus. The bar represents 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 548.*

Table 59.1 Togaviruses of veterinary significance

Virus	Arthropod vector	Significance of infection
Venezuelan equine encephalitis virus	Mosquito	Present in Central and South America with occasional outbreaks in southern USA. Causes disease in horses, donkeys and man
Eastern equine encephalitis virus	Mosquito	Present in eastern USA, Caribbean and South America. Causes disease in pheasants, horses and man
Western equine encephalitis virus	Mosquito	Present throughout much of the Americas. Causes disease in horses and man. Milder disease than EEEV
Getah virus	Mosquito	Sporadic cause of disease in horses; fever, urticaria and hind-limb oedema. Infection common in pigs but significance unclear. Present in Australia and southeast Asia

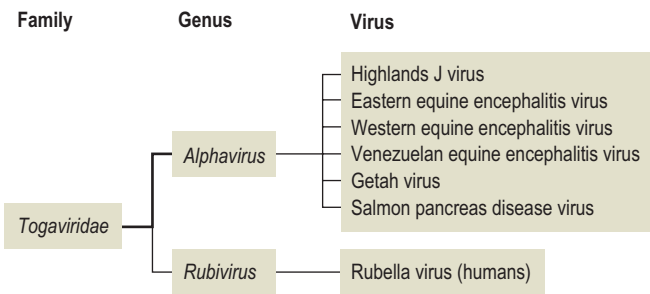

Figure 59.2 Classification of togaviruses of veterinary significance.

subtype IV as Pixuna virus. The viruses are maintained in sylvatic cycles involving rodents such as spiny rats (*Proechimys chrysaeolus*) and mosquitoes of the *Culex* (*Melanoconion*) subgenus in habitats characterized by mangrove swamps and brackish water (Carrara et al. 2005). Phylogenetic studies suggest the epizootic VEE viruses arise from mutation of enzootic progenitors (subtype I-D) and that this involves adaptation to *Aedes* (*Ochlerotatus) taeniorhynchus*, the most common mosquito vector in many coastal areas, as well as adaptation to horses which results in highly efficient amplification (Weaver et al. 1992, 2004). Between epizootics it is thought that virulent subtypes are maintained in nature in enzootic transmission cycles (Weaver et al. 1996). Epizootics of VEEV occurred regularly from 1962–1972 in the countries of northern South America and Central America, at one stage spreading as far north as Texas. Following a lull of 20 years, a small outbreak occurred in Venezuela in 1992 followed by a large-scale outbreak in Venezuela and Colombia in 1995. Outbreaks follow unusually heavy rains leading to increased mosquito vector populations. During epizootics humans, equines and several mosquito species become infected. Following inoculation with a virulent subtype horses develop a high-titred viraemia sufficient for subsequent transmission to feeding mosquitoes.

Eastern equine encephalitis virus (EEEV) is found principally in Atlantic coastal areas of the USA but has also been isolated in Canada, Michigan, the Caribbean Basin and South America. Two distinct lineages, North American and South American, are recognized. North American isolates are highly conserved and more pathogenic (Group I), while at least three subgroups (Groups IIA, IIB and III) are recognized within the South American lineage (Weaver et al. 1999). The virus is maintained in cycles of infection involving passerine birds and the irrigation ditch mosquito *Culiseta melanura* which inhabits freshwater swamps. A high-titred viraemia follows infection in birds and in many wild bird species the virus has no apparent effect. However, high mortality rates have been recorded in pheasants, whooping cranes and emus. Transmission of virus between pheasants can also occur by pecking and cannibalism. Accessory cycles of infection leading to transmission to man and horses involve other species of mosquito such as *Aedes sollicitans* and *Coquillettidia perturbans* which feed on both birds and mammals. Infection typically manifests itself as sporadic, localized outbreaks of encephalitis in man, horses and pheasants. Epizootics are not common and tend to occur in the summer/autumn and disappear with the first frosts. The over-wintering mechanism for virus maintenance is unclear as transovarial transmission in mosquitoes has not been demonstrated. The over-wintering reservoir is probably wild birds.

Western equine encephalitis virus (WEEV) has traditionally been associated with US states west of the Mississippi but is in fact present in most of the Americas. The cycle of infection involves mosquitoes, mainly *Culex tarsalis*, and wild birds. The infection is inapparent in indigenous wild bird species. Horses are infected incidentally and are dead-end hosts, developing only low titres of virus in the blood. Infections tend to occur regularly in certain areas. Epizootics are rare and studies have indicated that virulent epizootic virus arises from non-pathogenic enzootic virus. The overwintering mechanism of the virus is unclear but may involve birds, reptiles or mosquitoes over-wintering as adults. A related virus, Highlands J virus, occurs in the eastern United States and causes encephalitis in horses rarely.

Pathogenesis

The incubation period varies from one to nine days and clinical signs usually last four to nine days. Replication occurs near the site of inoculation by a feeding mosquito and also in the local draining lymph nodes. Viraemia ranging from barely detectable to an exceedingly high titre follows. This is accompanied by fever. In severe cases the virus invades the central nervous system resulting in neuronal necrosis, perivascular cuffing and interstitial mononuclear inflammatory infiltration.

Clinically, the diseases caused by the three viruses are very similar. They vary in severity from mild signs of fever and depression to a fatal febrile encephalomyelitis. The case fatality rate is highest for EEE at 90%. It is 50–80% for VEE and 20–40% for WEE. Recovered horses are usually normal but a few may have residual nervous signs.

Diagnosis

Clinical signs and associated history of previous cases in the same area at the same time of year may be suggestive but laboratory confirmation is usually required on account of the possibility of outbreaks occurring. Care must be taken in the collection and handling of specimens due to the possibility of serious human infection. Laboratory work with these viruses should only be carried out by immunized personnel in biosafety cabinets following containment level 3 procedures.

- The definitive diagnostic technique is virus isolation. Whole blood or serum are suitable specimens during the pyrexic phase. Brain or cerebrospinal fluid can be collected from horses that have died following nervous disease. EEEV can frequently be isolated from the brain of dead horses, however WEEV is rarely isolated. VEEV is recovered less frequently from the blood or brain tissue of encephalitic animals and isolation is best attempted using the blood or serum of febrile animals at an early stage of infection. Isolation is carried out in infant mice, in embryonated hens' eggs or in cell culture such as Vero, RK-13, BHK-21 or C6/36 (mosquito) cells. EEEV and WEEV produce a cytopathic effect in cell

culture. Virus can be identified using the complement fixation test or by immunofluoresence. Isolates of VEEV should be typed in a reference laboratory to distinguish virulent from non-virulent subtypes by the use of monoclonal antibodies or by nucleic acid sequencing (Sanchez-Seco et al. 2001).

- Reverse transcription-PCR protocols have been developed for the detection of viral RNA in tissues of affected animals and in mosquitoes (Pfeffer et al. 1997, Linssen et al. 2000).
- The detection of EEEV antigen in fixed brain sections using an immunohistochemical staining technique has been described (Patterson et al. 1996).
- Most cases of WEE and EEE are diagnosed by serology. Paired serum samples should be collected in order to demonstrate a rise in titre although horses in the acute stage of disease frequently already have antibody titres. Suitable testing methodologies include ELISA, plaque reduction neutralization assay, complement fixation and haemagglutination inhibition. Antibodies to WEEV and EEEV cross react in the CF and HI tests. Single serum samples have been tested using an IgM capture ELISA to provide evidence of recent infection (Sahu et al. 1994). The vaccination status of an animal must be considered when interpreting serological results. The serological diagnosis of VEEV is complicated by the possible presence of antibodies induced following infection with non-virulent subtypes.

REFERENCES

Carrara, A.S., Gonzales, M., Ferro, C., et al., 2005. Venezuelan equine encephalitis virus infection of spiny rats. Emerging Infectious Diseases 11, 663–669.

Linssen, B., Kinney, R.M., Aguilar, P., et al., 2000. Development of reverse transcription-PCR assays specific for detection of equine encephalitis viruses. Journal of Clinical Microbiology 38, 1527–1535.

Patterson, J.S., Maes, R.K., Mullaney, T.P., et al., 1996. Immunohistochemical diagnosis of eastern equine encephalomyelitis. Journal of Veterinary Diagnostic Investigation 8, 156–160.

Pfeffer, M., Proebster, B., Kinney, R.M., et al., 1997. Genus-specific detection of alphaviruses by a semi-nested reverse transcription polymerase chain reaction. American Journal of Tropical Medicine and Hygiene 57, 709–718.

Powers, A.M., Brault, A.C., Shirako, Y., et al., 2001. Evolutionary relationships and systematics of the alphaviruses. Journal of Virology 75, 10118–10131.

Sahu, S.P., Alstad, A.D., Pedersen, D.D., et al., 1994. Diagnosis of eastern equine encephalomyelitis virus infection in horses by immunoglobulin M and G capture enzyme-linked immunosorbent assay. Journal of Veterinary Diagnostic Investigation 6, 34–38.

Sanchez-Seco, M.P., Rosario, D., Quiroz, E., et al., 2001. A generic nested-RT-PCR followed by sequencing for detection and identification of members of the alphavirus genus. Journal of Virological Methods 95, 153–161.

Strauss, H.H., Strauss, E.G., 1994. The alphaviruses: gene expression, replication and evolution. Microbiological Reviews 58, 491–562.

Weaver, S.C., Bellew, L.A., Rico-Hesse, R., 1992. Phylogenetic analysis of alphaviruses in the Venezuelan equine encephalitis complex and identification of the source of epizootic viruses. Virology 191, 282–290.

Weaver, S.C., Salas, R., Rico-Hesse, R., et al., 1996. Re-emergence of epidemic Venezuelan equine encephalomyelitis in South America. Lancet 348, 436–440.

Weaver, S.C., Kang, W.L., Shirako, Y., et al., 1997. Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. Journal of Virology 71, 613–623.

Weaver, S.C., Powers, A.M., Brault, A.C., et al., 1999. Molecular epidemiological studies of veterinary arboviral encephalitides. Veterinary Journal 157, 123–138.

Weaver, S.C., Ferro, C., Barrera, R., et al., 2004. Venezuelan equine encephalitis. Annual Review of Entomology 49, 141–174.

Chapter 60

Orthomyxoviridae

The name orthomyxovirus is derived from two Greek words, *orthos* meaning correct or proper and *myxa* meaning mucus. The family includes the viruses responsible for influenza. Influenza is the Italian form of the Latin word *influentia* meaning influence because epidemics of infectious disease were believed to result from astrological or occult influences. Orthomyxoviruses are spherical or pleomorhic, enveloped viruses, 80–120 nm in diameter (Fig. 60.1). Extremely long filamentous forms also occur. The envelope is derived from the host cell membrane into which viral glycoproteins and non-glycosylated proteins have been incorporated. The glycoproteins form surface projections, termed 'spikes' or peplomers, and are of two types in influenza A and B viruses: a haemagglutinin (H) which is involved in virus attachment and envelope fusion and a neuraminidase (N) which enzymatically destroys viral receptors and promotes both entry and release of virus from infected cells. Influenza viruses haemagglutinate erythrocytes from a wide variety of species. Antibody to the H glycoprotein neutralizes infectivity. The nucleocapsid is helical in symmetry and the genome of linear, negative-sense, single-stranded RNA is segmented into six to eight pieces depending on the genus. Replication occurs in the nucleus of the cell with release by budding from the plasma membrane. Virions are very labile under ordinary environmental conditions, being sensitive to heat, lipid solvents, detergents, irradiation and oxidizing agents.

The family consists of five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* and *Isavirus* (Fig. 60.2). Influenza A virus is the most important member of the family and a significant pathogen of animals and man. Influenza B and C viruses are pathogens of man while Thogoto virus and Dhori virus are tick-borne arboviruses isolated from camels, cattle and humans in parts of Africa, Europe and Asia. The genus *Isavirus* has one member, infectious salmon anaemia virus, which is a cause of disease in Atlantic salmon.

Influenza A virus isolates are divided into subtypes on the basis of the H and N antigens. Seventeen H and nine N subtypes have been described to date (Fouchier et al. 2005). New variant influenza A virus isolates emerge at frequent intervals. Two mechanisms are involved in this process, point mutations (antigenic drift) which effect variation within a subtype and genetic reassortment (antigenic shift) which gives rise to novel subtypes. In order to assess the risk posed by the emergence of new variant viruses a precise system of classification of isolates, promoted by the World Health Organization, has been adopted. This system is based on the type, host, geographic origin, strain number, year of isolation and subtype. For example, the prototype equine influenza A virus subtype 1 isolate from 1956 is designated: influenza virus A/equine/Prague/1/56 (H7N7). Isolates with a wide range of H and N combinations have been detected in birds whereas only a few combinations circulate in mammalian species (Table 60.1). Aquatic birds, particularly ducks, are the reservoirs of influenza A virus providing a genetic bank for the generation of novel subtypes capable of infecting mammals. The viruses replicate in the intestinal tract of birds resulting in a faecal–oral transmission pattern. Migratory birds facilitate the dissemination of virus across borders and between continents. Influenza A virus isolates are generally species-specific, but there are many well-documented incidences of transfer between species. In particular, incidences of direct transfer of H7 and H5 subtypes from birds to humans have given rise to concern. Interest has also focused on low pathogenic H9N2 isolates (Lupiani and Reddy 2009). A new pandemic strain of H1N1 appeared in people in Mexico in 2009, spreading rapidly to other parts of the world. The virus contained

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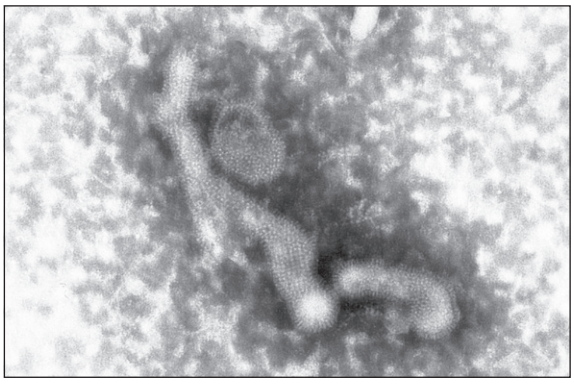

Figure 60.1 Electron micrograph of negatively stained particles of influenza A virus.

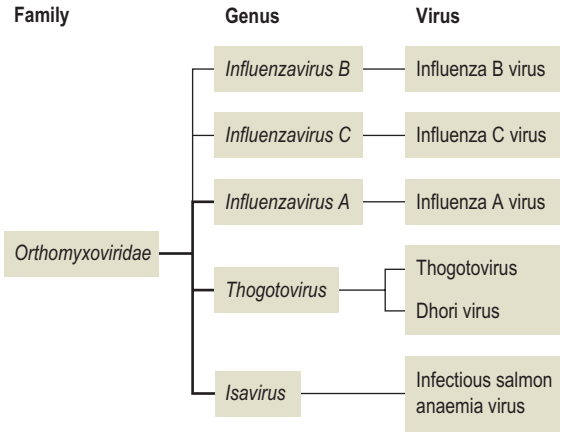

Figure 60.2 Classification of orthomyxoviruses.

genetic elements from avian, swine and human isolates. Pigs, the likely source of this virus, are susceptible to infection with this strain.

Interest has focused on southeast Asia due to the human pandemics in 1957 and 1967, referred to as 'Asian' and 'Hong Kong' influenza respectively, which arose in that part of the world. It is thought that the large human population present there and traditional agricultural practices which bring humans, ducks and pigs into close contact, facilitate mixed infections. Avian influenza viruses are capable of infecting humans but generally replicate poorly in humans. However, both human and avian influenza viruses replicate in pigs, which may serve as a 'mixing vessel' for genetic reassortment. Due to the segmented nature of the genome, mixed infections of influenza A virus subtypes frequently give rise to genetic reassortment. Novel subtypes generated in this way are responsible for the serious pandemics that occur in the human population at approximately 20-year intervals. There is little or no immunity in the human population to each new subtype which consequently spreads rapidly throughout the world, usually replacing the older subtype. In the period between pandemics more subtle antigenic changes occur probably due to the inherent error rate of the viral RNA polymerase and the accumulation of mutations. Variants produced in this way may become dominant if sufficient amino acid changes have occurred at antigenic sites of the H molecule, permitting escape from the effects of neutralizing antibody. Influenza outbreaks follow in the proportion of the population that is immunologically susceptible. Such outbreaks occur abruptly, typically in the winter months in temperate regions.

Table 60.1 Influenza A virus infections of veterinary significance

Host	Antigenic subtypes	Significance of infection
Birds	All subtypes, in most of the possible combinations of the 17 H and 9 N glycoproteins, have been isolated from birds. Highly pathogenic avian influenza (HPAI) isolates restricted to subtypes H5 and H7	Significance varies from inapparent intestinal tract infection to severe generalized infection with high mortality in chickens and turkeys (fowl plague). Wild birds, particularly ducks, act as reservoirs of infection
Pigs	Usually H1N1, H3N2 or H1N2 but other subtypes have also been described	Significant outbreaks of disease typically associated with emergence of new reassortant virus
Horses	H7N7, H3N8. The predominant circulating subtype is H3N8	Acute respiratory disease of young horses

AVIAN INFLUENZA

Influenza A virus subtypes have a worldwide distribution and are frequently recovered from clinically normal birds. Outbreaks of severe clinical disease accompanied by high mortality occur from time to time in chickens and turkeys, associated with H5 and H7 subtypes. The acute condition in these species is often referred to as fowl plague or highly pathogenic avian influenza (HPAI) and is designated as a listed disease by the OIE. Work with HPAI isolates should be carried out in facilities meeting OIE requirements for containment group 4 animal pathogens. In addition,

appropriate precautions should be taken to protect workers handling infectious material.

Infection is maintained in the avian population by low-level circulation within a large wild bird population. Waterfowl are considered the prime candidates for the spread of virus to domestic species during their migrations. Imported cage birds and live-bird markets are other possible sources of infection.

Pathogenesis

The ability of influenza virus to spread in the body is determined by the proteases present in a given tissue and the structure of the haemagglutinin molecule. The production of infectious virions in a tissue is dependent on post-translational cleavage of the precursor haemagglutinin molecule HA0 by host proteases. In the epithelium of the intestinal and respiratory tracts, trypsin and trypsin-like enzymes are capable of cleaving the haemagglutinin molecule of all influenza subtypes. In contrast, protease enzymes present in other tissues are only capable of cleaving the HA0 molecules of virulent subtypes which possess a multiple basic amino acid sequence at the cleavage site. Therefore only virulent subtypes are capable of producing generalized infection characterized by haemorrhages and multifocal necrosis. It is thought that HPAI isolates arise from low-virulence isolates by mutation and that such mutations only take place following the transfer of virus from the natural wild bird host to poultry (Alexander & Capua 2004).

Diagnosis

Clinical signs may vary from inapparent or mild to almost 100% mortality. The highly virulent subtypes cause sudden onset of high mortality. A wider range of clinical signs are seen in birds that survive for a few days. Respiratory signs, cessation of egg laying, greenish diarrhoea, oedema of the head, cyanosis, sinusitis and excessive lachrymation are frequent signs. The severe form of the disease could be confused with velogenic, viscerotropic Newcastle disease or fowl cholera while milder forms of the disease are indistinguishable clinically from other respiratory conditions. Diagnosis requires laboratory confirmation involving virus isolation and characterization.

- Suitable specimens include tracheal and cloacal swabs, faeces and pooled samples of organs. Suspensions in antibiotic solution are inoculated into nine- to 11-day-old specific pathogen-free or specific antibody-free embryonated hens' eggs. Allantoic fluid is harvested after four to seven days' incubation and tested for haemagglutinating activity.
- Confirmation of the presence of influenza A virus can be achieved with an immunodiffusion test using a suspension of chorioallantoic membrane from infected eggs and positive antiserum to the nucleocapsid or matrix antigens common to all influenza A viruses.
- Commercial antigen-detection immunoassays have been used to detect influenza A viruses in poultry (Slemons & Brugh 1998, Cattoli et al. 2004). The tests are rapid and should detect any influenza A virus as they are generally based on a monoclonal antibody against the conserved nucleoprotein. However, such assays are probably best used as a flock test as they may lack sensitivity.
- Isolates may be roughly typed by haemagglutination inhibition or immunodiffusion using broadly reactive antisera. Subtyping is carried out by reference laboratories using monospecific antisera prepared against the 16 haemagglutinin and nine neuraminidase subtypes.
- All of the highly pathogenic avian influenza isolates to date have possessed either H5 or H7. However, numerous isolations of low-virulence H5 and H7 subtypes have been made. In order to assess pathogenicity eight to 10 chicks at four to eight weeks of age are inoculated by the intravenous route. Isolates causing more than 75% mortality within eight days (intravenous pathogenicity index of greater than 1.2) are considered highly pathogenic. In addition, genomic sequencing can be used to determine the amino acid sequences at the cleavage site of the haemagglutinin molecule (Wood et al. 1993, Senne et al. 1996). Both HPAI and LPAI isolates of subtypes H5 and H7 are notifiable to the OIE.
- Reverse transcription-PCR techniques (Senne et al. 1996, Starick et al. 2000, Munch et al. 2001) as well as real time RT-PCR (Spackman et al. 2002) have been developed for the detection and subtype identification of virus in clinical samples. These methodologies are particularly useful for the rapid detection of subsequent outbreaks once the primary infected premises has been identified and the virus isolate fully characterized. Reverse transcription-PCR based on primers for conserved sequences of the matrix gene has proved useful for screening for all subtypes in samples from a range of different species (Fouchier et al. 2000). Rapid assays for the detection of H5 and H7 virus have also been developed (Slomka et al. 2007) including nucleic acid sequence-based amplification (NASBA) with electro-chemiluminescent detection (Collins et al. 2002, 2003).
- Serological testing for antibodies to influenza virus can be done using an agar gel immunodiffusion test, haemagglutination inhibition or competitive ELISA (Shafer et al. 1998). A neuraminidase inhibition test has been developed as part of a strategy to differentiate infected from vaccinated animals (DIVA) (Capua et al. 2003).

SWINE INFLUENZA

Swine influenza is a highly contagious, acute respiratory infection of pigs worldwide. Three subtypes are common in pigs, H1N1, H1N2 and H3N2. The H3N2 subtype crossed from humans to pigs in the early 1970s. In 1979 H1N1 isolates that were clearly distinguishable from 'classical' H1N1 isolates appeared in Europe. The haemagglutinins of these new isolates were more closely related to avian haemagglutinin. These avian-like swine H1N1 isolates are more pathogenic than the 'classical' H1N1 isolates, which they have largely replaced in Europe. In recent years further 'reassortant' viruses have been detected in pigs. An H1N2 subtype has been isolated from pigs with signs of acute respiratory disease in Japan (Ouchi et al. 1996) and the United Kingdom (Brown 1998). A H1N7 subtype derived from a human H1N1 virus and an equine H7N7 virus have also been isolated. Other subtypes identified in pigs include H3N1, H4N6 and H9N2.

Large quantities of virus are shed in the nasal secretions of infected pigs and spread is rapid within a herd. Outbreaks of disease are frequently described as explosive with abrupt onset and all pigs in a herd becoming ill almost simultaneously. Between outbreaks it is thought that virus circulates continuously in a herd without signs of disease and that some animals may be carriers for a number of months. Human infections with swine influenza virus can occur and laboratory work involving infectious material should be carried out in a class II biological safety cabinet.

Pathogenesis

Infection is limited to the respiratory tract. The severity of the illness varies from subclinical to acute and is strongly influenced by the strain of virus involved. Following aerosol infection the virus multiplies in nasal, tracheal and bronchial epithelium. Infection spreads throughout the respiratory tract resulting in cellular necrosis, consolidation, hyperaemia and the presence of exudates in the bronchi. Widespread alveolar atelectasis, interstitial pneumonia and emphysema ensue. Lesions are often limited to the apical and cardiac lung lobes. They continue to progress until about 72 hours post infection when virus replication diminishes and repair mechanisms commence, giving rise to proliferative lesions and alveolar epithelialization. Secondary bacterial infections frequently complicate the course of the infection. In most cases, after an illness of three to six days, pigs recover quickly.

Diagnosis

- Suitable samples for virus isolation include nasal mucus and lung tissue from early acute cases. Transport media and rapid transfer under moist, cool conditions to the laboratory are necessary due to the labile nature of the virus.
- Isolation is usually carried out in 10- to 11-day-old embryonated hens' eggs or in cell culture using Madin–Darby canine kidney (MDCK) cells or a primary porcine cell line. The allantoic fluid of inoculated eggs is tested for haemagglutinating activity after 72 hours. Similarly, the tissue culture medium from cell cultures displaying cytopathic effect can be tested for haemagglutinating activity.
- Additional methods for the detection of viral antigen or nucleic acid include immunofluorescence, immunohistochemistry (Vincent et al. 1997), ELISA (Lee et al. 1993b; Swenson et al. 2001), RT-PCR (Fouchier et al. 2000) and real time RT-PCR (Richt et al. 2004).
- A retrospective diagnosis can be made using the haemagglutination inhibition test or ELISA (Lee et al. 1993a). Paired serum samples collected two to three weeks apart can be used to demonstrate a four-fold rise in antibody levels to swine influenza virus.

EQUINE INFLUENZA

Equine influenza is an economically important, acute, respiratory disease of horses. It has a worldwide distribution except for Australia, New Zealand and Iceland. Two immunologically distinct subtypes of influenza A virus are described in horses. Influenza A virus was first isolated from horses in 1956 and designated A/equine/Prague/1/56 (H7N7) or A/equine 1. In 1963 the second subtype was isolated in America and designated A/equine/Miami/2/63 (H3N8) or A/equine 2. Infection or vaccination with one subtype will not confer protection against infection with the other. The last outbreak of disease attributed to A/equine 1 was in 1979 although there is serological evidence that this subtype still circulates. Antigenic drift has given rise to several genetic variants of A/equine 2 with two antigenically and genetically distinct lineages of the H3N8 subtype evolving in Europe and in the Americas (Oxburgh et al. 1998). In contrast the H3N8 subtype that appeared in horses in China in 1989 was much more avian-like than the H3N8 subtype circulating in horses elsewhere in the world and was probably transmitted from birds to horses. Outbreaks of respiratory disease in greyhounds in the USA have been associated with viral isolates closely related to H3N8 equine influenza virus (Crawford et al. 2005).

Disease outbreaks are associated with movement and assembly of horses. Equine influenza is highly contagious, spreading rapidly among susceptible horses. Large quantities of virus are aerosolized by the frequent coughing of affected animals. Humans can become infected with

equine influenza virus but such infections are uncommon and subclinical.

Pathogenesis

The incubation period is short, about one to two days. The virus replicates in the epithelium of the upper and lower respiratory tract resulting in inflammation, destruction of ciliated epithelium and hypersecretion by serous submucosal glands. Affected animals develop a high temperature, nasal discharge and frequent dry cough. Anorexia and depression are common but variable in degree. Eye discharge, limb oedema and stiffness may also be seen.

Diagnosis

The clinical signs can be suggestive but laboratory confirmation should be obtained.

- Nasopharyngeal swabs are suitable specimens for the isolation of virus in embryonated eggs and in cell culture (MDCK cells). The swabs should be collected during the acute phase of the infection. The use of a viral transport medium and rapid transit to the laboratory is required for this labile virus. The monitoring of new isolates is important for the detection of antigenic drift.
- The use of antigen-capture ELISA for the detection of H3N8 virus has been described (Cook et al. 1988, Chomel et al. 1989, Livesay et al. 1993). A commercial human diagnostic kit for the detection of influenza A viral nucleoprotein has been used successfully for the diagnosis of equine influenza (Chambers et al. 1994). However, it does not appear to be particularly sensitive (Quinlivan et al. 2004).
- Demonstration of viral nucleic acid can be achieved using RT-PCR (Donofrio et al. 1994, Oxburgh & Hagstrom, 1999, Fouchier et al. 2000) and real-time RT-PCR (Quinlivan et al. 2005, Lu et al. 2009).
- Serological diagnosis of equine influenza is possible using paired sera and haemagglutination inhibition (HI) or single radial haemolysis (Wood et al. 1983). Pretreatment of sera is required to remove non-specific inhibitors for the HI test.

REFERENCES

Alexander, D., Capua, I., 2004. Avian influenza. State Veterinary Journal 14, 3–8.

Brown, I., 1998 Swine influenza – a disease of increasing importance? State Veterinary Journal 8, 2–4.

Capua, I., Terregino, C., Cattoli, G., et al., 2003. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathology 32, 47–55.

Cattoli, G., Drago, A., Maniero, S., et al., 2004. Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. Avian Pathology 33, 432–437.

Chambers, T.M., Shortridge, K.F., Li, P.H., et al., 1994. Rapid diagnosis of equine influenza by the Directigen FLU–A enzyme immunoasay. Veterinary Record 135, 275–279.

Chomel, J.J., Thouvenot, D., Onno, M., et al., 1989. Rapid diagnosis of influenza infection of NP antigen using an immunocapture ELISA test. Journal of Virological Methods 25, 81–91.

Collins, R.A., Ko, L.S., So, K.L., et al., 2002. Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. Journal of Virological Methods 103, 213–225.

Collins, R.A., Ko, L.S., Fung, K.Y., et al., 2003. Rapid and sensitive detection of avian influenza virus subtype H7 using NASBA. Biochemical and Biophysical Research Communications 300, 507–515.

Cook, R.F., Sinclair, R., Mumford, J.A., 1988. Detection of influenza nucleoprotein antigen in nasal secretions from horses infected with A/equine influenza (H3N8) viruses. Journal of Virological Methods 20, 1–12.

Crawford, P.C., Dubovi, E.J., Castleman, W.L., et al., 2005. Transmission of equine influenza virus to dogs. Science 310, 482–485.

Donofrio, J.C., Coonrod, J.D., Chambers, T.M., 1994. Diagnosis of equine influenza by the polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 6, 39–43.

Fouchier, R.A., Bestebroer, T.M., Herfst, S., et al., 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. Journal of Clinical Microbiology 38, 4096–4101.

Fouchier, R.A.M., Munster, V., Wallensten, A., et al., 2005. Characterization of a novel influenza a virus hemagglutinin subtype H16 obtained from black-headed gulls. Journal of Virology 79, 2814–2822.

Lee, B.W., Bey, R.F., Baarsch, M.J., et al., 1993a. Subtype specific ELISA for the detection of antibodies against influenza A H1N1 and H3N2 in swine. Journal of Virological Methods 45, 121–136.

Lee, B.W., Bey, R.F., Baarsch, M.J., et al., 1993b. ELISA method for detection of influenza A infection in swine. Journal of Veterinary Diagnostic Investigation 5, 510–515.

Livesay, G.J., O'Neill, T., Hannant, D., et al., 1993. The outbreak of equine influenza (H3N8) in the United Kingdom in 1989; diagnostic use of an antigen capture ELISA. Veterinary Record 133, 515–519.

Lu, Z., Chambers, T.M., Boliar, S., et al., 2009. Development and evaluation of one-step Taqman real-time reverse transcription-PCR assays targeting

nucleoprotein, matrix, and hemagglutinin genes of equine influenza virus. Journal of Clinical Microbiology 47, 3907–3913.

Lupiani, B., Reddy, S.M., 2009. The history of avian influenza. Comparative Immunology. Microbiology and Infectious Diseases 32, 311–323.

Munch, M., Nielsen, L., Handberg, K., et al., 2001. Detection and subtyping H5 and H7 of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. Archives of Virology 146, 87–97.

Ouchi, A., Nerome, K., Kanege, Y., et al., 1996. Large outbreak of swine influenza in southern Japan caused by reassortant H1N2 influenza viruses: its epizootic background and characterization of the causative viruses. Journal of General Virology 77, 1751–1759.

Oxburgh, L., Akerblom, L., Fridberger, T., et al., 1998. Identification of two antigenically and genetically distinct lineages of H3N8 equine influenza virus in Sweden. Epidemiology & Infection 120, 61–70.

Oxburgh, L., Hagstrom, A.A., 1999. A PCR based method for the identification of equine influenza virus from clinical samples. Veterinary Microbiology 67, 161–174.

Quinlivan, M., Cullinane, A., Nelly, M., et al., 2004. Comparison of sensitivities of virus isolation, antigen detection and nucleic acid amplification for the detection of equine influenza virus. Journal of Clinical Microbiology 42, 759–763.

Quinlivan, M., Dempsey, E., Ryan, F., et al., 2005. Real-time reverse transcription PCR for detection and quantitative analysis of equine influenza virus. Journal of Clinical Microbiology 43, 5055–5057.

Richt, J.A., Lager, K.M., Clouser, D.F., et al., 2004. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. Journal of Veterinary Diagnostic Investigation 16, 367–373.

Senne, D.A., Panigrahy, B., Kawaoka, Y., et al., 1996. Survey of the haemagglutinin HA cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the cleavage site as a marker of pathogenicity potential. Avian Diseases 40, 425–437.

Shafer, A.L., Katz, J.B., Eernisse, K.A., 1998. Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Diseases 42, 28–34.

Slemons, R.D., Brugh, M., 1998. Rapid antigen detection as an aid in early diagnosis and control of avian influenza. In: Swayne, D.E., Slemons, R.D. (Eds.), Proceedings of the Fourth International Symposium on Avian Influenza. United States Animal Health Association, Athens, Georgia, USA, pp. 313–317.

Slomka, M.J., Coward, V.J., Banks, J., et al., 2007. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. Avian Diseases 51, 227–234.

Spackman, E., Senne, D.A., Myers, T.J., et al., 2002. Development of a real-time reverse transcriptase PCR asssy for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40, 3256–3260.

Starick, E., Romer-Oberdorfer, A., Werner, O., 2000. Type- and subtype-specific RT-PCR assays for avian influenza viruses. Journal of Veterinary Medicine B 47, 295–301.

Swenson, S.L., Vincent, L.L., Lute, B.M., et al., 2001. A comparison of diagnostic assays for the detection of type A swine influenza virus from nasal swabs and lungs. Journal of Veterinary Diagnostic Investigation 13, 36–42.

Vincent, L.L., Janke, B.H., Paul, P.S., et al., 1997. A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation 9, 191–195.

Wood, G.W., McCauley, J.W., Bashiruddin, J.B., et al., 1993. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Archives of Virology 130, 209–217.

Wood, J.M., Mumford, J.A., Folkers, C., et al., 1983. Studies with inactivated equine influenza vaccine 1: Serological responses of ponies to graded doses of vaccine. Journal of Hygiene 90, 371–384.

FURTHER READING

Alexander, D.J., 2008. Avian Influenza – Diagnosis. Zoonoses and Public Health 55, 16–23.

Chapter 61

Paramyxoviridae

Orthomyxoviruses and paramyxoviruses were originally grouped together and known as the 'myxoviruses'. The name paramyxovirus is derived from two Greek words, *para* meaning by the side of and *myxa* meaning mucus. Paramyxoviruses are pleomorphic though usually spherical in shape, 150 to 200 nm in diameter and enveloped (Fig. 61.1). The envelope is covered in glycoprotein spikes of two types, an attachment protein (G or H or HN) and a fusion protein (F). These glycoproteins are responsible for interaction with host cell surface receptors and for fusion of host cell membrane and virus envelope, respectively. They are of primary importance in eliciting virus-neutralizing antibodies and inducing immunity to reinfection. There is also an envelope-associated non-glycosylated matrix (M) protein. Paramyxoviruses can possess haemagglutinating, haemolytic and neuraminidase activities. The nucleocapsid has helical symmetry, is non-segmented, 13 to 18 nm in diameter, has a characteristic 'herringbone' appearance and has a single molecule of linear, negative-sense, single-stranded RNA. Replication occurs in the cell cytoplasm with release by budding from the plasma membrane at sites containing virus envelope proteins. Virions are labile, being sensitive to heat, desiccation, lipid solvents, non-ionic detergents and many disinfectants.

The families *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae* and *Bornaviridae* contain enveloped viruses with genomes consisting of a single molecule of negative-sense, single-stranded RNA and together form the order *Mononegavirales*. The family *Paramyxoviridae* has undergone several taxonomic changes including the creation of three new genera, *Metapneumovirus*, *Henipavirus* and *Avulavirus* and the renaming of the genus *Paramyxovirus* as *Respirovirus*. The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae* (Fig. 61.2). Members of the *Paramyxovirinae* are usually capable of haemagglutination and have been grouped in five genera; *Respirovirus*, *Rubulavirus, Henipavirus, Avulavirus* and *Morbillivirus*. The subfamily *Pneumovirinae* contains two genera; *Pneumovirus* and *Metapneumovirus*. Paramyxoviruses are genetically stable and do not undergo recombination. The only mechanism permitting some degree of variation is mutation.

Paramyxoviruses infect vertebrate hosts, mainly mammals and birds, and usually have a narrow host range (Table 61.1). Transmission occurs via close contact or aerosol. Primary replication typically occurs in the respiratory tract. Infection is generally cytolytic but temperate and persistent infections also occur. Syncytium formation and intracytoplasmic, acidophilic inclusions are characteristic features of infection. Morbilliviruses are capable of inducing intranuclear, acidophilic inclusions. The family contains several members responsible for the production of serious disease in animals and man. Paramyxoviruses have been shown to be of significance in wild animal populations. A number of new morbilliviruses including phocine distemper virus and cetacean morbillivirus have been discovered in marine mammals. Hendra virus was isolated following an outbreak of severe respiratory disease in horses in a stable in Brisbane, Australia, in 1994. Two in-contact humans were also affected. Fourteen horses and their trainer died. In 1999 a related virus, Nipah virus, was isolated in Malaysia following large outbreaks of disease in pigs and their human handlers. More than 100 human deaths due to a febrile encephalitic illness were recorded. Both viruses are capable of infecting cats and dogs. Human-to-human spread does not appear to occur. Fruit bats of the genus *Pteropus* are the natural hosts of both viruses.

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RINDERPEST

Rinderpest or cattle plague is an acute disease of cloven-hoofed animals that has been recognized for many centuries as a devastating disease of cattle and domestic buffalo. Epizootics of the disease in Europe provided the catalyst for the setting up of veterinary schools and state veterinary services as well as the foundation of the Office International des Epizooties (OIE), also known as the World Organization for Animal Health. It is classed as a listed disease by the OIE. A global eradication scheme by the Food and Agriculture Organization (FAO) of the United Nations (UN), begun in 1994, was officially declared a success in 2011. A number of factors made this a realistic goal including a single serotype, an excellent vaccine that confers lifelong immunity, good diagnostic tests, and the absence of a carrier state. On the basis of genetic sequencing, rinderpest virus isolates can be grouped into three distinct evolutionary lineages: African lineages 1 and 2 and Asian lineage 3. Towards the end of the eradication campaign, two reservoir sites of infection, the Indus River buffalo tract in Pakistan and the Somali pastoral ecosystem of southern Somalia and northern Kenya, were recognized (Roeder & Taylor 2002). The last clinical case was recorded in Kenya in 2001.

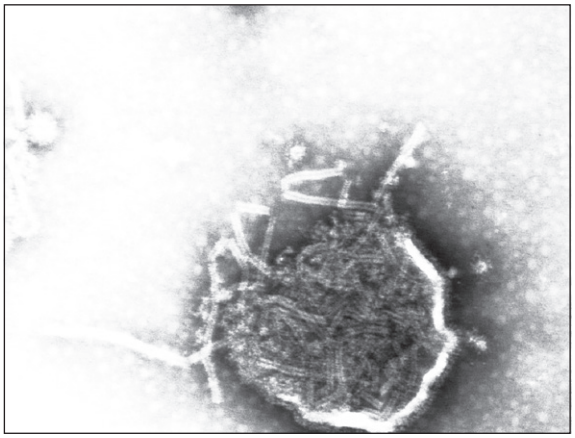

Figure 61.1 Electron micrograph of negatively stained particles of a paramyxovirus.

Domestic cattle and buffalo and several wildlife species including giraffe, warthog, buffalo and eland are highly susceptible. Virus is shed in all secretions and excretions, beginning a few days before the appearance of clinical signs. In endemic areas the disease tends to be mild and typically restricted to young cattle following the loss of maternal immunity. It is believed that the virus may undergo changes in virulence. A mild form of disease in cattle has been described associated with low-virulence strains of virus. In susceptible populations with multiple

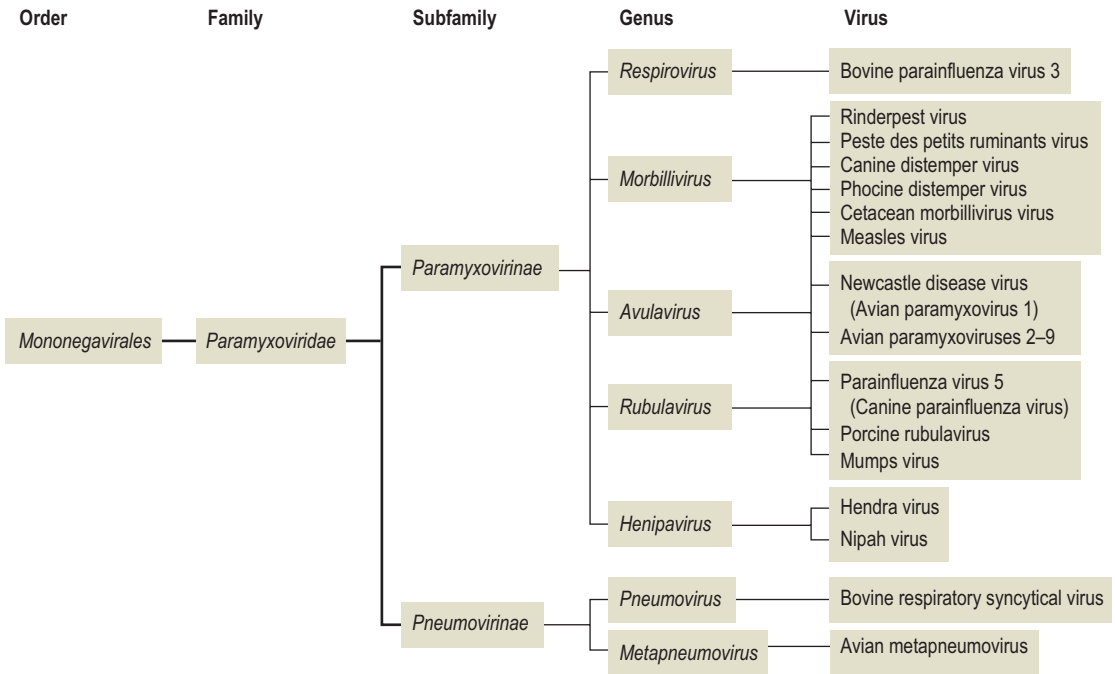

Figure 61.2 Classification of members of the family *Paramyxoviridae* of veterinary significance.

Table 61.1 Paramyxoviruses of veterinary significance

Virus	Host species	Disease
Rinderpest virus	Cloven-hoofed animals, particularly cattle and domestic buffalo	Highly contagious disease responsible for devastating outbreaks characterized by high morbidity and high mortality. First animal virus to be eradicated globally
Peste des petits ruminants virus	Small ruminants, particularly goats	Severe disease with many similarities to rinderpest. High morbidity and mortality rates seen in epizootics in goats. OIE listed disease
Bovine parainfluenza virus 3	Cattle, sheep	Produces subclinical or mild respiratory disease. Associated with shipping fever. Predisposes to secondary bacterial infection, particularly *Mannheimia haemolytica*. Worldwide distribution
Bovine respiratory syncytial virus	Cattle, sheep, goats	Common infection in cattle, worldwide. Associated with moderate to severe respiratory disease outbreaks in young cattle
Porcine rubulavirus	Pigs	Cause of blue eye disease. Only described in Mexico to date
Canine distemper virus	Wide range of domestic and wild carnivores	Worldwide, acute infection of dogs characterized by multisystemic involvement and moderate mortality
Parainfluenza virus 5 (Canine parainfluenza virus)	Dogs	Inapparent or mild respiratory disease in dogs. May be involved in kennel cough outbreaks
Feline morbillivirus (FmoPV)	Cats	Newly discovered virus of cats. Possible association with tubulointerstitial nephritis
Newcastle disease virus (Avian paramyxovirus 1)	Wide range of domestic and wild bird species including chickens, turkeys, pigeons, pheasants and waterfowl	Cause of Newcastle disease. Varying virulence isolates designated velogenic, mesogenic and lentogenic. Generalized infection characterized by respiratory, intestinal and/or nervous signs. OIE listed disease
Avian metapneumovirus	Turkeys, chickens	Associated with coryza in turkeys and swelling of sinuses (swollen head syndrome) in chickens

transmission opportunities the virus may regain its virulence for cattle. Epizootics result from the movement of animals and the introduction of an infected animal to susceptible animals. During an outbreak all ages of animals are affected, morbidity may be as high as 90% and mortality can approach 100%.

Pathogenesis

After entry via the nasopharynx, primary multiplication occurs in the pharyngeal and mandibular lymph nodes. Viraemia occurs within two to three days with further proliferation in lymphoid tissues, lungs and mucosa of respiratory and digestive tracts. Affected animals develop a fever and become anorexic and depressed from three to nine days after infection. Erosions appear within two to five days on the mucous membranes of the mouth and nasal passages. Salivation is profuse and there is a mucopurulent oculonasal discharge. Two to three days after the appearance of the mucosal erosions the fever begins to regress and a profuse diarrhoea appears. The faeces are dark brown, very fluid and contain excess mucus, necrotic debris and streaks of blood. There is rapid dehydration and wasting. Leukopenia and immunosuppression occur due to destruction of lymphoid tissue, secondary infections and reactivation of protozoal infections may result. Virus continues to be shed throughout the febrile period and for a few days after the fever regresses. Severely affected animals collapse and die during the diarrhoeic phase, about six to 12 days after the onset of illness.

Diagnosis

In enzootic areas or following the laboratory confirmation of an epizootic the clinical and pathological findings may be sufficient for a diagnosis. However, in regions where rinderpest is uncommon or absent laboratory confirmation is required to differentiate the condition from conditions such as bovine viral diarrhoea and malignant catarrhal fever. A pen-side test based on a rapid chromatographic strip test has been reported to be a useful tool for field personnel investigating outbreaks (Bruning et al.

1999). Specimens should be collected from several febrile animals early in the disease.

- Lesions are characteristically found throughout the alimentary tract. The longitudinal folds of the colonic and rectal mucosae often appear highly engorged with bloody streaks creating the appearance of 'zebra striping'. The base of the tongue, retropharyngeal lymph node and third eyelid are suitable tissues for histopathology and immunohistochemistry. Syncytia and intranuclear inclusion bodies may be evident in sections. Rinderpest virus antigens can be demonstrated using immunohistochemical staining.
- Suitable specimens for isolation of virus include the buffy coat from uncoagulated blood collected from pyrexic animals. Alternatively, 20% suspensions (w/v) of spleen, prescapular or mesenteric lymph nodes may be used. Specimens should be kept cool on ice and not frozen, followed by rapid transport to the laboratory. The use of glycerol as a preservative should be avoided as it inactivates the virus. Suitable cell lines include primary calf kidney and Vero cells. Rinderpest virus produces cytopathic effects in cell culture and its presence can be confirmed by immunofluorescence.
- Agar gel immunodiffusion or a counter-immunoelectrophoresis test are suitable as simple, rapid antigen detection assays (Foreman et al. 1983). The test is carried out at 4°C using anti-rinderpest rabbit hyperimmune serum. Suitable specimens include ocular secretions and exudates from the cut surface of spleen or lymph nodes. The test is not particularly sensitive or specific but is robust and easy to perform.
- A reverse transcription-polymerase chain reaction (RT-PCR) method capable of detecting and differentiating between rinderpest virus and peste des petits ruminants virus has been developed (Forsyth & Barrett 1995). Three primer sets are required; two 'universal' sets based on conserved regions of the phosphoprotein and nucleoprotein genes of morbilliviruses and a rinderpest virus-specific primer set based on fusion protein gene sequences.
- An immunocapture ELISA has been described for the rapid differentiation of rinderpest virus and peste des petits ruminants virus (Libeau et al. 1994).
- A competitive ELISA for the detection of serum antibodies to rinderpest virus has been developed and adopted by the OIE as the prescibed test for international trade (Anderson et al. 1991). The test is based on competition between antibodies in positive sera and a rinderpest anti-H protein monoclonal antibody for binding to rinderpest antigen. The virus neutralization test (Plowright & Ferris 1961) is considered the 'gold standard' serological test but is not as easy or cheap to perform (Roeder & Taylor 2002).

PESTE DES PETITS RUMINANTS

Peste des petits ruminants or goat plague is an acute contagious disease of small ruminants, particularly goats. It is caused by peste des petits ruminants virus (PPRV), closely related to other members of the morbillivirus genus. Isolates of PPRV can be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion protein gene (Dhar et al. 2002). The disease occurs in sub-Saharan Africa north of the equator, the Middle East, India and Pakistan. In West Africa, epizootics are associated with the onset of the rains when flocks are gathered together and surplus kids are sold. Infection rates are similar in sheep and goats but disease is generally more severe in goats. It is an OIE listed disease.

Pathogenesis

The incubation period is usually four to six days. The pathogenesis of the infection is similar to rinderpest with erosive mucosal and diarrhoeic phases. Virus is shed in all secretions and excretions. A severe leukopenia occurs facilitating secondary infections. Bronchopneumonia often develops in the later stages associated with *Mannheimia* or *Pasteurella* species infection. Pregnant animals may abort. Most acutely affected animals die within 10 days. Mortality rates in severe outbreaks typically range from 70 to 80%. Sheep tend to develop a subacute infection characterized by fever, nasal catarrh, mucosal erosions and intermittent diarrhoea. Affected animals usually recover after 10–14 days.

Diagnosis

A tentative diagnosis of PPR may be made based on clinical signs; however, laboratory confirmation is important in regions where rinderpest is also present.

- Specimens for laboratory confirmation should be obtained during the acute phase of the disease and include nasal and ocular swabs, unclotted blood and scrapings of buccal and rectal mucosae. Pieces of lung, spleen, mesenteric and bronchial lymph nodes and intestinal mucosae may also be collected from animals killed early in the course of the infection.
- Rapid antigen detection methods include immunocapture ELISA (Libeau et al. 1994), counter-immunoelectrophoresis and agar gel immunodiffusion. A rapid and inexpensive haemagglutination assay has been described for PPRV (Wosu 1991).

- Specific primers, designed to amplify the fusion and nucleocapsid protein genes (Forsyth & Barrett 1995, Couacy-Hymann et al. 2002), have been successfully applied in the RT-PCR technique. This technique has been shown to be very sensitive.
- Virus isolation may be carried out in primary lamb kidney or African green monkey kidney (Vero) cell lines. Blind passages may be required before CPE, consisting of cell rounding, aggregation and syncytia formation, becomes evident.
- Serological detection of antibodies can be carried out by virus neutralization or competitive ELISA (Libeau et al. 1995).

BOVINE PARAINFLUENZA VIRUS 3 DISEASE

Infection with bovine parainfluenza virus 3 (BPIV-3) is common in cattle around the world. Uncomplicated infections are frequently subclinical although mild respiratory disease may be seen. However, the virus is commonly isolated from more serious respiratory disease outbreaks, including enzootic calf pneumonia and shipping fever, along with other respiratory viruses and bacteria following various stressful incidences such as transportation, overcrowding or adverse environmental conditions.

Pathogenesis

The virus replicates in ciliated epithelium of the respiratory tract, alveolar epithelium and macrophages. The virus has been shown to cause destruction of ciliated epithelium, resulting in interference with the mucociliary clearance mechanism. In addition, there is depressed phagocytosis and bacterial killing by alveolar macrophages. Such changes predispose to secondary pulmonary bacterial colonization.

Diagnosis

Virus isolation can be attempted from nasal swabs or lung tissue in suitable bovine cell lines such as bovine kidney or lung. The virus produces cytopathic effects including syncytia, cell destruction and inclusion bodies. Samples should be taken from several animals early in an outbreak of respiratory disease and transported quickly to the laboratory in viral transport medium. Virus remains viable for longer in lung tissue than in nasal secretions. Direct immunofluorescence for the detection of viral antigen can also be carried out on nasal mucus samples or on cryostat sections of lung. Serological tests commonly used for the demonstration of a four-fold rise in antibody titre between acute and convalescent sera include haemagglutination inhibition, virus neutralization, ELISA and indirect immunofluorescence.

BOVINE RESPIRATORY SYNCYTIAL VIRUS

Bovine respiratory syncytial virus (BRSV), named after the characteristic syncytia produced in infected cells, is an important cause of respiratory disease in beef and dairy calves worldwide. Clinical signs in calves vary from mild to severe respiratory disease. Affected animals are typically three to nine months old. Infection occurs in cattle, sheep and goats. There is evidence from studies of the G surface glycoprotein of the virus that two subgroups exist in ruminants comprising isolates from cattle and goats and isolates from sheep respectively (Alansari et al. 1999).

Pathogenesis

The virus replicates primarily in ciliated epithelium in the respiratory airways giving rise to destruction of infected cells and a characteristic necrotizing bronchiolitis. Infection of type II pneumocytes may occur, resulting in pneumocyte hyperplasia, alveolar epithelialization and interstitial pneumonia. Up-regulation of pro-inflammatory cytokines occurs in the lung and it has been suggested that a lot of the pathology is due to the host's response to the viral infection (Valarcher & Taylor 2007). Secondary bacterial infection is common. The accumulation of cellular debris and exudate enhances bacterial proliferation. It is also thought that BRSV is immunosuppressive. The pathogenesis of the oedema and emphysematous lung lesions that occur in severe BRSV infections is unclear. It has been suggested that hypersensitivity reactions may play a role and account for similarities with cases of atypical interstitial pneumonia.

Diagnosis

Clinical signs and pathological findings may be sufficient to permit a presumptive diagnosis. Characteristic histopathological lung changes include bronchointerstitial pneumonia with severe bronchiolitis. The cranial and apical lobes are most severely affected. Emphysema and alveolar oedema are present in all lung lobes but are particularly prominent in the diaphragmatic lobe. Histopathology frequently reveals multinucleated cells with eosinophilic intracytoplasmic inclusions but such changes may also occur following BPIV-3 infection. Laboratory confirmation is required for a definitive diagnosis. Suitable specimens for testing include nasal swabs, bronchoalveolar lavage fluid, lung tissue and paired sera.

- The virus is thermolabile and must be transported rapidly in suitable transport medium to the laboratory. Samples should be collected from several animals in a group. Isolation is not routinely attempted as it is difficult, requiring several blind passages in suitable cell culture such as calf kidney or testicle cell culture.
- Commercial ELISA kits for the detection of viral antigen are available. Immunofluoresence has also been shown to be a rapid and useful technique. Improved rates of detection are obtained using lower respiratory tract specimens rather than nasal swabs.
- Amplification of the nucleoprotein gene (Valarcher et al. 1999) or the fusion protein gene (Larsen et al. 1999) by RT-PCR has been used to diagnose BRSV. A real time RT-PCR assay has been described (Willoughby et al. 2008).
- Serological tests such as virus neutralization and ELISA are suitable for the demonstration of a rising antibody titre. However, the acute serum sample needs to be obtained early in the infection as serum antibody levels rise rapidly.

CANINE DISTEMPER

Canine distemper is a highly contagious, acute or subacute viral infection of dogs and other carnivores. It has a worldwide distribution. Canine distemper virus (CDV) is pantropic producing a generalized infection involving the alimentary, respiratory and central nervous systems. The host range of CDV is extremely wide including members of the families *Canidae, Ailuridae, Hyaenidae, Mustelidae, Procyonidae, Ursidae, Viverridae* and *Felidae*. Disease outbreaks have been documented in several wildlife species including foxes, skunks, racoons, black-footed ferrets and lions (Appel & Summers 1995, Roelke-Parker et al. 1996). Infection spreads rapidly among unvaccinated, young dogs, usually at about three to six months of age, when maternal immunity wanes. Modified live vaccines are available commercially and generally provide excellent protection when administered to animals lacking interfering maternal antibody. Post vaccinal encephalitis has been reported occasionally. Some live CDV vaccines have been shown to produce disease in wildlife species that are particularly susceptible to CDV infection.

Pathogenesis

The incubation period is usually about one week but may last four or more weeks. The virus replicates locally in the upper respiratory tract spreading to the tonsils and bronchial lymph nodes. A cell-associated viraemia follows, resulting in spread to lymphoreticular tissues. Viral replication results in lymphocytolysis and leukopenia giving rise to immunosuppression. The secondary viraemia that follows and the extent of spread to target tissues is affected by the rapidity and effectiveness of the immune response. Dissemination and replication of the virus will occur in the respiratory, gastrointestinal, urinary and central nervous systems if the immune response is not sufficiently vigorous. Spread to the skin also occurs. The virus infects neurons and glial cells in the CNS and signs of nervous disease are usually seen one to three weeks after the appearance of the generalized illness. Nervous signs may not appear until several weeks or months later and may develop without preceding clinical disease. Old dog encephalitis, a rare progressive neurological condition of mature dogs, is associated with persistence of CDV in the CNS. It is analogous to a rare condition of man, subacute sclerosing panencephalitis, which is associated with persistent measles virus infection of the CNS. Prolonged persistence of CDV in the CNS is possible due to selective non-cytolytic viral spread from cell to cell without budding thus delaying detection by the immune system (Stettler et al. 1997). The presence of persistent viral antigen stimulates an anti-viral inflammatory response leading to severe, progressive neurological disease.

Diagnosis

Young dogs presenting with a febrile, catarrhal illness that has neurological sequelae are highly suggestive of canine distemper.

- Viral antigen may be demonstrable in conjunctival or vaginal impression smears or in buffy coat smears by immunofluorescence for up to three weeks following infection. An ELISA has been used to detect CDV antigen in serum (Soma et al. 2003). In animals that have died, cryostat sections of lymph nodes, urinary bladder and cerebellum are suitable for immunofluorescence.
- Reverse transcription-polymerase chain reaction (RT-PCR) has been shown to be suitable for the detection of CDV RNA in clinical samples (Shin et al. 1995, Frisk et al. 1999, Kim et al. 2001). A nested PCR protocol has been shown to be more sensitive than the one-step protocol (Shin et al. 2004, Jozwik & Frymus 2005), while a semi-nested RT-PCR has been developed for the retrospective diagnosis of CDV infection using formalin-fixed, paraffin-embedded tissue blocks (Stanton et al. 2002). A real-time RT-PCR protocol has been reported (Elia et al. 2006). It is possible to identify seven major genetic lineages; America-1 and -2, Asia-1 and -2, European-wildlife, Europe and Arctic-like, based on sequencing of the H gene (Martella et al. 2007, McCarthy et al. 2007). This information is helpful in distinguishing field and vaccine (America-1 lineage) CDV strains.

- Demonstration of eosinophilic inclusions in lymphoid, nervous or epithelial tissues by histopathology is reliable but time-consuming.
- Serological demonstration of virus-specific IgM or of a fourfold rise in titre between acute and convalescent sera may be carried out using virus neutralization, ELISA (von Messling et al. 1999) or indirect immunofluorescence. The presence of antibody in cerebrospinal fluid is usually confirmatory.
- Virus isolation is not routinely attempted due to the time required and technical difficulties. Suitable specimens include the buffy coat from heparinized blood in the live animal and bladder and brain post mortem.

CANINE PARAINFLUENZA VIRUS

Originally isolated from monkey cells this virus is also referred to as simian virus 5 or parainfluenza virus 5. It was subsequently found to be widespread in dog populations and associated with self-limiting, mild respiratory disease. Canine parainfluenza virus (CPIV) is frequently isolated along with other infectious agents in outbreaks of kennel cough. Diagnosis of CPIV is based on detection of the agent in nasal or throat secretions by virus isolation or RT-PCR (Erles et al. 2004). Demonstration of a rising antibody titre by virus neutralization, haemagglutination inhibition or ELISA is a useful aid to diagnosis.

AVIAN PARAMYXOVIRUSES

Avian paramyxovirus (APMV) isolates have been reported from a wide range of domestic and wild birds from around the world. Currently, nine species of avian paramyxoviruses are recognized in the genus *Avulavirus*. Each species is antigenically distinct with new isolates assigned to a species on the basis of their antigenic relatedness in haemagglutination inhibition tests. By far the most important member is Newcastle disease virus (avian paramyxovirus 1). The other avian paramyxoviruses have generally been associated with only mild or inapparent disease in wild and domestic birds. Respiratory disease in turkeys has been associated with APMV-2 and APMV-3 infections.

NEWCASTLE DISEASE

Newcastle disease is one of the most important diseases of poultry worldwide. It was first recognized in 1926 following large outbreaks in Java and in Newcastle, England. It is caused by Newcastle disease virus (avian paramyxovirus 1) and is an OIE listed disease. Three panzootics are considered to have occurred since the disease was first recognized. The first arose in southeast Asia in 1926, the second began in the Middle East in the late 1960s and the third, primarily affecting pigeons, started in the Middle East in the late 1970s. A wide range of domestic and wild bird species are susceptible to infection including chickens, turkeys, pigeons, pheasants, ducks and geese. Numerous strains of varying virulence exist. Isolates can be broadly categorized into five pathotypes on the basis of virulence and tissue tropism in poultry:

- Viscerotropic velogenic (Doyle's form), a highly pathogenic form characterized by haemorrhagic intestinal lesions.
- Neurotropic velogenic (Beach's form), an acute form chacarterized by respiratory and nervous signs and high mortality.
- Mesogenic (Beaudette's form), a less pathogenic form with mortality confined to young birds.
- Respiratory or lentogenic (Hitchner's form), characterized by mild or inapparent respiratory infection with low-virulence, lentogenic strains.
- Asymptomatic enteric form, this form is associated with intestinal infection by lentogenic strains.

There is evidence that virulent strains may arise from progenitor viruses of low virulence following passage in chickens (Shengqing et al. 2002). In addition to the influence of the strain of virus, the pathogenicity of NDV isolates varies with the host, dose, age of bird and environmental conditions.

Newcastle disease virus is probably enzootic in wild birds, especially waterfowl (Takakuwa et al. 1998). Virus is shed in all excretions and secretions, particularly respiratory discharges and faeces. Pigeons are susceptible to infection by all strains of APMV-1 and play an important role in the transmission of NDV. In the early 1980s mesogenic isolates, distinguishable from other APMV-1 isolates using monoclonal antibodies, appeared in racing pigeons in Europe. These isolates are often referred to as 'pigeon' paramyxovirus type 1 and are associated with clinical disease in pigeons resembling the neurotropic form of Newcastle disease. Infection is now considered to be worldwide in distribution. Outbreaks of disease in poultry in Great Britain in 1984 were associated with feed contaminated by infected feral pigeons. Newcastle disease virus can infect humans exposed to large quantities of virus, producing a transitory conjunctivitis.

Pathogenesis

The incubation period is about five days. Viral replication occurs initially in the mucosal epithelium of the respiratory and intestinal tracts. Spread to the spleen and bone marrow occurs via the bloodstream. Secondary viraemia

leads to infection of other target organs including lung, intestine and CNS. Invasion of the brain tends to occur after multiplication in non-nervous tissues. The extent of spread within the body largely depends on strain virulence which in turn is influenced by the amino acid sequence of the fusion (F) glycoprotein. The F glycoprotein of NDV is synthesized within an infected cell as a precursor F_0 molecule which is activated following cleavage to F_1 and F_2 subunits by host cell proteases. In the absence of cleavage non-infectious particles are produced. Additional basic amino acids are present at critical residues in the F_0 of virulent strains of NDV. This facilitates cleavage by proteases present in a wide range of host tissues. In contrast, the cleavage of F_0 of lentogenic strains only occurs in the respiratory and intestinal tracts where suitable trypsin-like enzymes are present.

Diagnosis

A presumptive diagnosis may be possible in severe outbreaks where characteristic signs and lesions are present. However, laboratory confirmation should be obtained by virus isolation and characterization. Virus-secure laboratory facilities are required for the handling of pathogenic isolates of NDV.

- Samples from live birds should include tracheal and cloacal swabs. Suitable specimens from recently dead birds include faeces, intestinal contents, tracheal material, intestine, spleen, brain and lung. Samples may be stored at 4°C for not more than four days.
- Virus isolation is carried out in 9- to 11-day-old embryonated SPF hens' eggs by inoculation of the allantoic cavity. Following incubation for four to seven days the allantoic fluid is tested for haemagglutination activity. Confirmation of the presence of NDV requires the use of specific antiserum in a haemagglutination inhibition test. An assessment of the virulence of an NDV isolate is important on account of the marked variation in virulence of isolates and the widespread use of live vaccines. A number of in vivo tests are available; the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) carried out in day-old and six-week-old SPF chicks respectively and the mean death time (MDT) carried out by inoculating the allantoic cavity of 9- to 10-day-old embryonated SPF hens' eggs. The MDT has been used to classify isolates as velogenic (less than 60 hours to kill), mesogenic (60 to 90 hours to kill) and lentogenic (more than 90 hours to kill). Following intracerebral or intravenous inoculation SPF chicks are observed each day and assigned a clinical score. The pathogenicity index is the mean score per bird per observation over an eight- or ten-day observation period. The pathogenicity of isolates can also be evaluated using RT-PCR and sequencing (Aldous & Alexander 2001). The current OIE definition of ND is infection of birds by avian paramyxovirus serotype 1 with either an ICPI ≥0.7 or with multiple (at least three) basic amino acids at the C-terminus of the F_2 protein and phenylalanine at residue 117 (N-terminus of the F_1 protein).
- Molecular techniques have been used to detect NDV in clinical specimens (Gohm et al. 2000, Creelan et al. 2002). Tracheal or orpharyngeal swabs are generally the samples of choice as some organ tissues and faeces may contain organic material that interferes with RNA recovery and amplification. Primers may be selected to cover that part of the F_0 protein gene which encodes the cleavage site, thus providing information on the virulence of the virus detected (Cavanagh 2001). A real time RT-PCR protocol has also been published (Wise et al. 2004).
- The demonstration of antibody to NDV is only of diagnostic value in unvaccinated flocks. The most widely used assay is the haemagglutination inhibition test. Several commercial ELISA kits are available.
- Viral antigen can be detected using immunofluorescence on tracheal sections or impression smears. It is less sensitive than virus isolation.

BLUE EYE DISEASE

This disease was first observed in pigs in1980 in La Piedad, Michoacan, Mexico. It is characterized by nervous disease, corneal opacity and reproductive failure. Blue eye disease has not been recognized outside of Mexico and is caused by porcine rubulavirus, also known as La Piedad-Michoacan-Mexico virus. Morbidity and mortality are highest in young piglets. Recovered animals are immune and the disease is self-limiting in closed herds. Diagnosis is based on clinical signs, histopathology, immunostaining for viral antigen and serological testing of paired serum samples. Suitable serological assays include haenagglutination inhibition, ELISA (Nordengrahn et al. 1999) and virus neutralization. Another rubulavirus, referred to as Menangle virus, was isolated in Australia in 1997 from stillborn piglets during an outbreak of reproductive disease at a large commercial piggery. In addition to pigs, this virus is capable of infecting fruit bats and humans.

REFERENCES

Alansari, H., Duncan, R.B., Baker, J.C., et al., 1999. Analysis of ruminant respiratory syncytial virus isolates by RNAase protection of the G glycoportein transcripts. Journal of Veterinary Diagnostic Investigation 11, 215–220.

Aldous, E.W., Alexander, D.J., 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathology 30, 117–128.

Anderson, J., McKay, J.A., Butcher, R.N., 1991. The use of monoclonal antibodies in competitive ELISA for the detection of antibodies to rinderpest and peste des petits ruminants. In: The Seromonitoring of Rinderpest Throughout Africa. Phase One. Proceedings of Final Research Co-ordination Meeting Joint FAO/IAEA (Food and Agriculture Organisation of the United Nations/International Atomic Energy Agency) Division, Vienna, Austria, 43–53.

Appel, M.J.G., Summers, B.A., 1995. Pathogenicity of morbilliviruses for terrestrial carnivores. Veterinary Microbiology 44, 187–191.

Bruning, A., Bellamy, K., Talbot, D., 1999. A rapid chromatographic test for the pen-side diagnosis of rinderpest virus. Journal of Virological Methods 81, 143–154.

Cavanagh, D., 2001. Innovation and discovery: the application of nucleic acid-based technology to avian virus detection and characterization. Avian Pathology 30, 581–598.

Couacy-Hymann, E., Roger, F., Hurard, C., et al., 2002. Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. Journal of Virological Methods 100, 17–25.

Creelan, J.L., Graham, D.A., McCullough, S.J., 2002. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. Avian Pathology 31, 493–499.

Dhar, P., Sreenivasa, B.P., Barrett, T., et al., 2002. Recent epidemiology of peste des petits ruminants virus (PPRV). Veterinary Microbiology 88, 153–159.

Elia, G., Decaro, N., Martella, V., et al., 2006. Detection of canine distemper virus in dogs by real-time RT-PCR. Journal of Virological Methods 136, 171–176.

Erles, K., Dubovi, E.J., Brooks, H., et al., 2004. Longitudinal study of viruses associated with canine infectious respiratory disease. Journal of Clinical Microbiology 42, 4524–4529.

Foreman, A.J., Rowe, L.W., Taylor, W.P., 1983. The detection of rinderpest antigen by agar gel diffusion and counterimmunoelectrophoresis. Tropical Animal Health and Production 15, 83–85.

Forsyth, M.A., Barrett, T., 1995. Evaluation of polymerase chain reaction for the detection and characterization of rinderpest and peste des petitis ruminants viruses for epidemiological studies. Virus Research 39, 151–163.

Frisk, A.L., Konig, M., Moritz, A., et al., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood and cerebrospinal fluid from dogs with distemper. Journal of Clinical Microbiology 37, 3634–3643.

Gohm, D.S., Thur, B., Hofmann, M.A., 2000. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. Avian Pathology 29, 143–152.

Jozwik, A., Frymus, T., 2005. Comparison of the immunofluorescence assay with RT-PCR in the diagnosis of canine distemper. Veterinary Research Communications 29, 347–359.

Kim, Y.H., Cho, K.W., Youn, H.Y., et al., 2001. Detection of canine distemper virus CDV through one step RT-PCR combined with nested PCR. Journal of Veterinary Science 2, 59–63.

Larsen, L.E., Tjornehoj, K., Viuff, B., et al., 1999. Diagnosis of enzootic pneumonia in Danish cattle: reverse transcription-polymerase chain reaction assay for detection of bovine respiratory syncytial virus in naturally and experimentally infected cattle. Journal of Veterinary Diagnostic Investigation 11, 416–422.

Libeau, G., Diallo, A., Colas, F., et al., 1994. Rapid differential diagnosis of rinderpest and peste des petits ruminants using an immunocapture ELISA. Veterinary Record, 134, 300–304.

Libeau, G., Prehaud, C., Lancelot, R., et al., 1995. Development of a competitive ELISA for detecting antibodies to the peste des petits ruminants virus using a recombinant nucleoprotein. Research in Veterinary Science 58, 50–55.

Martella, V., Elia, G., Lucente, M.S., et al., 2007. Genotyping canine distemper virus (CDV) by a hemi-nested multiplex PCR provides a rapid approach for investigation of CDV outbreaks. Veterinary Microbiology 122, 32–42.

McCarthy, A.J., Shaw, M.A., Goodman, S.J., 2007. Pathogen evolution and disease emergence in carnivores. Proceedings of the Royal Society B 274, 3165–3174.

Nordengrahn, A., Svenda, M., Moreno-Lopez, J., et al., 1999. Development of a blocking ELISA for screening antibodies to porcine rubulavirus, La Piedad Michoacan virus. Journal of Veterinary Diagnostic Investigation 11, 319–323.

Plowright, W., Ferris, R.D., 1961. Studies with rinderpest virus in cell culture III The stability of cultured virus and its use in neutralisation tests. Archiv fur die Gesamte Virusforsch 11, 516–533.

Roeder, P.L., Taylor, W.P., 2002. Rinderpest. Veterinary Clinics of North America: Food Animal Practice 18, 515–547.

Roelke-Parker, M.E., Munson, L., Packer, C., et al., 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature 379, 441–445.

Shengqing, Y., Kishida, N., Ito, H., et al., 2002. Generation of velogenic Newcastle disease viruses from a non-pathogenic waterfowl isolate by passaging in chickens. Virology 301, 206–211.

Shin, Y.J., Cho, K.O., Cho, H.S., et al., 2004. Comparison of one-step

RT-PCR and a nested PCR for the detection of canine distemper virus in clinical samples. Australian Veterinary Journal 82, 83–86.

Shin, Y.S., Mori, T., Okita, M., et al., 1995. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. Journal of Veterinary Medical Science 57, 439–445.

Soma, T., Ishii, H., Hara, M., et al., 2003. Detection of canine distemper virus antigen in canine serum and its application to diagnosis. Veterinary Record 153, 499–501.

Stanton, J.B., Poet, S., Frasca, S., et al., 2002. Development of a semi-nested reverse transcription polymerase chain reaction assay for the retrospective diagnosis of canine distemper virus infection. Journal of Veterinary Diagnostic Investigation 14, 47–52.

Stettler, M., Beck, K., Wagner, A., et al., 1997. Determinants of persistence in canine distemper viruses. Veterinary Microbiology 57, 83–93.

Takakuwa, H., Toshihiro, I., Takada, A., et al., 1998. Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations. Japanese Journal of Verterinary Research 45, 207–215.

Valarcher, J.F., Taylor, G., 2007. Bovine respiratory syncytial virus infection. Veterinary Research 38, 153–180.

Valarcher, J.F., Bourhy, H., Gelfi, J., et al., 1999. Evaluation of a nested reverse transcription-PCR assay based on the nucleoprotein gene for diagnosis of spontaneous and experimental bovine respiratory syncytial virus infections. Journal of Clinical Microbiology 37, 1858–1862.

von Messling, V., Harder, T.C., Moennig, V., et al., 1999. Rapid and sensitive detection of immunoglobulin M (IgM) and IgG antibodies against canine distemper virus by a new recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 1049–1056.

Willoughby, K., Thomson, K., Maley, M., et al., 2008. Development of a real time reverse transcriptase polymerase chain reaction for the detection of bovine respiratory syncytial virus in clinical samples and its comparison with immunohistochemistry and immunofluorescence antibody testing. Veterinary Microbiology 126, 264–270.

Wise, M.G., Suarez, D.L., Seal, B.S., et al., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42, 329–338.

Wosu, L.O., 1991. Haemagglutination test for diagnosis of peste des petits ruminants disease in goats with samples from live animals. Small Ruminant Research 5, 169–171.

FURTHER READING

Ellis, J.A., 2010. Bovine parainfluenza-3 virus. Veterinary Clinics of North America: Food Animal Practice 26, 575–593.

Ellis, J.A., 2012. A review of canine parainfluenza virus infection in dogs. Journal of the American Veterinary Medical Association 240, 273–284.

Woo, P.C., Lau, S.K., Wong, B.H., et al., 2012. Feline morbillivirus, a previously undescribed paramyxovirus associated with tubulointerstitial nephritis in domestic cats. Proceedings of the National Academy of Sciences USA 109, 5435–5440.

Chapter 62

Coronaviridae

The family *Coronaviridae* (Latin *corona* meaning crown) belongs to the Order *Nidovirales*, which also includes the family *Arteriviridae*. Coronaviruses are large, pleomorphic, enveloped viruses. Virions contain a single molecule of linear, positive-sense, single-stranded RNA. Coronaviruses have a crown-like appearance due to club-shaped glycoprotein peplomers projecting 15 to 20 nm from the envelope (Fig. 62.1). A peplomer is composed of a large trimeric viral glycoprotein (spike or S protein). These viral peplomers mediate viral attachment to specific cell receptors and fusion between the viral envelope and the cell membrane. The S protein is responsible for the induction of neutralizing antibodies during natural infection. Originally there were two genera in the family, *Coronavirus* and *Torovirus*. Coronaviruses have a helical nuclocapsid and are almost spherical with a diameter of 120–160 nm. Toroviruses have a tubular nucleocapsid, are approximately 130 nm × 40 nm and may appear disc-shaped, kidney-shaped or rod-shaped. Members of the genus *Coronavirus* form three genetic clusters or groups with distinct genetic and antigenic properties (Fig. 62.2). These clusters have formed the basis of additional genera in the family. The International Committee on Taxonomy of Viruses (ICTV) has divided the family into two subfamilies, *Coronavirinae* and *Torovirinae*, with the creation of new genera (Fig. 62.3), *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* and *Bafinivirus*. A number of viral species have been merged and renamed as follows; *Alphacoronavirus 1* (comprising the viruses of feline coronavirus, canine coronavirus and transmissible gastroenteritis virus (TGEV) including the respiratory variant of TGEV, porcine respiratory coronavirus); *Betacoronavirus 1* (comprising the viruses of human coronavirus OC43, bovine coronavirus, porcine haemagglutinating encephalomyelitis virus, equine coronavirus and the newly recognized canine respiratory coronavirus); *Avian coronavirus* (comprising the viruses of infectious bronchitis virus, turkey coronavirus, pheasant coronavirus, duck coronavirus, goose coronavirus and pigeon coronavirus).

Coronaviruses replicate in the cytoplasm of cells with new progeny virions acquiring their envelopes from the membranes of the endoplasmic reticulum and the Golgi complex. Following incorporation into vesicles, they are transported to the cell surface where the virions are released by exocytosis following fusion of the vesicles with the plasma membrane. Coronaviruses mutate readily giving rise to genetically divergent strains. In addition, genetic recombination can occur between related coronaviruses and is probably an important mechanism responsible for the genetic diversity of the viruses in nature.

Coronaviruses are sensitive to heat, lipid solvents, formaldehyde, oxidizing agents and non-ionic detergents. The stability of the virions at low pH values is variable with some species being stable at values as low as pH 3.0. Coronaviruses tend to be difficult to grow in cell culture, a notable exception being infectious bronchitis virus.

Coronaviruses display tropisms for respiratory and intestinal epithelium. Severe acute respiratory syndrome-related (SARS) coronavirus is a recently described member of the family, capable of producing serious disease in humans. There is evidence for an animal reservoir of SARS virus and Chinese horseshoe bats are suspected. The coronaviruses of veterinary importance are indicated in Table 62.1. Infections are usually mild or inapparent in mature animals but may be severe in young animals. Coronaviruses are aetiologically important in the common cold in humans. Feline coronavirus, canine coronavirus and transmissible gastroenteritis virus are closely related antigenically and genetically and are now referred to as alphacoronavirus 1. Evidence of torovirus infection has

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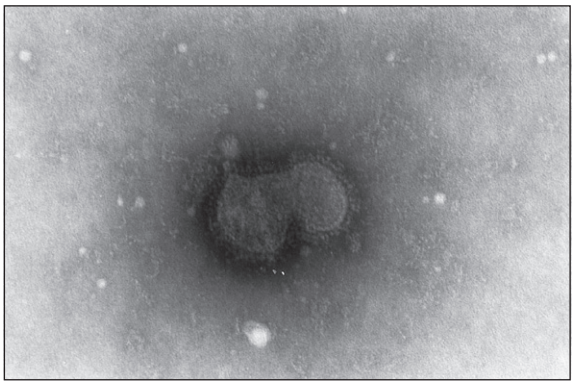

Figure 62.1 Negative stained electron micrograph of a coronavirus.

been found in horses, pigs, sheep, goats and cats (Muir et al. 1990). However, the clinical significance of these infections is questionable. Only bovine torovirus is recognized as capable of inducing enteric disease (Table 62.2).

FELINE INFECTIOUS PERITONITIS

Feline infectious peritonitis, caused by certain strains of feline coronavirus (FCoV), is an invariably fatal, sporadic disease of domestic cats and other members of *Felidae*. It has a worldwide distribution and is a major problem in mutilple-cat households. Feline coronavirus is comprised of two closely related biotypes which vary in pathogenicity. The term feline enteric coronavirus (FECV) is used to

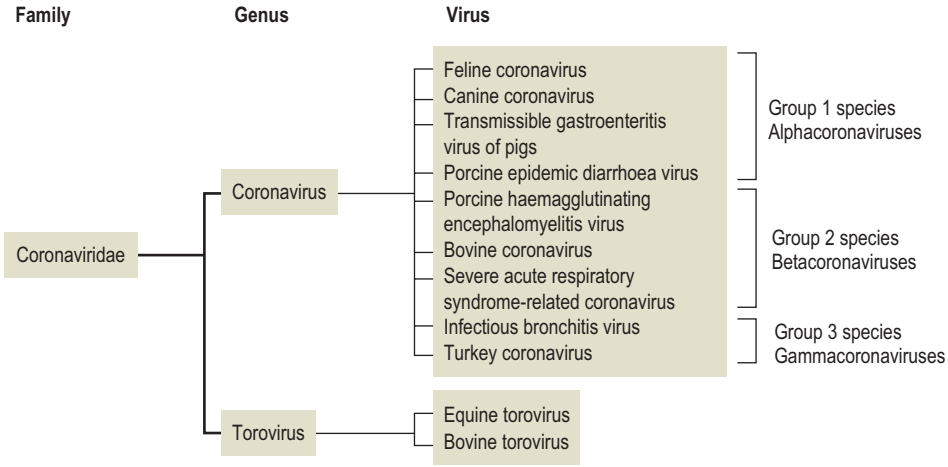

Figure 62.2 Old classification scheme for members of *Coronaviridae*.

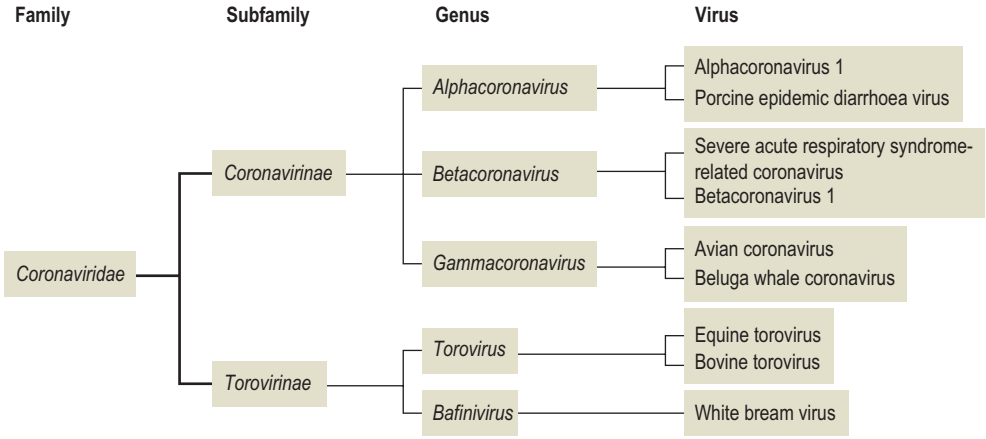

Figure 62.3 New classification scheme for members of *Coronaviridae*.

Table 62.1 Coronaviruses of animals

Virus	Host species	Disease
Feline coronavirus (FCoV)	Cats	Feline enteric coronavirus (FECV) is a strain of feline coronavirus which replicates in enterocytes. Relatively common cause of subclinical infection but may produce mild gastroenteritis in young kittens. Feline infectious peritonitis virus (FIPV) is a strain of feline coronovirus which initially replicates in enterocytes and subsequently in macrophages. Causes sporadic fatal disease of young cats typically presenting as an effusive peritonitis
Canine coronavirus	Dogs	Usually asymptomatic infection. Has also been isolated from dogs with diarrhoea; high morbidity but low mortality
Canine respiratory coronavirus	Dogs	Recently recognized as a cause of respiratory disease in kennelled dogs
Bovine coronavirus	Cattle	Important cause of diarrhoea in calves; associated with winter dysentery in adult cattle
Transmissible gastroenteritis virus (TGEV)	Pigs	Causes highly contagious infection. Infected piglets present with vomiting and diarrhoea; up to 100% mortality in newborn piglets. Porcine respiratory coronavirus, a deletion mutant of TGEV, induces partial immunity to the virulent virus
Porcine epidemic diarrhoea virus	Pigs	Enteric infection similar to TGE but with lower neonatal mortality
Porcine haemagglutinating encephalomyelitis virus	Pigs	Characterized by nervous disease or vomiting and emaciation (vomiting and wasting disease) in young pigs. Although infection is widespread, clinical disease is uncommon
Infectious bronchitis virus	Fowl	Causes acute, highly contagious respiratory infection in young birds and a drop in egg production in layers
Turkey coronavirus	Turkeys	Causes infectious enteritis (bluecomb disease)

Table 62.2 Toroviruses of animals

Virus	Host species	Disease
Equine torovirus (Berne virus)	Horses	Originally isolated from rectal swab of a horse with diarrhoea in Berne, Switzerland. No clear association with disease
Bovine torovirus (Breda virus)	Cattle	Associated with diarrhoea in neonates, particularly if the animals have been deprived of colostrum

describe strains that are present in virtually all multicat environments and cause little or no disease. The term feline infectious peritonitis virus (FIPV) is applied to those mutant strains of FECV responsible for cases of FIP (Vennema et al. 1998). Feline infectious peritonitis virus isolates display an enhanced ability to replicate in macrophages as a result of a mutation in the 3c gene (Pedersen 2009). Two serotypes of FCoV, distinguishable by neutralization tests, occur. Serotype 1 is the original FCoV, while serotype 2 is closely related to canine coronavirus and is probably a recombinant virus. Serotype 1 is the more prevalent serotype in Europe and North America, while serotype 2 is more prevalent in Japan. Both serotypes can cause clinically inapparent infections as well as FIP. Feline coronavirus serotype 1 strains are difficult to grow in cell culture, producing a slowly developing cytopathic effect. In contrast, serotype 2 strains grow more rapidly and give rise to a pronounced cytopathic effect.

Cats shed FCoV in the faeces and oronasal secretions. Infection is acquired by kittens at a young age from their mothers or from other adult cats (Addie & Jarrett 1992). Feline infectious peritonitis is a sporadic disease occurring in individual cats in catteries or multicat households. Although cats of any age may be affected, disease is most commonly seen in young cats from six months to three years of age. It is thought that cats with FIPV do not generally transmit this mutant virus to other cats or at least only under certain circumstances.

Pathogenesis

Infection with FCoV does not usually result in clinical disease. Factors considered important in the development of the disease include the age, immune status and genetic make-up of the host as well as the emergence of virulent virus strains (Addie et al. 1995). Infection with FECV is usually limited to enterocytes. In some animals a virulent FIPV strain capable of replication in macrophages and systemic invasion emerges. The development of effective cell-mediated immunity (CMI) may restrict viral replication and ultimately eliminate infection. When CMI is impaired or defective, virus replication continues leading to monocyte and perivascular macrophage activation, B cell activation and the production of non-neutralizing antibodies. The immune complexes formed from FIPV and the non-neutralizing antibodies are considered to be important in the development of immune-mediated vasculitis. However, the lesions are restricted to small- and medium-sized veins and there is evidence that the phlebitis is initiated by activated and FCoV-infected circulating monocytes (Kipar et al. 2005). The severity of this vasculitis influences the clinical presentation and the rate of progression of the disease. The incubation period ranges from weeks to months. The onset of clinical signs may be either sudden or slow and insidious. Early signs, which are generally non-specific, include anorexia, weight loss, listlessness and dehydration. Cats with the effusive form of the disease have fibrin-rich exudates in the abdominal or thoracic cavities. If the pleural effusion is marked, dyspnoea develops. The effusive form of the disease usually leads to death within eight weeks. In the non-effusive form of FIP, clinical findings are less characteristic. Signs associated with lesions in organs or tissues in the peritoneal cavity are present in about 50% of affected cats. Anterior uveitis, chorioretinitis and neurological signs may occur in up to 30% of cases. The course of the disease is usually protracted with animals surviving for several months.

Diagnosis

- Histological examination of affected tissues is the only procedure currently available for the definitive diagnosis of FIP. The demonstration of FCoV antigen in macrophages in the tissues by immunohistochemical means is confirmatory but obtaining appropriate tissue samples ante mortem is difficult.
- Diagnosis of FIP in the live cat is usually based on history, clinical signs and a number of suggestive abnormal laboratory findings (Addie et al. 2004).
- Pleural or peritoneal fluid from affected cats, which may contain fibrin strands, clots on standing and has a very high protein content with globulins comprising 50 to 82% (Sparkes et al. 1991). Cytological examination of the fluid typically reveals a predominance of macrophages and neutrophils consistent with the pyogranulomatous nature of the disease. Positive immunofluorescent staining of FcoV antigen in macrophages in the fluid is an excellent indication of FIP, but negative results can be obtained in cats that have FIP due to low numbers of macrophages. Rivalta's test is an inexpensive way to distinguish transudates from exudates in cats. A reagent tube is three-quarters filled with distilled water to which is added one drop of acetic acid and thoroughly mixed. A drop of effusion fluid is carefully layered on the surface. A positive result is obtained if the drop retains its shape, stays attached to the surface or slowly moves to the bottom. The test is defined as negative if the drop disappears and the solution remains clear. A positive result has a predictive value of 86% in FIP cases (Hartmann 2005).
- Haematological changes typical of the disease include neutrophilia, lymphopenia and, in chronic cases, a normocytic, normochromic anaemia.
- Hypergammaglobulinaemia with a consequent serum hyperproteinaemia is frequently present. The albumin-to-globulin ratio is considered to be a better diagnostic indicator than total serum protein or gamma-globulin concentration. An albumin-to-globulin ratio of less than 0.8 is highly suggestive of FIP. Serum liver enzymes and total bilirubin may be raised in affected cats. High levels (>3 mg/mL) of the acute phase protein alpha one acid glycoprotein (AGB) are suggestive of FIP.
- Diagnostic serological tests such as IFA and ELISA detect antibodies to FCoV. Titres must be interpreted with caution as many healthy cats have antibodies. However, they are of some value and considered indicative where high titres (>1/300) are detected in cats with clinical signs suggestive of FIP or in cats from a single-cat environment. Antibody titres by indirect immunofluorescence may be very high in some FIP cases but in other cases antibody titres have been found to be negligible (Sparkes et al. 1991). Serological testing is a valuable tool in identifying cats that are free of FCoV and for screening animals before introduction to a FCoV-free cattery.
- Reverse transcription-PCRs for the detection of FCoV genome have been developed for evaluating blood and peritoneal/pleural effusions (Gamble et al. 1997, Kita et al. 2002) but are considered too sensitive, resulting in false positives (Addie et al. 2004). It has not been possible to design primers capable of distinguishing enteric FCoV from FIP-causing FCoV. The most promising ante mortem test is detection of FCoV messenger RNA within circulating monocytes using RT-PCR (Simons et al. 2005).

CANINE CORONAVIRUS INFECTION

Serological studies indicate that infection is common, ranging from 6 to 75% in pet dogs. However, the importance of canine coronavirus (CCoV) as a cause of disease is uncertain as the virus can be isolated from normal dogs as well as those with diarrhoea (Tennant et al. 1993). A large number of strains occur with occasional highly virulent strains. Genotyping studies have identified two genetic types CCoV type 1 and CCoV type 2 (Decaro & Buonavoglia 2008). The virus is related antigenically to feline coronavirus. Canine respiratory coronavirus is a newly identified and genetically distinct virus associated with mild respiratory disease in groups of dogs (Erles & Brownlie 2008).

Pathogenesis

The incubation period is up to three days and infection is acquired from the faeces of infected animals. Canine coronaviruses are capable of withstanding the acid environment of the stomach. They infect the enterocytes in the duodenum. Infection spreads rapidly to other parts of the small intestine resulting in diarrhoea due to damage to mature enterocytes at the tips of villi and the loss of digestive and absorptive capacity in the small intestine. Recovery is generally rapid.

Diagnosis

- Detection of the virus in faeces by electron microscopy.
- Virus can be isolated in a number of cell lines, such as A-72 and Crandell feline kidney. However, the procedure is slow and unreliable.
- Nested-PCR assays have been described for the detection of canine coronavirus in faecal samples, utilizing primers to the gene encoding the transmembrane M protein (Pratelli et al. 1999) and primers to the spike (S) glycoprotein gene (Naylor et al. 2001).
- Serum neutralization or indirect immunofluorescence tests are suitable and can be used to demonstrate an increasing antibody titre.

BOVINE CORONAVIRUS INFECTION

Bovine coronavirus (BCV) is an important cause of calf diarrhoea. It is also associated with winter dysentery in adult housed cattle. In addition, there is evidence of its involvement in the bovine respiratory disease complex (Kapil & Goyal 1995). A single serotype of the virus occurs, capable of haemagglutinating the red cells of mice, rats and hamsters.

Infected calves often harbour BCV in both the enteric and respiratory tracts (McNulty et al. 1984). However, virus is mainly transmitted by the faecal–oral route. Infection is usually enzootic on farms, where it is maintained by clinically affected calves as well as persistently infected, clinically normal calves and cows. In calves, the incubation period is up to two days and clinical signs of profuse diarrhoea are usually observed between three to 21 days of age. The virus replicates and destroys mature enterocytes in the small intestine and colon. This results in a malabsorptive diarrhoea. The severity of disease is influenced by the age of the animal and the type of management.

The incubation period in adult animals is three to seven days and is followed by the sudden onset of diarrhoea accompanied by a dramatic drop in milk yield. Blood or blood clots may be evident in the faeces of some animals. Risk factors, which include changes in diet, cold temperatures, close confinement and the presence of other microorganisms such as *Campylobacter jejuni*, appear to be particularly important in the development and pathogenesis of winter dysentery.

Diagnosis

- Faeces or intestinal contents should be collected early in the course of the disease for laboratory testing.
- Characteristic coronavirus particles can be demonstrated in faecal samples by direct electron microscopy (EM) or using immune EM. The latter is preferable as it is more sensitive and specific. Other suitable viral detection methods include ELISA and reverse passive haemagglutination.
- Detection of viral antigen in cryostat sections of distal small intestine or colon can be achieved using immunofluorescence.
- Reverse transcription-PCR and nested-PCR assays targeting the nucleocapsid gene have been shown to be extremely sensitive in detecting bovine coronavirus in clinical samples (Cho et al. 2001). A real-time assay targeting the transmembrane protein gene has also been described (Decaro et al. 2008).
- Serological testing is not so useful for diagnosis because antibodies are widespread in cattle. Suitable assays include virus neutralization, ELISA and haemagglutination inhibition using mouse or chicken erythrocytes.
- The isolation of virus in tissue culture is difficult and usually not undertaken.

TRANSMISSIBLE GASTROENTERITIS

Transmissible gastroenteritis (TGE) is a highly contagious, enteric coronaviral disease of young pigs with a worldwide

distribution. There is a single serotype of transmissible gastroenteritis virus (TGEV) which is closely related antigenically to both feline and canine coronaviruses. Porcine respiratory coronavirus (PRCV) is a relatively non-pathogenic respiratory variant of TGEV, probably a deletion mutant, which was first recognized in 1984. Outbreaks of TGE have become more sporadic since the appearance of PRCV. Infection with PRCV is usually subclinical and provides cross-protection but complicates the diagnosis of TGE, particularly by serological means.

Pathogenesis

The incubation period is up to three days with transmission of the virus usually occurring by the faecal–oral route. The virus spreads rapidly in fully susceptible herds, infecting animals of all ages. Disease is most severe in newborn piglets. Outbreaks usually only last a few weeks provided no new susceptible animals are introduced into the herd. The virus replicates mainly in mature enterocytes at the tips of the villi in the small intestine, resulting in villous atrophy along the length of the small intestine. Severe disruption of digestion and the cellular transport of nutrients and electrolytes follow. This results in the accumulation of fluid in the intestinal lumen and diarrhoea. Young piglets in particular are susceptible to the ensuing dehydration and metabolic acidosis.

Diagnosis

Sudden onset, rapid spread of diarrhoea and a mortality rate approaching 100% amongst newborn pigs is highly suggestive of TGE.

- Detection of viral antigen in mucosal smears or cryostat sections of the small intestine by immunofluorescence is confirmatory. In order to obtain suitable specimens for immunofluorescence it may be necessary to sacrifice some piglets in the early stages of the disease process. Alternatively viral antigens can be detected in paraffin-embedded tissues by immunohistochemical staining (Shoup et al. 1996).
- Viral antigens can be demonstrated in faeces by ELISA (Bernard et al. 1986, Lanza et al. 1995).
- Sensitive RT-PCR methods for the direct detection of TGEV and differentiation from PRCV in clinical specimens have been described (Paton et al. 1997, Kim et al. 2000).
- Virus can be isolated from faeces or small intestine in a swine testis or pig kidney cell line, producing CPE after three to seven days. However, TGEV does not grow readily in cell culture and viral isolation is not routinely used for diagnosis.
- Serological testing for antibodies can be carried out. However, the virus neutralization test is not capable of distinguishing antibodies to TGEV from those induced by PRCV infection. In contrast, competitive blocking ELISAs, based on the use of monoclonal antibodies directed against TGEV-specific epitopes, will specifically identify TGEV-exposed animals (Callebaut et al. 1989, Sestak et al. 1999). A number of commercial ELISA kits are available.

PORCINE EPIDEMIC DIARRHOEA

Porcine epidemic diarrhoea (PED) is a clinically similar condition to TGE. It has been described in Europe and Asia but not in America. There is only one serotype of porcine epidemic diarrhoea virus (PEDV) and it is serologically unrelated to TGEV. Transmission of the virus occurs by the faecal–oral route. The spread of virus to susceptible herds occurs both directly through infected pigs and indirectly through contaminated fomites or vehicles. Compared to TGEV, the rate of spread of infection within a herd is slower.

Pathogenesis

The incubation period is up to four days. Virus replication in the enterocytes of the small intestine and colon damages the villi, but the damage is less marked than in TGEV infection.

Diagnosis

- Direct immunofluorescent staining of cryostat sections of small intestine is both sensitive and reliable. Samples are best obtained from pigs sacrificed during the acute diarrhoeic phase, particularly from newborn piglets.
- Viral antigen may be detected in faecal material (Rodak et al. 2005) or intestinal contents collected during the acute phase of the disease by ELISA.
- A duplex RT-PCR for the combined detection and differentiation of PEDV and TGEV has been published (Kim et al. 2001, 2007).
- Antibodies to PEDV can be detected using a blocking ELISA or alternatively by using an indirect immunofluorescent technique on PEDV-positive cryostat sections of intestine.

PORCINE HAEMAGGLUTINATING ENCEPHALOMYELITIS VIRUS INFECTION

This disease of young pigs, also referred to as vomiting and wasting disease, is caused by porcine haemagglutinating

encephalomyelitis virus. Only one serotype of the virus has been described and infection is common, probably worldwide. The virus is capable of agglutinating the red cells of several animal species. The virus is shed in nasal secretions and transmission occurs readily by aerosols with infection persisting on breeding farms as a subclinical respiratory condition. In those herds where infection is enzootic, piglets are protected by antibodies received in the colostrum until they have developed an age-related resistance.

Pathogenesis

After an incubation period of up to seven days, clinical signs are seen in pigs less than three weeks of age. Following replication in the upper respiratory tract and tonsils, the virus spreads via the peripheral nervous system to the medulla oblongata and to other parts of the central nervous system. The signs of vomiting and delayed gastric emptying are due to viral damage to the vagal sensory ganglion and to the intramural plexus of the stomach.

Diagnosis

- Samples of brain stem must be collected within two days of the onset of clinical signs to be suitable for virus isolation or for viral antigen demonstration in cryostat sections by immunofluorescence, Suitable cells for isolation include porcine thyroid cells.
- Lesions of non-suppurative encephalomyelitis may be evident upon histopathological examination of the brain.
- Reverse transcription and nested PCR assays have been described for the detection of viral RNA (Sekiguchi et al. 2004).
- A significant rise in antibody titre in paired serum samples is suggestive. Suitable tests include virus neutralization and haemagglutination inhibition.

INFECTIOUS BRONCHITIS

Infectious bronchitis is a highly contagious, economically important, disease of poultry with a worldwide distribution. Infection with infectious bronchitis virus (IBV) can affect the respiratory, reproductive and renal systems. Numerous serotypes, often with different virulence and tissue tropisms, have been recognized. The most important route of transmission is by aerosol. The spread of infection occurs rapidly among susceptible birds and morbidity may approach 100%.

Pathogenesis

The incubation period is short, up to 48 hours. The respiratory system is the primary site of virus replication followed by viraemia within one to two days of exposure. The virus becomes widely distributed throughout the body, in particular localizing in the oviducts, kidneys and bursa of Fabricius. The virulence of the infecting strain influences the distribution and severity of lesions in these tissues. Disease tends to be most severe in young birds, particularly when secondary infections are present. Layers show signs of rales. This is followed by a marked reduction in egg production which slowly returns to normal. Egg quality may be poor for several weeks. Nephrotropic strains of IBV are associated with interstitial nephritis and mild respiratory signs with mortality of up to 25% in broilers. Both field and vaccinal virus may persist in the intestinal tract with excretion in faeces for long periods.

Diagnosis

- Isolation and identification of the virus is required to confirm a clinical diagnosis (Ignjatovic & Sapats 2000). Detection methods for IBV have been thoroughly reviewed by De Wit (2000). Virus isolation is usually possible in the acute stage of the disease. Although specimens from the respiratory tract are those most suitable for virus isolation during acute respiratory disease, samples from kidney, oviduct, caecal tonsils and faeces should also be included in the case of chronic infections. This material is inoculated into the allantoic sac of nine- to 10-day-old embryonated eggs. Several passages may be required to produce the characteristic stunting and curling of the embryo. Identification of virus is usually by monoclonal antibody and ELISA or by use of RT-PCR and sequencing (Ramneek & McFarlane 2005), restriction fragment length polymorphism (Jackwood et al. 2005) or hybridization. Neutralization of virus in tracheal organ explant cultures from day-old specific-pathogen-free chicks is considered the best method for antigenic typing. A real-time RT-PCR protocol suitable for the detection and typing of isolates has been described (Callison et al. 2005).
- The use of live vaccines complicates diagnosis as there is no simple diagnostic assay that can differentiate field from vaccinal strains of virus. Discrimination of all IBV strains can be accomplished by nucleotide sequencing of the gene coding for the S1 subunit of the S protein (Ignjatovic & Sapats 2000).
- Suitable serological tests include virus neutralization, agar gel immunodiffusion, haemagglutination inhibition and ELISA. These tests can be used to demonstrate a rise in antibody titre between acute and convalescent serum samples. Commercial ELISAs are available.

REFERENCES

Addie, D.D., Jarrett, O., 1992. A study of naturally occurring feline coronavirus infections in kittens. Veterinary Record 130, 133–137.

Addie, D.D., Toth, S., Murray, G.D., et al., 1995. Risk of feline infectious peritonitis in cats naturally infected with feline coronavirus. American Journal of Veterinary Research 56, 429–434.

Addie, D.D., Patrinieri, S., Pedersen, N.C., 2004. Recommendations from workshops of the second international feline coronavirus/feline infectious peritonitis symposium. Journal of Feline Medicine and Surgery 6, 125–130.

Bernard, S., Lantier, I., Laude, H., Aynaud, J.M., 1986. Detection of transmissible gastroenteritis coronavirus antigens by a sandwich enzyme-linked immunosorbent assay technique. American Journal of Veterinary Research 47, 2441–2444.

Callebaut, P., Pensaert, M.B., Hooyberghs, J., 1989. A competitive inhibition ELISA for the differentiation of serum antibodies from pigs infected with transmissible gastroenteritis virus (TGEV) or with the TGEV-related porcine respiratory coronavirus. Veterinary Microbiology 20, 9–19.

Callison, S.A., Hilt, D.A., Jackwood, M.W., 2005. Rapid differentiation of avian infectious bronchitis virus isolates by sample to residual ratio quantitation using real-time reverse transcriptase-polymerase chain reaction. Journal of Virological Methods 124, 183–190.

Cho, K.O., Hasoksuz, M., Nielsen, P.R., et al., 2001. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Archives of Virology 146, 2401–2419.

De Wit, J.J., 2000. Detection of infectious bronchitis virus. Avian Pathology 29, 71–93.

Decaro, N., Buonavoglia, C., 2008. An update on canine coronaviruses: viral evolution and pathobiology. Veterinary Microbiology 132, 221–234.

Decaro, N., Elia, G., Campolo, M., et al., 2008. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. Journal of Virological Methods 151, 167–171.

Erles, K., Brownlie, J., 2008. Canine respiratory coronavirus: an emerging pathogen in the canine infectious respiratory disease complex. Veterinary Clinics of North America Small Animal Practice 38, 815–825.

Gamble, D.A., Lobbiani, A., Gramegna, M., et al., 1997. Development of a nested PCR assay for detection of feline infectious peritonitis virus in clinical specimens. Journal of Clinical Microbiology 35, 673–675.

Hartmann, K., 2005. Feline infectious peritonitis. Veterinary Clinics of North America, Small Animal Practice 35, 39–79.

Ignjatovic, J., Sapats, S., 2000. Avian infectious bronchitis virus. Revue Scientifique et Technique de l'Office International des Epizooties 19, 493–508.

Jackwood, M.W., Hilt, D.A., Lee, C.W., et al., 2005. Data from 11 years of molecular typing infectious bronchitis virus field isolates. Avian Diseases 49, 614–618.

Kim, L., Chang, K.O., Sestak, K., et al., 2000. Development of a reverse transcription-nested polymerase chain reaction assay for differential diagnosis of transmissible gastroenteritis virus and porcine respiratory coronavirus from faeces and nasal swabs of infected pigs. Journal of Veterinary Diagnostic Investigation 12, 385–388.

Kim, S.J., Song, D.S., Park, B.K., 2001. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhoea virus by duplex RT-PCR. Journal of Veterinary Diagnostic Investigation 13, 516–520.

Kim, S.H., Kim, I.J., Pyo, H.M., et al., 2007. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. Journal of Virological Methods 146, 172–177.

Kipar, A., May, H., Menger, S., et al., 2005. Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis. Veterinary Pathology 42, 321–330.

Kita, P., Frymus, T., Kapulkin, W., 2002. Detection of feline coronavirus RNA in ascitic fluid and blood of naturally infected cats by reverse transcriptase-PCR. Second International Feline Coronavirus/Feline Infectious Peritonitis Symposium, Glasgow, Scotland.

Kapil, S., Goyal, S.M., 1995. Bovine coronavirus – associated respiratory disease. Compendium on Continuing Education for the Practicing Veterinarian 17, 1179–1181.

Lanza, I., Shoup, D.I., Saif, L.J., 1995. Lactogenic immunity and milk antibody isotypes to transmissible gastroenteritis virus in sows exposed to porcine respiratory coronavirus during pregnancy. American Journal of Veterinary Research 56, 739–748.

McNulty, M.S., Bryson, D.G., Allan, G.M., et al., 1984. Coronavirus infection of the bovine respiratory tract. Veterinary Microbiology 9, 425–434.

Muir, P., Harbour, D.A., Gruffydd-Jones, T.J., et al., 1990. A clinical and microbiological study of cats with protruding nictitating membranes and diarrhoea: isolation of a novel agent. Veterinary Record 127, 324–330.

Naylor, M.J., Harrison, G.A., Monckton, R.P., et al., 2001. Identification of canine coronavirus strains from faeces by S gene nested PCR and molecular characterization of a new Australian isolate. Journal of Clinical Microbiology 39, 1036–1041.

Paton, D., Ibata, G., Sands, J., et al., 1997. Detection of transmissible gastroenteritis virus by RT-PCR and differentiation from porcine respiratory coronavirus. Journal of Virological Methods 66, 303–309.

Pedersen, N.C., 2009. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of Feline Medicine and Surgery 11, 225–258.

Pratelli, A., Tempestra, M., Greco, G., et al., 1999. Development of a nested PCR assay for the detection of

canine coronavirus. Journal of Virological Methods 80, 11–15.

Ramneek, M.N.L., McFarlane, R.G., 2005. Rapid detection and characterisation of infectious bronchitis virus IBV from New Zealand using RT-PCR and sequence analysis. New Zealand Veterinary Journal 53, 457–461.

Rodak, L., Valicek, L., Smid, B., et al., 2005. An ELISA optimized for porcine epidemic diarrhoea virus detection in faeces. Veterinary Microbiology 105, 9–17.

Sekiguchi, Y., Shirai, J., Taniguchi, T., et al., 2004. Development of reverse transcriptase PCR and nested PCR to detect porcine hemagglutinating encephalomyelitis virus. Journal of Veterinary Medical Science 66, 367–372.

Sestak, K., Zhou, Z., Shoup, D.I., et al., 1999. Evaluation of the baculovirus-expressed S glycoprotein of transmissible gastroenteritis virus (TGEV) as antigen in a competition ELISA to differentiate porcine respiratory coronavirus from TGEV antibodies in pigs. Journal of Veterinary Diagnostic Investigation 11, 205–214.

Shoup, D.I., Swayne, D.E., Jackwood, D.J., et al., 1996. Immunohistochemistry of transmissible gastroenteritis virus antigens in fixed paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation 8, 161–167.

Simons, F.A., Vennema, H., Rofina, J., et al., 2005. A mRNA PCR for the diagnosis of feline infectious peritonitis. Journal of Virological Methods 124, 111–116.

Sparkes, A.H., Gruffydd-Jones, T.J., Harbour, D.A., 1991. Feline infectious peritonitis: a review of clinico-pathological changes in 65 cases, and a critical assessment of their diagnostic value. Veterinary Record 129, 209–212.

Tennant, B.J., Gaskell, R.M., Jones, R.C., et al., 1993. Studies on the epizootiology of canine coronavirus. Veterinary Record 132, 7–11.

Vennema, H., Poland, A., Foley, J., Pedersen, N.C., 1998. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. Virology 243, 150–157.

FURTHER READING

Addie, D., Belák, S., Boucraut-Baralon, C., et al., 2009. Feline infectious peritonitis. ABCD guidelines on prevention and management. Journal of Feline Medicine and Surgery 11, 594–604.

Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. Veterinary Research 38, 281–297.

Clark, M.A., 1993. Bovine coronavirus. British Veterinary Journal 149, 51–70.

Hoet, A.E., Saif, L.J., 2004. Bovine torovirus (Breda virus) revisited. Animal Health Research Reviews 5, 157–171.

Olsen, C.W., 1993. A review of feline infectious peritonitis virus: molecular biology, immuno-pathogenesis, clinical aspects, and vaccination. Veterinary Microbiology 36, 1–37.

Siddell, S.G., 1995. The Coronaviridae. Plenum Press, New York.

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Chapter 63

Rhabdoviridae

Rhabdoviruses are named from the Greek word *rhabdos* meaning rod which refers to their distinctive morphology. The family *Rhabdoviridae* along with the families *Paramyxoviridae, Bornaviridae* and *Filoviridae* is part of the order *Mononegavirales*. Viruses within this order possess a linear non-segmented RNA genome of negative polarity in a helical ribonucleoprotein complex (RNP). Rhabdoviruses are enveloped and variable in length, 100–430 nm, and diameter, 45–100 nm. The animal viruses are bullet-shaped while plant rhabdoviruses may be bacilliform- or bullet-shaped (Fig. 63.1). It is a large family comprising six genera *Vesiculovirus, Lyssavirus, Ephemerovirus, Novirhabdovirus, Cytorhabdovirus* and *Nucleorhabdovirus* (Fig. 63.2). In addition there are a large number of rhabdoviruses which have not been assigned to any genus yet. Members of the family infect vertebrates, invertebrates and plants. The genera *Vesiculovirus, Lyssav*irus and *Ephemerovirus* contain viruses of vertebrates. The genus *Novirhabdovirus* contains infectious haematopoietic necrosis virus and related rhabdoviruses of fish.

Rhabdoviruses generally contain five structural proteins; 'large' protein that is responsible for RNA-dependent RNA polymerase (L), surface glycoprotein (G), nucleoprotein (N), co-factor of the viral polymerase (P) and matrix protein (M). The G protein forms surface peplomers which are responsible for binding to host cell receptors, resulting in endocytosis, and the subsequent fusion of the viral envelope and cell membrane as the pH decreases within the endosome. In addition the G protein induces virus-neutralizing antibodies and elicits cell-mediated immunity. The nucleoprotein has epitopes involved in inducing cell-mediated immunity. Replication occurs in the cytoplasm (with the exception of nucleorhabdoviruses) with budding of the newly synthesized nucleocapsids of animal rhabdoviruses from the plasma membrane. Rhabdoviruses are stable over a pH range of five to 10 but are rapidly inactivated at 56°C and by exposure to lipid solvents or UV light.

The family contains more than 150 viruses of vertebrates, invertebrates and plants, of which a number are of veterinary significance (Table 63.1). Transmission of rhabdoviruses frequently involves an arthropod vector. Arthropods are thought to be the original reservoir hosts from which adaptation to plants and vertebrates occurred. Transmission between vertebrate hosts can also occur by contact, aerosol, bite or venereally. The best known and most important member of the family is rabies virus which is a member of the genus *Lyssavirus*. The name of the genus is derived from the Greek word *lyssa* meaning rage or fury. More than 25 viruses isolated from vertebrates and invertebrates have been described in the genus *Vesiculovirus*. Several vesiculoviruses are known to infect domestic animals, of most importance are vesicular stomatitis Indiana virus (VSIV) and vesicular stomatitis New Jersey virus (VSNJV). Infection of man with these viruses is reasonably common and results in an influenza-like condition. Bovine ephemeral fever or three-day sickness is caused by bovine ephemeral fever virus, the type species of the genus *Ephemerovirus*. Rhabdoviruses are important causes of disease in fish including infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus, eel rhabdovirus, pike fry rhabdovirus and spring viraemia of carp virus. A number of these viruses are members of the genus *Vesiculovirus* while others have been assigned to the genus *Novirhabdovirus*.

RABIES

Rabies is a viral infection of the CNS of warm-blooded animals, including man, that usually ends in death of the host. The disease occurs in most parts of the world. All mammals are considered susceptible to infection. The vast majority of clinical cases are due to infection with rabies virus. Several genotypically distinct lyssaviruses are capable

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of producing rabies-like encephalitis but incidences of human disease due to these 'rabies-related' viruses are rare. Antigenic and genetic sequencing studies have allowed the differentiation of at least four serotypes and seven genotypes (Smith 1996, Gould et al. 1998, Marston et al. 2012). The genotypes are sufficiently different to be ascribed separate species status (Table 63.2). An additional five lysssaviruses have been isolated from a number of bat species and classified; West Caucasian bat virus, Shimoni bat virus, Aravan virus, Irkut virus and Khujand virus. Two further lyssaviruses, one from a bat (Bokeloh bat virus) and one from an African civet (Ikoma lyssavirus) have been detected (Marston et al. 2012).

Figure 63.1 Negative stain electron microscopy of vesicular stomatitis Indiana virus showing characteristic bullet-shaped virions. Bar: 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 431.*

All warm-blooded animals are susceptible to infection with rabies virus. However, there is species variation in susceptibility and several species-adapted variants of rabies virus occur. Each variant is more readily transmitted to members of the same species than to individual animals of a different species. In a particular geographical area rabies virus is usually maintained and transmitted by one or possibly two mammalian species. These species serve as reservoirs and vectors of the infection. Two important epidemiological cycles are recognized, canine (urban) rabies and sylvatic rabies. Canine rabies involves stray dogs and accounts for more than 95% of human cases in developing countries. Sylvatic rabies involves a range of wildlife species, principally small to medium-sized carnivores, that varies geographically and includes foxes, coyotes, racoons, skunks, jackals, mongooses and bats. Rabies virus exploits a number of traits common to these reservoir hosts including the fact that they often live at high population densities and have high intrinsic population growth rates which permit the rapid recovery of a population following a rabies epizootic. In Africa, Asia, South America and the Middle East canine rabies is important whereas in

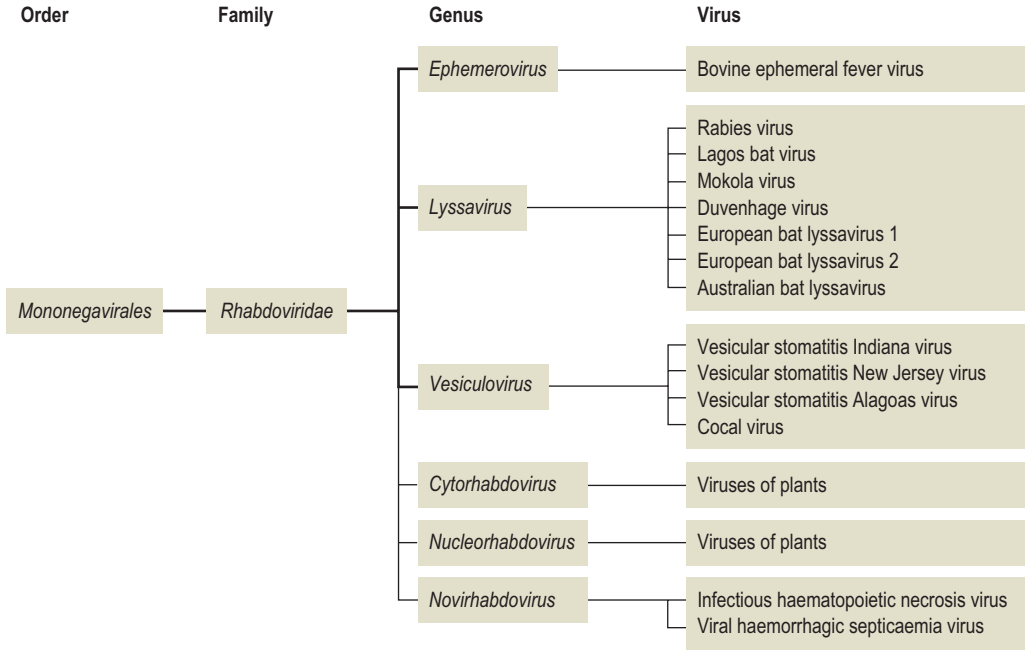

Figure 63.2 Outline of classification of rhabdoviruses.

Table 63.1 Members of *Rhabdoviridae* of veterinary significance

Virus	Host species	Significance of infection
Rabies virus	Wide host range	Present in a wide range of mammalian species. Canine (urban) and sylvatic cycles. Fatal central nervous disease. Worldwide except for Australia and island countries
Vesicular stomatitis Indiana virus	Cattle, sheep, horse, pig, man	Febrile vesicular disease similar to foot-and-mouth disease. Type virus of the genus
Vesicular stomatitis New Jersey virus	Cattle, sheep, horse, pig, man	Febrile vesicular disease similar to foot-and-mouth disease
Vesicular stomatitis Alagoas virus (Brazil virus)	Horse, mule, cattle, man	Originally isolated from mules in Alagoas, Brazil. Pathogenic for cattle and horses
Cocal virus (Argentina virus)	Arthropods, horses	First isolated from mites in Trinidad. Associated with disease in horses
Bovine ephemeral fever virus	Cattle	Febrile, arthropod-borne illness present in tropical and semi-tropical regions of Africa, Asia and Australia

developed countries vaccination and the control of strays have reduced its importance but increased awareness of wildlife reservoirs. In North America racoons, skunks, foxes and bats act as important reservoirs (Krebs et al. 1998). In Europe, the principal reservoir is the red fox. The vampire bat is an important reservoir in Central and South America. In Africa, the predominant rabies virus variants are the mongoose and canine biotypes.

Domestic animals and man are considered to be moderately susceptible to the virus while red foxes, wolves, coyotes and jackals are very susceptible. However, the variant involved in the infection is important in determining the development of disease. Transmission usually occurs through biting. Most infected animals excrete virus in their saliva, beginning a number of days before the onset of clinical signs. The virus may be widely disseminated through social grooming or aggressive encounters. Less common routes of transmission include scratching, licking or aerosol.

Pathogenesis

The incubation period is extremely variable, ranging from a few days to many months. Factors influencing its length include host species, virus strain, amount of inoculum and site of inoculation. Following subdermal inoculation the virus enters peripheral, unmyelinated nerve terminals at the site either immediately or following local replication in non-nervous tissue. The virus is transported to the CNS by retrograde axoplasmic flow. Within the CNS the virus becomes widely disseminated through intra-axonal spread. Clinical signs become apparent at this stage due to cell damage within the CNS caused by the multiplying virus. Subsequently the virus spreads centrifugally to peripheral sites within nerve cells. The virus is released from axon terminals and infects adjacent non-nervous tissue. Most tissues become infected, the most important being the salivary glands. Stimulation of an immune response is minimal due to the highly neurotropic nature of the virus which remains largely protected from immune cells. Neither antibody or virus is readily detectable until after the onset of clinical disease. The clinical course typically lasts from a few days to a few weeks and can be divided into three phases or forms; prodromal, furious (excitative) and paralytic (dumb). Death is usually due to respiratory failure.

Diagnosis

Laboratory confirmation of rabies is essential as clinical signs vary from animal to animal and there is no pathognomonic gross lesion. Following a biting incident, suspect animals should be isolated and observed for 10–14 days to see if clinical signs develop. Rapid laboratory confirmation is important to facilitate the early treatment of in-contact humans. Animals with clinical signs are killed and the brain examined for the presence of virus. A pool of brain tissue, of which the brain stem is the most important component, should be collected and tested. Samples should be kept refrigerated, placing brain material in a mixture of 50% glycerol in phosphate buffered saline will help to extend infectivity of the material for a few days at room temperature. Rabies virus is most abundant in the thalamus, pons and medulla of the brain. Laboratory

Table 63.2 Rabies and rabies-like lyssaviruses

Virus	Phylogroup	Genotype	Distribution	Significance
Rabies virus	I	1	All continents except Australia and Antarctica. Some island nations such as Japan, United Kingdom, New Zealand and Ireland are free	Recognized since antiquity. More than 50,000 human fatalities per year. Majority of cases occur in developing countries and are associated with rabid dogs
Lagos bat virus	II	2	Africa	Isolated from fruit bats originally. Some isolates from domestic animals. No human cases reported
Mokola virus	II	3	Africa	Original isolation from shrews. Several isolates from domestic animals. Evidence of infection in rodent species. Some human cases
Duvenhage virus	I	4	Africa	Original isolate from human bitten by insectivorous bat
European bat lyssavirus 1	I	5	Europe	Present in insectivorous bats (*Eptesicus serotinus*). Some human cases
European bat lyssavirus 2	II	6	Europe	Present in insectivorous bats (*Myotis dasycneme*). First isolated from Swiss bat biologist who died of rabies
Australian bat lyssavirus	I	7	Australia	Circulates in fruit and insectivorous bats. Human infection reported
West Caucasian bat virus	III		Eurasia	Isolated from bats in Russia (western Caucasus mountains)
Irkut virus	I		Eurasia	Isolated from bats in Russia (eastern Siberia)
Aravan virus	I		Eurasia	Isolated from a lesser mouse-eared bat (*Myotis blythi*) in Krygyzstan
Khujand virus	I		Eurasia	Isolated from a whiskered bat in northern Tajikistan
Shimoni bat virus	II		Africa	Isolated from the brain of a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Kenya
Bokeloh bat lyssavirus	I		Europe	Isolated from a Natterer's bat (*Myostis nattererii*) in Germany
Ikoma lyssavirus	–		Africa	Isolated from an African civet in Tanzania. Possibly originated in a bat species

personnel working with suspect material must be vaccinated against lyssaviruses.

- Characteristic histopathological lesions are confined to the brain and include non-suppurative encephalomyelitis, lymphoid perivascular cuffing and intracytoplasmic inclusions (Negri bodies). Negri bodies are found most consistently in the pyramidal cells of the hippocampus, but are not detectable in many cases. Histological methods are considered to have low sensitivity.
- The direct fluorescent antibody test (FAT) is the most widely used diagnostic test, recommended by both WHO and OIE. Brain smears, fixed in acetone, are stained with specific conjugate. The test is rapid and highly specific but may give false-negative results on degraded samples of brain. The use of modern, commercial preparations of anti-rabies antibody conjugates directed against conserved antigenic sites on the nucleocapsid proteins permit the detection of all lyssaviruses. A commercial rapid rabies enzyme immunodiagnosis test (RREID) is also available for the detection of viral antigen.
- The rabies tissue culture infection test (RTCIT) involves virus isolation in neuroblastoma or baby hamster kidney cells. It is a useful back-up test when the results of the FAT are inconclusive. Rabies virus is non-cytopathic and its presence is detected by FAT using a suitable conjugated antiserum.
- The mouse inoculation test involves the intracerebral inoculation of mice with brain material from a suspect case and observation over several days for the development of disease. The brains of clinically affected mice are tested by FAT to confirm the presence of rabies virus. This test has been largely supplanted by the RTCIT.
- Reverse transcription-polymerase chain reaction (RT-PCR) has been applied successfully to the detection of viral RNA in diagnostic rabies samples (Hughes et al. 2004). A useful aspect of this technique is its ability to distinguish between rabies virus variants and between rabies virus and 'rabies-related' viruses (Heaton et al. 1997, Black et al. 2002).
- A number of serological tests are available for the measurement of an animal's response to vaccination. Serological testing is rarely used for confirmation of diagnosis due to late seroconversion and the high mortality rate of host species. However, serological tests are commonly used for trade purposes. Two virus neutralization tests are internationally recognized, the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralization test (FAVN). Results are expressed in International Units relative to an international standard serum. Appropriate containment facilities must be available for the handling of live virus in laboratories carrying out these tests. An indirect ELISA (Cliquet et al. 2000) with rabies glycoprotein-coated plates has been shown to be useful as a rapid screening test to determine if vaccinated cats and dogs have seroconverted without the need to handle live virus. It is considered to be less sensitive than virus neutralization and negative results should be confirmed by FAVN or RFFIT.

VESICULAR STOMATITIS

Vesicular stomatitis is a febrile, vesicular disease that primarily affects horses, cattle and pigs. Other susceptible species include sheep, goats, camelids, several wildlife species and man. It is clinically similar to foot-and-mouth disease and is classified as a listed disease by the Office International des Epizooties (OIE). Several closely related but antigenically distinct members of the genus *Vesiculovirus* are capable of producing the disease. The vast majority of outbreaks are associated with vesicular stomatitis Indiana virus (VSIV) or vesicular stomatitis New Jersey virus (VSNJV). The type species of the genus is vesicular stomatitis Indiana virus but the more important and generally more severe cause of disease in livestock is vesicular stomatitis New Jersey virus. Cocal (Argentina) virus and vesicular stomatitis Alagoas (Brazil) virus are also referred to as subtypes 2 and 3 of VSIV respectively and have been isolated from a number of outbreaks involving horses and cattle in South America. Personnel in contact with infected animals should be warned of the risk of zoonotic infection which manifests as an influenza-like illness.

Infection is endemic in Central America, parts of South America and parts of the USA. Outbreaks of disease occur every two to three years in tropical/subtropical regions and every five to 10 years in temperate areas. Clinical cases are most common at the end of the rainy season and early in the dry season in tropical and subtropical regions. Spread beyond endemic areas occurs explosively in some summers. Disease outbreaks in temperate regions usually cease abruptly with the onset of winter frosts. The mode of transmission is not completely understood but direct contact and insect vectors are important. Virus is shed from lesions into saliva and contaminates water and feed troughs.

Pathogenesis

The incubation period is one to five days. Subclinical infection is common. Affected animals are usually more than one year of age. Virus probably enters the body through cuts in the skin or mucous membranes or following inoculation by a biting insect. Vesicles appear on the tongue and on the mucous membranes of the mouth,

often accompanied by excessive salivation. Secondary lesions may be found on the coronary band and teats. It is unclear if these result from viraemia or are due to environmental contamination (Clarke et al. 1996). Lesions generally heal within about two weeks provided secondary infection is not a problem. Economic losses due to poor weight gains, loss of production, culling and implementation of other disease control measures may be considerable (Hayek et al. 1998).

Diagnosis

Rapid laboratory confirmation of a diagnosis is important because of the clinical similarities between vesicular stomatitis, foot-and-mouth disease and swine vesicular disease. The presence of clinical cases in horses is indicative of infection with vesicular stomatitis virus. Specimens suitable for the isolation of virus or the detection of viral antigen include vesicular epithelium and vesicle fluid.

- Demonstration of viral antigen can be carried out by CFT or ELISA (Ferris & Donaldson 1988, Alonso et al, 1991).
- Virus isolation can be carried out in susceptible cell lines, mice or the allantoic sac of embryonated hens' eggs. Many cell lines are susceptible including Vero and baby hamster kidney (BHK-21). The virus is highly cytopathic. Fluorescent antibody test, ELISA, CFT or virus neutralization test (VNT) are suitable for confirmation of the identity of an isolate.
- Electron microscopy may be performed on original specimens or on passaged material.
- Reverse transcription-PCR assays have been described for the diagnosis of vesicular stomatitis (Hofner et al. 1994, Rasmussen et al. 2005).
- Suitable assays for the detection of antibodies in recovered animals include liquid-phase blocking ELISA, CFT, VNT, ELISA and IgM-specific capture ELISA. As CF and IgM-type antibodies do not persist as long as virus neutralizing antibodies, these assays are useful for identifying recent infections in endemic areas (Katz et al. 1997).

BOVINE EPHEMERAL FEVER

Bovine ephemeral fever is an arthropod-borne viral disease of cattle and water buffalo that occurs in tropical and subtropical regions of Asia, Australia and Africa. A wide range of other ruminant species including Cape buffalo, wildebeest, waterbuck and deer undergo subclinical infection with the virus.

There is good epidemiological evidence that insect vectors (mosquitoes and *Culicoides* species) are involved in transmission of the virus. In tropical areas subclinical infections are common and outbreaks are strongly associated with recent rainfall. In more temperate regions epizootics occur during the summer months and tend to terminate with the onset of winter.

PATHOGENESIS

The incubation period is four to eight days. Infected animals are viraemic for only a few days and it is during this period that blood-sucking insects acquire the infection. The virus multiplies in the body of the insect vector and is shed in the insect's saliva. The virus is inoculated into the wounds inflicted at subsequent feeds. Many of the disease signs observed are due to the infected animal's response rather than to direct viral damage. There is a 'left shift' neutrophilia, increase in plasma fibrinogen and decrease in plasma calcium levels. The means by which the virus induces these changes is unclear. Disease tends to be more severe in well-conditioned animals, high yielding dairy cows and in animals forced to exercise during the illness. Affected animals become depressed, anorexic, lame and constipated. There is muscle stiffness and ruminal stasis. In the majority of cases the animal recovers, often quite dramatically after a few days of illness. The mortality rate is usually 1–2% but can be higher.

DIAGNOSIS

The diagnosis of bovine ephemeral fever is frequently based solely on clinical signs. The blood picture and blood biochemistry can be helpful with increased plasma fibrinogen, decreased plasma calcium levels and neutrophilia often present. Paired serum samples should be obtained and tested for a rise in antibody titre using ELISA or virus neutralization test. The interpretation of serological tests is complicated by antibodies resulting from infection with related but non-pathogenic ephemeroviruses such as Kimberley virus and Berrimah virus. Isolation of BEFV is difficult but possible in insect cell lines. A quantitative RT-PCR assay is available (Stram et al., 2005).

REFERENCES

Alonso, A., Martins, M., Gomes, M.P.D., et al., 1991. Development and evaluation of an enzyme-linked immunosorbent assay for detection, typing and subtyping of vesicular stomatitis virus. Journal of Veterinary Diagnostic Investigation 3, 287–292.

Black, E.M., Lowings, J.P., Smith, J., et al., 2002. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. Journal of Virological Methods 105, 25–35.

Clarke, G.R., Stallknecht, D.E., Howerth, E.W., 1996. Experimental infection of swine with a sandfly (*Lutzomyia shannoni*) isolate of vesicular stomatitis virus, New Jersey serotype. Journal of Veterinary Diagnostic Investigation 8, 105–108.

Cliquet, F., Sagne, L., Schereffer, J.L., et al., 2000. ELISA tests for rabies antibody titration in orally vaccinated foxes sampled in the fields. Vaccine, 18, 3272–3279.

Ferris, N.P., Donaldson, A.I., 1988. An enzyme-linked immunosorbent assay for the detection of VSV antigen. Veterinary Microbiology 18, 243–258.

Gould, A.R., Hyatt, A.D., Lunt, R., et al., 1998. Characterization of a novel lyssavirus isolated from Pteropid bats in Australia. Virus Research 54, 165–187.

Hayek, A.M., McCluskey, B.J., Chavez, G.T., et al., 1998. Financial impact of the 1995 outbreak of vesicular stomatitis on 16 beef ranches in Colorado. Journal of the American Veterinary Medical Association 212, 820–823.

Heaton, P.R., Johnstone, P., McElhiney, L.M., et al., 1997. Hemi-nested PCR assay for detection of six genotypes of rabies and rabies-related viruses. Journal of Clinical Microbiology 35, 2762–2766.

Hofner, M.C., Carpenter, W.C., Ferris, N.P., et al., 1994. A hemi-nested PCR assay for the detection and identification of vesicular stomatitis virus nucleic acid. Journal of Virological Methods 50, 11–20.

Hughes, G.J., Smith, J.S., Hanlon, C.A., et al., 2004. Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. Journal of Clinical Microbiology 42, 299–306.

Katz, J.B., Eernisse, K.A., Landgraf, J.G., et al., 1997. Comparative performance of four serodiagnostic procedures for detecting bovine and equine vesicular stomatitis virus antibodies. Journal of Veterinary Diagnostic Investigation 9, 329–331.

Krebs, J.W., Smith, J.S., Rupprecht, C.E., et al., 1998. Rabies surveillance in the United States during 1997. Journal of the American Veterinary Medical Association 213, 1713–1728.

Marston, D., Horton, D.L., Ngeleja, C., et al., 2012. Ikoma lyssavirus, highly divergent novel lyssavirus in African civet. Emerging Infectious Diseases 18, 664–667.

Rasmussen, T.B., Uttenthal, A., Fernandez, J., et al., 2005. Quantitative multiplex assay for simultaneous detection and identification of Indiana and New Jersey serotypes of vesicular stomatitis virus. Journal of Clinical Microbiology 43, 356–362.

Stram, Y., Kuznetzova, L., Levin, A., et al., 2005. A real-time RT-quantative(q) PCR for the detection of bovine ephemeral fever virus. Journal of Virological Methods 130, 1–6.

FURTHER READING

Blanton, J.D., Robertson, K., Palmer, D., et al., 2009. Rabies surveillance in the United States during 2008. Journal of the American Veterinary Medical Association 235, 676–689.

Bowen-Davies, J., Lowings, P., 2000. Current perspectives on rabies: 1 The biology of rabies and rabies-related viruses. In Practice March, 120–124.

King, A.A., 1998. Rabies. In: Palmer, S.R., Soulsby, L., Simpson, DIH (Eds.) Zoonoses: Biology, Clinical Practice and Public Health Control. Oxford University Press, Oxford, pp. 437–458.

Ministry of Agriculture, Fisheries and Food, UK, 1998. Quarantine and rabies, a reappraisal. MAFF Publications, London.

Smith, J.S., 1996. New aspects of rabies with emphasis on epidemiology, diagnosis, and prevention of the disease in the United States. Clinical Microbiology Reviews 9, 166–176.

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Chapter 64

Bunyaviridae

This family is one of the largest, with more than 300 member species. Bunyaviruses derive their name from Bunyamwera, the location in Uganda where the type species *Bunyamwera virus* was first isolated. The viruses are spherical, 80 to 120 nm in diameter and enveloped. The envelope is studded with projecting glycoprotein peplomers and encloses three circular, helical nucleocapsids (Fig. 64.1). The genome is made up of three circular, negative-sense (ambisense in the case of members of the genera *Phlebovirus* and *Tospovirus*) single-stranded RNA segments, which differ in size and are designated small (S), medium (M) and large (L). Four structural proteins: a large (L) transcriptase protein, a nucleocapsid (N) protein and two external glycoproteins (Gn and Gc) are described. Non-structural (NS) proteins are coded for by the S segment of RNA (designated NSs) and by the M segment (designated NSm). Genetic reassortment occurs between closely related viruses. Replication occurs in the cytoplasm of host cells. The envelope is acquired by budding into the Golgi cisternae. Transport through the cytoplasm in vesicles and release at the cell surface by exocytosis completes the replication cycle.

Members of the *Bunyaviridae* family are grouped into five genera; *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus* and *Tospovirus*. Within each genus viruses are placed into serogroups on the basis of serological testing and antigenic relatedness. More than 40 serogroups/species have been described in the genus *Orthobunyavirus*. A relatively small number of viruses in the family are significant pathogens of vertebrates and are contained in the genera *Orthobunyavirus, Phlebovirus, Nairovirus* and *Hantavirus* while the genus *Tospovirus* contains viruses of plants (Fig. 64.2). The viruses are sensitive to heat, acid pH, lipid solvents, detergents and many disinfectants.

CLINICAL INFECTIONS

In all genera except *Hantavirus* the viruses are arboviruses and maintained in nature in complex life cycles involving replication in arthropod vectors and vertebrate hosts (Table 64.1). Infection of mammalian cells results in cytolysis while infection of invertebrate cells is non-cytolytic and persistent. Mosquitoes form the largest, most important group of vector species but ticks, sandflies and biting midges are important for certain virus species. Transovarial and venereal transmission of virus has been shown to occur in vector species. Vertebrate hosts infected during blood feeding by the vector serve to amplify the amount of virus in circulation and pass on infection to susceptible arthropods during a viraemic period of variable intensity and duration. Virus species tend to display a narrow host range, particularly in their arthropod vectors. In contrast, hantaviruses are maintained in nature by chronic, non-pathogenic infections of rodents. Hantavirus infections established in susceptible mammalian cell lines are typically non-cytolytic and persistent. Virus is shed in urine, faeces and saliva facilitating transmission between rodent hosts by aerosol and biting. Each hantavirus species is associated with a particular rodent species.

Outbreaks of disease associated with bunyaviruses are frequently seasonal, reflecting natural fluctuations in arthropod or rodent populations. Most bunyaviruses are capable of infecting humans and causing serious disease including California encephalitis, haemorrhagic fever with renal syndrome, Crimean–Congo haemorrhagic fever and hantavirus pulmonary syndrome. These human infections are generally considered accidental and usually do not result in amplification of the virus in nature.

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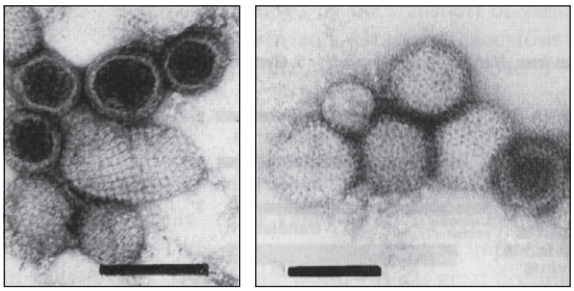

Figure 64.1 Negatively stained electron micrographs of; (left) Hantaan virus virions showing the pattern of peplomer placement in squares that is characteristic of all hantaviruses, (right) Rift Valley fever virus virions showing the delicate peplomer fringe. Bars represent 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Family* Bunyaviridae. *Page 471.*

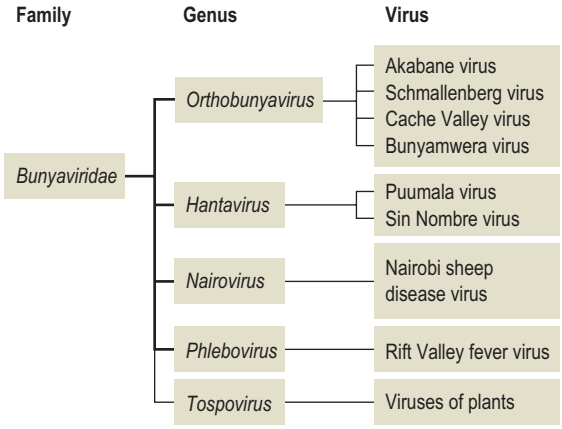

Figure 64.2 Summary outline of classification of members of *Bunyaviridae*.

Table 64.1 Bunyaviruses of veterinary significance

Genus	Virus	Host	Significance of infection
Phlebovirus	Rift Valley fever virus	Sheep, goats, cattle	Peracute and acute disease characterized by high mortality rates in neonates and abortion in pregnant animals. Endemic in southern and eastern Africa, transmitted by mosquitoes. Important zoonotic infection
Nairovirus	Nairobi sheep disease virus	Sheep, goats	Severe, often fatal disease in susceptible animals moved into enzootic areas. Present in central and east Africa. Transmitted by ticks
Orthobunyavirus	Akabane, Aino, Peaton, Tinaroo, Schmallenberg viruses	Cattle, sheep	Members of several serogroups, transmitted by mosquitoes and midges. Associated with congenital defects and abortion. Widespread distribution in Middle East, Asia, Australia and Africa. In 2011, Schmallenberg virus appeared in Germany and rapidly spread to several other European countries
Orthobunyavirus	Cache Valley virus	Sheep	Bunyamwera serogroup, transmitted by mosquitoes. Associated with occasional epizootics of congenital defects in flocks in North America

RIFT VALLEY FEVER

Rift Valley fever (RVF) is a peracute or acute disease of domestic ruminants in Africa characterized by high mortality rates in newborn animals and abortion in pregnant animals. The severity of the disease varies with the strain of virus involved and the susceptibility of the vertebrate species. A wide range of species are susceptible to infection but RVF occurs primarily in sheep, cattle and goats. Infections in indigenous African species and breeds tend to be less severe and may be inapparent. Rift Valley fever is an important zoonotic disease and must be handled with care in properly equipped laboratories.

Outbreaks of disease tend to occur in eastern and southern Africa at irregular intervals of five to 15 years or more. In recent years Rift Valley fever virus (RVFV) has spread to the Arabian Peninsula (Al-Afaleq et al. 2003). Outbreaks are associated with abnormally heavy rains and an explosive increase in vector populations. Transovarial transmission of RVFV occurs in *Aedes* species and the virus survives for very long periods in mosquito eggs dormant in the soil during dry periods. In an epidemic, RVFV is amplified in both domestic and wild ruminant species and can be

transmitted by many species of mosquito which become very numerous after heavy rains. Infected mosquitoes frequently transmit the infection to humans during such epidemics. A high level of viraemia occurs in infected ruminants for three to five days after infection and the blood and tissues of such animals are infectious. Abattoir workers and veterinarians are at particular risk.

Pathogenesis

The incubation period in lambs is 12 to 36 hours. Following infection by mosquito bite or through the oropharynx the virus spreads from the initial site of replication to target organs, principally the liver and spleen. An intense viraemia follows replication at target sites. There is widespread destruction of infected cells, particularly hepatocytes. Foetal death results from direct viral damage. A small proportion of the animals that survive the hepatic infection display nervous signs due to viral encephalitis. The mortality rate in lambs less than a week old is about 90%. Mortality rates in adult sheep may be as high as 60%, although five to 30% is more usual, while abortion rates approaching 100% are not unusual. The disease is not as devastating in cattle with mortality rates usually below 10% and abortion rates averaging 15 to 40%. Human RVFV infections are often inapparent but they may present as a moderate to severe influenza-like illness. In a small number of human cases the infection is fatal, characterized by haemorrhagic and/or encephalitic forms of the disease.

Diagnosis

- The histopathological lesions in lambs, and in particular the liver lesions, are considered to be pathognomonic. Confirmation can be obtained by immunohistochemical detection of viral antigen in fixed tissues.
- The virus can be isolated in susceptible cell cultures including Vero, baby hamster kidney and primary bovine or ovine cell lines. A cytopathic effect characterized by slight rounding of cells followed by the rapid destruction of the whole monolayer is observed in RVFV-positive cultures. The virus may also be isolated using laboratory animals or embryonated hens' eggs. Suitable specimens include blood from viraemic animals, foetal organs or liver, spleen and brain from animals that have died. The virus has caused disease in laboratory personnel.
- More rapid confirmatory tests include detection of viral antigen in impression smears of liver, spleen and brain by immunofluorescence or in the serum of febrile animals by ELISA. Viral antigen can also be detected in liver samples from affected animals using agar gel immunodiffusion.
- Detection of viral RNA in serum and tissues using RT-PCR and real time RT-PCR has been successful (Sall et al. 2001, Espach et al. 2002, Bird et al. 2007). Sequencing of the amplified product, the NS(S) protein coding region, has identified two distinct phylogenetic lineages of RVFV, Egyptian and sub-Saharan (Sall et al. 1997).
- A serological diagnosis may be achieved by demonstration of IgM by ELISA on single acute sera or demonstration of seroconversion by virus neutralization, ELISA (Paweska et al. 2003) or haemagglutination inhibition on paired sera (Paweska et al. 1995). The neutralization test is highly specific and sensitive but requires the use of live virus and is not recommended for use outside endemic areas.

NAIROBI SHEEP DISEASE

Nairobi sheep disease is a highly pathogenic, tick-borne infection of sheep and goats in central and east Africa. Ganjam virus of sheep and goats in India is considered to be a strain of Nairobi sheep disease virus (NSDV), while NSDV is now considered to be a strain of Dugbe virus. Although humans are susceptible to NSDV, infection which results in a mild influenza-like illness appears to be uncommon.

The brown ear tick *Rhipicephalus appendiculatus* is the principal invertebrate host and vector of the virus. Transovarial and transstadial infection occurs; infection can be transmitted by all stages of the brown ear tick. In enzootic areas lambs and kids are exposed to infection while still protected by maternal antibody and develop active immunity. Outbreaks of disease generally arise from the movement of susceptible animals into enzootic areas or following the incursion of infected ticks into NSD-free areas.

Pathogenesis

The incubation period varies from two to five days. Following inoculation of the virus by an infected tick, the virus reaches its target organs via the bloodstream. It has a predilection for vascular endothelium, replicating to high titres in the lungs, liver, spleen and other organs of the reticuloendothelial system. Pregnant animals commonly abort. The mortality rate varies from 30 to 90%, with death occurring from two to 11 days after the onset of clinical signs. The disease is more severe in native breeds of sheep than in exotic breeds.

Diagnosis

A history of recent movement of the flock into an enzootic area and high mortality rate is suggestive. Suitable specimens from febrile or dead animals for virus isolation in cell culture or inoculation of laboratory animals include

uncoagulated blood, mesenteric lymph nodes and spleen. The inoculation of susceptible sheep is the most sensitive method of detection. Susceptible cell lines include BHK-21, Vero and primary or secondary lamb or hamster kidney cells. Most strains of NSDV produce a cytopathic effect. Direct immunofluorescence is useful for identification of the virus in inoculated tissue cultures. Alternatively viral antigen can be detected directly in tissue specimens by AGID. The recommended serological test for the detection of antibodies to NSDV is the indirect immunofluorescence test.

AKABANE DISEASE

Orthobunyaviruses associated with congenital defects, principally arthrogryposis and hydranencephaly, as well as abortion in cattle and sheep include Akabane, Aino, Schmallenberg, Douglas, Tinaroo, Peaton and Cache Valley viruses. Akabane virus is the best studied and probably most pathogenic virus of the group. Serological studies indicate a widespread distribution in tropical and subtropical regions in the Middle East, Asia, Australia and Africa. Sporadic epizootics of developmental deformities associated with these viruses have been described in Japan, Australia, Israel and parts of Africa. Akabane virus is transmitted by insect vectors, midges and mosquitoes. Disease outbreaks tend to coincide with the introduction of susceptible animals into enzootic areas or the movement of the vector species. The primary pathological lesions produced in the foetus are encephalomyelitis and polymyositis. The extent of the damage is related to the stage of gestation at which infection occurs. In cattle most damage is seen in animals infected at 12 to 16 weeks of gestation when neural tissues are differentiating. Clinical signs are rare in the dam (Lee et al. 2002). Diagnosis is based on pathology and detection of specific neutralizing antibody in sera from aborted or newborn animals prior to suckling. Suitable serological tests include virus neutralization and ELISA. Virus isolation from foetal tissues is generally not used for diagnosis as viral clearance by the foetal immune response has usually occurred by the time of birth. Susceptible cell lines include Vero and BHK-21. Viral antigen may be detectable in brain and muscle of aborted foetuses by immunofluorescence. Conventional and real-time RT-PCR assays are available for the detection and differentiation of Akabane virus from other bunyaviruses (Akashi et al. 1999, Stram et al. 2004).

CACHE VALLEY VIRUS

Cache Valley virus (CVV) is a member of the Bunyamwera serogroup of the genus *Orthobunyavirus* and mainly affects sheep. It is endemic to North America and infects domestic ruminants, deer and horses. Most infections are subclinical but outbreaks in sheep flocks characterized by abortion and the birth of lambs with arthrogryposis, hydranencephaly and vertebral column deformities have been described. It is transmitted by mosquitoes. The virus cannot be isolated at birth from the foetus but can be isolated from viraemic adult animals or insect vector pools in BHK and Vero cells. Group-specific and virus-specific primers have been designed for use in RT-PCR (Kuno et al. 1996, Moreli et al. 2001). Virus neutralization and ELISA have been used to detect antibodies to CVV.

REFERENCES

Akashi, H., Onuma, S., Nagano, H., et al., 1999. Detection and differentiation of Aino and Akabane Simbu serogroup bunyaviruses by nested polymerase chain reaction. Archives of Virology 144, 2101–2109.

Al-Afaleq, A.I., Abu Elzein, E.M.E., Mousa, S.M., et al., 2003. A retrospective study of Rift Valley fever in Saudi Arabia. Revue Scientifique et Technique de l'Office International des Epizooties 22, 867–871.

Bird, B.H., Bawiec, D.A., Ksiazek, T.G., et al., 2007. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. Journal of Clinical Microbiology 45, 3506–3513.

Espach, A., Romito, M., Nel, L.H., et al., 2002. Development of a diagnostic one-tube RT-PCR for the detection of Rift Valley fever virus. Onderstepoort Journal of Veterinary Research 69, 247–252.

Kuno, G., Mitchell, C.J., Chang, G.J., et al., 1996. Detecting Bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. Journal of Clinical Microbiology 34, 1184–1188.

Lee, J.K., Park, J.S., Choi, J.H., et al., 2002. Encephalomyelitis associated with akabane virus infection in adult cows. Veterinary Pathology 392, 269–273.

Moreli, M.L., Aquino, V.H., Figueiredo, L.T., 2001. Identification of Simbu, California and Bunyamwera serogroup bunyaviruses by nested RT-PCR. Transcripts of the Royal Society for Tropical Medicine and Hygiene 95, 108–113.

Paweska, J.T., Barnard, B.J.H., Williams, R., 1995. The use of sucrose-acetone-extracted Rift Valley fever virus antigen from cell culture in an indirect enzyme-linked immunosorbent assay and haemagglutination-inhibition tests. Onderstepoort Journal of Veterinary Research 62, 227–233.

Paweska, J.T., Smith, S.J., Wright, I.M., et al., 2003. Indirect enzyme-linked immunosorbent assay for the

detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. Onderstepoort Journal of Veterinary Research 70, 49–64.

Sall, A.A., De Azanotto, P.M., Zeller, H.G., et al., 1997. Variability of the NS(S) protein among Rift Valley fever virus isolates. Journal of General Virology 78, 2853–2858.

Sall, A.A., Thonnon, J., Sene, O.K., et al., 2001. Single-tube and nested reverse transcriptase-polymerase chain reaction for the detection of Rift Valley fever virus in human and animal sera. Journal of Virological Methods 91, 85–92.

Stram, Y., Kuznetzova, L., Guini, I.M., et al., 2004. Detection and quantitation of akabane and aino viruses by multiplex real-time reverse-transcriptase PCR. Journal of Virological Methods 116, 147–154.

FURTHER READING

de la Concha-Bermejillo, A., 2003. Cache Valley virus is a cause of fetal malformation and pregnancy loss in sheep. Small Ruminant Research 49, 1–9.

Gerdes, G.H., 2004. Rift Valley fever. Revue Scientifique et Technique de l'Office International des Epizooties 23, 613–623.

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Chapter 65

Retroviridae

Retroviruses (Latin *retro*, backwards) are spherical, labile, enveloped RNA viruses with a diameter of 80 to 100 nm (Fig. 65.1). Two subfamilies comprising seven genera are currently recognized within the family (Fig. 65.2): *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus* and *Lentivirus* in the subfamily *Orthoretrovirinae* while the subfamily *Spumaretrovirinae* contains a single genus, *Spumavirus*. The family name refers to the presence in the virion of the enzyme reverse transcriptase which is encoded by the *pol* gene. The envelope is acquired from the plasma membrane of the host cell. It surrounds an icosahedral capsid which contains two identical linear, positive-sense, single strands of RNA and a number of core proteins including the enzymes reverse transcriptase and integrase. Historically, based on their appearance using electron microscopy, retroviruses were described as A-type, B-type, C-type and D-type particles. Retroviruses are sensitive to heat, lipid solvents and detergents. However, on account of their diploid genomes, they are relatively resistant to UV light.

Reverse transcriptase functions as an RNA-dependent DNA polymerase, transcribing from RNA to DNA. The four major genes are 5′-*gag-pro-pol-env*-3′. The *gag* (group-specific antigen) gene encodes internal structural proteins. The *pro* (protease) gene encodes the enzyme protease while the *pol* (polymerase) gene encodes the enzymes reverse transcriptase and integrase. The *env* (envelope) gene encodes surface (SU) and transmembrane (TM) envelope glycoproteins. Cell entry follows attachment of an envelope glycoprotein to specific cell receptors. Double-stranded DNA copies of the viral genome are synthesized in the cytoplasm of the host cell under the direction of the reverse transcriptase. During this process, repeat base sequences, containing several hundred base-pairs are added to the ends of the DNA transcripts. These sequences are called long terminal repeats (LTR). The DNA transcripts are integrated into the chromosomal DNA at random sites under the direction of the viral integrase and referred to as provirus. The nature and extent of host cell changes following infection are determined by the sites of proviral integration. Important promoter and enhancer sequences are located within the LTRs and these are involved in the transcription of mRNA and virion RNA from provirus. Release of mature virions typically occurs by budding from cell membranes. Insertion of a provirus of certain retroviruses close to host cell genes responsible for the regulation of cell division (insertional mutagenesis) may result in an increase in the rate of mitosis and give rise to an increased tendency for the cell to transform.

A high mutation rate is a feature of retroviral replication with errors occurring relatively frequently during reverse transcription. In addition, reverse transcriptase can transfer from the RNA template of one virus to that of another giving rise to recombination between retroviral genomes in multiply infected cells. As a consequence, antigenically distinct retroviruses or quasispecies emerge at frequent intervals making precise classification of species and subtypes difficult.

Retroviruses may be described as endogenous or exogenous retroviruses. Endogenous retroviruses occur widely among vertebrates. They are the result of infection of germline cells at some time in the past and are transmitted only as provirus in germ cell DNA from dam to offspring. The provirus genes are usually silent and are under the control of cellular genes. Endogenous retroviral genomes may contribute *env* genes to produce recombinant feline leukaemia viruses and avian leukosis viruses. It is sometimes possible to reactivate endogenous retrovirus elements by irradiation, mutagens or carcinogens, resulting

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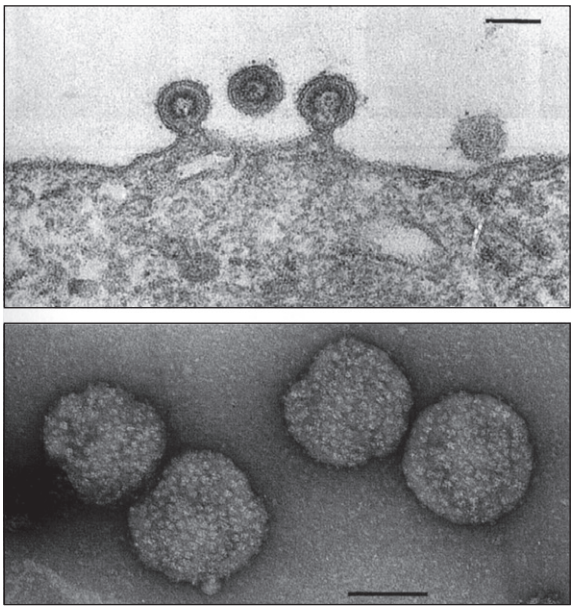

Figure 65.1 Top: Budding of typical type C retrovirus virions; Bottom: Retrovirus virions stained negatively with uranyl acetate showing peplomers on their surface. Bars represent 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 365.*

in the completion of replication and the production of new virions. There are fears that endogenous retroviruses of pigs may pose a danger to humans receiving xenotransplants.

Retroviruses in the genera *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus* and *Epsilonretrovirus* are frequently referred to as oncogenic retroviruses because they can induce neoplastic transformation in host cells. Exogenous oncogenic retroviruses are described either as slowly transforming (cis-activating) viruses or as rapidly transforming (transducing) viruses depending on the interval between exposure to the virus and tumour development. For example, slowly transforming retroviruses induce B cell, T cell or myeloid tumours after long incubation periods. The provirus must be integrated into the host cell DNA close to a cellular oncogene (c-*onc*, proto-oncogene), resulting in interference with the regulation of cell division (cis-activation) or alternatively the proviral LTR may increase the rate of mitosis and enhance the risk of neoplasia. Insertion of the provirus is largely random and it may be some time before the provirus is inserted in a position in the cell genome that gives rise to transformation of the cell. Rapidly transforming retroviruses, which can induce tumour formation after short incubation periods, contain viral oncogenes (v-*onc*). Viral oncogenes are cellular oncogenes which have been acquired by the virus through recombination. In the case of Rous sarcoma virus the oncogene has been integrated into the viral genome without loss of replicative virus genes. This retrovirus is described as replication-competent. However, it is more common for existing viral sequences necessary for replication to be deleted as a consequence of cellular oncogene integration into the viral genome, These retroviruses are referred to as replication-defective retroviruses, because they cannot multiply without helper viruses (related replication-competent viruses). They are rarely transmitted under normal field conditions but may cause a rapidly developing neoplastic disease in the host animal in which they arise. A third method of tumour induction by retroviruses has been described. Bovine leukaemia virus possesses a *tax* gene which encodes for a protein capable of up-regulating both viral LTR and cellular promoter sequences. This up-regulation can occur even when the provirus is integrated into a different chromosome from the one containing the up-regulated cellular sequences (transactivation).

The important oncogenic retroviruses of veterinary species are presented in Table 65.1. The genus *Epsilonretrovirus* contains viruses associated with neoplasia in fish. Lentivirus (Latin *lentus*, slow) infections are characterized by diseases with long incubation periods and insidious protracted courses. Examples of important animal and human lentivirus diseases include acquired immunodeficiency syndrome (AIDS), feline immunodeficiency, equine infectious anaemia and maedi-visna (Table 65.2). Spumaviruses (Latin *spuma*, foam) have been described in primates, cattle, horses and cats. They cause vacuolation of cultured cells, but are not associated with clinical disease.

AVIAN LEUKOSIS

Both replication-competent and replication-defective retroviruses make up the avian leukosis virus (ALV) group. These viruses are associated with neoplastic conditions in chickens including lymphoid, erythroid and myeloid leukoses, fibrosarcoma, haemangiosarcoma and nephroblastoma. Lymphoid leukosis, a B cell lymphoma, is the most common and economically important. Viral envelope glycoproteins determine virus neutralization properties, viral interference patterns and host range. Avian leukosis viruses can be divided into ten subgroups (A to J) on the basis of differences in these glycoproteins. Subgroups A, B, C, D, E and J contain the isolates from chickens. Isolates from outbreaks of disease in chickens generally belong to subgroup A. Endogenous avian leukosis viruses, subgroup E, are commonly present in chickens and are transmitted vertically in the germline cells. Subgroup J isolates are associated with myeloid leukosis in broilers and have arisen from recombination of a novel

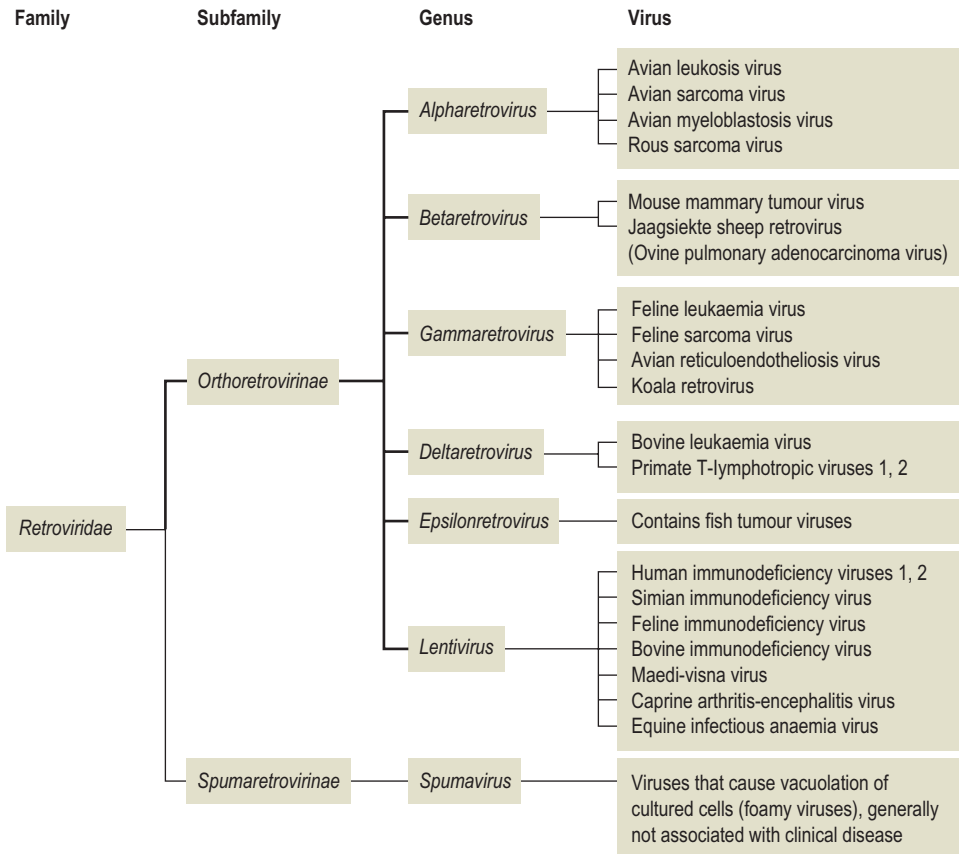

Figure 65.2 Outline of classification of retroviruses.

family of endogenous viruses (ev/J) and exogenous avian leukosis viruses (Benson et al. 1998).

There is usually an incubation period of months to years between natural infection with ALV and the development of neoplasia, because of the time required for the genetic events to occur that lead to transformation of cells to malignancy. Neoplastic conditions associated with ALV include lymphoid leukosis, myeloid leukosis, sarcomas, osteopetrosis and renal tumours. The generation of recombinant, rapidly transforming viruses, which have incorporated a cellular oncogene into their genome, often occurs on the pathway to transformation. Examples of such viruses isolated from tumours include avian erythroblastosis virus, avian myeloblastosis virus and Rous sarcoma virus.

Transmission of exogenous ALV occurs both vertically, through virus present in egg albumen, and horizontally, by close contact via saliva and faeces. The chicks that hatch from infected eggs are usually immunotolerant and persistently viraemic. As a result they represent the principal on-going source of virus in a flock. In contrast, the chicks infected after hatching usually develop a transient viraemia before they produce neutralizing antibodies. Some horizontally infected chicks may become persistently infected, particularly if maternal antibodies are absent and the birds are exposed very early in life. Neoplasms occur most frequently in the congenitally infected birds. In general, natural exposure of adult birds to infection does not result in virus shedding. Virus-neutralizing antibodies, passed from antibody-positive hens in the yolk sac to their chicks, provide protection for the first few weeks of life.

Pathogenesis

Lymphoid leukosis has an incubation period of four months or more. Following infection the virus spreads throughout the body with most tissues supporting viral replication. Avian leukosis virus transforms B cells in the bursa of Fabricius by integrating as provirus close to the c-*myc* gene which becomes abnormally expressed as a result of the influence of the viral LTR promoter. The

Table 65.1 Oncogenic retroviruses of veterinary importance

Genus	Virus	Hosts	Comments
Alpharetrovirus	Avian leukosis virus	Chickens, pheasants, partridge, quail	Endemic in commercial flocks, where both exogenous and endogenous transmission of virus can occur. May result in lymphoid leukosis in birds between five and nine months of age
Gammaretrovirus	Reticuloendotheliosis virus	Turkeys, ducks, chickens, quail, pheasants	Infection is usually subclinical but sporadic disease occurs with anaemia, feathering defects, impaired growth or neoplasia. Some outbreaks associated with use of vaccine contaminated with reticuloendotheliosis virus
Deltaretrovirus	Bovine leukaemia virus	Cattle	Cause of enzootic bovine leukosis in adult cattle, an important worldwide infection that results in lymphosarcoma in some infected individuals
Betaretrovirus	Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus)	Sheep	Slowly progressive but invariably fatal neoplastic lung disease of adult sheep. Worldwide, except Australasia
	Enzootic nasal tumour virus	Sheep, goats	Closely related to jaagsiekte sheep retrovirus. Causes adenocarcinoma of nares, low grade malignancy
Gammaretrovirus	Feline leukaemia virus	Cats	Important, worldwide viral infection of cats, producing chronic illness and death in young adult cats. Variable presentation including immunosuppression, enteritis, reproductive failure, anaemia and neoplasia

Table 65.2 Lentiviruses of veterinary importance

Virus	Hosts	Comments
Maedi-visna virus	Sheep	Lifelong infection with clinical signs in some infected animals. Signs of progressive respiratory disease and indurative mastitis in older sheep, rarely presents as progressive neurological disease
Caprine arthritis-encephalitis virus	Goats	Worldwide distribution, mostly seen in dairy herds. Lifelong infection associated with polyarthritis and indurative mastitis in adults; progressive nervous disease in kids
Bovine immunodeficiency virus	Cattle	Widely distributed but pathogenicity unclear
Equine infectious anaemia virus	Horses, donkeys, mules	Lifelong infection with recurring febrile episodes and anaemia
Feline immunodeficiency virus	Cats	Worldwide distribution. Lifelong infection characterized by persistent viraemia and immunosuppression in cats over five years of age

developing lymphoma metastasizes to the viscera. Less commonly ALV has been associated with erythroblastosis where the c-*erb*B gene in an erythroid cell is activated by insertional mutagenesis. Rapidly transforming viruses arise as a rare event in individual birds by the transduction and modification of a cellular proto-oncogene in transforming avian retroviruses. Multiple insertions of the v-*onc* gene into a host cell genomes give rise to excessive gene expression and over-production of a transformation-associated protein which may act as a hormone or growth factor receptor, a transcription factor in the nucleus or a kinase in a signal transduction pathway.

Disease tends to be sporadic in infected flocks. Affected birds become weak and emaciated with pale wattles. The

liver and bursa of Fabricius may be enlarged. Subclinical infections are associated with depressed egg production and fertility, decreased hatchability and growth rate, and increased death rates. The increased deaths in egg-laying and breeding birds between five and nine months of age accounts for most of the economic loss associated with ALV infection.

Diagnosis

- Post mortem findings and histopathological determination of tumour type are often sufficiently characteristic to be diagnostic.
- Differentiation from Marek's disease is important. It is based on the age of affected birds, the presence of bursal tumours, an absence of thickening of peripheral nerves and a histological assessment of neoplastic cell types.
- Virus isolation is difficult and generally not attempted.
- Commercial ELISA kits for the detection of ALV p27 group-specific antigen are available. However, ALVs are widespread in poultry flocks and may be present in the absence of tumour formation. Detection of ALVs in flocks is particularly useful in control programmes aimed at the elimination of ALV-positive birds from breeder flocks.
- The presence of flock infection can be demonstrated by detecting antibodies in serum or egg yolk. Suitable assays include virus neutralization, ELISA and indirect immunofluorescence.
- The RT-PCR assay has been developed for the detection of ALV (Smith et al. 1998, Cavanagh 2001). Sequencing of the amplicon can be used to determine the infecting subgroup and to differentiate endogenous ALV from exogenous ALV (Pham et al. 1999). Alternatively primers specific for particular subgroups may be used (Garcia et al. 2003, Silva et al. 2007).

ENZOOTIC BOVINE LEUKOSIS

This worldwide, retroviral disease of adult cattle is characterized by persistent lymphocytosis and the development of B cell lymphosarcoma in a number of infected animals. A number of countries have eradicated enzootic bovine leukosis (EBL), while other countries are embarking on eradication programmes. Bovine leukaemia virus (BLV) is labile and intimately cell-associated. Transmission occurs by direct contact or transplacentally through the transfer of blood or secretions such as colostrum and milk containing infected lymphocytes. Iatrogenic transmission is important. Transplacental transmission is not particularly efficient with less than 10% of calves born to infected dams being infected at birth. In addition, calves are protected for several months by maternally derived antibody. Typically animals are infected between six months and three years of age (Hopkins & DiGiacomo 1997). The prevalence of infection is higher in dairy cattle than in beef cattle. Susceptibility to infection is influenced by the animal's genotype.

Pathogenesis

Infections are lifelong but most animals remain subclinically infected. Approximately 30% of infected animals develop persistent lymphocytosis. A small percentage of infected cattle develop lymphosarcoma. The primary target cell for the virus is the B lymphocyte. Athough bovine leukaemia virus does not possess an oncogene, there are nucleic acid sequences at the 3′ end of the *env* gene (the X region) which encode the regulatory proteins Tax and Rex. These proteins are central to neoplastic transformation. Clinical disease occurs in adult animals between four and eight years of age. The presenting signs are determined by the sites of tumour formation and include enlargement of superficial lymph nodes, digestive disturbance, general debility and weight loss.

Diagnosis

Differentiation of EBL from sporadic bovine leucosis, which usually affects calves and young adult cattle, is necessary. Formerly, blood lymphocyte counts were used for laboratory diagnosis and for the eradication of EBL. However, lymphocytosis is not present in all cases and serological testing for virus-specific antibody is now generally used for diagnosis and eradication.

- Several serological tests such as AGID, ELISA and radioimmune assay can be used for the detection of antibodies to BLV, usually antibodies directed against the gp51 and p24 of the virus. Both indirect and blocking ELISAs are available commercially and may be designed for use with bulk milk samples or serum samples. Antibodies present in calves less than six months of age may be colostral in origin.
- Virus isolation, by cultivation of peripheral blood lymphocytes, is not performed routinely. Infected cells are usually co-cultivated with an indicator cell line such as bovine lung cells and infectious virus production is encouraged by the use of mitogens. The virus causes syncytial development in the cell sheet.
- The polymerase chain reaction has been utilized for the detection of provirus in peripheral blood lymphocytes (Ballagi-Pordany et al. 1992, Belak & Ballagi-Pordany 1993). It is useful as a confirmatory test in individual cases or as an adjunct to large-scale serological testing.

JAAGSIEKTE

Jaagsiekte (Afrikaaner word meaning 'panting sickness') is caused by jaagsiekte sheep retrovirus (JSRV), also known as ovine pulmonary adenocarcinoma virus. The disease is also called ovine pulmonary adenomatosis and is a slowly progressing neoplastic disease of adult sheep. With the exception of Australasia and Iceland, jaagsiekte has a worldwide geographical distribution. Infection occurs rarely in goats. About 20 copies of related endogenous betaretroviruses (enJSRV) occur in the genome of sheep and goats. Expression of these endogenous viruses during foetal ontogeny may be the reason for the apparent immune tolerance and lack of immune response in mature sheep to exogenous JSRV (Palmarini et al. 2004). Transmission of JSRV occurs by the respiratory route and close contact facilitates spread of infection. The disease incidence in an infected flock may be up to 20%, it is influenced by breed and the type of flock management.

Pathogenesis

The incubation period is highly variable ranging from several months up to two years. Virus replication occurs in two types of pulmonary cells, type II pneumocytes and non-ciliated bronchial cells. Tumours arising from these cell types progressively replace normal lung tissue. A viral oncogene has not been detected and the mechanism of neoplastic transformation is unclear. Studies have confirmed the transformation potential of the envelope protein in sheep (Caporale et al. 2006). Affected animals are usually three to four years of age. They are in poor bodily condition and display mouth breathing, particularly after exercise. By raising the hind legs and lowering the head (wheelbarrow test), a clear fluid can be seen to flow from the nostrils. At any one time only a single animal in an infected flock may be clinically affected. The course of the disease may extend over weeks or months and secondary pasteurellosis is common.

Diagnosis

Secondary infection can mask the characteristic clinical signs. Confirmation by histopathological examination of affected lung tissue is necessary. Attempts to culture JSRV have been unsuccessful to date. Although ovine herpesvirus 1 is frequently found in association with the tumours, there is no evidence of an aetiological role for this virus. It is possible to detect viral antigen by ELISA (Palmarini et al. 1995), while viral nucleic acid may be detected in tumour tissue and peripheral blood mononuclear cells by PCR (Bai et al. 1996, Palmarini et al. 1996, Gonzalez et al. 2001). Infected animals do not appear to develop a specific humoral immune response (Ortin et al. 1998).

SMALL RUMINANT LENTIVIRUS GROUP

Two closely related lentiviruses have been described in small ruminants: maedi-visna virus (MVV) affects sheep while caprine arthritis-encephalitis virus (CAEV) affects adult goats and kids. These viruses cause persistent infections and similar disease syndromes. Each virus can infect both sheep and goats. Genomic analyses suggest that they evolved from a common ancestral genotype. Small ruminant lentivirus isolates comprise a single, heterogeneous group with a variable host range and different pathogenic capabilities (Leroux et al. 1997a, Pasick 1998, Shah et al. 2004). For both viruses the most important antigens for serology are gp135, a viral envelope glycoprotein, and p28, a core protein. It has been suggested that a combination of serology and PCR is optimal for the diagnosis of these infections because PCR is capable of detecting infection in animals prior to seroconversion (de Andres et al. 2005).

Maedi-Visna

Maedi-visna, also known as ovine progressive pneumonia, la bouhite and zwoegersiekte, is present in many countries. Infection is lifelong and frequently subclinical but can cause chronic progressive disease in adult sheep. Maedi-visna caused significant losses in Icelandic sheep before its eradication in 1965. In fact, maedi and visna are Icelandic words meaning 'laboured breathing' and 'wasting' respectively. They refer to the two clinical forms described: the respiratory form and the rare nervous form. The severity of the clinical disease is influenced by viral virulence, the age of the host when exposed and other host factors. The virus is mainly transmitted via pulmonary exudates and colostrum or milk. Ovine lentiviruses occur in many countries except Iceland, Australia and New Zealand.

Pathogenesis

Clinical disease is rarely observed in animals less than two years of age. There is a chronic, progressive inflammatory reaction characterized by mononuclear cell infiltration and lymphoproliferation particularly in the lungs and mammary glands. Lesions may also be found in synovial membranes and in the brain. Progressive respiratory distress is the most common clinical presentation. Indurative mastitis resulting in a reduction in milk production and poor growth of lambs is a relatively common finding. Lameness has been described in some flocks. Neurological signs are relatively rare.

It is only when an infected monocyte develops into a macrophage that the provirus located in the genome is activated. This has been dubbed the 'Trojan horse'

mechanism, permitting the insidious spread of virus around the body in monocytes with minimal immune stimulation. A humoral and cell-mediated immune response occurs which is sufficient to restrict virus production to low levels, but does not eliminate infection. The period from infection to seroconversion is usually several weeks but may be delayed for months or years, reflecting the low level of viral antigen production (Brodie et al. 1998).

Diagnosis

A clinical diagnosis of maedi-visna may be possible and can be confirmed serologically.

- Commonly used serological assays include AGID (Cutlip et al. 1977), ELISA (Houwers & Schaake 1987, Simard & Briscoe 1990, Zanoni et al. 1994) and Western blotting. Commercial ELISAs are available (de Andres et al. 2005). Serological testing is best conducted on a flock basis. The time required for seroconversion may be long and unpredictable but, once antibody production is initiated, it continues.
- Histological examination of appropriate tissues including lung, CNS, udder and joints usually reveals an inflammatory reaction consisting of an interstitial mononuclear cell reaction that may be accompanied by accumulations of lymphoid cells and follicle formation.
- Virus isolation is possible but time-consuming, expensive and usually not required. In the live animal it is carried out by co-cultivating peripheral blood or milk leukocytes with ovine choroid plexus cells. Tissue samples collected at post mortem can be established as explant cultures. The cultures should be maintained over a period of several weeks with regular examination for CPE, characterized by the appearance of refractile stellate cells and syncytia. The presence of viral antigen can be confirmed by immunolabelling.
- The two principal difficulties regarding nucleic acid detection are strain variability and low virus load in vivo. Primers directed against more conserved regions of the genomes, such as the *pol* or LTR regions, will help to minimize the effects of strain variation. Proviral nucleic acid can be detected in peripheral blood and in tissues by PCR (Johnson et al. 1992, Wagter et al. 1998) while RT-PCR has been used to detect virus in milk (Leroux et al. 1997b). Quantitative RT-PCR has also been described (Gudmundsson et al. 2003).

Caprine Arthritis-Encephalitis

This worldwide disease of goats is characterized by polyarthritis in adults. Rarely cases of leuko-encephalomyelitis have been described in kids. Caprine arthritis-encephalitis virus (CAEV) gives rise to a persistent infection. Clinical disease is uncommon even though infection is common in dairy goats. Typically the virus is acquired by kids through the ingestion of colostrum or milk from infected does.

Pathogenesis

The pathogenesis is similar to that of maedi-visna. Persistent infection and non-protective immune mechanisms are considered to be responsible for lesion development. The most common presentation in adult animals is slowly progressive arthritis. Chronic mastitis and reduced milk production also occur in affected does.

Diagnosis

- Laboratory confirmation is based on detection of virus-specific antibodies. The most commonly used assays are AGID (Knowles et al. 1994) and ELISA (Rimstadt et al. 1994, Herrmann et al. 2003). Commercial ELISAs are available (de Andres et al. 2005).
- Virus isolation is possible by co-cultivating leukocytes from blood or milk with goat synovial membrane cells. Following detection of CPE, similar to that described for MVV, the presence of viral antigen can be confirmed by immunolabelling. Explant cultures established using synovial membrane from affected joints collected at post mortem may also be attempted.
- Nucleic acid detection of CAEV by PCR in blood and tissues has been described (Reddy et al. 1993, Barlough et al. 1994).

EQUINE INFECTIOUS ANAEMIA

Equine infectious anaemia, also called swamp fever, affects horses, mules and donkeys in many countries. Equine infectious anaemia virus (EIAV), a lentivirus, is transmitted mechanically on the mouthparts of haematophagous insects particularly *Tabanus* species and *Stomoxys* species. Transmission occurs during the summer in low-lying swampy areas close to woodlands. Iatrogenic transmission is possible through contaminated needles or surgical instruments.

Pathogenesis

The incubation period is usually one to three weeks. Infected animals may present with fever, depression and petechiae on mucous membranes and conjunctivae. The clinical signs are largely attributable to the host's immune response rather than to direct viral damage. The virus replicates in macrophages, monocytes and Kupffer cells with

dissemination throughout the body (Oaks et al. 1998). Infected horses fail to eliminate the virus and become persistently infected despite mounting a strong immune response. Mutations frequently arise in the viral genome during these persistent infections resulting in the emergence of new virus strains exhibiting antigenic variation in envelope glycoproteins gp45 and gp90 (antigenic drift). The emergence of each new strain is marked by febrile episodes and marked immune stimulation. The number and severity of recurring disease episodes varies widely in individual animals. Most occur during the first year after infection and decline in number thereafter. Non-neutralizing antibodies are produced against the virus early in the course of infection and lead to the formation of immune complexes, which activate complement contributing to the signs of fever, anaemia, thrombocytopenia and glomerulonephritis. In most animals, the infection is brought under control and clinical episodes eventually cease, probably as a consequence of a broad-based neutralizing response against a wide range of viral epitopes. Infected horses, which may appear clinically normal, remain lifelong viraemic carriers. However, a few horses go on to develop a chronic form of the disease characterized by weight loss, anaemia, ventral oedema and debilitation, leading eventually to death.

Diagnosis

Laboratory confirmation of infection usually relies on the demonstration of serum antibodies to the virus core protein p26.

- The AGID test (Coggins test) is the serological test that is recognized for international trade (Coggins et al. 1972). Although the ELISA (Suzuki et al. 1982) is a sensitive assay, positive results need to be confirmed by the more specific AGID test. Positive results can also be confirmed by immunoblotting. Early in the course of the disease it may not be possible to detect antibodies. False-positive results due to the presence of colostral antibodies may be encountered in foals up to six months of age.
- The presence of virus in blood can be demonstrated by intravenously inoculating a susceptible horse with blood from the suspect horse. The clinical status and antibody response of the inoculated animal are then monitored for at least 45 days.
- Virus can be isolated in monocyte-derived macrophage cultures prepared from the blood of susceptible horses free of infection. Virus isolation is rarely attempted or required due to the time and expense involved.
- Proviral DNA may be detected in peripheral blood by nested PCR (Nagarajan & Simard 2001). A real-time reverse transcription-PCR assay has been described for the quantification of viral RNA in the blood of infected horses (Cook et al. 2002).

FELINE LEUKAEMIA AND ASSOCIATED CLINICAL CONDITIONS

Infection with feline leukaemia virus (FeLV) occurs worldwide and is associated with feline leukaemia as well as a variety of other clinical conditions. Isolates of FeLV are assigned to one of four subgroups (A, B, C and T) on the basis of differences in the gp70 envelope glycoprotein. Feline leukaemia virus A (FeLV-A), the predominant subgroup, is present in all FeLV-infected cats. Viruses of subgroup B arise through recombination between the *env* genes of FeLV-A and endogenous FeLV-related proviral DNA. Subgroup B viruses are present in about 50% of isolates and are only transmitted in conjunction with FeLV-A. The continuing occurrence of FeLV-B in a cat population depends upon the generation of new recombinants in cats persistently infected with FeLV-A. FeLV-C isolates arise de novo in FeLV-A persistently infected cats through mutations in the receptor-binding region of the FeLV-A *env* gene. They rapidly cause a fatal anaemia and are not transmitted to other cats. FeLV-T is a T-cell-tropic, cytopathic virus that arises from FeLV-A as a result of an insertion and mutations in the *env* gene. It is capable of inducing immunodeficiency.

FeLV causes tumours by similar means to ALV, including insertional mutagenesis and recombination to produce rapidly transforming, replication-defective viruses such as feline sarcoma virus (FeSV). Feline sarcoma virus is associated with rare multicentric fibrosarcomas in young cats and is not transmitted under natural conditions. Feline leukaemia virus is an important cause of feline mortality. Transmission requires close contact due to the labile nature of the virus. The virus is shed in saliva with smaller quantities in tears, urine, milk and faeces. Infection is typically acquired by licking, grooming and through bite wounds. The incidence of infection is related to population density with the highest infection rates being found in catteries and multicat households. Young kittens are more susceptible to infection than adults and a significant proportion of cats exposed before 14 weeks of age become persistently infected. Such animals constitute the main reservoir of FeLV and are likely to develop an FeLV-related disease. Kittens born to persistently infected queens also develop persistent infection.

Pathogenesis

The incubation period varies from months to years. Following oronasal exposure, the virus replicates in the lymphoid tissues of the oropharyngeal region before spreading to other lymphoreticular tissues and bone marrow. In most cats, cell-mediated immunity and neutralizing antibodies result in virus elimination. In persistently infected cats extensive virus production occurs in the bone marrow

and the virus is disseminated to both glandular and mucosal epithelia. Tissues with high mitotic activity such as bone marrow and epithelia are targeted by the virus. Viral replication in haemolymphatic tissues can lead to depletion of lymphoid and myeloid cells producing immunosuppression and anaemia. The majority of persistently infected cats die within three years of infection. Approximately 80% of these cats die from non-neoplastic FeLV-associated disease, while about 20% of infected cats develop tumours, usually lymphosarcoma. Thymic, alimentary, multicentric and leukaemic forms of lymphosarcoma are described. Fibrosarcoma and myeloid tumours occur less frequently.

Cats may develop a transient fever, malaise and lymphadenopathy following initial infection. A long asymptomatic period follows. Non-specific clinical signs usually appear in young adult cats between two and four years of age and can include anaemia, reduction in reproductive performance, enteritis and a variety of secondary infections due to the immunosuppressive effects of the virus. In the case of cats with neoplasia, the clinical signs are variable and related to the location of the tumours.

Diagnosis

Detection of viral antigen in blood is commonly used for the laboratory diagnosis of feline leukaemia. Virus isolation is expensive and time-consuming, being mainly used as a confirmatory test or for research purposes. Guidelines are available to assist test selection and interpretation of diagnostic FeLV assays (Richards 2003).

- Commercial ELISA and rapid immunochromatographic tests, designed to detect soluble antigens such as the major capsid protein (p27), are available. Serum or plasma are the preferred samples for testing (Barr 1996). These tests vary in their sensitivity and specificity (Hartmann et al. 2001).
- The immunofluorescent antibody test can be used for the detection of cell-associated viral antigen in the cytoplasm of leukocytes in blood smears. It is more sensitive and specific than ELISA and commonly employed as a confirmatory test.
- Both conventional (Jackson et al. 1996, Miyazawa & Jarrett, 1997) and real time PCR (Hofmann-Lehmann et al. 2001) assays have been described for the detection of proviral FeLV DNA in peripheral blood samples. Cats which have overcome FeLV viraemia may continue to be provirus-positive whereas detection of viral RNA in saliva, plasma or faeces using RT-PCR is a reliable indicator of viraemia (Tandon et al. 2005, Gomes-Keller et al. 2006).
- Both recovered and persistently viraemic cats may have anti-FeLV antibodies. As a result serological testing for antibodies is not used for diagnosis. However, the demonstration of virus-neutralizing antibodies indicates that a cat is immune and resistant to infection.
- Feline oncovirus-associated cell membrane antigen (FOCMA) is expressed on all FeLV- and FeSV-transformed cells and development of anti-FOCMA antibodies provides protection against FeLV-associated neoplasia.

FELINE IMMUNODEFICIENCY VIRUS INFECTION

Following its description in 1987, feline immunodeficiency virus (FIV) infection is now recognized worldwide as an important cause of disease in domestic cats. The infection is sometimes referred to as 'feline AIDS' on account of similarities to acquired immunodeficiency syndrome (AIDS). Five subtypes or clades (A to E) of FIV have been identified based on diversity in the envelope gene sequences with most isolates belonging to subtypes A or B. Related lentiviruses have been isolated from a number of wild *Felidae*, including pumas and lions. Animals are infected for life and persistently viraemic. The virus is shed in saliva and transmission usually occurs through bites. Infection rates are highest in free-roaming, adult male cats. During the acute phase of infection queens may transmit infection to kittens in utero or via milk.

Pathogenesis

Different stages in the disease have been described: an acute phase, a prolonged asymptomatic phase, a phase characterized by vague clinical signs and a terminal phase with marked immunodeficiency (Hartmann 1998). The virus replicates mainly in CD4+ (helper) T lymphocytes, some replication occurs in macrophages, astrocytes and microglial cells. Humoral responses are normal but there is progressive deterioration in cell-mediated immunity due to depletion of CD4+ T lymphocytes. The reduction in CD4+ lymphocyte numbers, increased production of virus, the emergence of variants with increased virulence and infection with opportunistic pathogens all contribute to the development of clinical immunodeficiency. The prevalence of clinical disease is highest in cats over six years of age and is marked by recurrent fever, leukopenia, anaemia, weight loss, lymphadenitis, chronic gingivitis and behavioural changes. Opportunistic infections are frequent in the terminal phase of the disease and may manifest as chronic respiratory, enteric and skin infections. Neurological signs, as a result of direct viral damage in the CNS, develop in a small number of infected cats. Not all infected cats develop clinical disease during their lifetime.

Diagnosis

- The main method for confirming infection is through serological testing for antibodies to FIV. Commercial ELISA and immunochromatographic kit sets are available. These tests vary in their sensitivity and specificity (Hartmann et al. 2001). Alternative and confirmatory tests include immunoblotting and indirect immunofluorescence. Cats typically develop antibodies to FIV within 60 days of infection. Some cats fail to produce antibodies for several months following infection, while antibody levels may become undetectable in terminally ill cats. The presence of maternal antibodies in the kittens of infected queens may result in seropositivity for up to five months.
- Virus isolation from blood or saliva is possible but is laborious and not considered suitable for routine diagnostic purposes. Typically peripheral blood lymphocytes are harvested from fresh heparinized blood and co-cultivated with primary feline T cells for two to three weeks. Viral growth is monitored by measuring the levels of viral core proteins in the tissue culture fluids.
- Proviral DNA can be detected using the polymerase chain reaction (Lawson et al. 1993) but for diagnostic purposes the assay appears to be of variable reliability, particularly in FIV-vaccinated cats (Bienzle et al. 2004, Crawford et al. 2005). In general such assays detect subtype A viruses well but results are more variable with the other subtypes of the virus.

REFERENCES

Bai, J., Zhu, R.-Y., Stedman, K., et al., 1996. Unique long terminal repeat U3 sequences distinguish exogenous jaagsiekte sheep retroviruses associated with ovine pulmonary carcinoma from endogenous loci in the sheep genome. Journal of Virology 70, 3159–3168.

Ballagi-Pordany, A., Klintevall, K., Merza, M., et al., 1992. Direct detection of bovine leukaemia virus infection: practical applicability of a double polymerase chain reaction. Journal of Veterinary Medicine (B) 39, 69–77.

Barlough, J., East, N., Rowe, J.D., et al., 1994. Double-nested polymerase chain reaction for detection of caprine arthritis-encephalitis virus provirus DNA in blood, milk and tissues of infected goats. Journal of Virological Methods 50, 101–113.

Barr, M.C., 1996. FIV, FeLV and FIPV – interpretation and misinterpretation of serological test results. Seminars in Veterinary Medicine and Surgery (Small Animal Practice) 11, 144–153.

Belak, S., Ballagi-Pordany, A., 1993. Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Molecular and Cellular Probes 7, 241–248.

Benson, S.J., Ruis, B.L., Fadly, A.M., et al., 1998. The unique envelope gene of the subgroup J avian leukosis virus derives from ev/J proviruses a novel family of avian endogenous viruses. Journal of Virology 72, 10157–10164.

Bienzle, D., Reggeti, F., Wen, X., et al., 2004. The variability of serological and molecular diagnosis of feline immunodeficiency virus infection. Canadian Veterinary Journal 45, 753–757.

Brodie, S.J., de la Concha-Bermejillo, A., Snowder, G.D., et al., 1998. Current concepts in the epizootiology, diagnosis and economic importance of ovine progressive pneumonia in North America: a review. Small Ruminant Research 27, 1–17.

Caporale, M., Cousens, C., Centorame, P., et al., 2006. Expression of the jaagsiekte sheep retrovirus envelope glycoprotein is sufficient to induce lung tumours in sheep. Journal of Virology 338, 144–153.

Cavanagh, D., 2001. Innovation and discovery: the application of nucleic acid-based technology to avian virus detection and characterization. Avian Pathology 30, 581–598.

Coggins, L., Norcross, N.L., Nusbaum, S.R., 1972. Diagnosis of equine infectious anaemia by immunodiffusion test. American Journal of Veterinary Research 33, 11–18.

Cook, R.F., Cook, S.J., Li, F.L., et al., 2002. Development of a multiplex real-time reverse transcriptase-polymerase chain reaction for equine infectious anaemia virus (EIAV). Journal of Virological Methods 105, 171–179.

Crawford, P.C., Slater, M.R., Levy, J.K., 2005. Accuracy of polymerase chain reaction assays for diagnosis of feline immunodeficiency virus infection in cats. Journal of the American Veterinary Medical Association 226, 1503–1507.

Cutlip, R.C., Jackson, T.A., Laird, O.A., 1977. Immunodiffusion test for ovine progressive pneumonia. American Journal of Veterinary Research 38, 1081–1084.

de Andres, D., Klein, D., Watt, N.J., et al., 2005. Diagnostic tests for small ruminant lentiviruses. Veterinary Microbiology 107, 49–62.

Garcia, M., El-Attrache, J., et al., 2003. Development and application of reverse transcriptase nested polymerase chain reaction test for the detection of exogenous avian leukosis virus. Avian Diseases 47, 41–53.

Gomes-Keller, M.A., Gonczi, E., Tandon, R., et al., 2006. Detection of feline leukaemia virus RNA in saliva from naturally infected cats and correlation of PCR results with those of current diagnostic methods. Journal of Clinical Microbiology, 44, 916–922.

Gonzalez, L., Garcia-Goti, M., Cousens, C., et al., 2001. Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the pre-clinical period of sheep

pulmonary adenomatosis. Journal of General Virology 82, 1355–1358.

Gudmundsson, B., Bjarnadottir, H., Kristjansdottir, S., et al., 2003. Quantitative assays for maedi-visna genetic sequences and mRNA's based on RT-PCR with real-time FRET measurements. Virology 307, 135–142.

Hartmann, K., 1998. Feline immunodeficiency virus infection: an overview. Veterinary Journal 155, 123–137.

Hartmann, K., Werner, R.M., Egberink, H., et al., 2001. Comparison of six in-house tests for the rapid diagnosis of feline immunodeficiency and feline leukaemia virus infections. Veterinary Record 149, 317–320.

Herrmann, L.M., Cheevers, W.P., Marshall, K.L., et al., 2003. Detection of serum antibodies to ovine progressive pneumonia virus in sheep by using a caprine arthritis-encephalitis virus competitive-inhibition enzyme-linked immunosorbent assay. Clinical Diagnostic Laboratory Immunology 10, 862–865.

Hofmann-Lehmann, R., Huder, J.B., Gruber, S., et al., 2001. Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats. Journal of General Virology 82, 1589–1596.

Hopkins, S.G., DiGiacomo, R.F., 1997. Natural transmission of bovine leukaemia virus in dairy and beef cattle. Veterinary Clinics of North America: Food Animal Practice 13, 107–128.

Houwers, D.J., Schaake, J., 1987. An improved ELISA for the detection of antibodies to ovine and caprine lentiviruses, employing monoclonal antibodies in a one-step assay. Journal of Immunological Methods 98, 151–154.

Jackson, M.L., Haines, D.M., Taylor, S.M., et al., 1996. Feline leukemia virus detection by ELISA and PCR in peripheral blood from 68 cats with high, moderate, or low suspicion of having FeLV-related disease. Journal of Veterinary Diagnostic Investigation 8, 25–30.

Johnson, L.K., Meyer, A.L., Zink, M.C., 1992. Detection of ovine lentivirus in seronegative sheep by in situ hybridization, PCR and co-cultivation with susceptible cells. Clinical Immunology and Immunopathology 65, 254–260.

Lawson, M., Meers, J., Blechynden, L., et al., 1993. The detection and quantification of feline immunodeficiency provirus in peripheral-blood mononuclear cells using the polymerase chain reaction. Veterinary Microbiology 38, 11–21.

Leroux, C., Chastang, J., Greenland, T., et al., 1997a. Genomic heterogenetity of small ruminant lentiviruses: existence of heterogeneous populations in sheep and of the same lentiviral genotypes in sheep and goats. Archives of Virology 142, 1125–1137.

Leroux, C., Lerondelle, C., Chastang, J., et al., 1997b. RT-PCR detection of lentiviruses in milk or mammary secretions of sheep or goats from infected flocks. Veterinary Research 28, 115–121.

Knowles, D.P., Evermann, J.F., Schropshire, C., et al., 1994. Evaluation of agar gel immunodiffusion serology using caprine and ovine lentiviral antigens for detection of antibody to caprine-arthritis encephalitis virus. Journal of Clinical Microbiology 32, 243–245.

Miyazawa, T., Jarrett, O., 1997. Feline leukaemia virus proviral DNA detected by polymerase chain reaction in antigenaemic but non-viraemic ('discordant') cats. Archives of Virology 142, 323–332.

Nagarajan, M.M., Simard, C., 2001. Detection of horses infected naturally with equine infectious anemia virus by nested polymerase chain reaction. Journal of Virological Methods 94, 97–109.

Oaks, J.L., McGuire, T.C., Ulibarri, C., et al., 1998. Equine infectious anaemia virus is found in tissue macrophages during subclinical infection. Journal of Virology 72, 7263–7269.

Ortin, A., Minguijor, E., Dewar, P., et al., 1998. Lack of a specific immune response against a recombinant capsid protein of jaagsiekte sheep retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. Veterinary Immunology and Immunopathology 61, 229–237.

Palmarini, M., Dewar, P., Delasheras, M., et al., 1995. Epithelial tumor-cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for jaggsiekte retrovirus. Journal of General Virology 76, 2731–2737.

Palmarini, M., Cousens, C., Dalziel, R.G., et al., 1996. The exogenous form of Jaagsiekte retrovirus (JSRV) is specifically associated with a contagious lung cancer of sheep. Journal of Virology 70, 1618–1623.

Palmarini, M., Mura, M., Spencer, T.E., 2004. Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation. Journal of General Virology 85, 1–13.

Pasick, J., 1998. Maedi-visna virus and caprine arthritis-encephalitis virus: distinct species or quasispecies and its implications for laboratory diagnosis. Canadian Journal of Veterinary Research 62, 241–244.

Pham, T.D., Spencer, J.L., Traina-Dorge, V.L., et al., 1999. Detection of exogenous and endogenous avian leukosis virus in commercial chicken eggs using reverse transcription and polymerase chain reaction assay. Avian Pathology 28, 385–392.

Reddy, P.G., Sapp, W.J., Heneine, W., 1993. Detection of caprine arthritis-encephalitis virus by polymerase chain reaction. Journal of Clinical Microbiology 31, 3042–3043.

Richards, J., 2003. 2001 Report of the American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel on Feline Retrovirus Testing and Management. Journal of Feline Medicine and Surgery 5, 3–10.

Rimstad, E., East, N., Derock, E., et al., 1994. Detection of antibodies to caprine arthritis/encephalitis virus using recombinant gag proteins. Archives of Virology 134, 345–356.

Shah, C., Boni, J., Huder, J.B., et al., 2004. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. Virology 319, 12–26.

Silva, R.F., Fadly, A.M., Taylor, S.P., 2007. Development of a polymerase chain reaction to differentiate avian

leucosis virus (ALV) subgroups: detection of an ALV contaminant in commercial Marek's disease vaccines. Avian Diseases 51, 663–667.

Simard, C.L., Briscoe, M.R., 1990. An enzyme-linked immunosorbent assay for detection of antibodies to maedi-visna virus in sheep. A simple technique for production of antigen using sodium dodecyl sulfate treatment. Canadian Journal of Veterinary Research 54, 446–450.

Smith, L.M., Brown, S.R., Howes, K., et al., 1998. Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. Virus Research 54, 87–98.

Suzuki, T., Ueda, S., Samejina, T., 1982. Enzyme-linked immunosorbent assay for diagnosis of equine infectious anaemia. Veterinary Microbiology 7, 307–316.

Tandon, R., Cattori, V., Gomes-Keller, M.A., et al., 2005. Quantitation of feline leukaemia virus viral and proviral loads by TaqMan real-time polymerase chain reaction. Journal of Virological Methods 130, 124–132.

Wagter, L.H., Jansen, A., Bleumink-Pluym, N.M., et al., 1998. PCR detection of lentiviral GAG segment DNA in the white blood cells of sheep and goats. Veterinary Research Communications 22, 355–362.

Zanoni, R.G., Vogt, H.R., Pohl, B., et al., 1994. An ELISA based on whole virus for the detection of antibodies to small-ruminant lentiviruses. Journal of Veterinary Medicine B 41, 662–669.

FURTHER READING

Caney, S., 2000. Feline immunodeficiency virus: an update. In Practice 22, 397–401.

European Advisory Board on Cat Diseases, 2007. ABCD guidelines on feline leukaemia virus and feline immunodeficiency virus. http://abcd-vets.org/Pages/guidelines.aspx (accessed 11 January 2013).

Knowles, D.P., 1997. Laboratory diagnostic tests for retrovirus infections of small ruminants. Veterinary Clinics of North America: Food Animal Practice 13, 1–11.

Leroux, C., Girard, N., Cottin, V., et al., 2007. Jaagsiekte sheep retrovirus (JSRV): from virus to lung cancer in sheep. Veterinary Research 38, 211–228.

Pépin, M., Vitu, C., Russo, P., et al., 1998. Maedi-visna virus infection in sheep: a review. Veterinary Research 29, 341–367.

Peterhans, E., Greenland, T., Badiola, J., et al., 2004. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. Veterinary Research 35, 257–274.

Sparkes, A.H., 1997. Feline leukaemia virus: a review of immunity and vaccination. Journal of Small Animal Practice 38, 187–194.

Zenger, E., 2000. FIP, FeLV, FIV: Making a diagnosis (Reprinted from Proceedings of the 16th ACVIM Forum). Feline Practice 28, 16–18.

Chapter 66

Bornaviridae

The family *Bornaviridae* belongs to the order *Mononegavirales*. There is a single genus, *Bornavirus*, with a single member species, Borna disease virus (BDV). However, proventicular dilatation disease of parrots has been linked to an avian bornavirus (Honkavuori et al. 2008). Borna disease virus, which has a nondescript appearance, is spherical and about 90 nm in diameter (Fig. 66.1). An envelope surrounds an inner, spherical core, 50 to 60 nm in diameter, which contains a single molecule of negative-sense, single-stranded RNA. Unusual among non-segmented, negative-sense RNA animal viruses, replication of BDV occurs in the nucleus of host cells with budding at the cell surface. The virus spreads by cell-to-cell contact without producing cytopathic effects. The virus is labile, being sensitive to heat, lipid solvents and acid pH.

BORNA DISEASE

Borna disease was first described in southern Germany and owes its name to the town of Borna in Saxony where many horses died in an epidemic of the disease in 1885. It has primarily been associated with persistent infection and fatal neurological disease in horses and sheep. The disease occurs sporadically in Germany and Switzerland but sero-epidemiological studies suggest a much wider distribution. Antibodies reactive with BDV have been found in the sera of human patients with neuropsychiatric disorders and interest has focused on a possible link between the virus and human neurological disorders (Boucher et al. 1999).

Natural infections have been described in horses, ruminants, rabbits, cats and ostriches. It is thought that virus is transmitted through saliva and nasal secretions with susceptible animals becoming infected by direct contact or through contaminated food or water. Most cases of Borna disease occur in spring and early summer with cases being more common in some years than others. Persistent infections can be established in rats and there is evidence that rodents may act as a reservoir host species (Hilbe et al. 2006, Puorger et al. 2010).

Pathogenesis

The incubation period is highly variable from days to several months but is usually about four weeks. The virus is highly neurotropic and following infection by the oronasal route, gains entry to the CNS by migrating intra-axonally through the olfactory nerve or nerve endings in the oropharyngeal and intestinal regions. Viral spread throughout the CNS and centrifugally in peripheral nerves occurs by intra-axonal transport. Distribution of the virus in the brain suggests a preference for the limbic system, probably determined by the presence of certain neuroreceptors. Viral antigens on the surface of infected cells in the CNS elicit a cell-mediated immunopathological reaction involving cytotoxic $CD8^+$ and $CD4^+$ helper T lymphocytes and lysis of infected neurons. Antibody titres in infected animals are relatively low and are non-protective but are not involved in the pathogenesis of the disease. Clinical signs do not occur in immunocompromised animals despite the establishment of persistent infection. The disease course lasts one to three weeks with mortality rates of 37 to 94% in affected horses. Surviving horses have permanent CNS deficits and may undergo recurrent episodes. 'Staggering disease' in cats has been shown to be associated with BDV infection (Lundgren et al. 1995).

Diagnosis

The confirmation of a diagnosis of Borna disease (BD) is difficult and requires evaluation of clinical signs, serological findings and histopathology supported by positive RT-PCR findings in brain tissue. No single diagnostic method is sufficiently sensitive or specific enough to be used in isolation. The clinical signs of BD may be confused with rabies, tetanus or equine herpesvirus infection. Histopathology reveals a severe non-suppurative

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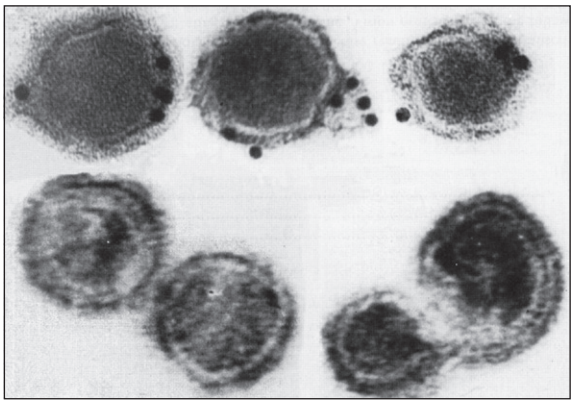

Figure 66.1 Negative stain electron micrograph with immunogold label for Borna disease virus. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 456.*

polioencephalomyelitis. The distribution of the lesions in the CNS is characteristic and eosinophilic intranuclear inclusions (Joest–Degen bodies), if present, are diagnostic. Viral antigen can be demonstrated in the brain by immunohistochemical methods. The demonstration of antibodies to BDV in the serum or preferably in cerebrospinal fluid (CSF) by Western blot, indirect immunofluorescence or ELISA is supportive of the diagnosis. Indirect immunofluorescence is considered to be the most reliable assay for detection of BDV-specific antibodies. Antibody titres are low in BDV-infected animals and are particularly difficult to detect in animals that are not acutely affected. Reverse transcription-PCR for the demonstration of BDV RNA in brain tissue has been shown to be a valuable diagnostic tool (Katz et al. 1998, Legay et al. 2000, Grabner et al. 2002). Real-time RT-PCR protocols are also available (Schindler et al. 2007, Wensman et al. 2007). Virus isolation can be achieved from brain tissue homogenates or CSF in cell cultures derived from embryonic rabbit or rat brain but viral isolation is of low sensitivity as a diagnostic tool due to the low numbers of virions present in brain tissue. The virus can be adapted to and cultivated in Vero and MDCK cells following co-cultivation with infected brain cells.

REFERENCES

Boucher, J.-M., Barbillon, E., Cliquet, F., 1999. Borna disease: a possible emerging zoonosis. Veterinary Research 30, 549–557.

Grabner, A., Herzog, S., Lange-Herbst, H., et al., 2002. Ante mortem diagnosis of Borna disease (BD) in equids. Pferdeheilkunde 18, 579–586.

Hilbe, M., Herrsche, R., Kolodziejek, J., et al., 2006. Shrews as reservoir hosts of Borna disease virus. Emerging Infectious Diseases 12, 675–677.

Honkavuori, K.S., Shivaprasad, H.L., Williams, B.L., et al., 2008. Novel Borna virus in psittacine birds with proventricular dilatation disease. Emerging Infectious Diseases 14, 1883–1886.

Katz, J.B., Alstad, D., Jenny, A.L., et al., 1998. Clinical, serologic, and histopathologic characterization of experimental Borna disease in ponies. Journal of Veterinary Diagnostic Investigation 10, 338–343.

Legay, V., Sailleau, C., Dauphin, G., et al., 2000. Construction of an internal standard used in RT-nested-PCR for Borna disease virus RNA detection in biological samples. Veterinary Research 31, 565–572.

Lundgren, A.-L., Zimmermann, W., Bode, L., et al., 1995. Staggering disease in cats: isolation and characterization of the feline Borna disease virus. Journal of General Virology 76, 2215–2222.

Puorger, M.E., Hilbe, M., Muller, J.-P., et al., 2010. Distribution of Borna disease virus antigen and RNA in tissues of naturally infected bi-colored white-toothed shrews, *Crocidura leucodon*, supporting their role as reservoir host species. Veterinary Pathology 47, 236–244.

Schindler, A.R., Vögtlin, A., Hilbe, M., et al., 2007. Reverse transcription real-time PCR assays for detection and quantification of Borna disease virus in diseased hosts. Molecular and Cell Probes 21, 47–55.

Wensman, J.J., Thorén, P., Hakhverdyan, M., et al., 2007. 2007 Development of a real-time RT-PCR assay for improved detection of Borna disease virus. Journal of Virological Methods 143, 1–10.

FURTHER READING

Dauphin, G., Legay, V., Pitel, P.-H., et al., 2002. Borna disease: current knowledge and virus detection in France. Veterinary Research, 33, 127–138.

Lipkin, W.I., Briese, T., Hornig, M., 2011. Borna disease virus – fact and fantasy. Veterinary Research 162, 162–172.

Ludwig, H., Bode, L., 2000. Borna disease virus: new aspects on infection, disease, diagnosis and epidemiology. Revue Scientifique et Technique de l'Office International des Epizooties 19, 259–288.

Chapter 67

Prions (proteinaceous infectious agents)

Several neurodegenerative diseases of animals and man belong to a group known as transmissible spongiform encephalopathies (TSEs), which are viewed as part of a larger group of neurodegenerative diseases including Alzheimer disease and Parkinson disease in humans referred to as 'protein-folding diseases'. These diseases share a number of characteristic features including appearance late in life, neuronal loss and the accumulation of deposits of misfolded protein aggregates in the CNS (Soto & Estrada 2008). Transmissible spongiform encephalopathies are caused by prions (Prusiner 1982) and have several common features including: prolonged incubation period, progressive course, invariably fatal outcome, similar neuropathological changes, accumulation of an abnormally folded host-derived prion protein. Prions are defined as proteinaceous, infectious particles that resist inactivation by procedures which modify nucleic acids. They appear to be composed exclusively of a modified isoform of a host-derived protein, prion protein (PrP), and are non-immunogenic. The normal or cellular form of the prion protein referred to as PrP^{C} is composed of about 208 amino acids and occurs on the surface of many types of cells, particularly neurons and lymphocytes. The function of PrP^{C} is still largely unknown. The disease-associated or scrapie form of the prion protein, in reference to the prion disease of sheep, is denoted PrP^{Sc} and accumulates as aggregates called scrapie-associated fibrils to form deposits in the brain and lymphatic tissue of affected individuals (Fig. 67.1). Both PrP^{C} and PrP^{Sc} are encoded by a single copy chromosomal gene. Differences between PrP^{C} and the corresponding PrP^{Sc} reside in their tertiary structure and not in amino acid composition. Much of the polypeptide chain of PrP^{C} is coiled up into structures termed α-helices. Conversion of PrP^{C} into PrP^{Sc} involves a post-translational change resulting in a largely β-sheet structure. This structural transition produces profound changes in physico-chemical properties including loss of solubility in non-denaturing detergents and a dramatic increase in resistance to proteases.

Substantial advances have been made in understanding the molecular features of TSE agents (Silveira et al. 2004). A number of theoretical models of PrP^{Sc} formation have been proposed, in particular the heterodimer model (Prusiner 1998) and the nucleation (seed)-dependent polymerization model (Jarrett & Lansbury 1993). These two models can overlap, for example auto-catalysis and the formation of a metastable PrP^{C} folding intermediate may be features of both. It has been shown in vitro that conditions of mild acidification and reduction, similar to those of endosomes or lysosomes, promote the unfolding and rearrangement of PrP^{C} resulting in a monomeric form rich in β-sheets (Jackson et al. 1999, Zou & Cashman 2002). Co-factors appear to play an important role in prion propagation and infectivity (Ma 2012). The β-sheet-rich form of PrP (β-PrP) may convert back to the α-conformation or may form a stable 'seed' that promotes the abnormal folding and polymerization of PrP^{C}. If several β-PrP molecules bind together to form a multimer they lock irreversibly in the β-conformation of PrP^{Sc}. During the process fission occurs whereby the long PrP^{Sc} polymers fragment resulting in an increase in the number of effective nuclei to direct further conversion of PrP^{C}. The conditions required for the conversion process may occur during recycling of PrP^{C}. Most membrane glycoproteins destined for degradation or recycling are transported to lysosomes via endosomes. Studies indicate that PrP^{Sc} is formed in caveolae-like domains before they fuse with endosomes and that PrP^{Sc} accumulates in cytoplasmic vesicles particularly lysosomes (Prusiner et al. 1999). Prion replication may be initiated:

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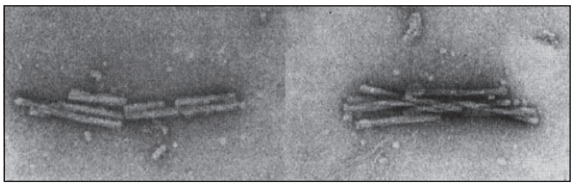

Figure 67.1 Electron micrograph of extensively purified scrapie-associated fibrils (SAF), composed of prion protein, PrP. Negatively stained with uranyl formate. Bar represents 100 nm. *Reprinted with permission: Veterinary Virology Third Edition (1999). Murphy et al., Academic Press. Page 575.*

- Following exposure to a 'seed' of PrP^{Sc} as seen in acquired disease cases.
- As a result of a rare stochastic event whereby there is spontaneous conversion of PrP^{C} to β-PrP as occurs in sporadic prion disease cases.
- As a result of a mutation in the PrP gene which gives rise to PrP^{C} pre-disposed to form β-PrP as seen in inherited prion disease cases.

PrP^{Sc} has a half-life of years compared to PrP^{C} which has a half-life of hours. The development of clinical signs is associated with the accumulation of PrP^{Sc} in the CNS. It is thought that an intermediate form of PrP^{Sc} has a toxic effect on neurons, facilitated or mediated by a PrP^{Sc} effect on microglia, which results in loss of neurons.

Prions are classified into two types, mammalian and fungal. The agents of spongiform encephalopathies are distinguished by their disease association and host species. The primary amino acid sequence and thus the species of a particular prion is determined by the sequence of the chromosomal PrP gene of the animal in which it last replicated. The resistance of some animals to the inoculated prions from another species is termed the 'species barrier'. It is primarily governed by differences in the amino acid sequence and conformation. According to the conformational selection model, ease of transmission between species is determined by the degree of overlap or compatability between the PrP^{Sc} types permitted by the host PrP^{C} and the introduced PrP^{Sc} (Collinge & Clarke 2007). The inoculation of a prion species from one host into another host species results in prolonged incubation times during the first passage. Subsequent passage in this second host species results in a shortening of the incubation time. The 'species barrier' has been used to explain why humans have not contracted scrapie from sheep.

Strains of prions that 'breed true', particularly scrapie strains, have been described based on the results of bioassays in mice. The determination of a strain is achieved using the incubation period and mortality pattern in inbred mice of particular known genotypes, the distribution and extent of spongiform lesions and prion protein plaques in the brain ('lesion profile') and the titre of infectivity in the brain. Although the primary structure of PrP is an important determinant of the tertiary structure of PrP^{C}, PrP^{Sc} is believed to act as a template for the conversion of PrP^{C} into nascent PrP^{Sc}, thereby determining the tertiary structure of nascent PrP^{Sc} molecules. Prion diversity is thought to be enciphered in the conformation and glycosylation pattern of PrP^{Sc} such that prion strains represent different conformers of PrP^{Sc}. The existence of strains has been cited as evidence of the involvement of nucleic acid and the 'protein only' theory of prions is still debated (Chesebro 1998).

Prions are stable over a very wide pH range and remarkably resistant to physical or chemical inactivation. Underestimation of the resistance of these agents has resulted in a number of tragic human cases as well as the dissemination of scrapie agent to 18,000 sheep in a louping ill vaccine prepared from formolized suspensions of brain, spinal cord and spleen (Greig 1950). In fact the treatment of prions with alcohols or aldehydes that fix proteins helps to protect these agents from inactivation and to enhance their thermostability. Extensive research has been carried out into physical methods of inactivating the agents of BSE and scrapie due to the importance of disposing of carcases of affected animals and the importance of meat and bone meal as a source of infection for ruminants. Autoclaving at temperatures of 132 to 138 °C does not guarantee inactivation. In fact, under certain conditions the effectiveness of autoclaving actually declines as the temperature is increased. The most effective methods under worst-case conditions are hypochlorite solutions containing 2% available chlorine or heated 2N sodium hydroxide (Taylor 2000). The inclusion of a formic acid step in formaldehyde fixation of brain tissue has been shown to dramatically reduce the infectivity of scrapie, BSE and CJD agents without significantly affecting the quality of histological sections. The effectiveness of formic acid is most likely due to its solubilizing effect on proteins.

CLINICAL INFECTIONS

Prion diseases are significantly affected by the genome of the host. In the case of the rare human diseases fatal familial insomnia and Gerstmann–Straussler–Scheinker disease (GSS) the condition is inherited in an autosomal dominant manner. Three different manifestations of prion diseases are described (Prusiner 1997) and best exemplified by Creutzfeldt–Jakob disease (CJD) in man which presents as an infectious, sporadic and inherited disorder:

- Slow infection. Kuru is a slowly progressive disease transmitted by ritualistic cannibalism formerly practised by the Fore people, inhabiting the Eastern Highlands of Papua New Guinea. A number of cases of CJD (iatrogenic CJD) have been associated with the use of human cadaver tissues or extracts such as

Table 67.1 Transmissible spongiform encephalopathies of veterinary significance

Disease	Host	Significance of infection
Scrapie	Sheep, goats	Exact mode of transmission unknown, probably maternal transmission in pre or early post natal life. Long incubation period and progressive, nervous disease of adult sheep. Pruritis is a common feature. Recognized in Europe since 1700s. Worldwide distribution, except Australia and New Zealand
Bovine spongiform encephalopathy	Cattle	First described in 1986 in Great Britain at start of major epizootic. Principally confined to Great Britain with much lower incidence in many other European countries. Controlled by effective exclusion of ruminant-derived protein from diet of cattle. Novel form of CJD described in man in 1996 associated with the epizootic
Chronic wasting disease	Mule deer, white-tailed deer and elk	Present in farmed and wild deer populations in North America. Mode of transmission unclear but probably through environmental contamination with infected saliva and faeces. Affects adult animals. Characterized by wasting, behavioural changes and nervous signs
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx and other exotic ungulates in zoological collections	Infection associated with feeding of meat and bone meal derived from BSE-affected animals
Transmissible mink encephalopathy	Mink	Sporadic disease of ranched mink. Infection associated with feeding of meat and offal from prion-infected sheep and cattle carcases. Control readily achieved by exclusion of foodstuffs derived from sheep, cattle or other mink
Feline spongiform encephalopathy	Cats	Infection associated with feeding of tissues derived from BSE-affected animals. Long incubation period with clinical course of several months characterized by nervous signs. Majority of cases reported in Great Britain. Control readily achieved by exclusion of contaminated bovine tissue from cat food

corneal transplants and human growth hormone. Several prion diseases of veterinary importance involving slow infection with very long incubation periods are described (Table 67.1). Certain polymorphisms of the PrP gene in sheep are strongly associated with the incidence of scrapie to the extent that some researchers have inferred from breeding studies that scrapie is an exclusively genetic disease. However, Australia and New Zealand are free of scrapie despite the presence of animals with scrapie-associated PrP alleles (Hunter et al. 1997). The epizootic of bovine spongiform encephalopathy (BSE) in Great Britain is believed to have resulted from changes in rendering practices permitting the preservation of prion proteins, possibly derived from scrapie-infected sheep, in meat and bone meal (Wilesmith 1993). New variant CJD cases in man were first reported in Great Britain in the mid 1990s and are believed to result from the ingestion of beef containing the agent of BSE.

- Sporadic disease. The majority of CJD cases occur in a sporadic manner in the human population at a rate of about one case per million people each year. These cases are thought to arise from a somatic mutation in the PrP gene or following a spontaneous conformational change in PrP^{C}.
- Genetic disorder. Familial CJD, GSS and fatal familial insomnia are all associated with heritable, germline mutations in the PrP gene.

SCRAPIE

Scrapie is an insidious, fatal disease affecting the CNS of adult sheep, goats and moufflon. It has been reported worldwide with the notable exceptions of Australia and New Zealand. The precise mode of transmission among sheep in a flock or between flocks is not known. Possible routes include ingestion of infected material, entry through skin abrasions and maternal transmission from ewe to lamb. Scrapie is frequently transmitted along family lines in flocks but whether transmission occurs prenatally or postnatally is still unclear. There is evidence to suggest that

transmission tends to occur during the perinatal period and that exposure to placental material from affected ewes may be important. Pastures where affected animals have grazed appear to remain contaminated for a number of years.

There is a strong association between the sequence of the PrP gene and susceptibility to scrapie. The PrP gene coding sequence in sheep is highly polymorphic. The frequency of particular polymorphisms and their influence on survival times varies somewhat from sheep breed to sheep breed. The most significant amino acid substitutions are those affecting codons 136, 154 and 171. In many breeds the valine136, arginine154, glutamine171 (denoted VRQ) allele is strongly associated with susceptibility to scrapie while the AA136RR154RR171 genotype has been linked to resistance. However, an atypical form of scrapie, termed Nor98, has been detected in recent years in Europe and the United States. This form has a different tissue distribution and can occur in animals shown to be genetically resistant to classical scrapie (Benestad et al. 2003).

Pathogenesis

Scrapie occurs predominantly in sheep of breeding age with a peak incidence at about three-and-half years of age. Cases of the disease are rare before 12 months of age. Following natural infection PrP^{Sc} is first detected in tissues of the lymphoreticular system, including the spleen, retropharyngeal lymph node, mesenteric lymph node and the palatine tonsil. In lymph nodes, follicular dendritic cells appear to be the target cells for replication and deposition of PrP^{Sc}. It is thought that the enteric nervous system at the level of the duodenum and ileum acts as a portal of entry to neural tissues following oral exposure. The scrapie agent then spreads in a retrograde fashion through sympathetic and parasympathetic efferent fibres of the autonomic nervous system to the spinal cord and medulla oblongata respectively (van Keulen et al. 1999).

The onset of clinical signs is insidious with affected animals showing signs of restlessness or nervousness, particularly after sudden noise or movement. Fine tremors of the head and neck and a lack of coordination with a tendency to move at the trot or hop like a rabbit are characteristic signs. Intense pruritis with wool loss and areas of skin rubbed raw is common but not present in all cases. There is progressive deterioration in the animal's condition with emaciation, ataxia and hind limb paralysis. Overt illness can last from two weeks to six months. The condition is invariably fatal.

Diagnosis

Diagnosis is based on clinical signs, histopathological examination of the CNS and demonstration of the accumulation of PrP^{Sc}. Although the biohazard of scrapie diagnostic testing appears to be very low, the adoption of containment procedures similar to those used for BSE and for human TSEs is recommended due to the fact that sheep are susceptible to BSE (Jeffrey et al. 2001).

- Brain tissue for histological examination should be fixed in 10% formal saline for at least one week. Initial sampling may be confined to the medulla at the level of the obex with additional samples of all major brain regions being processed as required given that strain-specific targeting of other parts of the brain has been described (Benestad et al. 2003). Characteristic microscopic changes include neuronal vacuolation and degeneration, spongy vacuolar transformation of the neuropil and astrocytosis, particularly in the medulla. The lesions are non-demyelinating and non-inflammatory. The lesions usually have a bilaterally symmetrical distribution.
- Methods suitable for the detection of PrP^{Sc} include immunohistochemical staining for PrP^{Sc}, immunoblotting to detect proteinase-K-resistant PrP or electron microscopy to detect scrapie-associated fibrils (SAF) in detergent-treated extracts of brain. Immunohistochemical detection of PrP^{Sc} is best carried out on sections from blocks processed after primary formalin fixation for three to five days. A number of suitable epitope demasking techniques and antibodies to PrP have been described (Mohri et al. 1992, Miller et al. 1994). The presence of detectable PrP^{Sc} precedes vacuolation and clinical signs, making immune-based detection methods highly sensitive. Such an approach also assists in the characterization of the prion protein that is present, permitting discrimination of disease phenotypes, particularly between classical scrapie, atypical scrapie and BSE. A wide range of antisera and monoclonal antibodies to PrP are available, some commercially. Certain antibodies are not suitable for the detection of ovine PrP (Hardt et al. 2000). Ante mortem detection methods based on demonstration of PrP^{Sc} by immunohistochemial staining of lymphoid tissues such as the palatine tonsil or nictitating membrane have been reported (O'Rourke et al. 1998, Schreuder et al. 1998). However, a peripheral phase of replication does not appear to occur in certain genotypes of sheep (van Keulen et al. 1995, Andreoletti et al. 2000).
- Detection of PrP^{Sc} by Western blotting is based on detection of proteins of molecular mass 27 to 30 kDa. A detergent extract of fresh brain material is ultracentrifuged to concentrate the PrP and then treated with proteinase K to digest any PrP^{C} leaving only PrP^{Sc} to be immunostained using specific antibody (Stack et al. 1996). A number of rapid immunodiagnostic tests in the form of Western blot methods or ELISA-based methods have been

developed for BSE and are now available for the diagnosis of scrapie. Positive or inconclusive results are generally subjected to examination by confirmatory immunohistochemical or Western blot methods.

- A morphological form of PrP^{Sc} known as scrapie-associated fibrils (SAF) can be detected in fresh brain material from scrapie cases by negative stain electron microscopy (Gibson et al. 1987). A modified method for SAF detection in fixed material has also been described (Chaplin et al. 1998). Both immunoblotting and demonstration of scrapie-associated fibrils can be carried out on brain material that is autolysed or has been frozen.
- Demonstration of the infectivity of brain tissue from scrapie animals can be achieved by the injection of laboratory rodents, usually mice. However, incubation periods of one to two years may be required and transmission is not always successful. Neuroblastoma cells are susceptible to certain prion strains and have been used to develop a quantitative, cell-based infectivity assay, the scrapie cell assay (SCA) in which infected cells are detected using an enzyme-linked immunospot (ELISPOT) plate (Klöhn et al. 2003). The sensitivity of the assay can be improved by the use of steel wires (Edgeworth et al. 2009).
- Serological testing is not useful as antibodies are not elaborated by the host against the scrapie agent.

BOVINE SPONGIFORM ENCEPHALOPATHY

Bovine spongiform encephalopathy (BSE) is a progressive, fatal, nervous disease of adult cattle that was first recognized in Great Britain in 1986 (Wells et al. 1987). A common source epizootic followed involving more than 180,000 confirmed cases and an estimated one million infected animals. The numbers of cases have fallen dramatically since the outbreak peaked in 1992–1993 when more than 600 new cases were being identified each week. Several countries have reported cases in animals imported from Great Britain. In addition, cases in indigenous cattle have occurred in many European countries including Switzerland, Ireland, France and Portugal. BSE is a notifiable disease in the European Union (EU) with active surveillance schemes using rapid diagnostic tests in place. Cases have also been reported in Asia and North America. A single major strain of BSE was responsible for the outbreak in Great Britain. As a result of large-scale active surveillance studies, atypical forms of BSE have been identified based on variant features of pathology and/or molecular characteristics. One type known as the 'H-type' is characterized by higher-molecular-mass fragments than classical BSE. A second type has a lower molecular mass and is referred to as 'L-type' or bovine amyloidotic spongiform encephalopathy (BASE). Bovine spongiform encephalopathy is not particularly species-specific and natural infection has been reported in cats and exotic ungulate species in zoological collections following ingestion of foodstuffs derived from contaminated bovine tissues. The 'promiscuous' nature of BSE is thought to be due to a thermodynamically favoured PrP^{Sc} conformation that is readily imprinted on PrP^{C} of multiple species.

In 1996 a novel form of human prion disease termed variant CJD (vCJD) was recognized in Great Britain. Molecular strain-typing studies and experimental transmission into transgenic and conventional mice indicate that variant CJD and BSE are caused by the same prion strain. Although the number of cases of vCJD have been relatively low to date, uncertainties about risk factors and the length of the incubation period mean that it will be some years before the scale of any epidemic in the human population can be properly estimated (Collinge 1999). All of the patients with vCJD analysed to date have been homozygous for methionine at codon 129 of the PrP gene.

The BSE epizootic in Great Britain started simultaneously at several geographical locations. The source was traced to contaminated meat and bone meal (MBM) which is a protein dietary supplement prepared from rendered slaughterhouse offal. It is suspected that the agent of scrapie may have crossed the species barrier into cattle in the early 1980s following the termination of the use of organic solvents in the rendering process in the preparation of MBM. An acceleration of the outbreak then occurred due to recycling of infected bovine tissues prior to recognition of the disease and the imposition of control measures. A number of factors are probably responsible for the fact that the epizootic has largely been confined to Great Britain including a high ratio of sheep to cattle, a high rate of enzootic scrapie, a heavy reliance on the feeding of MBM to dairy cattle and changes in the rendering process (Nathanson et al. 1999). The feeding of MBM of ruminant origin to ruminants was banned in Great Britain in 1988 and resulted in a marked decline in the numbers of cases from 1993 onwards. However, cases have continued to occur in animals born after the ban. These have been ascribed to ruminant-MBM still in circulation, cross-contamination in feed mills of ruminant rations by those produced for pigs and poultry, and the feeding of monogastric rations to cattle. More stringent regulations were introduced in Great Britain in 1996 banning all mammalian MBM in farm animal food. Since 2001 the use of mammalian MBM and fishmeal has been prohibited in the EU. Horizontal transmission does not appear to occur in affected herds. Maternal transmission may occur at a low rate but it is considered that it has negligible impact on the waning epizootic. No sex or breed disposition and no genotypic variation in susceptibility to BSE have been reported in cattle.

Pathogenesis

The mean incubation period is estimated to be five years. The pathogenesis of BSE is thought to be similar to that of scrapie with initial replication in the lymphoreticular system followed by migration via peripheral nerves to the CNS. The agent of BSE has been found in the Peyers patches of the ileum of orally inoculated cattle. This site is closely associated with the nervous tissue of the enteric nervous system. The onset of disease is insidious but the range and intensity of signs increase over time. Neurological signs including changes in behaviour and deficits in posture and movement occur together with non-specific signs of weight loss and decreased milk yield. There is considerable individual variation in clinical presentation and signs may include tremors, apprehension, hyperaesthesia, bruxism, head tossing, exaggerated menace reflex, head shyness and vocalization. Locomotory signs, including ataxia, hypermetria, generalized paresis and increased falling, tend to become more prominent as the condition progresses. The clinical course may extend over many days or months but most animals have reached a terminal state after two to three months.

Diagnosis

Bovine spongiform encephalopathy is considered a zoonosis and requires special precautions for the handling and disposal of BSE material. Diagnosis is based on herd history, clinical signs, histopathological and immunohistochemical examination of the brain.

- Brain material for histological examination should be fixed in 10% formol saline for about two weeks. It is possible to restrict examination to a single coronal section of the medulla oblongata at the level of the obex on account of the consistent neuropathological picture obtained in cases of BSE (Wells et al. 1989). Characteristic changes include neuropil vacuolation (Fig. 67.2) and astrocytosis. The astrocytosis can be clearly demonstrated via immunohistochemical detection of increased amounts of glial fibrillary acidic protein (GFAP). In the absence of lesions or where results are inconclusive, immunohistochemistry should be performed. A number of protocols have been successfully developed for the detection of PrP^{Sc} (Haritani et al. 1994, Graber et al. 1995). Suitable monoclonal antibodies are available commercially and from the OIE Reference Laboratory at Weybridge, United Kingdom. In contrast to the diagnosis of scrapie, the limited detection of PrP^{Sc} in lymphoid tissues in BSE cases precludes preclinical diagnosis by biopsy techniques.
- Additional confirmatory methods include detection of PrP^{Sc} by immunoblotting to detect proteinase-K resistant PrP, ELISA or electron microscopy to detect characteristic fibrils in detergent-treated extracts of brain. Fresh brain tissue should be collected from the medulla, caudal to the obex, from the cervical spinal cord and the lateral hemisphere of the cerebellum. Automated immunoblotting and ELISA techniques have been developed to permit large-scale screening of animals and are now commercially available (Moynagh & Schimmel 1999). Characteristic fibrils, analogous to SAF, can be demonstrated in detergent extracts of brain or spinal cord by negative-stain electron microscopy (Scott et al. 1990). The detection of fibrils is considered to be a less sensitive diagnostic method than detection of PrP^{Sc} by immunological means. The development of more sensitive assays for the testing of pre-symptomatic animals for food safety reasons is currently an area of intense research. One promising approach is the application of protein misfolding cyclic amplification (PMCA) technology to increase the amount of PrP^{Sc} in the sample (Saborio et al. 2001, Soto et al. 2005).
- Bioassay in mice involving challenge with brain tissue from terminally affected cattle is used for research purposes but is impractical for routine diagnosis due to the long incubation periods involved.

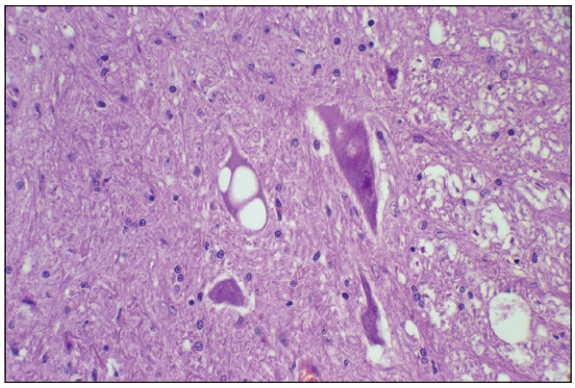

Figure 67.2 Multilocular vacuolation of a neuron characteristic of the spongiform encephalopathies. Section of the brain stem from a sheep with scrapie. (H&E stain, ×400)

REFERENCES

Andreoletti, O., Berthon, P., Marc, D., et al., 2000. Early accumulation of PrP^{Sc} in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. Journal of General Virology 81, 3115–3126.

Benestad, S.L., Sarradin, P., Thu, B., et al., 2003. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. Veterinary Record 153, 202–208.

Chaplin, M.J., Aldrich, A.D., Stack, M.J., 1998. Scrapie associated fibril detection from formaldehyde fixed brain tissue in natural cases of ovine scrapie. Research in Veterinary Science 64, 41–44.

Chesebro, B., 1998. BSE and prions: uncertainties about the agent. Science 279, 42–43.

Collinge, J., 1999. Variant Creutzfeldt–Jakob disease. Lancet 354, 317–323.

Collinge, J., Clarke, A.R., 2007. A general model of prion strains and their pathogenicity. Science 318, 930–936.

Edgeworth JA, Jackson GS, Clarke AR et al. 2009 Highly sensitive, quantitative cell-based assay for prions adsorbed to solid surfaces. Proceedings of the National Academy of Sciences USA doi: 101073/pnas0813342106.

Gibson, P.H., Somerville, R.A., Fraser, H., et al., 1987. Scrapie associated fibrils in the diagnosis of scrapie in sheep. Veterinary Record 120, 125–127.

Graber, H.U., Meyer, R.K., Fatzer, R., et al., 1995. In situ hybridization and immunohistochemistry for prion protein (PrP) in bovine spongiform encephalopathy (BSE). Journal of the Veterinary Medical Association 42, 453–459.

Greig, J.R., 1950. Scrapie in sheep. Journal of Comparative Pathology 60, 263–266.

Hardt, M., Baron, T., Groschup, M.H., 2000. A comparative study of immunohistochemical methods for detecting abnormal prion protein with monoclonal and polyclonal antibodies. Journal of Comparative Pathology 122, 43–53.

Haritani, M., Spencer, Y.I., Wells, G.A.H., 1994. Hydrated autoclave pretreatment enhancement of prion protein immunoreactivity in formalin-fixed bovine spongiform encephalopathy-affected brain. Acta Neuropathologica (Berlin) 87, 86–90.

Hunter, N., Cairns, D., Foster, J.D., et al., 1997. Is scrapie solely a genetic disease? Nature 386, 137.

Jackson, G.S., Hosszu, L.L.P., Power, A., et al., 1999. Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations. Science 283, 1935–1937.

Jarrett, J.T., Lansbury, P.T., 1993. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73, 1055–1058.

Jeffrey, M., Ryder, S., Martin, S., et al., 2001. Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE): 1 Onset and distribution of disease-specific PrP accumulation in brain and viscera. Journal of Comparative Pathology 124, 280–289.

Klöhn, P.-C., Stoltze, L., Flechsig, E., et al., 2003. A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. Proceedings of the National Academy of Sciences USA 100, 11666–11671.

Ma, J., 2012. The role of co-factors in prion propagation and infectivity. PLoS Pathogens 8, e1002589.

Miller, J.M., Jenny, A.L., Taylor, W.D., et al., 1994. Detection of prion protein in formalin-fixed brain by hydrated autoclaving immunohistochemistry for the diagnosis of scrapie in sheep. Journal of Veterinary Diagnostic Investigation 6, 366–368.

Mohri, S., Farquhar, C.F., Somerville, R.A., et al., 1992. Immunodetection of a disease specific PrP fraction in scrapie-affected sheep and BSE-affected cattle. Veterinary Record 131, 537–539.

Moynagh, J., Schimmel, H., 1999. Tests for BSE evaluated. Bovine spongiform encephalopathy. Nature 400, 105.

Nathanson, N., Wilesmith, J., Wells, G.A., et al., 1999. Bovine spongiform encephalopathy and related diseases. In: Prusiner, S.B. (Ed.), Prion Biology and Diseases. Cold Spring Harbor Laboratory Press, New York, pp. 431–463.

O'Rourke, K.I., Baszler, T.V., Parish, S.M., et al., 1998. Pre-clinical detection of PrP^{Sc} in nictitating membrane lymphoid tissue of sheep. Veterinary Record 142, 489–491.

Prusiner, S.B., 1982. Novel proteinaceous infectious particles cause scrapie. Science 216, 136–144.

Prusiner, S.B., 1997. Prion diseases and the BSE crisis. Science 278, 245–251.

Prusiner, S.B., 1998. Prions. Proceedings of the National Academy of Science USA 95, 13363–13383.

Prusiner, S.B., Peters, P., Kaneko, K., et al., 1999. Cell biology of prions. In: Prusiner, S.B. (Ed.), Prion Biology and Diseases. Cold Spring Harbor Laboratory Press, New York, pp. 349–391.

Saborio, G.P., Permanne, B., Soto, C., 2001. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411, 810–813.

Schreuder, B.E.C., van Keulen, L.J.M., Vromans, M.E.W., et al., 1998. Tonsillar biopsy and PrPSc detection in the preclinical diagnosis of scrapie. Veterinary Record, 142, 564–568.

Scott, A.C., Wells, G.A.H., Stack, M.J., et al., 1990. Bovine spongiform encephalopathy: detection and quantitation of fibrils, fibril protein PrP and vacuolation in brain. Veterinary Microbiology 23, 295–304.

Silveira, J.R., Caughey, B., Baron, G.S., 2004. Prion protein and the molecular features of transmissible spongiform encephalopathy agents. In: Harris, D. (Ed.), Mad Cow Disease and Related Spongiform Encephalopathies. Springer-Verlag, Berlin, pp. 1–50.

Soto, C., Estrada, L., 2008. Protein misfolding and neurodegeneration. Archives of Neurology 65, 184–189.

Soto, C., Anderes, L., Suardi, S., et al., 2005. Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. FEBS Letters 579, 638–642.

Stack, M.J., Keyes, P., Scott, A.C., 1996. The diagnosis of bovine spongiform encephalopathy and scrapie by the detection of fibrils and the abnormal protein isoform. In: Baker, H., Ridley, R.M. (Eds.), Methods in Molecular Medicine: Prion Diseases. Humana Press, Totowa, New Jersey, USA, pp. 85–103.

Taylor, D.M., 2000. Inactivation of transmissible degenerative encephalopathy agents: a review. Veterinary Journal 159, 1–7.

van Keulen, L.J.M., Schreuder, B.E.C., Meloen, R.H., et al., 1995. Immunohistochemical detection and localisation of prion protein in brain tissue of sheep with natural scrapie. Veterinary Pathology 32, 299–308.

van Keulen, L.J.M., Schreuder, B.E.C., Vromans, M.E.W., et al., 1999. Pathogenesis of natural scrapie in sheep. In: Proceedings of Characterization and Diagnosis of Prion Diseases in Animals and Man, Tubingen.

Wells, G.A.H., Scott, A.C., Johnson, C.T., et al., 1987. A novel progressive spongiform encephalopathy in cattle. Veterinary Record 121, 419–420.

Wells, G.A.H., Hancock, R.D., Cooley, W.A., et al., 1989. Bovine spongiform encephalopathy: diagnostic significance of vacuolar changes in selected nuclei of the medulla oblongata. Veterinary Record 125, 521–524.

Wilesmith, J.W., 1993. Epidemiology of bovine spongiform encephalopathy and related diseases. Archives of Virology 7, 245–254.

Zou, W.Q., Cashman, N.R., 2002. Acidic pH and detergent enhance in vitro conversion of human brain PrP^{C} to a PrP^{Sc}-like form. Journal of Biological Chemistry 277, 43942–43947.

FURTHER READING

Gavier-Widen, D., Stack, M.J., Baron, T., et al., 2005. Diagnosis of transmissible spongiform encephalopathies in animals: a review. Journal of Veterinary Diagnostic Investigation 17, 509–527.

Harman, J.L., Silva, C.J., 2008. Bovine spongiform encephalopathy. Journal of the American Veterinary Medical Association 234, 59–72.

Novakofski, J., Brewer, M.S., Mateus-Pinilla, N., et al., 2005. Prion biology relevant to bovine spongiform encephalopathy. Journal of Animal Science 83, 1455–1476.

Prusiner, S.B. (Ed.), 1999. Prion Biology and Diseases. Cold Spring Harbor Laboratory Press, New York.

Sejvar, J.J., Schonberger, L.B., BelayBelay, E.D., 2008. Transmissible spongiform encephalopathies. Journal of the American Veterinary Medical Association 233, 1705–1712.

Soto, C., 2004. Diagnosing prion diseases: needs, challenges and hopes. Nature Reviews, Microbiology 2, 809–819.

Section 5

Zoonoses

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Chapter 68

Zoonoses

Human populations encounter animals with varying frequency depending on their occupation, geographical location and the prevailing culture of the country. Whether living in an urban or rural environment, animals are constantly present and humans may have close contact with animals on their farms (food-producing animals), in their homes (dogs, cats, caged birds), through leisure activities (horses, wildlife) or by virtue of their occupation as veterinarians or animal nurses.

Apart from their obvious benefits as a source of food, draught power, transportation and companionship, animals may occasionally have a negative impact on the human population through pollution of the environment, as a cause of traffic accidents, injury to humans through bite wounds and attacks on other susceptible species such as dogs attacking sheep. Health hazards associated with animals are related to communicable diseases. The term zoonoses is applied to those diseases and infections which are naturally transmitted between vertebrate animals and man.

Transmission of disease may be direct, simply by contact with an animal, or indirect, through food, non-edible products, secretions or excretions (Fig. 68.1). Apart from food-borne zoonoses the importance of particular zoonotic diseases often varies with a person's occupation, the nature and type of animals present and the diseases prevalent in the animal population in a particular geographical region. The more frequent and direct the contact with animals, the greater the risk of acquiring a zoonotic infection. Farmers, owners of companion animals, workers in slaughterhouses or by-product processing plants, veterinarians and laboratory staff dealing with infectious material, workers in zoos and circuses and personnel engaged in servicing sanitary services are more likely to acquire zoonotic diseases than workers who have infrequent contact with animals.

An analysis of 335 emerging infectious disease (EID) events occurring between 1940 and 2004 revealed a number of interesting global trends (Jones et al. 2008). More than 60% were zoonoses with the majority of these (71.8%) originating in wildlife. The analysis also found that 54% of EIDs were due to bacteria (particularly drug-resistant bacteria) and the threat of emerging infectious diseases to global health has been increasing over time. As international travel and trade have proliferated, disease-causing agents now have the ability to move around the globe at faster rates.

Zoonoses may be classified according to their aetiology as being of bacterial, fungal, viral or parasitic origin (Tables 68.1, 68.2 and 68.3). Selected zoonotic diseases in each category are briefly reviewed.

Taenia saginata Infection (Beef Tapeworm)

Animals vary in their ability to act as reservoirs of zoonotic diseases and also in their ability to transmit infectious agents to humans. Rarely, humans act as definitive hosts of parasites which infect animals. The beef tapeworm *Taenia saginata*, which is found in the human small intestine and occurs worldwide, is an example of such a parasite. The adult tapeworm which may be up to 10 metres in length, is composed of segments or proglottides which may contain up to 100,000 eggs. Gravid segments may contaminate pasture through sewage disposal, indiscriminate defecation by infected humans in the vicinity of cattle, or by flooding of pasture land with contaminated water. Embryonated eggs which are immediately infective

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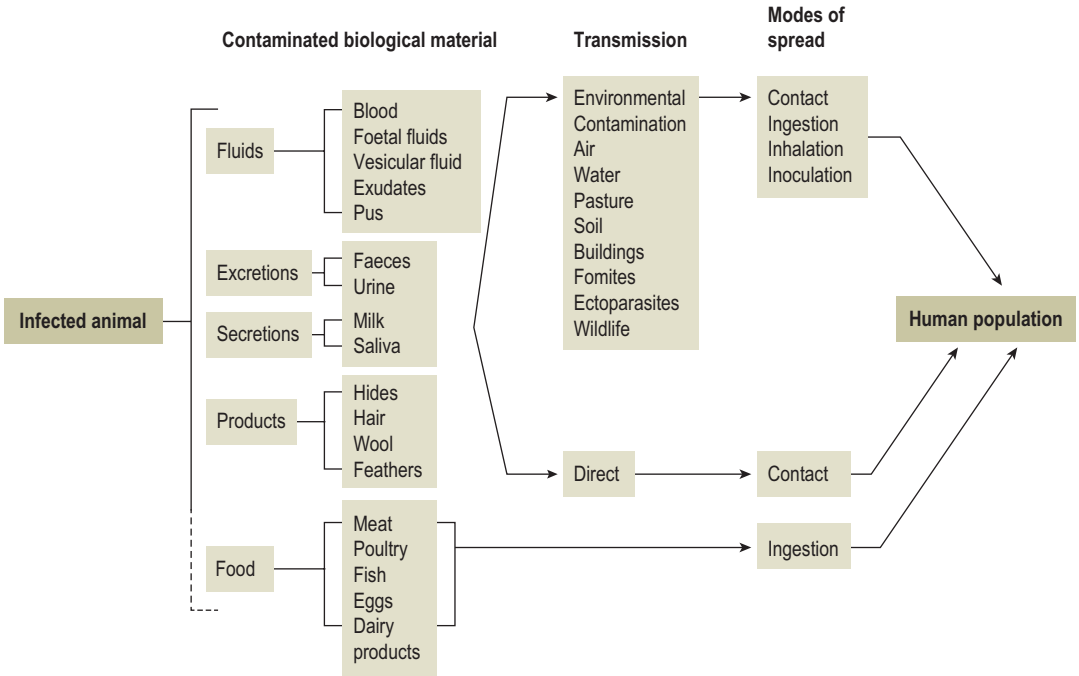

Figure 68.1 Direct and indirect transmission of infectious agents from animals to the human population. Human diseases acquired in this manner are referred to as zoonoses.

Table 68.1a Viral and prion zoonoses

	Infectious agent	Reservoir/mode of transmission	Distribution	Disease in humans
Borna disease	Borna disease virus (*Bornavirus, Bornaviridae*)	Rodents (especially shrews) form a wild animal reservoir/Predominantly affects horses and sheep. Transmitted via nasal fluid, saliva and tears or by contamination of food or water	Primarily central Europe. Seropositive horses in Israel, Japan, Iran and USA	Possible link to psychiatric illness and to fatal meningoencephalitis
B-virus disease of monkeys	*Macacine herpesvirus 1* (*Simplexvirus, Herpesviridae*)	Asian macaques and other primates, monkey-cell cultures/Monkey bites (infected saliva) or scratches; contact with infected material or by inhalation	Wherever infected monkeys are present or where their tissues are used in laboratories	Usually fatal, ascending encephalomyelitis; occasional recoveries with considerable residual CNS damage
Cowpox	(*Orthopoxvirus, Poxviridae*)	Dairy cattle, domestic cats, rodents/Direct contact during milking or handling infected cats or rodents	Endemic infection of certain rodents in Europe and Asia	Local erythema followed by vesicle, pustule and scab formation. Self-limiting occupational disease

Table 68.1a Viral and prion zoonoses—cont'd

	Infectious agent	Reservoir/mode of transmission	Distribution	Disease in humans
Ebola virus and Marburg virus haemorrhagic fevers	Two antigenically distinct genera of viruses (*Ebolavirus* and *Marburgvirus, Filoviridae*)	Presumed animal reservoir – fruit bats, rodents may also play a role. Primates are susceptible/Contact with infected monkeys, apes or their tissues. Person-to-person transmission occurs by direct contact, infected blood, semen or secretions, highly contagious	Africa	Severe haemorrhagic fever, CNS involvement, profuse diarrhoea, vomiting, uraemia, internal and external haemorrhages and organ failure. Fatal cases show necrotic foci in many organs including the brain. Case fatality rates for Ebola infections range from 50–90%. Case-fatality rates for Marburg infection > 80%
Foot-and-mouth disease	Foot-and-mouth disease virus (*Aphthovirus, Picornaviridae*)	Cattle, sheep, pigs and many wildlife species/ Contact	Africa, Asia, South America, some European countries	Mild disease, very uncommon in humans. Vesicles on mucous membranes and on the skin
Hendra virus	(*Henipavirus, Paramyxoviridae*)	Fruit bats (reservoir). Horses are susceptible/ Humans in contact with infected secretions from horses such as veterinarians	Australia	Flu-like; fever, aching muscles, sore throat, dizziness, drowsiness. Haemorrhage, oedema of lungs, respiratory and renal failure, meningitis and death
Hepatitis E	Hepatitis E virus (*Hepevirus, Hepeviridae*)	Humans, pigs, chickens/ Oral route	Worldwide	Predominantly transmitted through faecally contaminated water in endemic regions. Sporadic cases occur in developed countries linked to ingestion of contaminated food, particularly pork
Influenza	Influenza A virus (*Influenzavirus A, Orthomyxoviridae*)	Pigs and birds/Airborne spread	Worldwide	Self-limiting acute upper respiratory tract infection. Fever, joint pain, sore throat, cough, lethargy, loss of appetite and nasal discharge
Menangle virus	(*Rubulavirus, Paramyxoviridae*)	Fruit bats (reservoir)/Pigs and people involved in pig farming	Australia	Influenza-like symptoms
Monkey pox	Monkeypox virus (*Orthopoxvirus, Poxviridae*)	Primarily apes and monkeys. Squirrels, rats, mice, rabbits and prairie dogs may become infected/Transmission occurs through bite or scratches from infected animal as well as contact with bodily fluids	Africa. USA due to importation of infected animals from Africa	Mimic smallpox but milder. High fever, myalgia, headache, lymph node swelling and lethargy. Later vesicular rash appears. Pneumonia. Encephalitis and organ failure may occur

Continued

Table 68.1a Viral and prion zoonoses—cont'd

	Infectious agent	Reservoir/mode of transmission	Distribution	Disease in humans
Newcastle disease	Newcastle disease virus (*Avulavirus, Paramyxoviridae*)	Domestic and wild birds/ Direct contact. Primarily people working in the poultry industry, slaughterhouse and laboratory workers	Europe, Far East, America	Occasional human infection; conjunctivitis, fever, laryngitis, pharyngitis and tracheitis
Nipah virus	(*Henipavirus, Paramyxoviridae*)	Reservoir is fruit bat/Pigs and people involved in pig farming or associated activities (believed to be aerosol-borne). Cats are also susceptible	Malaysia, Bangladesh, India	Sensory and cerebral dysfunction, seizure, muscle spasm. Encephalitis (10–32% fatality rate)
Orf (contagious pustular dermatitis)	Orf virus (*Parapoxvirus, Poxviridae*)	Sheep and goats/Direct contact with an infected animal, infected wool or orf vaccine (live)	Worldwide	Usually a solitary lesion up to 3 cm in diameter on hands, arms or face. The ulcerative lesion regresses after four to six weeks
Pseudo-cowpox (milker's nodule)	Pseudocowpox virus (*Parapoxvirus, Poxviridae*)	Cattle/Direct contact	Worldwide	Lesion on hands or arms; rarely systemic with fever, rash and gastrointestinal upset. Mild, self-limiting disease
Rabies	Several lyssaviruses but especially rabies virus (*Lyssavirus, Rhabdoviridae*)	Wildlife reservoirs of rabies virus include foxes, skunks, racoons, jackals, bats/People generally contract virus by bite wounds and scratches from domestic animals – particulary dogs and cats. Possible airborne transmission in caves with large populations of bats. Organ transplantation	North, South and Central America, Africa, Asia, many European countries	An invariably fatal acute encephalomyelitis. The disease progresses to paresis or paralysis; the fear of water (hydrophobia) arises because of the difficulty in swallowing
Bat rabies	European bat lyssavirus-1 and -2, Australian bat lyssavirus (*Lyssavirus, Rhabdoviridae*)	Bats/People contract by bite wounds and scratches	Europe and Australia	Symptoms similar to classical rabies
Severe acute respiratory syndrome (SARS)	SARS-related virus (*Coronavirus, Coronaviridae*)	Bats seem to be reservoir species with racoon dogs, palm civets and ferret badgers also infected/Transmission route is unknown	China – original outbreak in 2003–2004 when virus spread to more than 30 countries in five continents	Maculopapular rash on trunk, hepatitis, jaundice, pancreatitis, anorexia, altered mental state, haemorrhage, fluid loss and organ failure

Table 68.1a Viral and prion zoonoses—cont'd

	Infectious agent	Reservoir/mode of transmission	Distribution	Disease in humans
Simian foamy virus	(*Spumavirus, Retroviridae*)	Bites, scratches or bodily fluids from infected apes	Africa and Indonesia	None
Vesicular stomatitis	Several vesiculoviruses (*Vesiculovirus, Rhabdoviridae*)	Horses, cattle, pigs and sheep/Insect vectors and direct transmission	The Americas	Influenza-like signs in laboratory workers and people in contact with infected animals
	Prions			
Transmissible spongioform encephalopathy (TSE)	Prion Agents	Cattle (bovine spongiform encephalopathy)/ Ingestion of meat likely to be contaminated with infected brain material. Infected blood and blood products	Worldwide	Human variant Creutzfeld-Jakob disease (vCJD) sufferers display dementia, muscle spasm, tremor and a distinctive EEG pattern. Behavioural changes, loss of motor function, paralysis and wasting. Long incubation period, average duration of illness is two years

Table 68.1b Viral zoonoses: arthropod-borne and rodent-borne infections

	Infectious agent	Reservoir/vectors and mode of transmission	Distribution	Disease in humans
	Arenaviridae			
Guanarito virus	(*Arenavirus*)	Cane rat/Direct contact (bodily fluids)	Guanarito is a municipality of Barinas state, Venezuela	Fever, malaise, sore throat, abdominal pain, diarrhoea, haemorrhagic manifestations, convulsions and death
Junin virus	(*Arenavirus*)	Infected rodents/Direct contact or with their urine/ faeces. Person-to-person transmission	Junin region, east of Buenos Aires	Haemorrhagic fever. Up to 30% fatality rate
Lassa fever	Lassa virus (*Arenavirus*)	Wild rodents (in Africa, a house rat *Mastomys natalensis*)/Direct or indirect contact with infected rodents or their urine/faeces. Patient-to-patient transfer through contaminated blood	Endemic in west Africa. Imported cases have been reported in several countries worldwide	Majority asymptomatic but produces a serious febrile illness in approximately 20% of cases; mucosal bleeding, vomiting, haemorrhagic diarrhoea, meningitis, case fatality rate variable (1–50%)

Continued

Table 68.1b Viral zoonoses: arthropod-borne and rodent-borne infections—cont'd

	Infectious agent	Reservoir/vectors and mode of transmission	Distribution	Disease in humans
Lymphocytic choriomeningitis	Lymphocytic choriomeningitis virus (*Arenavirus*)	Endemic in house mice worldwide. Pet rodents may become infected probably oral or respiratory route/ Direct or indirect contact with infected rodents or their urine/faeces	Europe, Americas	Influenza-like symptoms, occasionally meningoencephalitis. Fatalities in immunocompromised individuals. Abortion and birth defects
Machupo virus	(*Arenavirus*)	Inhaling aerosols of rodent urine/faeces or contamination of open wounds	Bolivia	Fever, lethargy, headache, muscle pains, headache. Bleeding from gums and nose may follow. Vomiting of blood. Fatality rate 3–30%
Mobala virus	(*Arenavirus*)	Rodents (soft-fur rats)/ Transmission pathway unclear	Central African Republic	Fever, facial swelling, fatigue, conjunctivitis, mucosal bleeding
Sabia virus	(*Arenavirus*)	Unknown (believed to be rodents)/Transmission is believed to be by aerosolized bodily fluids	Brazil	Fever, headache, myalgia, nausea, vomiting, conjunctivitis, acute hepatitis, diarrhoea, gastrointestinal haemorrhage
	Bunyaviridae			
Cache Valley virus	(*Orthobunyavirus*)	Cattle, deer, sheep and horses act as a reservoir/ mosquito-borne	North America	Meningitis, encephalitis
Crimean–Congo haemorrhagic fever	Crimean–Congo haemorrhagic fever virus (Nairovirus)	Cattle, goats, sheep, rabbits and hares act as reservoir/ Transmitted by *Ixodes* ticks which also serve as a reservoir	Eastern Europe, Balkans, Greece, Turkey, central Asia, Africa, China, Middle East, India and former Soviet Union	Severe headache, elevated body temperature, arthralgia, muscle pain, altered mental state, jaundice, haematomas, bleeding from nose and throat. Fatality rate of 9–50%
La Crosse	La Crosse virus (California serogroup, *Orthobunyavirus*)	Squirrels and chipmunks act as reservoir/mosquito-borne	North America	Mainly affects children. Non-specific symptoms that may progress to coma, paralysis and brain damage
Hantaviruses	*Hantavirus* – different viruses within the genus, named after the location where they were first identified such as Sin Nombre virus and Puumala virus	Rodents are the reservoir but the virus has been identified in cats and birds/Inhalation of infected aerosols of rodent saliva, urine and faecal matter. Person-to-person transmission	Asia, Europe and the Americas	Haemorrhagic fever with renal syndrome (HFRS) primarily in Eurasia. Hantavirus pulmonary syndrome (HPS) in the Americas. 35% fatality rate recorded in USA

Table 68.1b Viral zoonoses: arthropod-borne and rodent-borne infections—cont'd

	Infectious agent	Reservoir/vectors and mode of transmission	Distribution	Disease in humans
Nairobi sheep disease	Nairobi sheep disease virus (*Nairovirus*)	Sheep and goats/Transmitted by ticks	Eastern Africa, India	Fever, influenza-like
Oropouche virus	(*Orthobunyavirus*)	Transmitted by mosquitoes to humans from sloths	Amazon region of South America, Caribbean and Panama	Fever, chills, headache, anorexia, muscle and joint pain, vomiting and meningitis
Rift Valley fever	Rift Valley fever virus (*Phlebovirus*)	Sheep, goats, camels and cattle/Arthropod vectors, primarily mosquitoes or by direct contact with infected animals. Consumption of infected meat or milk	Africa, Middle East and Eurasia	Fever, headache, arthralgia, myalgia, visual impairment haemorrhages, encephalitis. Fatality rates of up to 50%
	Reoviridae			
Colorado tick fever	Colorado tick fever virus (*Coltivirus*)	Small animals such as ground squirrels and chipmunks/Transmitted by ticks	High altitude (above 2000 metres) Western USA and Western Canada	Acute, febrile diphasic disease. Symptoms include headache, muscle and joint pains, usually of short duration
	Togaviridae			
Chikungunya	Chikungunya virus (*Alphavirus*)	Monkeys are the reservoir/ Transmitted to humans by infected mosquitoes	Primarily Africa and Asia. Several cases in Italy	Muscle pain, rash, high fever, joint pain and arthritis
Eastern equine encephalomyelitis (EEE)	Eastern equine encephalitis virus (*Alphavirus*)	Birds and horses/Mosquito-borne *(Culiseta melanura)*	Canada, Eastern USA, Central and South America, Caribbean islands	Encephalitis, often severe infection; case fatality rate may be high (50%–75%) and survivors may have persistent neurological damage
Mayaro virus	(*Alphavirus*)	Rats and mice. Primates/ Mosquito-borne	Latin America	Influenza-like disease and possibly encephalitis
Western equine encephalomyelitis (WEE)	Western equine encephalitis virus (*Alphavirus*)	Birds are the reservoir/ Mosquito-borne	Western and central USA, Canada, Central and South America	Encephalitis, less severe than EEE and most patients recover. Can be severe in children. Fatality rate may be moderate (3%–14%)
Venezuelan equine encephalomyelitis (VEE)	Venezuelan equine encephalitis virus (*Alphavirus*)	Rodents and horses/ Mosquito-borne. Outbreaks in people associated with epizootics in horses	South America, Central America, southern USA	Influenza-like disease with a diphasic course; some patients may show encephalitis. Mortality rate is low

Continued

Table 68.1b Viral zoonoses: arthropod-borne and rodent-borne infections—cont'd

	Infectious agent	Reservoir/vectors and mode of transmission	Distribution	Disease in humans
	Flaviviridae			
Japanese encephalitis	Japanese encephalitis virus (*Flavivirus*)	Pigs and birds are the reservoir/Mosquito-borne	Southeast Asia – Philippines, Japan, China, Korea, India and Indonesia	Many cases are subclinical. Encephalitis, altered perception, confusion and coma. Case fatality rate of up to 30%, highest in children. Residual neurological damage may occur in 30% of patients
West Nile virus and Kunjin virus	Closely related viruses (*Flavivirus*)	Birds are the reservoir/ Mosquito-borne. Contaminated blood products and organs	Africa, the Americas, the Middle East and Europe. Kunjin virus is found in Australia and Asia	Initially similar to influenza. Stiff neck, vomiting, fever, headache, rash conjunctivitis, diarrhoea. Possible onset of meningitis or encephalitis. Highest mortality in the elderly
Kyasansur Forest disease	Kyasansur Forest disease virus (*Flavivirus*)	Reservoir possibly monkeys/ Transmitted by ticks	Kyansur Forest area of Karnatha, India	Febrile and haemorrhagic phases followed by CNS involvement. Symptoms include rash, conjunctivitis and pneumonia
Louping ill	Louping ill virus (*Flavivirus*)	Sheep, occasionally cattle, horses, deer and grouse/ Transmitted by ticks. Risk to veterinarians at postmortem and abattoir workers handling infected tissues. Consumption of unpasteurized sheep's and goats' milk	UK, Ireland and parts of western Europe	Usually mild but can be associated with residual neurological problems. Fatalities are rare. Biphasic disease, first phase is influenza-like, followed by a meningoencephalitic syndrome
Omsk haemorrhagic fever	Omsk haemorrhagic fever virus (*Flavivirus*)	Rodents (especially muskrats)/Transmission by *Dermacentor* ticks and by mosquitoes. Capable of being water-borne	Western Siberia	Febrile and haemorrhagic phases followed by CNS involvement. Symptoms include rash, conjunctivitis and pneumonia
Powassan virus	Flavivirus	Rodents, especially skunks and woodchucks/ Transmitted by *Ixodes* ticks	North America	Gastrointestinal disturbances, decreased kidney function, anaemia, altered mental states, joint stiffness, muscle weakness and encephalitis. Case fatality rate of 10–15%

Table 68.1b Viral zoonoses: arthropod-borne and rodent-borne infections—cont'd

	Infectious agent	Reservoir/vectors and mode of transmission	Distribution	Disease in humans
Tick-borne encephalitis	Tick-borne encephalitis virus (*Flavivirus*)	Mammals including a range of wildlife species/ Transmitted by ticks	Former Soviet Union (Russian spring-summer encephalitis), Asia	Often biphasic diseases, initial febrile stage, fever and meningoencephalitis. May result in residual neurological damage
St Louis encephalitis	St Louis encephalitis virus (*Flavivirus*)	Birds and bats are the reservoir hosts/Mosquito-borne	USA, Canada, Central and parts of South America	Encephalitis, occasionally hepatitis; case fatality rate is approximately 10% but higher in older age groups
Yellow fever	Yellow fever virus (*Flavivirus*)	Mainly primates (possibly marsupials)/Mosquito-borne	Africa, Central and South America	Acute febrile disease of varying severity; cell destruction in liver, spleen, kidney, bone marrow and lymph nodes. Jaundice and haemorrhages occur. Case fatality rate may approach 50% in epidemics

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Anthrax	*Bacillus anthracis*	Most commonly cattle, sheep and goats. Also horses, pigs and wildlife/ Contact with contaminated animal products including hides and wool or soil. Also inhalation and ingestion	Worldwide	Cutaneous form – papule becomes vesicular and necrotic, extensive oedema Pulmonary form (wool sorter's disease) – influenza-like illness, respiratory failure, may be fatal Intestinal form – severe diarrhoea and high fatality rates
Botulism*	*Clostridium botulinum*	Poultry, cattle and horses/ Food-borne (home-preserved meat), wounds	Worldwide	Neurotoxin: difficulty walking, impaired vision, dysphagia, respiratory distress, muscle weakness, constipation and systemic paralysis

Continued

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Boutonneuse fever	*Rickettsia conorii*	Dogs, rodents and other animals/Ticks (*Rhipicephalus sanguineas* in Mediterranean countries; *Amblyomma, Rhipecephalus, Boophilus* and *Hyalomma* species in South Africa)	Countries adjacent to Mediterranean Caspian and Black seas; Africa and India	Febrile illness, lesion at site of tick bite. Rash on palms and soles may be present for approximately seven days. Case fatality rate is low
Brucellosis	*Brucella abortus, Brucella melitensis, Brucella suis, Brucella canis*	Cattle (*B. abortus*), goats, sheep (*B. melitensis*), pigs (*B. suis*), dogs (*B. canis*)/Contact with infected biological material such as aborted foetus. Ingestion of milk and dairy products from infected animals, airborne infection occasionally	Worldwide especially Mediterranean countries, Africa, Asia and Central and South America	Disease spectrum is influenced by the species; often insidious with malaise, chills, fever and profuse sweating, headaches, arthralgia. Fever may be intermittent (undulant fever) May develop into a chronic disease with many non-specific manifestations. Case fatality rate, even without treatment, is low. Septicaemic form occasionally seen particularly in abattoir workers
Campylobacter enteritis	*Camplyobacter jejuni, C. coli*	Poultry, cattle, and other domestic animals; unpasteurized or recontaminated milk; faecal contamination of carcasses especially poultry; contamination of cooked meat by contact with raw meat/ Ingestion of contaminated food, milk or water; contact with infected pets, often involving young children	Worldwide, especially in temperate zones	Acute enteric disease of variable severity which usually lasts for about one week. Diarrhoea (sometimes with blood), abdominal pain, fever, nausea and vomiting are common manifestations
Cat-scratch disease	*Bartonella henselae*	Cats are carriers but show no ill effects / Humans are infected by scratch, bite or licking of a wound by an infected cat. Also fleas	Worldwide but uncommon; seasonal pattern may occur (late summer, autumn or winter)	A benign self-limiting illness. A primary skin lesion may occur at site of a scratch or bite, followed by development of regional lymphadenopathy More serious cases may result in CNS damage, osteomyelitis, liver damage and involvement of the respiratory tract particularly in immuno-compromised patients

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Chlamydial infections	*Chlamydophila abortus*	Sheep, particularly flocks with enzootic abortion of ewes (EAE)/ Inhalation, ingestion	Many countries	Seen in pregnant women following contact with flocks with EAE. Abortion and serious disease in mother with meningitis and disseminated intravascular coagulation has been recorded
Clostridium perfringens	*Clostridium perfringens*	Occurs in gastrointestinal tract of humans and animals. Found in foodstuffs as a result of faecal contamination Bacterial replication and production of exotoxin linked to inadequate cooking and reheating/ ingestion	Worldwide	Abdominal cramps, diarrhoea and fever. High toxin levels associated with necrotizing enteritis and septicaemia
Cryptococcosis	*Cryptococcus neoformans*	Birds, mammals, plants and soil. Debris of pigeon roosts and pigeon droppings (birds are not affected)/ Inoculation of wounds, inhalation	Worldwide	Pneumonia; lesions in the skin, kidneys, bone and liver may occur; meningitis is frequently seen, especially in immuneosuppressed individuals, and may terminate fatally if not treated
Dermatophilosis	*Dermatophilus congolensis*	Many animals including cattle, sheep, horses and wildlife/Contact	Worldwide	Usually occurs as discrete, cutaneous pustules on the hands and forearms or legs
Erysipeloid	*Erysipelothrix rhusiopathiae*	Pigs, fish, meat or poultry/ Contact (skin abrasions). Butchers, farmers, fish handlers, poultry workers and veterinarians are at greatest risk of infection	Worldwide	Infection is usually limited to the skin at the site of trauma, most commonly on the fingers or hands. The lesion is raised and red or purple with a burning sensation (erysipeloid). Rarely endocarditis occurs
Escherichia coli	*Escherichia coli* 0157/ H7	Consumption of meat, milk or water contaminated with faeces primarily of ruminant animals such as cattle, sheep and goats. The bacterium is also carried by horses, pigs, dogs and wild rabbits	Worldwide	Fluid loss and diarrhoea More serious symptoms that can occur include haemorrhagic colitis, haemolytic-uraemic syndrome and thrombocytopenic purpura (often fatal)
Glanders	*Burkholderia mallei*	Horses, mules and donkeys, goats, cats, dogs and zoo animals/ Contact and inhalation, also laboratory accidents	Africa, Asia, Central and South America, Middle East	Usually pustules and ulcers of the skin or mucous membranes. Inhalation may lead to fatal pneumonia. Septicaemia may be fatal unless treated promptly

Continued

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Leptospirosis	*Leptospira* Icterohaemorrhagiae *L.* Hardjo *L.* Canicola *L.* Pomona	Rodents, dogs Cattle, horses Dogs Pigs, cattle/Ingestion of water contaminated with infected animal urine	Worldwide	Phase 1: fever, headache, fatigue. Phase 2: occurrence depends on severity, after apparent recovery there may be liver and kidney failure, delusion, meningitis, hypotension. In second phase the symptoms continue until recovery or death
Listeriosis	*Listeria monocytogenes*	Soil, pasture, water, silage, infected domestic and wild animals/Ingestion of unpasteurized milk, dairy products or contaminated uncooked vegetables	Worldwide	Mild, febrile, influenza-like syndrome, but may cause congenital infection in pregnant women leading to abortion or meningitis in the baby. In older age groups meningitis may occur, especially in immunosuppressed individuals. Rarely, endocarditis may follow the bacteraemia
Lyme disease	*Borrelia burgdorferi*	Wild rodents, hedgehogs, deer and other animals/Ticks (*Ixodes* species) are main vector and also an infectious reservoir	Most countries	Undulant disease. Clinical signs include distinctive skin lesions and systemic symptoms (early) with arthritis, neurological and occasionally, cardiac involvement (later). Skin lesions occur in a high percentage of patients. Other findings include fatigue, fever, chills, musculoskeletal pain, eye problems and lymphadenopathy. In untreated patients, complications may persist
Melioidosis	*Burkholderia pseudomallei*	The bacterium is a saprophyte in soil and a variety of domestic and wild animals can become infected/Contact with infected material or by inhalation of contaminated dust or soil	Southeast Asia, Australia, Africa, Central and South America	Diabetes, thalassemia and renal disease predispose to disease A wide range of clinical manifestations are reported including fever, bone and joint pain, pulmonary disease, fatal septicaemia and chronic abscess formation. Mortality rate is high in untreated cases

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Murine typhus	*Rickettsia typhi*	Rodents and possibly small mammals and domestic animals may transport infected fleas, thus facilitating contact with humans/Rat flea (*Xenopsylia cheopis*) contamination of bite wound by flea faeces or following inhalation of dried infective flea faeces	Worldwide	Sudden onset with headaches, chills, fever and general pains. After the sixth day macular eruptions appear on chest and abdomen. The course of the disease is about three weeks. Case fatality rate is usually less than 1% but increases with age
Pasteurellosis	*Pasteurella canis* and *P. dagmatis*	Cats, dogs/Bite or scratch wounds	Worldwide	Local infections resulting from animal bites; cellulitis, swelling and local pain frequently occur at site of wound Lymphadenitis and abscess formation occur in occasional cases. Septicaemia in immunocompromised patients
	P. multocida	Pigs, birds, dogs, cats and horses/Ingestion of contaminated water		
Plague (urban and sylvatic)	*Yersinia pestis*	Urban plague: rats/Fleas (*Xenopsylla cheopis*) Sylvatic plague: squirrels, prairie dogs, rabbits, rats/Fleas	Areas of USA, former Soviet Union, the Middle East, Africa, Indonesia, China, Vietnam and parts of South America	In previous centuries *Y. pestis* produced pandemics of 'black death' with millions of fatalities. In bubonic plague, the bacteria reach the regional lymph nodes and infected nodes become inflamed, tender and may suppurate (buboes). Dissemination via the bloodstream may lead to pneumonia and meningitis Secondary pneumonic plague is of particular importance as aerosolized droplets may allow person-to-person transfer to occur and this form of disease is invariably fatal. Case fatality rate in untreated bubonic plague is about 50%
Psittacosis/ ornithosis	*Clamydophila psittaci*	Avian species especially parrots (healthy birds may be carriers)/ Inhalation from desiccated droppings or directly from infected birds	Worldwide	Initially mild, influenza-like disease; later headache, fever, myalgia and arthralgia may occur. Pulmonary changes include consolidation and an unproductive cough Encephalitis and mycocarditis are occasional complications, especially in older patients

Continued

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Q fever	*Coxiella burnetii*	Cattle, sheep, goats and cats and perhaps feral rodents/Often airborne infection but can also be acquired by direct contact with contaminated material and by ingestion of raw milk. Transmission via ticks, lice or fleas has been shown	Worldwide	There is considerable variation in the duration and severity of this disease. Usually acute, self-limiting, febrile illness resembling influenza Pneumonitis and pericarditis occur frequently. Rarely, subacute or chronic endocarditis may present years after the initial infection
Rat-bite fever	*Streptobacillus moniliformis*	Rats, gerbils, squirrels and weasels/Bite wounds or secretions of animals carrying the bacterium in their upper respiratory tracts. Milk or water contaminated with rat urine may transmit infection	North America	Abrupt onset with fever, petechial rash, headache and polyarthritis; endocarditis may occur. In untreated cases mortality rate may be up to 10%. When not associated with a rat bite, the disease is often referred to as Haverhill fever
Rat-bite fever (Sodoku)	*Spirillum minus*	Rats/Bite wounds	Africa and Asia	The bacterium invades the local lymph node causing lymphadenitis, rash and a relapsing fever
Relapsing fever (tick-borne)	*Borrelia recurrentis*	Wild rodents/ticks (*Ornithodoros* species)	North, Central and South America, Africa, Middle East	After an abrupt onset with fever, muscle aches and headache, large numbers of borreliae may be present in the blood and urine. Fever then declines and the borreliae disappear from the blood for about one week. Fever returns and the cycle is repeated up to ten or more times, usually with decreasing severity. The case fatality rate is usually less than 5% in untreated cases

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Ringworm (dermato-mycoses)	*Microsporum* species, *Trichophyton* species	Direct contact with infected cat, dog, poultry, pig, horse, cattle or other animal most common/Contact with contaminated fomites	Worldwide	The dermatophytes infect only epidermis, hair and occasionally nails. Lesions tend to expand equally in all directions with raised borders. Irritation and itching may be a feature of the disease Redness, oedema, scaling and vesicle formation may occur. If hair is invaded, hair shafts become fragile and break off a short distance above the skin leaving short stubs, usually in a balding circular patch (permanent in some cases)
Rocky Mountain spotted fever	*Rickettsia rickettsii*	Rodents, dogs/Ticks *Dermacentor* and *Amblyomma* species	Mexico, North and South America	Characterized by a sudden onset of fever which may persist for up to three weeks; muscle pain, severe headache and a maculopapular rash which appears about the third day. Damage to small blood vessels leads to a vasculitis Myocarditis and intravascular coagulation may occur at a later stage. The case fatality rate is up to 20% in the absence of specific therapy
Salmonellosis	Numerous *Salmonella* serotypes such as *Salmonella* Typhimurium and *Salmonella* Enteritidis	Domestic and wild animals including poultry *Salmonella* Enteritidis is associated with eggs and poultry and *S.* Typhimurium is mainly associated with cattle/ Ingestion of contaminated food (raw or undercooked) of animal origin, especially processed meats, poultry, eggs and raw milk. Contaminated water may also be a source of human infection	Worldwide	Gastroenteritis usually follows ingestion of contaminated food or drinking water. Signs of illness begin 12–24 hours after consumption of the food. Fever, nausea, vomiting and abdominal cramps occur, with profuse diarrhoea which may persist up to one week. There may be septicaemia and even death in older patients
Streptococcal infection	*Streptococcus suis* Type 2	Pigs/Cuts and abrasions, handling of infected carcasses, eating infected meat	Western Europe, China	Fever, meningitis, arthritis, septicaemia and occasional deaths

Continued

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Tetanus	*Clostridium tetani*	Soils or fomites contaminated with faeces, especially horse faeces/Puncture or other wounds contaminated with soil, faeces or dust	Worldwide	The incubation period may range from days to weeks. The neurotoxin produces convulsive tonic contractions of voluntary muscles, especially of the jaw ('lockjaw'), facial spasms and opisthotonos. External stimuli may precipitate a tetanic seizure. The case fatality rate may approach 90% in babies and older patients
Tuberculosis	*Mycobacterium bovis* (*Mycobacterium avium* in immuno-suppressed individuals)	Cattle (*M. bovis*), poultry, wild and caged birds (*M. avium*), occasionally other species/Direct contact with infected animals or birds, inhalation (infected dust or aerosols) or ingestion (raw milk, unpasteurized dairy products)	Countries where *Mycobacterium bovis* infection in cattle is endemic Uncommon in countries with advanced eradication schemes	A chronic progressive disease with early lesions in cervical or mesenteric lymph nodes if ingested; dissemination to bones and joints occurs at a later stage, especially in individuals suffering from AIDS. If inhaled, pulmonary tuberculosis results, indistinguishable from that caused by *M. tuberculosis* Ultimately the disease may lead to emaciation and death if not treated
Tularaemia	*Francisella tularensis*	Numerous wild animals, especially rabbits, hares, muskrats, beavers, rodents and some domestic animals; water polluted by carcasses of infected rodents/Bites of infected arthropods – deer fly (*Chrysops discalis*), mosquito (*Aedes* species), ticks (*Dermacentor* and *Amblyomma* species); animals (trappers); insufficiently cooked rabbit or hare meat; drinking contaminated water. Laboratory infections have also been reported	North America, former Soviet Union, Europe, China, Japan	Often presents as an ulcer in the skin at the site of introduction. The organisms are carried to the regional lymph node which enlarges, becomes painful and may suppurate. Intermittent headaches and malaise. Inhalation of infectious material may be followed by pneumonic disease. Typhoidal tularaemia follows ingestion of the organisms. This condition resembles typhoid fever with diarrhoea, vomiting and fever. The case fatality rate for typhoidal or pulmonary disease is about 10%

Table 68.2 Bacterial, chlamydial, rickettsial and fungal zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Typhus fever	*Rickettsia prowazekii*	Flying squirrels, human louse/Transmission may be possible through contact with flying squirrel nest, faeces, lice or fleas	USA and cold mountainous regions of Africa and South America	Headache, acute fever, haematuria, joint pain and vomiting
Yersiniosis	*Yersinia enterocolitica*	Pigs and other species/ Ingestion of raw or undercooked pork, food products or water that has been contaminated with faeces or urine	Worldwide	Most often young children – an acute but self-limiting gastroenteritis Occasionally joint pain and skin rash

*Not strictly a zoonosis, occurs due to ingestion of preformed toxin, or, rarely, due to elaboration of toxin within the body following contamination by spores from the environment

Table 68.3 Parasitic zoonoses

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Angio-strongyliasis	*Angiostrongylus cantonenis, Angiostrongylus costaricensis*	Rats (*Rattus* species)/ Ingestion of shrimps, crabs or large edible snails containing infective larvae. Ingestion of contaminated water or unwashed vegetables	Hawaii, many Pacific islands, Vietnam, Malaysia, China, Indonesia, Philippines, parts of Australia, east Africa, southern USA and Central America	Larvae of *A. cantonensis* migrate to the brain, spinal cord or eye where they become immature adults. Severe headache, neck stiffness and facial paralysis may occur. Meningitis or meningoencephalitis may cause permanent CNS damage or be fatal. The small intestine is the usual site of infection of *A. costaricensis* where it produces a granulomatous inflammation
Anisakiasis	*Anisakis simplex, Pseudoterranova decipiens* and a number of other larval nematodes	Saltwater fish, squid or octopus/Consumption of uncooked, smoked or marinated fish or fish products	Japan, Netherlands, Scandinavia, USA, Central America	The larval stage of *P. decipiens* rarely penetrates the stomach or intestinal wall, but the larval stage of *A. simplex* is capable of penetrating the wall of the stomach or intestine, leading to abdominal pain, nausea, vomiting, diarrhoea and eosinophilic granuloma. The motile larvae may migrate to the oropharynx causing a cough. Allergic reactions including anaphylaxis

Continued

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Babesiosis	*Babesia divergens* (Europe) *Babesia microti* and other *Babesia* species	Cattle (*B. divergens*) probably rodents (*B. microti*)/ticks (*Ixodes dammini* – USA; *Ixodes ricinus* – Europe)	USA, France, Ireland, Scotland, former Soviet Union, former Yugoslavia	Uncommon but a severe and potentially fatal disease (especially in patients who have been splenectomized). Fever, fatigue and haemolytic anaemia are features of the disease
Balantidiasis	*Balantidium coli*	Pigs, guinea pigs, primates and perhaps other animals/ Ingestion of faecally contaminated water or food	Worldwide in areas of poor environmental sanitation	Abdominal pain, nausea, vomiting, diarrhoea and dysentery are frequently observed, but asymptomatic carriage of *B. coli* is also reported
Capillariasis	*Capillaria philippensis, Capillaria hepatica*	Fish-eating birds/ Ingestion of raw or inadequately cooked fish containing infective larvae	Philippines, Thailand, Japan, Egypt, Iran	Diarrhoea, malabsorption syndrome, dehydration and progressive emaciation are features of the disease. Large numbers of worms may accumulate in the small intestine and produce serious disease. Case fatality rate may be up to 10%
Cheyletiella infection	*Cheyletiella parasitivoraz, Cheyletiella yasguri, Cheyletiella blakei*	Rabbit (*C. parasitivorax*), dog (*C. yasguri*), cat *(C. blakei*)/Contact	Worldwide	Usually a mild disease. Pruritic skin lesions on arms and body with papular eruptions and occasionally severe irritation
Ciguatera	*Gambierdiscus toxicus*	Consumption of farmed salmon and carnivorous species of fish such as shark, grouper, snapper. Especially when undercooked	Tropical and subtropical regions	Perioral numbness and tingling. Tooth pain, nausea, vomiting and diarrhoea may follow. Neurological symptoms include temperature sensitivity, paraesthesia, arthralgia, myalgia. Bradycardia, tachycardia and hypotension can also occur
Clonorchiasis	*Clonorchis sinensis*	Cats, dogs, pigs as well as humans/Ingestion of undercooked or raw freshwater fish or crayfish containing larvae	Southeast Asia, USA	Chronic disease. Loss of appetite, diarrhoea, liver enlargement and progressive ascites may occur. Sometimes asymptomatic

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Cryptosporidiosis	*Cryptosporidium* species	Cattle, sheep, deer, pigs, also infective humans/ Ingestion of food (offal, milk, meat products) or water containing sporulated oocysts. Recreational water such as swimming pools	Worldwide	Immunologically competent humans develop a profuse diarrhoea, nausea and abdominal pain which may last two weeks. Pancreatitis can occur. Immunosuppressed people develop a severe, long-lasting diarrhoea which may persist for many months with life-threatening consequences
***Dermanyssus* infection**	*Dermanyssus gallinae* (red mite of poultry)	Birds, both wild and domestic/Contact, direct attack by mites	Worldwide	Dermatitis with intense pruritis. The northern fowl mite *Ornithonysus sylviarum* and the tropical mite *Ornithonyssus bursa* may leave the nests of birds in buildings and also attack humans
Diphyllobothriasis (fish tapeworm infection)	*Diphyliobothrium latum*	Humans, also dogs, bears and other fish-eating mammals/ Ingestion of raw undercooked freshwater fish containing the larval (plerocercoid) stage	Scandinavia, former Soviet Union, northern Europe, Japan, Canada, Alaska	May produce diarrhoea and mild abdominal pain. A vitamin B12 deficiency is said to occur in some people infected with this tapeworm
Dipylidiasis (dog tapeworm infection)	*Dipylidium caninum* (the most common and widespread adult tapeworm of dogs and cats)	Dogs and cats/Accidental ingestion of the dog flea (*Ctenocephalides canis*) or cat flea (*Ctenocephalides felis*) containing the cysticercoids of *D. caninum*	Worldwide	Usually occurs in young children. Diarrhoea and restlessness
Dirofilariasis	*Dirofilaria immitis* (dog heartworm)	Dogs and other *Canidae*/ Mosquito bite	USA, Japan, Asia, Australia	These worms may lodge in the small pulmonary arteries, producing infarcts. Common symptoms include chest pain, cough and haemoptysis
Echinococcosis	*Echinococcus granulosus*	Dogs, wolves, dingoes and other *Canidae*/ Accidental ingestion (hand to mouth) of tapeworm eggs	Middle East, Africa, Australia, New Zealand, Asia. Parts of Europe, Canada, Alaska, South America	Cystic echinococcosis: hyatid cysts may develop in many organs and tissues of the body. They continue to grow until of sufficient size to cause clinical symptoms. Cysts in vital organs such as the brain may cause severe symptoms and even death

Continued

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
	E. multilocularis	Dogs, foxes and other *Canidae*/Accidental ingestion (hand to mouth)	Arctic regions, parts of temperate Europe	Alveolar echinococcosis (multilocular hydatid disease): growth in man simulates neoplasia. Because of the metastases and infiltrative process in organs, surgery is rarely successful
Fascioliasis	*Fasciola hepatica* (liver fluke)	Sheep and cattle/Human infection is acquired by ingestion of aquatic vegetation such as watercress in salads on which metacercariae have encysted	Sheep- and cattle-raising areas of Europe, South America, Caribbean, Australasia and Middle East	Metacercariae migrate from the intestine to the bile ducts by passing through the wall of the intestine into the abdominal cavity and entering the liver through its outer surface. Liver damage and enlargement occur, particularly if large numbers of flukes are present. Biliary colic and obstructive jaundice may occur. Eosinophilia is a feature of the disease
Fleas	*Ctenocephalides canis* (dog flea), *Ctenoceph alides felis* (cat flea), *Xenopsylia cheopis* (rat flea), *Echidnophaga gallinacea* (sticktight flea of poultry)	The rat flea (*X. cheopis*) may be a vector of murine typhus (*Rickettsia typhi*) and plague (*Yersinia pestis*). The dog flea (*C. canis*) is an intermediate host for the dog tapeworm	Worldwide	Fleas may cause irritation when they attack humans if their preferred host is no longer available. They may also transmit serious infections to the human population such as plague and murine typhus. Many humans become sensitized to flea bites (salivary antigen) and develop an immediate type hypersensitivity reaction at the bite site
Giardiasis	*Giardia lamblia*	Beavers, dogs, a variety of birds and also humans/Ingestion of food or water contaminated with faeces containing *Giardia* cysts	Worldwide	Intestinal symptoms include diarrhoea, abdominal cramps, fatigue and weight loss. Immunosuppressed patients are more liable to massive infection. Conversely some infections with this parasite may be asymptomatic
Gnathostomiasis	*Gnathostoma spinigerum*	Cats and dogs/Ingestion of undercooked fish or poultry containing third-stage larvae (fish and poultry may act as second intermediate hosts)	Southern Europe, Africa, Asia and Australia	Larvae migrate through the tissues (visceral larva migrans) forming transient inflammatory lesions, including abscesses, in various parts of the body. Larvae may invade the brain producing focal cerebral lesions

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Heterophyiasis	*Heterophyes heterophyes*	Dogs and cats/Ingestion of infected raw or undercooked fish containing the metacercariae	Middle East, Asia	Generally a mild infection. In occasional severe cases haemorrhagic diarrhoea may develop
Hookworm disease (cutaneous larva migrans)	*Ancylostoma caninum, Ancylostoma braziliense, Uncinaria stenocephala*	Dogs and cats/Contact with damp, sandy soil contaminated with dog and cat faeces	Worldwide. predominantly tropical and subtropical regions	Most common in children. Larvae enter the skin and migrate intracutaneously producing dermatitis ('creeping eruptions'). Intense itching is a feature of the disease, which is usually self-limiting after several weeks
Hymenolepiasis	*Hymenolepsis nana*	Rodents (intermediate hosts, infected insects)/Ingestion in food or water of larvae-bearing insects or eggs	Southern USA, Latin America, Australia, Mediterranean countries, Near East, India	Abdominal pain, enteritis, vague symptoms such as weight loss and weakness
Leishmaniasis (cutaneous)	*Leishmania mexicana, Leishmania tropica* and many other species	Humans, wild rodents, carnivores including dogs, marsupials and other unknown hosts/ Bites of infected sandflies of the genus *Phlebotomus*	Pakistan, Middle East, Africa, Mexico, South America	The disease which may present in many forms usually starts as an ulcer on the skin. Lesions may be single or multiple and may last from weeks to months. Recurrence after cure may occur
Leishmaniasis (visceral)	*Leishmania donovani* and other species	Believed to be wild *Canidae*, domestic dogs, rodents and humans/Bites of infected sandflies	Pakistan, China, former Soviet Union, Middle East, parts of Africa, Central and South America	A chronic systemic disease characterized by fever, hepatosplenomegaly, lymphadenopathy, anaemia and progressive emaciation. Untreated, the disease may be fatal
***Metagonimus* infection**	*Metagonimus yokogawai*	Dogs, cats, pigs and humans/Ingestion of infected raw or undercooked freshwater fish containing the metacercariae	Asia, Egypt, Turkey, Balkan states	Mild diarrhoea and abdominal pain, often asymptomatic
Paragonimiasis (lung fluke disease)	*Paragonimus westermani* and other species	Humans, dogs, cats, pigs and wild carnivores/ Ingestion of infected raw or undercooked freshwater crabs or crayfish containing the metacercariae	Asia, particularly Korea and Japan. Philippines, Africa, Central and South America; occasionally North America	The immature fluke penetrates the small intestine, migrates to the pleural cavity and penetrates the lung tissue. Symptoms include a cough, chest pains and haemoptysis. The parasite may migrate to other sites including the CNS

Continued

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Racoon roundworm	*Baylisascaris procyonis*	Soil or other materials containing raccoon faeces contaminated with worm eggs/ Ingestion	North America	Rare but can cause fatal encephalitis
Sarcocystis infection	*Sarcocystis hominis, Sarcocystis suihominis* and other species	Cattle, sheep, pigs/ Ingestion of raw or undercooked beef, lamb or pork containing cysts of the parasite	Worldwide	Nausea, abdominal pain and vomiting. Subcutaneous swellings and eosinophilia have been attributed to infection with this parasite
Scabies (acariasis)	*Sarcoptes scabiei*	Dogs, pigs, cattle/ Contact	Worldwide	Skin lesions on the hands, arms and occasionally on the body. Lesions usually consist of papules or vesicles and may be intensely pruritic
Schistosomiasis	*Schistosoma japonicum*	Many species of animals including dogs, cats, pigs, primates, wild rodents and humans/ Contact with water containing free-swimming cercariae	Africa, Asia, parts of South America	Symptoms include abdominal pain, diarrhoea, fever, fatigue and hepatosplenomegaly. Chronic infections give rise to liver fibrosis and portal hypertension. The larvae of some schistosomes of birds and mammals may penetrate the human skin and cause dermatitis sometimes referred to as 'swimmer's itch'
Shellfish poisoning	Dinoflagellate toxins	Ingestion of shellfish	Worldwide	Depending on toxin, paralysis, diarrhoea, cramps, numbness, loss of speech
Sparganosis	*Spirometra* species	Carnivores (adult worms), frogs, snakes and other animals (larval forms)/Humans infected by drinking contaminated water, eating intermediate hosts or by applying native poultices such as raw frog tissue	North and South America, Eastern Asia	Disease is characterized by the presence of large larvae in muscles and subcutaneous tissues, causing oedema and inflammation
Strongyloidiasis	*Strongyloides stercoralis, Strongyloides fulleborni*	Many species of animals including dogs, cats, non-human primates/ Contact with moist soil contaminated with faeces	Tropical and temperate regions of the world	Transient dermatitis, pneumonitis and abdominal symptoms, depending on the stage and severity of disease. Dissemination may occur in immunosuppressed individuals

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Taeniasis	*Taenia saginata* (beef tapeworm)	Humans (cattle are intermediate hosts)/ Ingestion of raw or undercooked beef containing viable cysticerci	Worldwide	Mild abdominal pain and diarrhoea may occur occasionally. Weight loss and intestinal obstruction can occur
	Taenia solium (pork tapeworm)	Humans (pigs are the intermediate hosts)/ Ingestion of infected raw or undercooked pork containing viable cysticerci ('measly pork')	Central America, Africa, southeast Asia and Eastern Europe	Intestinal infection in man follows ingestion of cysticerci in raw or undercooked pork (development of adult tapeworms in the intestine). Human cysticercosis occurs following ingestion of the eggs of *T. solium* and is a more serious form of infection. The cysticerci may develop in any organ or tissue in the body. Serious disease may develop from localization in the CNS or the eye
Ticks	Many species including *Ixodes Dermacantor, Rhipicephalus, Amblyomma*	Ticks may transmit bacterial disease such as Lyme disease (*Borrelia burgdorferi*), protozoan diseases such as babesiosis (*Babesia divergens*), rickettsial diseases such as Rocky Mountain spotted fever (*Rickettsia rickettsii*) and viral diseases such as louping ill (*Flavivirus*)	Worldwide	Apart from the disease they transmit, ticks cause skin irritation while feeding on their hosts. Some are reported to cause flaccid paralysis due to injection of toxin
Toxocariasis (visceral larva migrans)	*Toxocara canis, Toxocara cati*	Dog (*T. canis*), cat (*T. cati*)/Ingestion of *Toxocara* eggs from contaminated soil, uncooked vegetables, or from the coat of the animal shedding eggs	Probably worldwide	Primarily a disease of young children. Larvae penetrate the wall of the intestine but are unable to complete their life cycle. They migrate through their host's tissues, producing eosinophilic granulomas. Systemic toxocariasis (visceral larva migrans) results in hepatosplenomegaly, pulmonary infiltrates and sometimes seizures. Larvae migrating to the eye (ocular larva migrans) or CNS may produce serious disease

Continued

Table 68.3 Parasitic zoonoses—cont'd

	Infectious agent	Reservoir/vectors or mode of transmission	Distribution	Disease in humans
Toxoplasmosis	*Toxoplasma gondii*	Cats or other feline animals, various intermediate hosts/ Ingestion of: • Tissue cysts in raw or undercooked meat, particularly mutton or pork • Contaminated cat faeces from the environment (sand boxes, soil, litter trays) • Bradyzoites while assisting at lambing if *Toxoplasma* abortions are occurring in the flock • Tachyzoites present in raw goat's milk (rare)	Worldwide	Only a small proportion of infected people show evidence of infection. The disease may be severe, even fatal, in immunosuppressed individuals, and primary infection during pregnancy can lead to congenital infection with serious consequences. Lymphadenopathy, myalgia and many non-specific symptoms may occur postnatally. If cysts form in the eye, CNS or myocardium, the outcome may be serious
Trichinosis	*Trichinella spiralis, T. native, T. britovi*	Pigs and many other species of meat-eating animals including wild animals and marine mammals. *Trichinella spiralis* is not host-specific/Ingestion of raw or insufficiently cooked meat, usually pork, containing encysted larvae	Worldwide	Initially there may be fever, non-specific gastroenteritis, myositis and circumorbital oedema. If the infection is severe, cardiac and neurological complications may result
Trypanosomiasis (Chagas' disease)	*Trypanosome cruzi*	Many species of domestic and wild animals, including dogs, cats, rodents and humans/Infection occurs when infected blood-sucking vectors (triatomid insects) contaminate wounds with infected faeces after feeding	Mexico, South America and Southern USA	Acute disease occurs usually in children. An inflammatory response at the site of infection (chagoma) may last for two months. Unilateral bipalpebral oedema presents in a high proportion of acute cases. Fever, malaise, lymphadenopathy and hepatosplenomegaly are features of the disease. Occasionally, myocarditis and meningoencephalitis may occur
Trypanosomiasis (Sleeping sickness)	*Trypanosoma brucei gambiense, T. brucei rhodesiense*	Wild and domestic animal reservoirs, especially cattle, bush buck and impala/Bite of an infected tsetse fly (*Glossina* species)	Tropical Africa	Fever, intense headache and generalized lymphadenopathy may occur. CNS signs may be evident a few weeks after infection. Without treatment, the disease may be fatal within weeks

must be eaten by the only intermediate host, cattle, to develop further. Following ingestion by a susceptible bovine animal, the oncosphere, after hatching in the small intestine, travels via the blood to striated muscle and various organs including the heart, tongue and liver. Within three months the cysticerci which develop are infective for humans (Fig. 68.2).

The cysticerci may survive from months to years. Human infection follows the ingestion of raw or inadequately cooked beef, which has not been frozen, containing viable cysticerci. The immature tapeworm evaginates its scolex, attaches to the mucosa of the jejunum, and develops into a mature tapeworm in about 10 weeks. Eggs may remain viable for several months in the environment and the adult worm may remain in the small intestine for many years. In humans, the presence of an adult tapeworm may produce mild discomfort such as light abdominal pain and diarrhoea. Infection with this parasite is also of concern for public health and aesthetic reasons. An economic aspect of this disease is carcase condemnation arising from heavy infestation with the cysticerci of *T. saginata* as well as the cost of inspecting meat for the parasite.

Control measures include education of the public on measures relating to the prevention of infection in cattle. As the adult *T. saginata* has a long life span, identification and treatment of infected human carriers is an essential step in prevention of infection. High standards of human sanitation, thorough cooking of beef, inspection of beef carcases for cysticeri followed by condemnation of those heavily infected and freezing of lightly infected carcases at −10 °C for 10 days are essential preventive measures.

Figure 68.2 *Taenia saginata*: viable cysticerci dissected from the carcase of a heifer experimentally infected with eggs of *T. saginata* seven months earlier.

Toxocariasis (*Toxocara canis*)

Human infection, particularly in young children, caused by the accidental ingestion of eggs of *Toxocara canis*, the common dog roundworm, results in a disease syndrome termed visceral larva migrans or toxocariasis. The worm's life cycle is completed only in its canine host. Most dogs are infected in utero by larvae that have been reactivated in the pregnant bitch. Larvae which have migrated across the placenta mature in pups after birth. Toxocariasis is primarily acquired by exposure to soil contaminated with dog faeces. Eggs, which require about two weeks to become infectious, can survive in soil for many months. When eggs are ingested, larvae penetrate the wall of the intestine, but are unable to complete their life cycle and migrate through the host's tissues, producing eosinophilic granulomas. The life cycle of *T. canis*, the environmental contamination which may result from faecal dispersal, transmission of infection and its possible sequelae are shown in Figure 68.3.

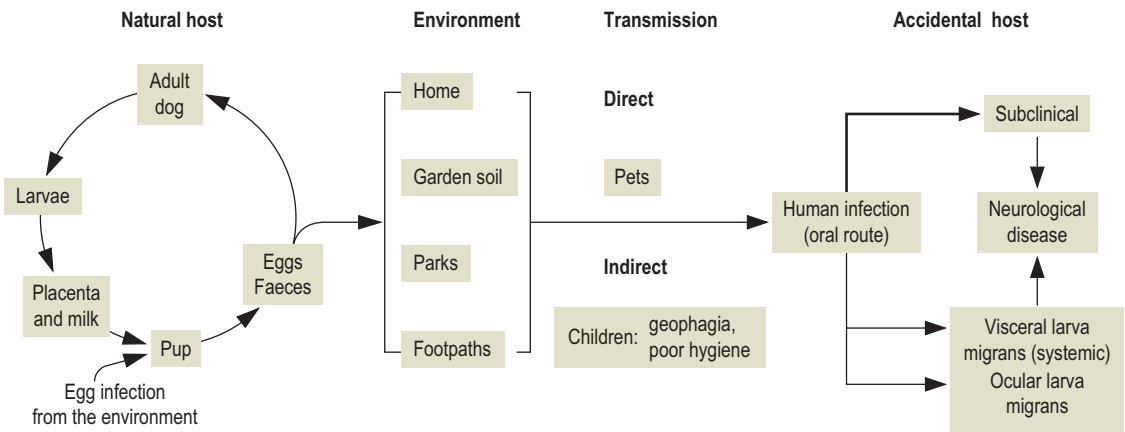

Figure 68.3 The life cycle of *Toxocara canis* showing how the dispersal of eggs can lead to environmental contamination. Young children in close contact with young dogs shedding eggs are particularly at risk. Children may also acquire infection from contaminated soil in gardens or parks, from sand boxes or from fouled pavements.

Systemic toxocariasis, which is termed visceral larva migrans, results in hepatosplenomegaly, pulmonary infiltrates, seizures and sometimes behavioural disorders. Eosinophilia is a prominent sign and usually persists for several months. Ocular larva migrans, an infrequent but serious disease, usually presents as unilateral reduced vision without systemic symptoms or eosinophilia. Involvement of the central nervous system, or myocardium, the most serious form of disease produced by *T. canis*, is rare but may be fatal.

Prevention and control of visceral larva migrans requires the cooperation of the veterinary and medical professions. Education of pet owners on matters relating to *T. canis* infections and their prevention, combined with advice to parents of young children on the hazards of poor hygiene and geophagia in gardens frequented by dogs, would greatly reduce the probability of infection. Strict enforcement of legislation relating to fouling of public places by dogs would decrease the risk of exposure in parks and playgrounds. The largest reservoir of environmental contamination, pups and nursing bitches, should be routinely treated with effective anthelmintics.

The prevalence of intestinal parasites in dogs is high and the common association of children with pups tends to increase the possibility of human infection. Young children should be instructed to routinely wash their hands after playing with dogs (and other animals). Pet shops offering young dogs for sale should be required to treat them with anthelmintics and maintain a good standard of hygiene to prevent reinfection. Elimination of stray dogs, especially in urban areas, is an important step in the prevention of faecal environmental contamination by parasitic ova including the eggs of *T. canis*.

Cryptosporidiosis (*Cryptosporidium* species)

Cryptosporidium species have a wide host range and appear to exhibit little species specificity. *Cryptosporidium parvum* is the species most often associated with zoonotic infections. These coccidian parasites are found in close association with epithelial cells of many species of animals including man. The gastrointestinal tract is most commonly affected in young ruminants and this parasite is thought to be of considerable importance in the calf diarrhoea complex. Major outbreaks of cryptosporidiosis have been reported in calves, lambs, goats and other species with large numbers of oocysts being shed in the faeces of animals (Fig. 68.4). Cryptosporidiosis may be acquired from infected animals and from faeces, contaminated food or water. Mild causes of disease have been reported in farm workers. Immunosuppressed persons, the very young and the very old, should avoid contact with this parasite as it may cause severe, and in some instances intractable diarrhoea.

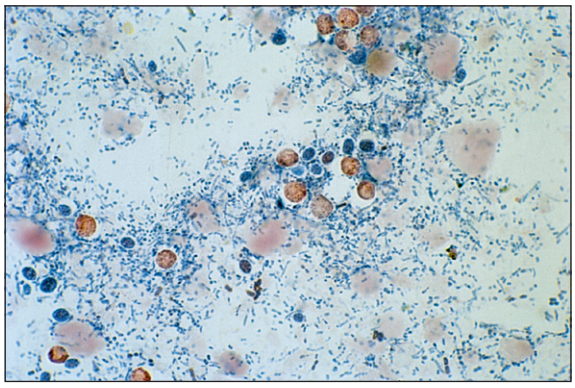

Figure 68.4 Oocysts of *Cryptosporidium* species in calf faeces appearing as round, reddish structures when stained with safranin-methylene blue stain. (×1000)

Figure 68.5 Lamb with lesions of orf (contagious pustular dermatitis) caused by a parapoxvirus. Orf is transmitted to humans by direct contact with an infected animal, or with the vaccine which contains live virus.

Orf (*Parapoxvirus*, *Poxviridae*)

A number of infectious diseases including ringworm, orf and leptospirosis can be readily acquired when handling infected animals. Lesions of orf are frequently seen in sheep and goat populations as scabby lesions, often involving the muzzle and lips, which bleed after mild trauma (Fig. 68.5). Occasionally the gums and tongues of lambs may be affected and the teats of ewes. Human infection can occur among persons occupationally exposed, particularly sheep handlers. Infection of humans occurs usually as a single lesion on a finger, hand, forearm or face. Rarely, generalized disease has been reported. The orf vaccine contains fully virulent live virus and is capable of infecting persons handling it.

Figure 68.6 Leptospiral jaundice in a young dog.

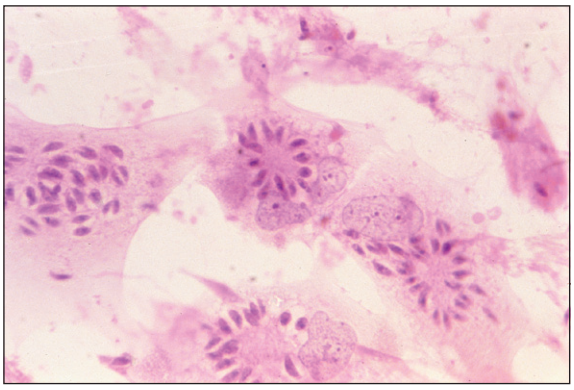

Figure 68.7 Tachyzoites of *Toxoplasma gondii* growing in a monolayer. In some heavily parasitized cells, the tachyzoites are arranged in a rosette manner. Monolayers usually disintegrate within a few days of exposure to this parasite. (H&E stain, ×1000)

Leptospirosis (*Leptospira interrogans* serovars)

Dogs, cattle, pigs, rats and a number of other animal species shed leptospires in their urine. Direct contact with urine or tissues of infected animals such as dogs (Fig. 68.6) can lead to human infection. Leptospires may penetrate the skin, especially if abraded, or mucous membranes, sometimes leading to severe systemic disease. Common features of leptospirosis are fever with sudden onset, headache, severe myalgia, meningitis, haemolytic anaemia and jaundice, depending on the serovar involved. Diagnosis can be confirmed by isolation of leptospires from the blood (first week of illness), by serology using the microscopic agglutination test or immunofluorescence, and by detection of leptospires in the urine during the second week of infection.

Toxoplasmosis (*Toxoplasma gondii*)

Toxoplasmosis is of major importance in many countries as a cause of perinatal mortality in sheep. It also causes sporadic disease in a wide range of animals including humans. *Toxoplasma gondii* is an intracellular protozoan parasite which occurs worldwide. Domestic cats and occasionally other members of the cat family (*Felidae*) are believed to be responsible for the widespread transmission of this parasite through faecal contamination of pasture, soil (including garden soil), feed concentrates and occasionally water. Although infection with *T. gondii* is relatively common in the human population, disease arising from infection with this parasite is uncommon. In immunosuppressed patients, such as cancer patients or victims of AIDS, toxoplasmosis is a serious disease which may be fatal. Congenital infection which occurs only after a woman acquires a primary infection while pregnant, may produce serious defects in the developing foetus. Abnormalities may include bilateral retinochoroiditis, hydrocephalus and other neurological changes. Infections postnatally are usually less severe and many human infections are asymptomatic.

The definitive host of *T. gondii* is the domestic cat or feral cats. Feline infection usually begins with cats eating raw meat or prey with tissue cysts containing bradyzoites. Occasionally, faecal oocysts may be responsible for feline infections, or tachyzoites in an acutely infected prey animal. Following the development of sexual forms in the intestinal epithelium of the cat (the only species in which this occurs), oocysts are shed in the faeces but are not infective for about 72 hours. Faecal oocysts are highly resistant to environmental conditions, are shed in large numbers especially by young cats, and are capable of infecting a wide range of animals including man. Tissue cysts containing bradyzoites are the forms most frequently seen in tissues of animals, but in acute toxoplasmosis, tachyzoites may be present. The tachyzoites can be cultured in monolayers (Fig. 68.7).

Prevention of infection in the human population is concerned with awareness of the role of the cat in the cycle of infection and implementation of suitable hygienic measures. Pregnant women should avoid contact with cats unless the animals are fed canned or cooked food and kept indoors. Cat litter boxes should be emptied daily before oocysts sporulate and the litter disposed of in a safe manner. Pregnant women and other people in high risk categories should not assist at lambing time, particularly if abortions have occurred in the flock. As meat, especially mutton or pork, may occasionally contain cysts of *T. gondii*, it should be adequately cooked. The faecal oocysts are resistant to many household disinfectants. Boiling water and iodophors should be used where contamination with this parasite is suspected.

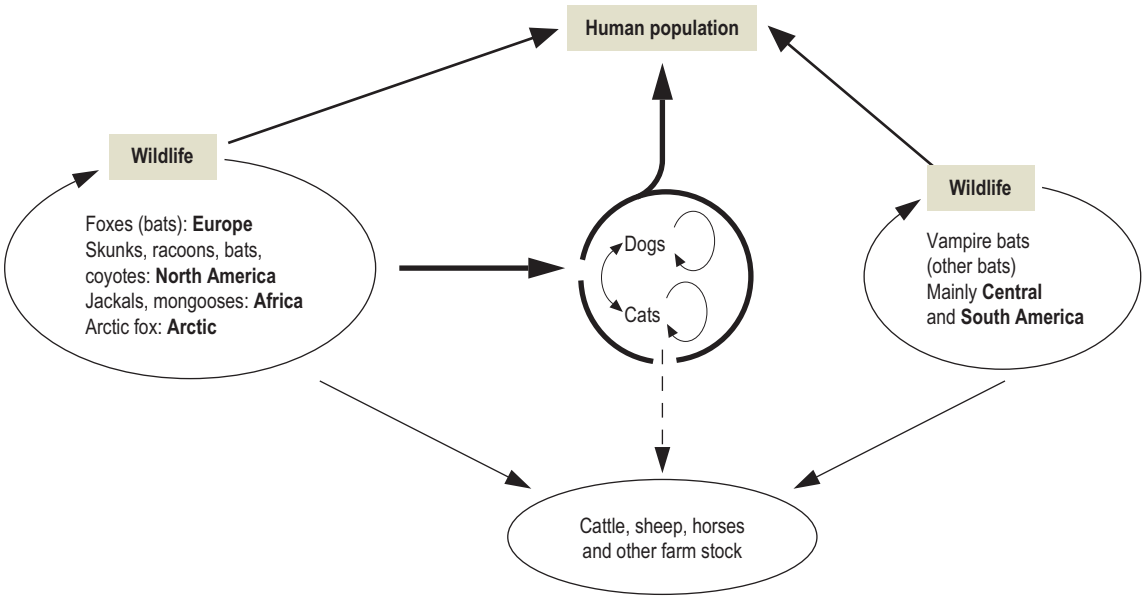

Figure 68.8 Transmission of rabies from wildlife reservoirs to humans and to domestic animals.

Rabies (*Lyssavirus, Rhabdoviridae*)

Several species of animals may cause injuries to humans through biting, scratching or kicking. Biting is the most common injury caused by domestic animals, mainly by dogs. A high proportion of cat-bite wounds become infected by *Pasteurella* species and other pathogenic bacteria. In countries where rabies is endemic, bite wounds from dogs and cats may assume life-threatening proportions until it has been definitively established that the virus was not present in the animal's tissues. Rabies virus enters the body through skin abrasions or bite wounds contaminated by virus-laden saliva from rabid animals. Occasionally, scratches by infected animals, or aerosols in caves frequented by bats harbouring the virus may transmit infection. Rabies is widely distributed throughout the world with the exception of Australia, New Zealand, Japan, a number of European countries and some Caribbean islands. Wild mammals serve as a large and mainly uncontrollable reservoir of **sylvatic** rabies which is an increasing threat to the human population and to domestic animals in many countries (Fig. 68.8).

The principal wildlife reservoir of disease in Europe is the red fox, while in North America bats, coyotes, skunks, raccoons and foxes (the Arctic fox in polar regions) are important. In several countries of Central and South America vampire-bat rabies is a threat to livestock and to the human population. The mouth parts, particularly the incisor teeth of a vampire bat (Fig. 68.9), allow it to collect blood from the puncture wounds it inflicts on its victims,

Figure 68.9 Close-up of the razor-sharp incisor teeth of a vampire bat. The bat punctures the skin of its victim with the incisor teeth and laps the blood from the puncture wound with its tongue.

often cattle, horses (Fig. 68.10), and other species of animals, thus facilitating the spread of the rabies virus. Infected dogs are responsible for most of the human rabies cases recorded annually and **urban** rabies is of particular importance in many developing countries such as India.

The control of rabies in a given country depends on whether it is free of the disease, has land frontiers or is

an island. Strict quarantine of animals imported from rabies regions for up to six months has maintained the disease-free status of a number of countries such as Australia and New Zealand. A pet passport/travel scheme has been successfully introduced in several European Union countries. However, once rabies is introduced into a region, especially if wildlife becomes infected, eradication is difficult and protracted. Immunization of dogs and cats is an effective measure where disease is endemic and attenuated, live virus vaccines are usually efficacious and safe. Control of urban rabies requires the elimination of stray dogs and cats combined with mass vaccination of all domestic pets. Vaccination has been successful in controlling the spread of rabies in wildlife. An attenuated rabies virus strain or vaccinia recombinant virus is given orally in fat or fish meal baits.

Figure 68.10 Horse bitten by a vampire bat.

Box 68.1 **Prevention of zoonoses acquired from companion animals**

- Education of pet owners and farmers so that they are aware of zoonotic diseases; increase awareness of potential zoonotic infections from exotic pets
- Practical hygiene, especially as it relates to children and those immediately at risk of infection
- Control of infectious diseases through strategic use of anthelmintics, chemotherapy and vaccination
- Isolation of sick animals, especially children's pets
- Control of stray animals, particularly in urban areas, by appointment of dog wardens, etc.
- Exclusion of pets from food shops and restaurants (except guide dogs)
- Prevention of indiscriminate fouling of pavements, gardens and parks by dogs (and cats)
- Mandatory identification of all pets by name tags or other appropriate systems
- Quarantine measures and pet travel schemes should be strictly enforced to exclude exotic zoonoses such as rabies
- Continuing education of the public through the mass media on matters relating to the care of animals, the diseases they acquire and disease prevention

Control

Control and prevention of zoonoses requires consideration of the infectious agent, its epidemiology, the nature and severity of the disease it produces in humans and the frequency of its occurrence in animal and human populations. Boxes 68.1 and 68.2 outline measures appropriate for the prevention of zoonoses acquired from companion animals and food-producing animals respectively.

Box 68.2 **Prevention of zoonoses acquired from food-producing animals**

- Education of farmers and producers on the measures appropriate for the prevention of zoonotic diseases in farm stock, irrespective of the species of animal involved – cattle, sheep, goats, pigs or poultry
- All farms should have hand washing facilities. On open/petting farms these facilities should be available close to eating areas
- Buildings should be rodent-proof and well-maintained
- Hygiene standards in meat plants should minimize the possibility of cross-contamination
- Carcases should be inspected for evidence of zoonotic disease and where necessary detained for confirmatory tests
- Pasteurization plants should operate to high efficiency and retain their records for periodic inspection
- In the event of an outbreak of a zoonotic disease in dairy cows, unpasteurized milk consumed on the farm should be heated to 100°C
- Vigilance is required to ensure that wholesome food, which is produced hygienically, is also stored properly, preferably at 4°C, and not recontaminated before it reaches the consumer
- Utensils, cutlery and other items used for preparing raw meat dishes or eggs should **not** be used for cooked meat unless thoroughly washed
- Adequate cooking is an essential step in the prevention of meat-borne zoonoses such as trichinosis and toxoplasmosis
- Slurry generated on dairy, beef and pig farms should be stored before dispersal. If zoonotic diseases have occurred on the farm of origin, longer storage and dispersal on land for tillage should be considered

REFERENCE

Jones, K.E., Patel, N.G., Levy, M.A., et al., 2008. Global trends in emerging infectious diseases. Nature 451, 990–994.

FURTHER READING

Benenson, A.S., 1990. Control of Communicable Diseases in Man. American Public Health Association, Washington, DC.

Colville, J.L., Berryhill, D.L., 2007. Handbook of Zoonoses, Identification and Prevention. Mosby, Elsevier, St Louis.

Palmer, S.R., Soulsby, L., Torgerson, P., Brown, D.W.G., 2011. Oxford Textbook of Zoonoses, second ed. Oxford University Press, Oxford.

Rabinowitz, P.M., Conti, L.A., 2010. Human-Animal Medicine: Clinical Approaches to Zoonoses, Toxicants and Other Shared Health Risks. Saunders-Elsevier Health Sciences, Missouri.

Shakespeare, M., 2009. Zoonoses, second ed. Pharmaceutical Press, London.

Webber, R., 2009. Communicable Disease Epidemiology and Control: A Global Perspective, third ed. CAB INternational, Wallingford.

Section 6

A systems approach to infectious diseases on a species basis

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Chapter 69

Infectious diseases

A microbiological approach to infectious diseases of domestic animals is presented in this chapter. The material has been organized on a species and systems basis. Species of veterinary importance are dealt with in the following order: cattle (Table 69.1), sheep/goats (Table 69.2), pigs (Table 69.3), horses (Table 69.4), dogs (Table 69.5), cats (Table 69.6) and domestic birds (Table 69.7). It incorporates in a tabular form clinical aspects of the more important infectious diseases of domestic animals and mentions confirmatory tests appropriate for each disease. More complete information on the diagnosis of individual infectious agents will be found in the appropriate chapters. Diseases where sudden death occurs do not lend themselves to description on a systems basis. Such diseases include anthrax and the clostridial enterotoxaemias in cattle and sheep (Chapters 14 and 16). Relevant information on chemotherapy relating to the bacterial and fungal diseases is presented in Chapter 6.

Abbreviations

Ab	antibody
Ag	antigen
AGID	agar gel immunodiffusion test
BA	blood agar
BAL	bronchoalveolar lavage
CAM	chorio-allantoic membrane
CFT	complement fixation test
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
DCF	dilute carbol fuchsin stain
Dd	differential diagnosis
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
FA	fluorescent antibody technique
HA	haemagglutination
HAI	haemagglutination inhibition test
IB	inclusion body
i/c	intracerebral
IFA	indirect fluorescent antibody test
IHA	indirect haemagglutination test
IN	intranuclear
i/p	intraperitoneal
i/v	intravenous
KOH	potassium hydroxide
LI	large intestine
l/n	lymph node
MAT	microscopic agglutination test
MZN	modified Ziehl – Neelsen stain
PCR	polymerase chain reaction
PM	post mortem
RIA	radioimmunoassay
RT-PCR	reverse transcription PCR
SI	small intestine
SMEDI	stillbirth, mummification, embryonic death, infertility
UN	United Nations
TC	tissue culture
URT	upper respiratory tract
VN	virus neutralization test
WBC	white blood cell
ZN	Ziehl Neelsen stain

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Table 69.1 Principal infectious diseases of cattle

Buccal cavity: cattle

Disease	Agent(s)	Comments	Diagnosis
ACTINOBACILLOSIS (WOODEN OR TIMBER TONGUE)	*Actinobacillus lignieresii*	Wooden tongue is a characteristic form of the disease. A hard, granulomatous mass develops in the substance of the tongue. There is anorexia, excess salivation and the tongue is hard and 'wooden'	• Clinical signs • Microscopy and culture if exudate is present
ACTINOMYCOSIS	*Actinomyces bovis*	Actinomycosis of the jaw can predispose to suppurative alveolar periostitis, often involving the fourth or third molars. The condition should be considered when loose cheek teeth occur in cattle	• Microscopy on sulphur granules in pus or exudate • Culture, if necessary
BLUETONGUE (BT)	Bluetongue virus (*Orbivirus*; *Reoviridae*)	In endemic areas infections often subclinical or mild in cattle, serotype 8 infections more pronounced. Occasionally well-developed lesions in mouth, encrusted muzzle ('burnt' appearance), nasal discharge, laminitis (severe) and a patchy dermatitis are present	• History of endemic area • RT-PCR • Virus isolation: i/v inoculation of 10–12-day-old embryonated eggs • Serology: VN, AGID, ELISA, modified CFT
BOVINE PAPULAR STOMATITIS	Bovine papular stomatitis virus (*Parapoxvirus; Poxviridae*)	Papular and erosive lesions occur in buccal mucosa and on muzzle of animals under six months of age. No systemic involvement	• History of milker's nodules in humans feeding calves • Lesions and no illness • EM on biopsy of lesions
BOVINE VIRAL DIARRHOEA (BVD) AND MUCOSAL DISEASE	Bovine viral diarrhoea virus 1, 2 (*Pestivirus*; *Togaviridae*)	BVD: Oral lesions are seen in about 75% of animals with the diarrhoeal syndrome. There is diffuse reddening of mucosa, followed by focal lesions that develop into discrete, rounded, shallow erosions (1–2 cm) Mucosal disease: Erosive stomatitis is characteristic. The oral lesions are discrete, rounded, sharply defined depressions. Disease occurs in 12- to 36-month-old animals. There is also diarrhoea and often lameness. These animals are immunotolerant (Ab –ve) but have a persistent viraemia (Virus +ve)	• Clinical signs • FA on frozen sections or buffy coat • Virus isolation: buffy coat • Viral antigen detection: ELISA, immunohistochemistry ('ear notch') • RT-PCR • Serology: ELISA or VN on paired sera
CALF DIPHTHERIA (NECROTIC STOMATITIS)	*Fusobacterium necrophorum*	Predisposing cause may be rough foodstuffs. Usually seen in calves under six months old. Necrotic lesions occur in buccal cavity or laryngeal region (dyspnoea)	• Clinical signs • Gram-stained smear on necrotic debris • Culture if necessary
DERMATOPHILOSIS (STREPTOTHRICOSIS)	*Dermatophilus congolensis*	Lesions can occasionally occur on the tongue as a result of the animal licking skin lesions	• Clinical signs • Giemsa-stained or Gram-stained smear of scabs • Culture, if necessary • Sequencing of rRNA gene for identification

Table 69.1 Principal infectious diseases of cattle—cont'd

Buccal cavity: cattle

Disease	Agent(s)	Comments	Diagnosis
EPHEMERAL FEVER (THREE-DAY-SICKNESS)	Bovine ephemeral fever virus (*Ephemerovirus*; *Rhabdoviridae*)	Signs include apparent pain in the throat region, accompanied by dysphagia and hypersalivation. Some animals have impaired swallowing reflexes and if death occurs it is often attributable to inhalation pneumonia. Other signs include fever, sudden drop in milk production and lameness, either constant or shifting. Usual course is one to three days. Milk production may be depressed for that lactation	• Usually based on clinical signs • Haematology: neutrophilia is a constant finding • RT-PCR • Serology: VN for rising Ab titre • Virus isolation: TC or mouse inoculation
GLOSSOPLEGIA **BOTULISM** **LISTERIOSIS** **ACTINOBACILLOSIS**	 *Clostridium botulinum* *Listeria monocytogenes* *Actinobacillus lignieresii*	Partial or complete loss of function of the tongue may be peripheral or central in origin. The aetiology can be traumatic or infectious. The unilaterally affected tongue is deviated towards the non-affected side. The bilaterally affected tongue is limp and protrudes from relaxed jaws	• Diagnosis of the specific condition and removal of any predisposing causes
EPIZOOTIC HAEMORRHAGIC DISEASE (IBARAKI DISEASE)	Epizootic haemorrhagic disease virus (*Orbivirus*; *Reoviridae*)	Occurs in North America, Far East and southeast Asia, ten serotypes. Acute arthropod-borne disease characterized by fever, ulcerative stomatitis and dysphagia that leads to dehydration and emaciation. Seasonal, in late summer and autumn	• Histopathology • Virus isolation in TC or fertile eggs and identification by VN • RT-PCR
INFECTIOUS BOVINE RHINOTRACHEITIS	Bovine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Systemic disease in neonatal calves with rhinitis, conjunctivitis, erosions of soft palate, bronchopneumonia, often encephalitis and high mortality. In young adults the URT form is characterized by inflamed nares and ulcers in the nasal mucosa. In severe cases lesions occur in pharynx, larynx and trachea	• Clinical signs • ELISA for Ag • FA for Ag on frozen sections • Virus isolation • PCR • Serology: ELISA, VN
MALIGNANT CATARRHAL FEVER	Ovine herpesvirus 2 Alcelaphine herpesvirus 1 (*Macavirus; Herpesviridae*)	Hyperaemia and diffuse, superficial necrosis of oral and nasal mucosa are constant findings in this disease	• Clinical signs • Histopathology • PCR
RABIES (PHARYNGEAL PARALYSIS)	Rabies virus (*Lyssavirus; Rhabdoviridae)*	Pharyngeal paralysis is usually a sign of encephalitis and occurs in cattle with rabies. The animal is unable to swallow, salivation is noticed with gurgling noises from the pharynx	• History of rabies being endemic • Clinical signs • Histopathology of brain: Negri bodies • FA (brain): viral antigen • RT-PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Buccal cavity: cattle

Disease	Agent(s)	Comments	Diagnosis
RINDERPEST	Rinderpest virus (*Morbillivirus*; *Paramyxoviridae*)	Erosive stomatitis and gastroenteritis are characteristic for this disease. There are punched-out lesions on gums, lips, tongue and hard palate. UN declared eradicated in 2011	• History of endemic area and clinical signs • AGID or CFT on lymph node biopsy for viral antigen • Histopathology • RT-PCR • Virus isolation • Serology: CFT, ELISA, VN, HAI
VESICULAR DISEASES **Foot-and-mouth disease (FMD), Vesicular stomatitis (VS)**	Foot-and-mouth disease virus (*Aphthovirus*; *Picornaviridae*) Vesicular stomatitis virus (*Vesiculovirus*; *Rhabdoviridae*)	Vesicular lesions on tongue, buccal mucosa, teats (milking cows) and interdigital cleft. Lesions on feet and teats less constant in VS than in FMD	• ELISA or CFT for Ag in vesicular fluid • Virus isolation • RT-PCR • Serology: ELISA, CFT, VN
Gastrointestinal tract: cattle			
ANTIBIOTIC-INDUCED DIARRHOEA	*Pseudomonas* sp., *Proteus* sp., or *Candida albicans*	Calves treated for diarrhoea with a prolonged course of oral antibiotics. Normal flora destroyed predisposing to a chronic diarrhoea with poor response to treatment and progressive weight loss	• History • Isolation of secondary invaders from faeces in heavy growth
BOVINE VIRAL DIARRHOEA (BVD) AND MUCOSAL DISEASE	Bovine viral diarrhoea virus 1, 2 (*Pestivirus*; *Togaviridae*)	BVD: Young cattle six to 24 months show mild depression, oculonasal discharge and occasionally shallow ulcers in buccal cavity. Diarrhoea occurs in susceptible herds. High morbidity but zero mortality Mucosal disease: Low morbidity/100% mortality. BVD-immunotolerant, 6- to 24-month-old animals at risk (virus +ve/Ab –ve). Severe lameness (laminitis), profuse diarrhoea and buccal cavity lesions extending to intestines occur	• Herd history and clinical signs • Clinical signs • FA on frozen sections or buffy coat • Virus isolation: buffy coat • Viral antigen detection: ELISA, immunohistochemistry ('ear notch') • RT-PCR • Serology: ELISA or VN on paired sera
CLOSTRIDIAL ENTEROTOXAEMIA	*Clostridium perfringens* types B and C	Occurs in young well-nourished calves up to 10 days of age. Severe haemorrhagic enterotoxaemia with rapid death. Uncommon	• Gross pathology and histopathology • Gram-stain on mucosa: large numbers of thick Gram +ve rods • Mouse tests or ELISA for toxin in small intestine
COLIBACILLOSIS AND COLISEPTICAEMIA	*Escherichia coli*	Neonates under seven days of age. Colostral immunity determines survival. Acute profuse diarrhoea, dehydration and acidosis	• Isolation of *E. coli* • Enteropathogenicity tests (see Chapter 17)

Table 69.1 Principal infectious diseases of cattle—cont'd

Gastrointestinal tract: cattle

Disease	Agent(s)	Comments	Diagnosis
CRYPTOSPORIDIOSIS	*Cryptosporidium parvum*	Outbreaks of diarrhoea in 5- to 35-day-old calves. Both affected and clinically normal calves shed large numbers of oocysts in faeces. Villous atrophy and enlargement of crypts occurs	• Safranin-methylene blue stain on faecal smears • Auramine-O technique on faecal smear • PCR
JOHNE'S DISEASE	*Mycobacterium avium* subsp. *paratuberculosis*	Seen in cattle over two years of age, although infection is acquired soon after birth. Not all infected cattle become clinical. Chronic disease with emaciation, profuse diarrhoea and eventual death. Corrugated and reddened ileocaecal valve area is characteristic	• ZN smear of rectal scraping or mucosa of ileocaecal valve area • Culture: Herrold's egg-yolk medium: up to 16 weeks' incubation • Quantitative PCR on faeces • PCR detection of IS900 for identification • Histopathology
MALIGNANT CATARRHAL FEVER (MCF)	Ovine herpesvirus 2 (OHV 2) Alcelaphine herpesvirus 1 (AHV 1, Africa) (*Macavirus; Herpesviridae*)	Low morbidity/high mortality. Corneal opacity, enlarged lymph nodes of head and neck, ragged erosions in buccal cavity. Terminal diarrhoea and encephalitis. Reservoirs are sheep (OHV 2) and wildebeest (AHV 1)	• Histopathology • Virus isolation from buffy coat cells: calf thyroid cell line for wildebeest derived-MCF only • PCR
RINDERPEST	Rinderpest virus (*Morbillivirus*; *Paramyxoviridae*)	High morbidity/high mortality. Very contagious. Profuse diarrhoea, dehydration, weakness, buccal erosions ('punched-out' ulcers) extending into the intestinal tract, 'zebra-striping' of terminal large intestine. UN declared eradicated in 2011	• History and clinical signs • Detection of Ag in tissue: CFT, AGID • Histopathology • RT-PCR • Virus isolation • Serology: VN, ELISA, CFT
ROTAVIRUS AND CORONAVIRUS INFECTIONS	*Rotavirus* (*Reoviridae*) Bovine coronavirus (*Betacoronavirus*; *Coronaviridae*)	Neonates five to 21 days old. Explosive outbreaks of profuse watery diarrhoea. Extensive villous atrophy (most severe with coronavirus). Calves that recover may be unthrifty until villi regenerate	• EM (faeces) • ELISA, latex agglutination for Ag detection • Virus isolation is difficult • RT-PCR
SALMONELLOSIS	*Salmonella* serotypes	Animals of all ages are susceptible. Young calves often develop the septicaemic form of disease. May be stress-induced. Acute diarrhoea/dysentery and fever are present. Deaths can occur in young animals	• Culture for salmonellae • PCR
WINTER DYSENTERY ('BLACK SCORUS')	Bovine coronavirus (*Betacoronavirus*; *Coronaviridae*)	Once thought to be due to *Campylobacter jejuni*. Explosive outbreaks of diarrhoea/dysentery in mature housed cattle. Outbreak lasts 24 hours, is usually non-febrile and there are dark watery faeces with a fetid odour. High morbidity/low mortality	• Virus isolation is difficult • Virus detection in faeces: EM, passive haemagglutination, ELISA • RT-PCR • Serology: VN, HAI

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Liver: cattle

Disease	Agent(s)	Comments	Diagnosis
BACILLARY HAEMOGLOBINURIA	*Clostridium haemolyticum*	Ingested spores lodge in the liver. Migrating liver fluke cause tissue damage and provide conditions suitable for germination of spores. Sudden death or fever, abdominal pain, 'port-wine' urine and infarcts in liver. The infarcts are pale, raised and surrounded by a bluish-red zone. Disease affects cattle and occasionally sheep	• History of a liver fluke area • Clinical or post mortem findings • FA on smears from liver lesion
BOVINE LIVER ABSCESSES	*Fusobacterium necrophorum* and *Trueperella pyogenes*	Usually no clinical signs but the lesions are discovered at slaughter. Most common in feedlot cattle. Similar lesions have been reported in pigs	• Pathology discovered at slaughter. Most common in feedlot cattle. Similar lesions have been reported in pigs • Direct microscopy: Gram-stained smear • Culture of pathogens
FACIAL ECZEMA (MYCOTOXICOSIS)	Sporidesmin in spores of *Pithomyces chartarum* (mycotoxin)	Bile duct obstruction, liver fibrosis, jaundice and failure to excrete phylloerythrin results in photosensitization seen on unpigmented or bare skin	• Clinical signs • Spore count on pastures • Gross and histopathology of livers
LISTERIOSIS (SEPTICAEMIC/ VISCERAL)	*Listeria monocytogenes*	Occurs in many species of young animals and in birds. There is fever, anorexia, depression and death in one to three days. Necrotic foci are seen throughout the liver and other body organs at post mortem examination	• Direct Gram-stained smears from lesions • Culture of pathogen • PCR
RIFT VALLEY FEVER	Rift Valley fever virus (*Phlebovirus*; *Bunyaviridae*)	Hepatitis and high mortality occur in lambs, kids and calves; severe disease and abortions in adult sheep and goats, but only mild or subclinical infections in cattle with a high percentage of abortions. Transmitted by mosquitoes	• Clinical signs • Gross and histopathology • Antigen detection: FA, ELISA • Virus isolation: TC or i/p mouse inoculation • RT-PCR • Serology: VN, CFT, ELISA, or HAI
Genital system: cattle			
AKABANE DISEASE	Akabane virus (*Orthobunyavirus*; *Bunyaviridae*)	Japan, Australia, South Africa and Israel. Cattle, sheep and goats. Solid immunity after infection. Transmitted by mosquitoes • Severe damage to foetus with death and abortion • Congenital abnormalities: hydranencephaly and arthrogryposis	• Pathology • Virus isolation: suckling mice or TC • Serology: VN • RT-PCR

Table 69.1 Principal infectious diseases of cattle—cont'd

Genital system: cattle

Disease	Agent(s)	Comments	Diagnosis
ANAPLASMOSIS (GALL SICKNESS)	*Anaplasma marginale*	Arthropod-transmitted disease of ruminants in tropics and subtropics. Clinical disease seen in introduced, non-immune, adult cattle: fever, anaemia, icterus (but not haemoglobinuria), weakness and abortion in pregnant animals. Death, recovery or chronic disease with emaciation may ensue	• History of endemic area • Giemsa-stained blood smears for rickettsiae • Serological tests for carriers and chronic cases • PCR
BLUETONGUE	Bluetongue virus (*Orbivirus*; *Reoviridae*)	Sheep, deer and cattle are affected but only about 5% of infected cattle show clinical signs. If cattle are infected during gestation: • Abortion • Congenital abnormalities: cerebellar hypoplasia, arthrogryposis or hydranencephaly Vector: *Culicoides* spp.	• Isolation: i/v inoculation of 10–12-day embryonated eggs • RT-PCR • Serology: ELISA, VN, AGID
BOVINE CONGENITAL CAMPYLOBACTERIOSIS (VIBRIOSIS)	*Campylobacter foetus* ss. *venerealis.* (*C. foetus* ss. *foetus*)	Venereal infection, bulls are carriers. Non-immune cows suffer mild metritis, salpingitis and embryonic death with irregular cycles at 28–35 days. Self-limiting disease and natural immunity in three to five months with destruction of *Campylobacter*. Occasional carrier cow (vagina) Sporadic abortions with *C. foetus* ss. *foetus*	• Isolation of pathogen from abomasal contents of foetus • Direct microscopy using DCF or FA • PCR
BOVINE VIRAL DIARRHOEA (BVD)	Bovine viral diarrhoea virus 1, 2 (*Pestivirus*; *Togaviridae*)	Syndromes include: • Neonatal calves: immunosuppression • Young cattle six to 24 months: diarrhoea and erosions of buccal mucosa • Adult pregnant cows, depending on stage of gestation when infected: 1. 50–100 days: foetal death and abortion or mummification 2. 100–150 days: congenital defects in foetus 3. Before 120 days of pregnancy (with non-CPE strain): immunotolerance (virus +ve/Ab –ve) and calf is at risk from mucosal disease when six to 24 months old 4. Virus in semen: fertilization failure and a 'repeat breeder' problem	• Clinical signs • FA on frozen sections or buffy coat • Virus isolation: buffy coat • Viral antigen detection: ELISA, immunohistochemistry ('ear notch') • RT-PCR • Serology: ELISA or VN on paired sera

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Genital system: cattle

Disease	Agent(s)	Comments	Diagnosis
BRUCELLOSIS	*Brucella abortus* (*B. melitensis* and *B. suis*)	Abortion storms occur in non-immune herds. A cow usually only aborts once but remains infected and excretes brucellae at subsequent parturitions. Organisms can be excreted in milk. Infection is usually by oral route. Notifiable disease in many countries	• Serology: many tests used on a herd basis as part of a national eradication scheme • Isolation of brucellae • Direct microscopy: MZN-stained smears • PCR-based methods for detection and identification
CHLAMYDIAL ABORTION	*Chlamydophila abortus*	Similar to enzootic abortion of ewes	• Isolation • Impression smears from cotyledons • Serology: CFT, IFA, ELISA • PCR
EPIZOOTIC BOVINE ABORTION (FOOTHILL ABORTION)	*Borrelia coriaceae*	Abortion or full-term weak calves. Vector is *Ornithodoros* tick. No signs of illness in cows but abortions in 10–90% of susceptible cattle in third trimester. Incubation period 90–150 days. Cows usually abort only once and are normal at next pregnancy. Occurs in Western USA	• Gross and histopathology • Microscopy: darkfield (foetal blood) • PCR
INFECTIOUS BOVINE RHINOTRACHEITIS/ INFECTIOUS PUSTULAR VULVOVAGINITIS (IBR/IPV)	Bovine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	IBR: Incubation period two to six days. Syndromes include: • Young adults: respiratory disease ('red nose') • Abortions up to 90 days after infection with or without previous respiratory signs. Abortion most common between four to seven months of pregnancy. Infertility is not a sequel of IBR. No gross lesions in foetus but microscopic foci of necrosis in many organs with IN inclusions • Neonates: diarrhoea and/or encephalitis IPV: venereal infection, localized in genitalia of both sexes. No viraemia occurs so abortion is not seen. Self-limiting infection. Many subclinical cases occur	• Histopathology (foetal tissues) • Virus isolation: vaginal and preputial swabs • FA: foetal tissues • PCR • Serology: VN or ELISA
LEPTOSPIROSIS	*Leptospira interrogans* serovars	Abortion storms common with some serovars but are sporadic with others. Abortion occurs six to 12 weeks after infection and is most common in seventh month of pregnancy. Other signs include infertility, weak calves and an agalactia syndrome. Carrier state common with leptospires excreted in urine	• FAT on foetal tissues • PCR Herd tests: • Darkfield or FA microscopy on urine • Culture (difficult)/PCR on urine or kidneys • Serology: MAT, CFT, ELISA

Table 69.1 Principal infectious diseases of cattle—cont'd

Genital system: cattle

Disease	Agent(s)	Comments	Diagnosis
LISTERIOSIS	*Listeria monocytogenes* (*L. ivanovii*: abortion in cattle and sheep only)	Syndromes include: • Visceral or septicaemic listeriosis in many young animals and birds • Neural listeriosis (circling disease) in cattle, sheep and goats • Abortion in cattle, sheep and goats. Usually sporadic and in late gestation. No systemic illness in dam and no infertility. Listeriae are shed in milk and uterine discharges for some months after infection. Minor outbreaks occur associated with silage feeding to pregnant animals • Ocular form: a self-limiting iritis often with corneal opacity. Associated with silage feeding Circling disease and abortions do not usually occur together on same property. Focal necrosis of foetal liver occurs but may be masked by autolysis	• Isolation and identification of pathogen • PCR • Histopathology
MYCOTIC ABORTION	*Aspergillus fumigatus* or *Mortierella wolfii*	Abortions sporadic and usually between six to nine months of pregnancy. Characteristic findings: • 'Wooden' appearance of placenta as some maternal caruncles detach and adhere to cotyledons • Ringworm-like lesions on foetus: pathognomonic when present • Placenta often retained *M. wolfii* abortions (in 5% of cases) can be followed within 48 hours by peracute pneumonia and death of cow	• Histopathology on cotyledons or foetal lesions • Isolation of fungal pathogen
RIFT VALLEY FEVER	Rift Valley fever virus (*Phlebovirus*; *Bunyaviridae*)	Epidemics in cattle, sheep and goats in South and East Africa. • Hepatitis and high mortality in young animals • Severe disease and 90–100% abortions in sheep and goats • Mild disease in cattle but 100% abortion rate • Vector: mosquitoes. Reservoir: wild ruminants • Influenza-like disease in man	• Histopathology: liver necrosis. • Virus isolation: lab. animal inoculation or TC • RT-PCR • Serology: VN, CFT, ELISA, HAI
SALMONELLOSIS	*Salmonella* serotypes (especially *S.* Dublin)	Sporadic abortions and the cow may or may not show systemic illness. Many abortions may be seen in a herd where an outbreak of enteric disease has occurred. Faecal–oral transmission and carrier state is common. Stress can convert a carrier state to a clinical case	• Isolation of salmonellae from placenta or foetus • PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Genital system: cattle

Disease	Agent(s)	Comments	Diagnosis
TICK-BORNE FEVER	*Anaplasma phagocytophilum*	Seen in Western Europe and Finland. Relatively mild enzootic disease with dullness, fever, and immunosuppression. Abortions and stillbirths in cattle and sheep. Rickettsial organism has predilection for neutrophils Vector: *Ixodes ricinus*	• Direct microscopy. Giemsa-stained blood smear: purple bodies 0.3–0.7 μm • PCR
TRICHOMONIASIS	*Tritrichomonas foetus*	Early embryonic death (infertility) and occasionally abortions or pyometra. Venereal infection: bull carrier in prepuce and can infect 90% of cows served. Foetus dies 50–100 days after conception and irregular oestrous cycles follow. Uterine discharge often purulent and contains large numbers of protozoan parasites (maximum numbers three to seven days before oestrus)	• Direct microscopic examination of uterine discharges. Specimens should be kept warm or protozoan parasite will become non-motile • Culture • PCR

Urinary system: cattle

ABSCESSES IN KIDNEYS	Pyogenic bacteria such as: streptococci, *Staphylococcus aureus, Trueperella pyogenes*	If a bacteraemia occurs, these pyogenic bacteria can lodge and multiply in the kidneys and in other body organs	• Isolation of the pathogenic bacteria • Histopathology
BACILLARY HAEMOGLOBINURIA	*Clostridium haemolyticum*	Associated with liver damage due to migrating liver fluke. The urine is 'port-wine'-coloured and foams when voided. Sudden deaths are common. On post mortem, the liver infarct is pathognomonic: raised, light in colour and outlined by a bluish-red zone of congestion	• History: liver fluke area • Clinical signs • Pathology • FA on smears from liver lesions • Isolation of pathogen • PCR • Demonstrate toxin in peritoneal cavity fluid using ELISA/mouse bioassay
ENZOOTIC HAEMATURIA	Bovine papillomavirus 1 (*Deltapapillomavirus; Papillomaviridae*)	Associated with infection in bladder, exposure to bracken fern and progression to neoplasia in bladder wall	• History: bracken fern ingestion • Clinical signs: blood in urine • Pathology
ENZOOTIC BOVINE LEUKOSIS	Bovine leukaemia virus (*Deltaretrovirus*; *Retroviridae*)	Tumours (B lymphocyte infiltration) can occur in the kidneys and in other body organs	• History of the disease in the herd • Clinical signs • Histopathology (dead animals or biopsy) • Serology: AGID or ELISA (herd test)

Table 69.1 Principal infectious diseases of cattle—cont'd

Urinary system: cattle

Disease	Agent(s)	Comments	Diagnosis
LEPTOSPIROSIS	*Leptospira interrogans* serovar Pomona and other serovars	Some serovars such as Pomona, produce a haemolysin resulting in haemoglobinuria in calves and occasionally older animals. There is usually accompanying fever, icterus and anorexia	• Clinical signs • Urine for dark-field microscopy • FA technique on urinary deposits • PCR • Isolation of leptospires (difficult)
PYELONEPHRITIS (BOVINE)	*Corynebacterium renale* group (streptococci and other bacteria often present)	Disease of mature cows. Often precipitated by pregnancy and dystocia. Urine cloudy with blood clots in advanced cases. There is frequent micturition, uneasiness, hunched back and enlarged kidney may be felt on palpation	• History and clinical signs • Isolation of a member of the *C. renale* group
'WHITE SPOTTED KIDNEY' IN CALVES	*Escherichia coli, Leptospira interrogans* serovars or other bacteria	Focal interstitial nephritis following a bacteraemia or septicaemia. Often only detected at slaughter	• Isolation of the pathogens may be difficult at this stage in the disease • PCR • Fixed tissue for histopathology may assist in the diagnosis
Eyes and ears: cattle			
INFECTIOUS BOVINE KERATOCONJUNCTIVITIS (IBK)	*Moraxella bovis*	Predisposing causes are irritants, flies and sunlight. Acute disease with highest incidence in animals under two years of age. Initial signs are photophobia, blepharospasm, lacrimation and conjunctivitis. Ulcers, corneal oedema and opacity occur with vascularization in severe cases. Healing stage involves granulation tissue projecting from the ulcer as a characteristic 'red-cone'. Condition resolves completely or leaves a white corneal scar	• History of outbreak • Clinical signs • Isolation of *M. bovis* from lacrimal secretions (within two hours of collection) • PCR
INFECTIOUS BOVINE RHINOTRACHEITIS	Bovine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Conjunctivitis occurs as part of the acute syndrome and in mild cases may be the only sign present. In acute and severe disease, signs include fever, depression, nasal discharge and inflamed nares ('red nose'). Ulcers develop in the nasal mucosa. There is dyspnoea, mouth breathing and excessive salivation	• Clinical signs • FA: frozen sections • Virus isolation • PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Eyes and ears: cattle

Disease	Agent(s)	Comments	Diagnosis
LISTERIOSIS (CORNEAL OPACITY)	*Listeria monocytogenes*	Corneal opacity is often unilateral when it occurs in neural listeriosis. Listerial iritis can be associated with feeding of big bale silage. The condition may progress to corneal opacity and blindness. Systemic signs are usually absent	• History of silage feeding • Clinical signs • Attempted isolation of listeriae from eyes in ocular form
MALIGNANT CATARRHAL FEVER (CORNEAL OPACITY)	Ovine herpesvirus 2 Alcelaphine herpesvirus 1 (*Macavirus; Herpesviridae*)	Bilateral corneal opacity is a constant finding in this sporadic but usually fatal disease	• Clinical course of disease • Histopathology • PCR
MYCOPLASMAL CONJUNCTIVITIS	*Mycoplasma bovoculi*	Causes conjunctivitis and transient corneal opacity. A concurrent infection with *Moraxella bovis* may increase the severity of IBK	• Isolation of *M. bovoculi* • PCR
Nervous system: cattle			
ABSCESSES OR GRANULOMAS (SPINAL OR BRAIN)	*Trueperella pyogenes*, *Fusobacterium necrophorum*, *Actinomyces bovis*, or *Mycobacterium bovis*	Usually occurs in young animals. May arise from direct trauma such as dehorning or as an extension of otitis media, paranasal infections or lesions of the meninges. There is rotation or deviation of neck, ataxia, circling, blindness or nystagmus in one eye	• Gross pathology • Histopathology • CSF: high neutrophil count • Isolation of pathogen from CSF or lesion • PCR
BOTULISM	*Clostridium botulinum*	History of cattle eating toxin-containing foods (baled silage, processed poultry litter or carrion), but wound infections (toxico-infections) can occur. Toxin causes progressive muscular paralysis with dysphagia, recumbency and respiratory failure. There is no fever. Mild cases may recover	• Type of foodstuffs • Clinical signs • Demonstration of toxin in serum: mouse inoculation or ELISA
ENZOOTIC BOVINE LEUKOSIS	Bovine leukaemia virus (*Deltaretrovirus*, *Retroviridae*)	Tumours can occur anywhere in the body including the brain or spinal canal. Clinical signs will depend on the site of the tumour	• Serology: AGID or ELISA on a herd basis • Histopathology
BOVINE SPONGIFORM ENCEPHALOPATHY (BSE)	Prion	Highest incidence in adults three to six years old. Long incubation period two to eight years. Onset is insidious. There is apprehension, low head carriage, irritability and excessive ear movement. Later ataxia, falling and recumbency occur. Occasionally there is aggression towards other animals. Course usually one to three months	• Clinical signs • Histopathology: characteristic changes, immunohistochemistry for confirmation • EM: scrapie-associated fibrils in brain tissue • PrP^{Sc} detection: ELISA, immunoblotting
CONGENITAL CNS LESIONS	Akabane virus (*Bunyaviridae*) Bovine viral diarrhoea virus (*Flaviviridae*)	Hydranencephaly and/or arthrogryposis Hydranencephaly, hydrocephalus, cerebellar hypoplasia, cataract or arthrogryposis	• Serology: test dam for high antibody titres to the appropriate virus • Virus isolation • RT-PCR

Table 69.1 Principal infectious diseases of cattle—cont'd

Nervous system: cattle

Disease	Agent(s)	Comments	Diagnosis
HEARTWATER	*Ehrlichia ruminantium* Vector: *Amblyomma* ticks	Endemic in southern Africa, Madagascar and some West Indian islands. Clinical signs occur in non-immune animals: lip-licking, high-stepping, recumbency and death during a galloping convulsion. Course of the disease is three to six days	• History of endemic area • Giemsa-stained smears of cerebral grey matter to visualize rickettsiae • PCR
INFECTIOUS BOVINE RHINOTRACHEITIS (IBR)	Bovine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Infection in neonatal calves can cause conjunctivitis, pneumonia and nervous signs such as excitement, tremor, ataxia and recumbency. A severe non-suppurative meningoencephalitis/myelitis with marked vascular cuffing and gliosis is present	• Clinical signs • Histopathology: tissue damage and IBs • Virus isolation • FA: frozen sections • PCR
VIRAL ENCEPHALITIS	Bovine herpesvirus 5 (*Varicellovirus; Herpesviridae*)	Sporadic cases of encephalitis described in calves in several countries	• Histopathology • Virus isolation • FA: frozen sections • PCR
LISTERIOSIS	*Listeria monocytogenes*	Neural form: occurs in all ages but most common in adults. Signs include drooling, facial hypalgesia, head tilt and unilateral drooping ear. Loss of blink reflex can lead to keratitis and corneal ulceration. Ataxia, circling and occasionally mania and bellowing occur. Course is less than 14 days. Other syndromes are abortion, septicaemic (visceral) form in young animals and ocular form	• History and clinical signs • Histopathology: microabscesses and perivascular cuffing in brain • Isolation of listeriae from brain (cold enrichment) • PCR
LOUPING ILL	Louping ill virus (*Flavivirus*; *Flaviviridae*)	The disease in cattle is usually mild and seen mainly in calves, as adults acquire an immunity in endemic areas. Signs include excitement, tremors, incoordination and ataxia. A non-suppurative meningoencephalitis is present mainly affecting the lower brain stem and cerebellum. Vector: *Ixodes ricinus*	• History of endemic area with vector present • Clinical signs • Histopathology • Virus isolation: TC or i/c inoculation of mice • RT-PCR • Serology: HAI, AGID, CFT, VN, IFA
MALIGNANT CATARRHAL FEVER	Ovine herpesvirus 2 or Alcelaphine herpesvirus 1 (Africa) (*Macavirus; Herpesviridae*)	Sporadic disease, usually in adults. Reservoir is sheep (OHV 2) or wildebeests (AHV 1). Generalized disease with fever, encrusted muzzle, diffuse erosions in buccal cavity, corneal opacity, cervical lymphadenopathy, deep depression, incoordination, head-pressing and eventually paralysis and death. Mortality 100%. A non-suppurative encephalomyelitis is present	• History of contact with sheep or wildebeests • Clinical signs: sporadic cases and usually fatal • Histopathology • Virus isolation: AHV 1 but not OHV 2 from buffy coat • PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Nervous system: cattle

Disease	Agent(s)	Comments	Diagnosis
MENINGITIS (BACTERIAL)	*Staphylococcus aureus, Histophilus somni, Escherichia coli*, streptococci or others	Seen in young calves and condition often results from a bacteraemia following infection of the umbilicus. There is fever, neck rigidity, opisthotonos, nystagmus, extensor spasms, clonus and coma. Thickening of meninges and congestion of vessels occurs	• Histopathology • Isolation of pathogen from CSF or meninges
POLIO-ENCEPHALOMALACIA (CEREBROCORTICAL NECROSIS)	Attributed to thiaminases produced by bacteria in the rumen	Thiamine deficiency. Animals are afebrile, have decreased mobility, blindness with active pupillary and corneal reflexes, recumbency with extensor spasms and 'paddling' can occur. Rapid response to thiamine early in condition	• Clinical signs • Swift response to thiamine • Histopathology: focal necrosis of cortex
PSEUDORABIES	Porcine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	History of close contact with pigs or rats. Signs include intense focal pruritis often in flank area, dog-sitting position, bellowing, teeth grinding, salivation, pharyngeal paralysis but no aggression is seen. Death from respiratory or cardiac failure	• Virus isolation • FA on cryostats of brain tissue • History: association with pigs
RABIES	Rabies virus (*Lyssavirus*; *Rhabdoviridae*)	The dumb form is most common in cattle with salivation, tenesmus, constipation, ataxia and paralysis. Rarely the furious form is seen with bellowing, mania and aggression. A non-suppurative encephalitis is present with Negri bodies in neurons of cerebellum and hippocampus	• History of dog or fox bites (vampire bats in South America) • Clinical signs • FA on brain • Histopathology of brain for Negri bodies • RT-PCR
SPORADIC BOVINE ENCEPHALOMYELITIS (BUSS DISEASE)	*Chlamydophila (Chlamydia) pecorum*	Described in USA, Europe, Japan, Australia and South Africa. Incubation period six to 31 days. Chlamydiae excreted in faeces and urine. Depression, fever, salivation and dyspnoea. Recovery can occur at this stage but CNS signs usually develop: stiff, staggering gait, circling and falling over small obstacles. Limbs become weaker and general paralysis follows. Low morbidity but mortality >50%. All ages affected but most common in young animals. In chronic cases, serofibrinous exudates occur in body cavities. Course of disease usually 10–14 days	• Pathology: peritonitis • Isolation: yolk sac or TC • PCR • Serology: group antigen. Rising titre, CFT or ELISA
TETANUS	*Clostridium tetani*	Spores enter via traumatized tissue following dystocia, wounds, injections and umbilicus. Signs include muscle stiffness ('saw horse' stance), tremors, trismus, hyperaesthesia, raised tail-head, bloat, tetanic convulsions, opisthotonos and death from respiratory paralysis	• History: predisposing cause • Clinical signs • Gram-stained smear from deep wound if present

Table 69.1 Principal infectious diseases of cattle—cont'd

Disease	Agent(s)	Comments	Diagnosis
Nervous system: cattle			
THROMBOEMBOLIC MENINGOENCEPHALITIS (TEME)	*Histophilus somni*	Septicaemic form of infection ('sleeper syndrome') is seen most often in young feedlot cattle in autumn and winter. There is fever, stiffness, extension of head, lingual paralysis, ataxia, stupor, opisthotonos, occasional circling and blindness. Haemorrhagic infarcts occur in brain and retina	• Clinical signs • Gross pathology of brain • Histopathology • Isolation of *H. somni* • PCR
TREMORGEN STAGGERS	Heterogeneous group of mycotoxins produced by *Penicillium* spp., *Aspergillus* spp., *Neotyphodium lolii, Claviceps paspali*	Often seen in calves. There is stiffness, ataxia, trembling in large muscle masses, falling and convulsions if hurried. Animals should be moved slowly and gently from suspect pasture	• Clinical signs • Recovery on removal from suspect pasture
VERTEBRAL OSTEOMYELITIS	*Salmonella* serotypes, *Actinomyces bovis*	Signs dependent on site of lesion. Ataxia, hemiplegia or paraplegia can occur due to either pressure on spinal cord or extension to and inflammation of the spinal meninges	• Gross pathology • Isolation of pathogen
Musculoskeletal system: cattle			
BLACKLEG	*Clostridium chauvoei*	Sudden death usually occurs, especially if heart muscle is involved. Muscle masses of hind quarters commonly affected and age range is three to 24 months. The muscles are dry, dark, spongy with small gas bubbles and have a sweet, rancid odour. Crepitation can be felt. Usually an endogenous infection in cattle	• History: endemic area • Clinical signs • FA on muscle or bone marrow from a rib • Isolation of pathogen, if necessary • PCR
BOTULISM	*Clostridium botulinum*	Usually an intoxication from toxin-containing baled silage, processed poultry litter or carrion eating. Less commonly wounds are contaminated by spores and toxico-infectious botulism occurs. Signs include progressive weakness, tongue paralysis, inability to swallow, flaccid paralysis and death from cardiac or respiratory paralysis	• History of foodstuffs • Clinical signs • Demonstration of toxin in serum by mouse inoculation
CHLAMYDIAL POLYARTHRITIS	*Chlamydophila (Chlamydia) pecorum*	Can involve all ages but calves four to 30 days of age are most severely affected. Lameness is pronounced but calves remain alert and will suck if aided. Limb joints are swollen and painful. There is no navel involvement	• Clinical signs • Cytological examination of joint fluid for elementary bodies or inclusions • Isolation of pathogen • PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Musculoskeletal system: cattle

Disease	Agent(s)	Comments	Diagnosis
CONGENITAL DISEASES **BLUETONGUE, AKABANE, BOVINE VIRAL DIARRHOEA**	*Orbivirus, Orthobunyavirus, Pestivirus*	Congenital abnormalities can occur if each of these viruses infects cows at a critical point in gestation. In all three infections, common abnormalities in the calves include arthrogryposis and hydrocephaly	• Serology: detection of antibodies to each of these viruses in the dam • Virus isolation
EPHEMERAL FEVER	Bovine ephemeral fever virus (*Ephemerovirus, Rhabdoviridae*)	Characterized by a sudden onset, biphasic fever, drop in milk production, depression, muscle stiffness and lameness. Usually recovery is dramatic and complete in three days ('three-day-sickness'). Vectors are mosquitoes and *Culicoides* spp.	• Clinical signs in an endemic region • Haematology: neutrophilia • Serology: VN (specific) or IFA • Virus isolation: TC or mouse inoculation • RT-PCR
ERGOTISM	*Claviceps purpurea* (mycotoxins: Ergotamine, ergometrine, ergocristine)	Lameness is the first sign and occurs two to six weeks after ingestion of ergot alkaloids. All ages can be affected. There is tenderness and swelling in the fetlock and pastern joints, followed in about one week by loss of sensation and dry gangrene of the distal part of the limb	• History of pasture or foodstuffs contaminated by ergots • Clinical signs
FOOTROT (INTERDIGITAL NECROBACILLOSIS, FOUL-OF-THE-FOOT)	*Fusobacterium necrophorum, Porphyromonas levii, Prevotella melaninogenica* and *Trueperella pyogenes*	Infection is usually confined to one foot. There is acute lameness. Necrosis and foul-smelling exudate in the interdigital space. Infection can spread to involve joints if not treated	• Clinical signs • Gram-stained smear from pus • Isolation of pathogens if necessary
JOINT-ILL	*Escherichia coli, Staphylococcus aureus*, streptococci or others	Lodgement of pathogenic bacteria in joints following an omphalitis or a septicaemia. In the acute phase there is fever, severe lameness and swollen joints	• Clinical signs • Isolation of pathogen from aspirated joint fluid
LAMENESS (GENERALIZED DISEASES) **FOOT-AND-MOUTH DISEASE** **VESICULAR STOMATITIS** **MUCOSAL DISEASE**	*Aphthovirus* *Vesiculovirus* *Pestivirus*	In foot-and-mouth disease and vesicular stomatitis, lameness is due to interdigital vesicular lesions, and all four feet are usually affected. Lameness occurs in some animals with mucosal disease and is due to laminitis, coronitis or erosive lesions of skin in the interdigital cleft. All four feet are usually affected	• Tests for detection of viral Ag, virus isolation, RT-PCR and demonstration of antibodies to each virus
MALIGNANT OEDEMA	*Clostridium septicum, C. sordellii* or *C. novyi* type A	Animals of all ages affected. If alive, there is fever and soft swelling around a wound that spreads to muscle masses. The swelling pits on pressure and if muscle is incised the tissue is dark. Exudate and gas are present	• Clinical signs • FA in muscle or bone marrow from a rib • Isolation if necessary • PCR

Table 69.1 Principal infectious diseases of cattle—cont'd

Musculoskeletal system: cattle

Disease	Agent(s)	Comments	Diagnosis
MYCOPLASMAL ARTHRITIS	*Mycoplasma bovis*	Recognized most frequently in feed-lot cattle, six to eight months of age. There is moderate fever, stiffness, lameness and progressive weight loss. Swelling of joints and distension of tendon sheaths occurs associated with fibrinous synovitis and synovial fluid effusions	• Clinical signs • Isolation of *M. bovis* from joint fluid (transport medium is required) • PCR
OSTEOMYELITIS	*Salmonella* serotypes; *Brucella abortus; Actinomyces bovis*	Vertebrae are often affected leading to pressure on the spinal cord or extension of infection to meninges. There is often ataxia and eventually hemiplegia or paraplegia depending on the site of the lesion. Lumpy jaw *(A. bovis)* is a specific disease affecting bone and soft tissue in the jaw region	• Clinical signs and radiographic examination • Pathology • Isolation of pathogen from CSF or lesion
TERMINAL DRY GANGRENE	*Salmonella* Dublin	Ischaemic necrosis of tips of ears, tail and distal part of hind limbs can follow a few weeks after recovery from acute diarrhoeal disease. The condition characteristically occurs in young calves and is thought to be a localized form of disseminated intravascular coagulation	• History of previous illness • Differentiate from ergotism
TETANUS	*Clostridium tetani*	Tetanus may occur in cows following dystocia, calves can be infected via the umbilicus, castration or dehorning wounds, and occasionally all ages via deep wounds or injections. There is muscle stiffness, raised tail-head, bloat, tetanic spasms and opisthotonos	• History and clinical signs • Gram-stained smear of necrotic tissue deep in wound
THROMBOEMBOLIC MENINGOENCEPHAL-ITIS	*Histophlus somni*	Bacterial colonization of the meningeal vessels produces a thrombotic vasculitis leading to encephalitis and meningitis. There is fever, and with CNS involvement, motor and behavioural abnormalities develop such as stiffness, ataxia, stupor and opisthotonos	• Clinical signs • Pathology • Isolation of *H. somnus* from brain lesions
Respiratory system: cattle			
CALF DIPHTHERIA	Trauma (coarse feed) and *Fusobacterium necrophorum*	Necrotic lesions in oral, pharyngeal or laryngeal mucosa. Fever, anorexia and salivation are seen and the condition can lead to pneumonia	• Gram-stained smear on scrapings from lesions • Isolation (anaerobic)
CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP)	*Mycoplasma mycoides* ss. *mycoides* (small colony type)	**Acute**: 'Marbling' of lungs and a large volume of fluid in thorax **Chronic**: Necrosis and walling-off of portion of lungs. These animals can be latent carriers or 'lungers' for up to three years. In time the lesion may break down and mycoplasmas are shed	• Gross pathology and histopathology • Isolation from lung or pleural fluid • Serology: ELISA, CFT and AGID • PCR

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Respiratory system: cattle

Disease	Agent(s)	Comments	Diagnosis
ENZOOTIC PNEUMONIA	Complex involves some or all of the following pathogens: Bovine parainfluenza 3 (P13) virus; bovine respiratory syncytial virus; bovine viral diarrhoea (BVD) virus; infectious bovine rhinotracheitis (IBR); *Mycoplasma bovis; M. dispar; Mannheimia haemolytica; Pasteurella multocida* and *Histophilus somni*	Predisposing causes are important in this disease complex and morbidity can reach 100%. Mainly a problem of intensively reared calves two to six months of age. There is fever, depression, increased respiratory rate and coughing. Gradual recovery unless a severe bacterial pneumonia develops. Lesions usually in anteroventral portion of lungs	• Isolation and identification of pathogen(s). Pasteurellae are isolated in later stages, by then the isolation of viruses and mycoplasmas is difficult • FA: for mycoplasmas, viruses • PCR • Serology: for viruses
'FARMER'S LUNG' IN CATTLE	Sensitization to spores of thermophilic actinomycetes such as: *Saccharopolyspora rectivirgula*	Immediate type hypersensitivity. Condition due to mouldy hay fed in an enclosed area. Clinical signs of respiratory distress are seen at the end of winter. A few acute cases occur but the condition is usually chronic	• Usually only 1 or 2 acute cases which occur in cattle over five years old • Serology: AGID antibodies to *S. faeni*
HAEMORRHAGIC SEPTICAEMIA	*Pasteurella multocida* serotype B:2 (Asia) or E:2 (Africa)	An acute pasteurellosis characterized by a rapid course, oedematous swelling in the head-throat-brisket area, respiratory distress, swollen and haemorrhagic lymph nodes and subserous petechiation. Cattle and water buffaloes are susceptible	• History of endemic area • Isolation of pathogen from heart-blood, liver, spleen or lymph nodes • Serotype identification • PCR
INFECTIOUS BOVINE RHINOTRACHEITIS (IBR)	Bovine herpes virus 1 (*Varicellovirus; Herpesviridae*)	Most common in young cattle under stress such as in feedlots. Nasal discharge, 'red nose', ulcers of mucous membranes in nasal passages, conjunctivitis, mouth breathing. Recovery four to five days without secondary bacterial invasion. Abortions three to four weeks after respiratory disease in pregnant adults	• Virus isolation or FA: nasal and eye swabs or nasopharyngeal aspirate early in disease • FA: frozen sections from aborted fetuses • PCR • Histopathology (IBs transitory, occasionally seen) • Serology: VN or ELISA
INHALATION PNEUMONIA	Anaerobes from rumen	Following milk fever or general anaesthesia	• History • PM findings

Table 69.1 Principal infectious diseases of cattle—cont'd

Respiratory system: cattle

Disease	Agent(s)	Comments	Diagnosis
LUNG ABSCESSES	*Trueperella pyogenes*	Occurs alone or as a complication of other respiratory diseases, especially in pasteurellosis of young cattle	• Direct microscopy • Isolation and identification
MYCOTIC PNEUMONIA	*Aspergillus fumigatus* or *Mortierella wolfii*	Usually chronic except for peracute and fatal *M. wolfii* pneumonia that occurs within 48 hours of abortion. The lungs are wet, oedematous with a large volume of fluid in thorax	• Histopathology • Isolation and identification of pathogenic fungi
SHIPPING FEVER (BOVINE PNEUMONIC PASTEURELLOSIS)	Bovine parainfluenza virus 3 (PI3), *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*	Aetiology involves stress + virus + bacteria. All ages can be affected and deaths are usually due to an overwhelming *M. haemolytica* infection. The disease varies from mild pneumonia to a fulminating bronchopneumonia	• Isolation and identification of bacteria • PCR • Serology for PI3: IFA, HAI, VN (rising Ab titre)
TUBERCULOSIS (BOVINE)	*Mycobacterium bovis*	Tubercles in lymph nodes, lungs and pleural cavity. 'Open cases' can create an aerosol of *M. bovis*	• Tuberculin test • ZN-smears on tissue • Isolation and identification of organism. Molecular typing methods for identification and epidemiological investigation • IFN-γ assay
Skin: cattle			
ACTINOBACILLOSIS	*Actinobacillus lignieresii*	Classical disease is a granulomatous infection of the tongue: wooden or timber tongue. The pathogen can also cause granulomatous lesions of skin with purulent exudates from fistulae anywhere on body, including the jaw area. Bone not involved and prognosis is usually good	• Microscopy of crushed granules from pus: Gram –ve rods • Culture: aerobic
BACTERIAL ABSCESSES	*Staphylococcus aureus*, streptococci, or *Trueperella pyogenes*	Infection usually via abrasions and other trauma such as injections by a non-aseptic technique	• Gram-stained smear from pus • Culture for pathogen
BOVINE PAPILLOMATOSIS	Bovine papillomaviruses 1, 3, 5 (several genotypes described; *Papillomaviridae*)	Cutaneous warts, teat warts and warts in bladder or intestines. Affects all ages but highest incidence in calves and yearlings. Skin warts range from small nodules to cauliflower-like growths. Most common on head and neck. Self-limiting condition	• Clinical signs • EM • Histopathology

Continued

Table 69.1 Principal infectious diseases of cattle—cont'd

Skin: cattle

Disease	Agent(s)	Comments	Diagnosis
DERMATOPHILOSIS (streptothricosis; mycotic dermatitis)	*Dermatophilus congolensis*	Reservoir often small foci on carrier animal. Condition most common in young animals. Predisposing causes include: wet conditions (lesions along backline), abrasions from vegetation (muzzle, face and limbs) and tick infestation (tick predilection sites). Exudative dermatitis with extensive scab formation occurs and scab comes away with tufts of hair leaving a raw bleeding surface	• Gram or Giemsa-stained smears from scab. Filamentous and branching with zoospores at least two across; 'tram-track' appearance • Culture: 10% CO_2 • PCR
FACIAL ECZEMA	Sporidesmin in spores of *Pithomyces chartarum* (mycotoxin)	Mycotoxicosis of grazing cattle and sheep. Fungus grows in pasture litter under moist, warm conditions. Liver damage and biliary obstruction occurs that restricts excretion of bile pigments and jaundice can occur. Failure to excrete phylloerythrin leads to photosensitization with lesions in non-pigmented skin including the udder and ears	• Spore counts on pasture • Gross pathology: liver • Histopathology
LUMPY JAW	*Actinomyces bovis*	Granulomatous lesions in jaw region with abscesses and fistulous tracts exuding pus. Bone is attacked and once rarefying osteitis becomes extensive the prognosis is poor	• Microscopy of crushed sulphur granules from pus: Gram +ve filaments • Culture: anaerobic
LUMPY SKIN DISEASE	*Lumpy skin disease virus* (Neething virus) (*Orthopoxvirus*; *Poxviridae*)	Limited to Africa. Nodules in skin all over body with general lymphadenitis, oedema of the limbs, nasal discharge and internal organs including lungs are involved. There is fever and anorexia. Mortality only 1–2% but animals remain debilitated for long periods. Nodules ulcerate and heal slowly leaving scars and alopecia	• Histopathology on biopsy: IBs • EM: demonstration of poxvirus particles • Isolation: CAM or TC • PCR • Antigen trapping ELISA
PSEUDOCOWPOX (MILKER'S NODULE)	Pseudocowpox virus (*Parapoxvirus; Poxviridae*)	Mild teat infection with characteristic pox lesions, ring- or horse-shoe-shaped scabs	• EM: demonstration of poxvirus particles
PSEUDOLUMPY SKIN DISEASE/BOVINE ULCERATIVE MAMMILLITIS	Bovine herpesvirus 2 (Allerton virus) (*Simplexvirus; Herpesviridae*)	This syndrome seen mainly in Africa but can occur elsewhere. The virus also causes bovine ulcerative mammillitis in Europe and North America. Pseudolumpy skin disease is a comparatively mild disease with fever and skin nodules over the body. The nodules undergo necrosis with central depression but no scar. Mortality very low	• EM • Histopathology on biopsy: IN inclusion bodies • Virus isolation • PCR • Serology: VN, CFT, AGID, IFA

Table 69.1 Principal infectious diseases of cattle—cont'd

Skin: cattle

Disease	Agent(s)	Comments	Diagnosis
PSEUDORABIES ('MAD ITCH')	Porcine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Reservoir is usually pigs with Aujeszky's disease. Rats may take virus from farm to farm. Infection mainly by ingestion and less commonly by inhalation or via wounds (pig bites). Dominant sign is an intense pruritis; mainly flanks and hind limbs. Incessant licking, biting and rubbing so infected areas become abraded. Progressive involvement of CNS with frenzy and bellowing but not aggression. Death may occur within a few hours to a maximum of six days after first signs	• History of association with pigs • Clinical signs • Diagnosis of Aujeszky's disease in pigs • FA: frozen sections • PCR • Virus isolation
RINGWORM	*Trichophyton verrucosum*	Usually seen in calves or yearlings. The lesions are most common on the face, around the eyes and on the neck. They are circular and later develop a grey-white crust. Self-limiting disease	• Microscopy on hairs in 10–20% KOH to visualize arthrospores • Culture at 27°C and 37°C, for six weeks
SKIN TUBERCULOSIS	Acid-fast bacterium (unspecified)	Chronic indurative nodules associated with the presence of acid-fast bacteria. Usually occurs on the lower limbs. The affected animals often react to the tuberculin test	• Clinical signs • ZN-stained smears
VESICULAR DISEASES	Foot-and-mouth-disease virus (*Aphthovirus, Picornaviridae*) Vesicular stomatitis virus (*Vesiculovirus, Rhabdoviridae*)	Vesicular lesions on muzzles, buccal cavity, interdigital cleft and on teats of milking animals	• Viral Ag detection: CFT, ELISA (vesicle fluid or epithelial material) • Virus isolation • RT-PCR • Serology: VN, ELISA, CFT

Table 69.2 Principal infectious diseases of sheep (goats)

Buccal cavity: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
BLUETONGUE	Bluetongue virus (*Orbivirus; Reoviridae*)	The highest losses are in growing lambs. There is depression, fever, oedema of lips, tongue and throat. The buccal mucosa is erythematous or cyanotic and erosions appear on the dental pad, tongue, gums and lips. The muzzle is usually encrusted. Stiffness and lameness occur	• History of endemic area • Virus isolation • Virus detection: RT-PCR, ELISA • Serology: VN, ELISA, AGID, modified CFT
CONTAGIOUS PUSTULAR DERMATITIS (ORF)	Orf virus (*Parapoxvirus; Poxviridae*)	Scab formation is usually restricted to the lips and nostrils but, as there is no colostral immunity, the disease can be severe in young lambs and kids. With inflamed, sensitive lips and sometimes lesions in the buccal cavity, the young animals cannot suck or graze	• Clinical signs • EM for parapoxvirus particles • Histopathology on biopsy. • Virus isolation • PCR
FOOT-AND-MOUTH DISEASE	Foot-and-mouth disease virus (*Aphthovirus; Picornaviridae*)	Lameness, usually in all four feet due to vesicular lesions of the interdigital clefts, is the constant finding in sheep. Occasionally, vesicular lesions occur in the buccal cavity	• Clinical signs • Viral Ag: CFT, ELISA on vesicular fluid • Virus isolation • RT-PCR • Serology: ELISA, CFT, VN
PESTE DES PETITS RUMINANTS	Peste des petits ruminants virus (*Morbillivirus; Paramyxoviridae*)	Highly contagious, systemic disease of sheep and goats (West Africa), although many infections are subclinical. Signs include fever, anorexia, necrotic stomatitis with gingivitis and diarrhoea. Mortality in goats can reach 95%, sheep slightly less susceptible	• Gross and histopathology • ELISA, AGID and CFT for viral Ag detection • Virus isolation • RT-PCR • Serology: VN, HAI, AGID
RINDERPEST	Rinderpest virus (*Morbillivirus; Paramyxoviridae*)	Sheep and goats are susceptible to infection but disease is usually mild. A few large and serious outbreaks have been reported with signs similar to those in cattle: severe diarrhoea and shallow erosions of lips, dental pads and gums. Global eradication scheme	• AGID and CFT for viral Ag • Histopathology • Virus isolation • RT-PCR • Serology: CFT, ELISA, VN, HAI
SHEEPPOX (GOATPOX)	Sheeppox/Goatpox virus (*Capripoxvirus; Poxviridae*)	The disease affects all ages. There is fever, rhinitis, anorexia and generalized pox eruptions on skin and mucosa of buccal cavity and pharynx within one to two days of first signs. Caseous nodules and catarrhal pneumonia occur in the lungs. Mortality varies from 5–50%	• Clinical signs • EM • Viral Ag detection: ELISA, AGID • Histopathology • Virus isolation or PCR • Serology: VN, ELISA
Gastrointestinal tract: sheep (goats)			
COLIBACILLOSIS AND COLISEPTICAEMIA	*Escherichia coli*	Most commonly seen in neonatal lambs in crowded lambing sheds. Predisposing causes: mismothering or other causes of insufficient colostrum. Acute diarrhoea, septicaemia and sudden deaths may occur	• History of predisposing causes • Isolation of *E. coli* • Tests for enterotoxigenicity

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Gastrointestinal tract: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
CRYPTOSPORIDIOSIS	*Cryptosporidium* sp.	Lambs seven to 10 days of age are affected. There is dullness, anorexia and diarrhoea but no fever. Death can occur in two to three days and the survivors may be unthrifty. Not common in lambs	• Safranin-methylene-blue-stained smears of faeces • Auramine-O technique or FA on faecal smears
JOHNE'S DISEASE	*Mycobacterium avium* subspecies *paratuberculosis*	Main sign is emaciation. As diarrhoea may not occur in sheep and goats, the disease is often not diagnosed. Mesenteric lymph nodes are best specimens for culture, rather than ileocaecal valve	• ZN-stained smear of faeces, mucosal scrapings or lymph nodes • Culture from mesenteric lymph nodes • PCR-based methods for detection and typing
LAMB DYSENTERY	*Clostridium perfringens* type B	A clostridial enterotoxaemia with death a few hours after signs of dysentery, abdominal pain and continuous bleating. Seen in newborn lambs up to three weeks of age	• Clinical signs • Gross pathology and histopathology • Mouse neutralization test/ELISA for toxins in small intestine • Anaerobic culture, PCR typing
RINDERPEST and PESTE DES PETITS RUMINANTS	Closely related morbilliviruses (*Paramyxoviridae*)	These viruses have an affinity for lymphoid tissue and epithelium of intestines. There is fever, necrotic stomatitis and gastroenteritis. Infections can be subclinical in cases of peste des petits ruminants, but not in rinderpest	• Pathology • Virus isolation • RT-PCR • Detection of viral antigen in tissues: AGID, ELISA and CFT • Serology: AGID, HAI
ROTAVIRUS INFECTION	*Rotavirus* (*Reoviridae*)	Occurs in neonatal to four-week-old lambs. Intestinal villi are shortened and this affects lactose digestion. Usually recover if no complications such as a concurrent *E. coli* infection	• Electron microscopy on faeces • Latex agglutination, ELISA for Ag capture • Virus isolation and identification
SALMONELLOSIS	*Salmonella* serotypes	The disease can be a problem in young lambs, adult ewes in late pregnancy or as a flock problem after mustering. Acute diarrhoea/dysentery, septicaemia and/or abortion may occur	• Clinical signs • Gross and histopathology • Isolation of salmonellae from faeces or bone marrow • PCR
'WATERY MOUTH' OF LAMBS	Associated with *E. coli*	Occurs in lambs up to three days old and almost always in those born in enclosed lambing pens. The lamb is dull and ceases to suck. There is saliva-drooling, abomasal tympany and a bloated appearance. Death occurs within six to 24 hours. Thought to be due to endotoxaemia	• Clinical signs • History of colostrum deprivation • Isolation of *E. coli* from body organs

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Liver: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
BACILLARY HAEMOGLOBINURIA	*Clostridium haemolyticum*	Less common in sheep than in cattle. Liver infarcts are pale, raised and surrounded by a bluish-red zone	• History of a liver fluke area • PM findings • FA on smears from liver lesion • PCR
FACIAL ECZEMA (MYCOTOXICOSIS)	Sporidesmin in spores of *Pithomyces chartarum*	Mycotoxicosis with bile duct obstruction, liver damage, jaundice and photosensitization (non-excretion of phylloerythrin). Photosensitization seen on non-woolled areas of face and ears. There is erythema and oedema of affected skin. Livers are initially enlarged and icteric and later atrophied and fibrotic	• Spore count on pasture • Gross and histopathological appearance of livers
INFECTIOUS NECROTIC HEPATITIS (BLACK DISEASE)	*Clostridium novyi* type B	Endogenous infection with spores present in liver tissue. Migration of liver fluke larvae produces conditions suitable for spore germination and toxin production. Usually sudden death. Post mortem findings include greyish-yellow foci in liver, excess fluid in body cavities and extensive blood-stained oedema under the skin. The skin appears black (black disease)	• History of a liver fluke area • Clinical or post mortem signs • FA on smears from lesions • PCR • Demonstration of alpha toxin in body cavity fluid or liver lesion
LISTERIOSIS (SEPTICAEMIC/ VISCERAL)	*Listeria monocytogenes*	Occurs in lambs and kids. There is fever, anorexia, depression and death in one to three days. Necrotic foci occur in the liver and other body organs	• Gram-stained smears from lesions • Culture of the pathogen • PCR
OVINE GENITAL CAMPYLOBACTERIOSIS ('VIBRIOSIS')	*Campylobacter fetus* subsp. *fetus* (*C. jejuni*)	Round (1–3 cm), grey necrotic foci in liver of aborted foetus are pathognomonic, if present. Abortion usually occurs in the last eight weeks of pregnancy	• Characteristic lesions in foetus • Direct microscopy on foetal abomasal contents • Isolation from abomasal contents • PCR
RIFT VALLEY FEVER	Rift Valley fever virus (*Phlebovirus; Bunyaviridae*)	South and East Africa: sheep, goats, cattle and man. Syndromes: • Hepatitis and high mortality in lambs, kids and calves • Severe disease and 90–100% abortion in sheep and goats • Mild or subclinical in cows but 100% abortions Vector: mosquitoes	• Histopathology: liver necrosis • Virus isolation: lab. animals or TC • RT-PCR • Serology: VN, CFT, ELSIA, HAI

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Liver: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
WESSELSBRON DISEASE	Wesselsbron virus (*Flavivirus; Flaviviridae*)	Clinical disease and epidemiology (mosquito-borne) resemble Rift Valley fever. Occurs in sub-Saharan Africa. Sheep are the most susceptible species, with signs of fever, hepatitis with jaundice, subcutaneous oedema and abortion. Mortality high in pregnant ewes and neonatal lambs. Cattle, horses and pigs are infected subclinically	• History of endemic area and clinical signs • Gross and histopathology • Virus isolation: i/c inoculation of mice • Serology: VN, CFT, HAI
Genital system: sheep (goats)			
AKABANE DISEASE	Akabane virus (*Orthobunyavirus, Bunyaviridae*)	Occurs in Japan, Australia, South Africa and Israel. Teratogenic virus (congenital abnormalities). Severe damage to foetus leads to abortion, otherwise arthrogryposis and/or hydranencephaly occur Vector: mosquitoes and gnats	• Virus isolation: suckling mice or TC • RT-PCR • Serology: VN
BLUETONGUE	Bluetongue virus (*Orbivirus; Reoviridae*)	Incubation period six to eight days. Strains of virus vary greatly in virulence. Seasonal incidence dependent on vector. Acute disease: hyperaemia and swelling of mucosa of lips, tongue and dental pad, laminitis with severe lameness, fever and leukopenia. Abortion and congenital abnormalities can also occur. Vector: *Culicoides imicola*	• Virus isolation: i/v inoculation of 10–12-day eggs • Virus detection: RT-PCR, ELISA • Serology: ELISA, VN, AGID, modified CFT
BORDER DISEASE (HAIRY SHAKER DISEASE)	Border disease virus (*Pestivirus; Flaviviridae*)	Infection in pregnant ewes: 1 Abortion and/or congenital abnormalities 2 Birth of hairy-shaker lambs, ataxia and muscle tremors due to defective myelination of CNS nerve fibres 3 Normal but immunotolerant lambs that can shed virus	• Virus isolation: blood clots or tissue • FA: frozen sections • RT-PCR
BRUCELLOSIS (OVINE)	*Brucella ovis*	Unique to sheep. Transmission venereal, ram–ram or ram–ewe–ram. Infected rams shed brucellae in semen intermittently for four years or more. Syndromes are: • Epididymitis, orchitis and impaired fertility in rams • Occasionally placentitis and abortion in ewes	• Isolation of *B. ovis* from semen, foetus or placenta • Direct microscopy (MZN-stained smears): foetal abomasal contents or placenta • PCR • Serology: ELISA or CFT
	Brucella abortus or *B. melitensis*	Ingestion main route but infection via vagina and conjunctiva can occur. Abortions usually in fourth month of pregnancy. Orchitis and arthritis occur rarely. Organisms shed in milk, placental fluids and placenta. Infection with *B. abortus* usually occurs due to contact with cattle Goats are natural host of *B. melitensis*	• Direct microscopy (MZN-stained smears) • Isolation of brucellae • PCR

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Genital system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
ENZOOTIC ABORTION OF EWES (CHLAMYDIAL ABORTION)	*Chlamydophila (Chlamydia) abortus*	Lambs may become infected soon after birth (oral) and infection remains latent until conception. The agent invades placenta and abortion occurs. Ewes affected in late pregnancy may not abort until the next gestation. No systemic effect on ewe but placentitis and thickening of intercotyledonary areas occurs. Solid flock immunity does not develop	• Isolation • Impression smears • Serology: CFT, IFA, ELISA • PCR
EPIDIDYMITIS (SUPPURATIVE)	*Actinobacillus seminis*	An acute unilateral (occasionally bilateral) suppurative epididymitis of young rams. In advanced cases the contents of the scrotal sac consists largely of purulent material. Polyarthritis may also occur	• Clinical signs • Isolation and identification of the pathogen • PCR
LISTERIOSIS	*Listeria monocytogenes* (*L. ivanovii:* abortion only)	Syndromes include: • Visceral or septicamic listeriosis in lambs and kids • Neural listeriosis (circling disease) • Abortions in cattle, sheep and goats (humans). Sporadic and late in gestation. No illness in dam except, rarely, a fatal septicaemia secondary to a metritis. Infection may be associated with silage feeding	• Isolation of listeriae from foetus or placenta • PCR • Histopathology
NAIROBI SHEEP DISEASE	Nairobi sheep disease virus (*Nairovirus; Bunyaviridae*)	Central Africa: sheep and goats. Acute disease with fever, haemorrhagic gastroenteritis and nasal discharge. Mortality 30–90%. Abortions in ewes. Vector: brown dog tick	• Histopathology • Virus isolation: TC or mouse inoculation • AGID: detection of viral antigen • PCR: detection of viral genome • Serology: VN, CFT, IFA
OVINE GENITAL CAMPYLOBACTERIOSIS ('VIBRIOSIS')	*Campylobacter fetus* ss. *fetus* or *Campylobacter jejuni*	Oral route of transmission. Carrier animals main source of infection. Incubation period 10–50 days. After an outbreak of abortion there is flock immunity for three years but 10–70% abortion rate in susceptible, introduced ewes. Abortion usually occurs in last eight weeks of pregnancy. No illness in ewe and fertility normal at subsequent mating. Round (1–3 cm) grey necrotic foci can occur in foetal livers that are pathognomonic, if present	• Gross pathology: foetal liver lesions • Direct microscopy: DCF or FA technique (foetal abomasal contents) • Isolation of pathogen • PCR

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Genital system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
Q-FEVER	*Coxiella burnetii*	Infection is usually subclinical in cattle, sheep and goats but anorexia and abortion can occur rarely in sheep and goats. The organism is shed in placental fluids at parturition and is excreted in milk. A biological cycle occurs in ticks. Humans are most commonly infected by aerosols and less commonly by ingestion of the pathogen in milk	• Serology: CFT, IFA • Giemsa-stained smears from placenta • Culture in fertile eggs. Laboratory culture is a human health hazard • PCR
RIFT VALLEY FEVER	Rift Valley fever virus (*Phlebovirus; Bunyaviridae*)	South and East Africa: sheep, goats, cattle and man. Syndromes: • Hepatitis and high mortality in lambs, kids and calves • Severe disease and 90–100% abortion in sheep and goats • Mild or subclinical in cows but 100% abortions Vector: mosquitoes	• Histopathology: liver necrosis • Virus isolation: lab, animal or TC • RT-PCR • Serology: VN, CFT, ELISA, HAI
SALMONELLOSIS	*Salmonella* serotypes	Infection most common in sheep after mustering or during periods of drought and feed shortages. Abortions may occur with enteric infections but some serotypes can cause abortion without apparent clinical signs in ewes	• Isolation of salmonellae from foetus or placenta • PCR
SCHMALLENBERG VIRUS	*Orthobunyavirus*, Bunyaviridae	Cause of teratogenic effects in sheep and cattle across Europe since 2011. Transmitted by *Culicoides* species	• RT-PCR • Serology: ELISA
TICK-BORNE FEVER	*Anaplasma phagocytophilum*	Occasionally abortions and stillbirths in ewes Occurs in Europe Vector: *Ixodes ricinus*	• Direct microscopy: Giemsa-stained smears (blue coccobacilli in cytoplasm of neutrophils) • PCR
TOXOPLASMOSIS	*Toxoplasma gondii*	Congenital toxoplasmosis is a major cause of abortion in sheep (occasionally goats and pigs). Source of infection: oocytes in cat faeces on pasture or feed. Oocytes are resistant and survive for over one year. Lesions on foetal cotyledons are characteristic: white foci of necrosis up to 2 mm. Flock immunity develops after infection	• Gross lesions on cotyledons • Histopathology • Serology: IFA, IHA
WESSELSBRON DISEASE	Wesselsbron virus *(Flavivirus; Flaviviridae)*	Mosquito-borne infection of sheep, cattle and man in southern Africa. The virus is both teratogenic and abortigenic. Occasionally abortion storms in ewes and hepatitis in neonatal lambs may mimic Rift Valley fever	• Histopathology: IBs in hepatocytes • Virus isolation from liver or brain • Serology: ELISA, VN

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Urinary system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
KIDNEY ABSCESSES	*Staphylococcus aureus,* streptococci, or *Trueperella pyogenes*	In tick pyaemia of lambs (*S. aureus*), abscesses may occur in many body organs	• Tick pyaemia: rough grazing with tick infestation (*Ixodes* spp.) • Post mortem findings • Isolation and identification of bacterial pathogen
OVINE POSTHITIS ('PIZZLE ROT')	*Corynebacterium renale*	Condition is precipitated by high urinary urea. Ulcers around preputial orifice and a brown crust develops. Total occlusion of the preputial orifice can occur, if the condition is not treated	• Clinical findings • Isolation of principal pathogen
PULPY KIDNEY	*Clostridium perfringens* type D	One feature of this enterotoxaemia is the rapid autolysis of the kidneys after death	• History of unvaccinated animal and/or lush pasture • Clinical signs or sudden death • Demonstration of epsilon toxin in ileal contents • Histopathology: focal symmetrical encephalomalcia (pathognomonic when present)
'WHITE SPOTTED KIDNEY' IN LAMBS	*Escherichia coli, Leptospira interrogans* serovars or other bacteria	Focal interstitial nephritis. Often only discovered at slaughter	• Attempted isolation of the bacterial pathogen • Darkfield microscopy may reveal leptospires in kidneys • PCR • Histopathology
Eyes and ears: sheep (goats)			
CONTAGIOUS AGALACTIA (SHEEP AND GOATS)	*Mycoplasma agalactiae*	Ingestion is the main route of entry. There is a transient 'bacteraemia' and the *Mycoplasma* localizes in eyes, joints, lungs and pleura. Signs include conjunctivitis, fever, arthritis and mastitis. The eye lesions can vary from conjunctivitis to hypopyon and rarely perforation of the eyeball	• Clinical signs in an endemic region • Isolation of pathogen • Serology: ELISA, CFT • PCR
INFECTIOUS OVINE KERATOCONJUNCTIVITIS	*Mycoplasma conjunctivae (Acholeplasma oculi, Chlamydophila (Chlamydia) pecorum)*	Initial signs are conjunctivitis, lacrimation, blepharospasm or blinking. The disease may progress to involve the cornea, usually at the periphery, but can be more extensive. Ulceration, if it occurs, is usually superficial and complete healing results	• Clinical signs • Isolation of pathogen(s). Swab rubbed vigorously over conjunctiva of early cases and placed in mycoplasmal and chlamydial transport medium • PCR

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Nervous system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
ABSCESSES (BRAIN OR SPINAL)	*Staphylococcus aureus*	Most common as a sequel of tick pyaemia. Usually occurs in lambs under one year of age. Variety of clinical signs occur, depending on the location of the abscess and its size	• Gross and histopathology • Isolation of pathogen
BOTULISM	*Clostridium botulinum*	Sheep do not show the typical flaccid paralysis until the final stages of the intoxication. There is stiffness when walking, incoordination and some excitement in the early stages. The head tends to bob up and down when walking. In terminal stages there is abdominal breathing, limb paralysis and death	• History: feedstuffs • Clinical signs • Attempt demonstration of toxin in serum/feedstuffs by mouse protection assay or ELISA
CAPRINE ARTHRITIS-ENCEPHALITIS (CAE)	Caprine arthritis-encephalitis virus (*Lentivirus; Retroviridae*)	Three main syndromes: • Encephalomyelitis in kids two to four months of age. Kids show progressive wasting, tremors and a poor hair coat but are alert, afebrile and have a normal appetite. There is a progressive ascending paralysis with paresis in hind limbs seen first. Terminally, after a course of months, there is deviation of head and neck, paddling, paralysis and death • Arthritis and indurative mastitis in adults (two to nine years old) • Encephalitis in adults (one to five years old) manifested as a slow progressive paralysis. Least common syndrome. It is often associated with interstitial pneumonia	• Histopathology: focal malacia of white matter and demyelination • Serology: ELISA, AGID • Virus isolation: buffy coat • RT-PCR
COENUROSIS (STURDY OR GID)	*Taenia multiceps* (*Coenurus cerebralis*)	Eggs in canine faeces are ingested by sheep. Embryos from eggs pass from intestines to bloodstream. Only those embryos that lodge in brain or spinal cord survive, where they mature and cause clinical signs in sheep (occasionally cattle). These include unilateral blindness, ataxia, tremors, deviation of head, circling, excitement and collapse. If the skull is palpated caudal to the horn buds, bone rarefaction may be detected. Surgery is possible in these cases	• Clinical signs: palpate skull • Necropsy findings
CONGENITAL CNS LESIONS	Akabane virus (*Bunyaviridae*)	Hydranencephaly and arthrogryposis (infected midpregnancy)	• Virus isolation • RT-PCR • Serology: VN
	Border disease virus (*Flaviviridae*)	Hairy shaker lambs with excessively hairy, pigmented fleece and tremors due to defective myelination of CNS nerve fibres. A few recover but are carriers of virus	• Virus isolation • FA: frozen sections • RT-PCR

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Nervous system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
FOCAL SYMMETRICAL ENCEPHALOMALACIA	*Clostridium perfringens* type D	Occurs in pulpy kidney disease. The lambs are often found dead with the peracute form. In subacute intoxications with the epsilon toxin, there can be salivation, abdominal pain, excitement followed by depression, head-pressing, recumbency, paddling, opisthotonos and coma. Symmetrical haemorrhages and malacia are found in white matter of brain	• History: vaccination? • Histopathology • Mouse neutralization or ELISA: demonstration of epsilon toxin in SI contents
LISTERIOSIS	*Listeria monocytogenes*	Circling disease in sheep, goats and cattle that has a rapid course of four to 48 hours. Often associated with silage feeding. Head is held to side of lesion and circling occurs in one direction. There is fever, hemiplegia of facial muscles, ataxia, blindness, depression and paralysis. Other syndromes: abortion and septicaemic (visceral) listeriosis in young animals including lambs and kids	• Clinical and history • Histopathology • Isolation from brain (cold enrichment) • PCR
LOUPING ILL	Louping ill virus (*Flavivirus; Flaviviridae*)	Seen in sheep under two years old in endemic areas as the older sheep are immune. Goats can excrete the virus in high titre in milk. Following a viraemia there can be a subclinical infection or CNS signs. The latter include fine muscular tremors, nervous nibbling, hind limb incoordination (louping gait), in severe cases collapse and death in one to three days. Non-suppurative polioencephalomyelitis is present Vector: *Ixodes ricinus*. Reservoir: grouse	• Histopathology: brain • Virus isolation: i/c inoculation of mice or TC • RT-PCR • Serology: HAI, AGID, CFT, VN and IFA
POLIOENCEPHALOMALACIA (CEREBROCORTICAL NECROSIS)	Bacterial thiaminases in rumen and intestines, or dietary thiaminases	Sporadic occurrence in young sheep. Sudden onset of blindness, opisthotonos and convulsions. There is a rapid response to thiamine injections early in condition. Acute cerebral oedema and laminar necrosis of cerebral cortex occur. Condition seen in goats (two to 36 months of age) and is associated with milk-replacer diets in kids and concentrate feeding in older goats	• Dietary causes? • Response rapid to thiamine injection in early cases • Histopathology
PSEUDORABIES	Porcine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Rare in sheep probably because of husbandry methods. Clinically the disease is similar to that in cattle with intense pruritis and a short course. Reported in goats housed with infected pigs. There may be no pruritis in goats. Goats lie down and get up frequently and there is plaintive crying, profuse sweating, spasms, paralysis and death, which is rapid	• History of association with pigs and diagnosis of Aujeszky's disease in pigs • Virus isolation • FA: cryostats of brain tissue • PCR

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Nervous system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
RABIES	Rabies virus (*Lyssavirus*; *Rhabdoviridae*)	In sheep the disease tends to occur in a number of animals at the same time, resulting from an attack on the flock by a rabid dog or fox. Incubation period is from two weeks to several months. It is clinically similar to that in cattle. Most affected sheep have dumb or paralytic rabies, they are quiet, anorexic, with salivation and muscle tremors. A minority show aggression and violent exertion. There is no excessive bleating. In goats, aggression and continuous bleating are more common	• FA for Ag in brain • Histopathology of brain: Negri bodies • RT-PCR
SCRAPIE	Prion	Incubation period several months to three years and the course of disease averages about six months. In early stages there is a stilted gait, loss of condition but no fever and normal appetite. Intense pruritis develops, always bilateral and usually affecting rump, thighs and tail base. There is loss of fleece from these areas. Small stimuli cause the nibbling reflex, there are fine muscle tremors, incoordination and a staring gaze. Terminally there is extreme emaciation, sternal and then lateral recumbency and death	• Histopathology: astrocytosis, vacuolation of neurons, confirmation by immunohistochemistry • EM: scrapie-associated fibrils in brain tissue • PrP^{Sc} detection: immunoblotting, ELISA
TETANUS	*Clostridium tetani*	Entry is often via navel, castration and docking wounds, uterus after dystocia or deep puncture wounds. Average incubation period is 10–14 days, then stiffness of muscles and hyperaesthesia are seen. Mild stimuli cause sheep or goats to fall to the ground with tetanic spasms and opisthotonos. Mortality is about 80%	• History • Clinical signs • Gram-stained smear from deep in wound
TREMORGEN STAGGERS	Heterogeneous group of mycotoxins produced by *Penicillium* spp., *Aspergillus* spp., *Neotyphodium lolii, Claviceps paspali*	Stiffness, ataxia and tremor in large muscle masses. The animals fall and have convulsions if chased or are excited. Recovery usually complete on removal from pasture, where the tremorgen-producing fungi are growing in the pasture litter	• Recovery when moved from suspect pasture
VISNA	Maedi/visna virus (*Lentivirus; Retroviridae*)	Visna is a wasting disease with meningoencephalitis and an incubation period of two years. There is an insidious onset with a progressive course, weight loss, paresis leading to paralysis and death. It is non-febrile and the course can be one to two years. Only sheep are affected	• Histopathology: brain • CSF: mononuclear cells 200/ml • RT-PCR • Serology: ELISA, AGID

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Musculoskeletal system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
BLACKLEG	*Clostridium chauvoei*	Occurs in all ages and usually follows trauma such as docking, castration or vulval damage from parturition. There can be extensive local lesions and severe lameness if limb muscles are involved. Fever, anorexia and depression occur and often rapid death	• History of trauma and clinical signs • FA on smears from affected tissue or bone marrow
BOTULISM	*Clostridium botulinum*	Sheep tend not to show typical flaccid paralysis until the final stages of the disease. Stiffness, ataxia and salivation occur and in terminal stages there is limb paralysis, abdominal breathing and rapid death	• History of contaminated foods or eating carcases of small rodents (protein or phosphorus deficiency) • Demonstration of toxin in serum
CAPRINE ARTHRITIS-ENCEPHALITIS (goats only)	Caprine arthritis-encepahlitis virus (*Lentivirus; Retroviridae*)	Chronic arthritis is the most common sign in adult goats. Carpal joints often involved and lameness is usually intermittent	• Serology: ELISA and AGID tests (seropositive four to five months after infection) • Virus isolation: buffy coat • RT-PCR
CHLAMYDIAL POLYARTHRITIS	*Chlamydophila (Chlamydia) pecorum*	Can be common in lambs in feedlots and also at pasture. There is stiffness, lameness, depression, fever and conjunctivitis. Signs relating to lesions in other body systems may also occur. Morbidity rate is up to 80% but mortality very low	• Clinical signs • Examination of joint fluid for elementary bodies or inclusions • Isolation of pathogen • PCR
CONTAGIOUS AGALACTIA (sheep and goats)	*Mycoplasma agalactiae*	The main signs in this generalized disease include fever, conjunctivitis, arthritis and mastitis. Morbidity about 25% but mortality is low	• Clinical signs in an endemic area • Isolation of pathogen • PCR • Serology: ELISA, CFT
CONTAGIOUS (VIRULENT) FOOTROT	*Dichelobacter nodosus, Fusobacterium necrophorum, Trueperella pyogenes* ± treponemes	Lameness present early when only other signs are warmth and tenderness of claw and mild inflammatory reaction in inner aspect of digit. Separation of sole occurs rapidly, initially at the heel. There is necrosis of soft tissues under the horny sole and a little dark, watery, foetid pus is present between the layers of separating tissue. If untreated, involvement of deep tissues and joints occurs and claw becomes misshapen	• Clinical signs • Gram-stained smear of pus • Isolation of *D. nodosus* • PCR
ERYSIPELAS IN SHEEP	*Erysipelothrix rhusiopathiae*	***Polyarthritis in lambs:*** non-suppurative polyarthritis and signs appear about 14 days after birth (navel) or docking. Sudden lameness but minor swelling of affected joints. Can involve up to 50% of the flock and chronic disease occurs in about 5% of affected lambs	• Clinical signs • Isolation of pathogen from aspirated joint fluid

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Musculoskeletal system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
ERYSIPELAS IN SHEEP (*continued*)		***Post-dipping lameness (cellulitis/ laminitis):*** infection from contaminated sheep-dip through skin abrasions. A cellulitis with extension to laminae of feet occurs but without joint involvement. Severe lameness with one or more feet affected. Legs are hot and slightly swollen often to the metacarpus. Recovery good with treatment	• History and clinical signs • Isolation of pathogen from aspirated joint fluid
FOOT ABSCESS	*Fusobacterium necrophorum* and *Trueperella pyogenes*	Usually confined to one foot. Acute lameness is the first sign and affected claw is hot and painful. Paring of horn of sole liberates pus, otherwise the pus may break-out at the coronet	• Clinical signs • Gram-stained smear of pus
JOINT-ILL	*Staphylococcus aureus, Escherichia coli, Trueperella pyogenes*, streptococci or others	Localization of pathogens in joints following a navel infection or bacteraemia/ septicaemia	• Clinical signs • Isolation of pathogen from aspirated joint fluid
LAMENESS (GENERALIZED DISEASES)	Foot-and-mouth disease virus (*Aphthovirus*) Bluetongue virus (*Orbivirus*)	Acute lameness in all four feet. Vesicular lesions in interdigital cleft In acute disease laminitis and coronitis, manifested by lameness and recumbency, occur in a few sheep. A dark purple band in the skin just above the coronet is diagnostic. In endemic areas milder disease can be seen such as 'range stiffness in lambs' (Texas)	• Tests for virus detection: ELISA, RT-PCR, CFT • Serology for antibodies for virus, in each disease
MAEDI-VISNA (sheep only)	Maedi/visna virus (*Lentivirus; Retroviridae*)	Arthritis may occur in visna together with meningo-encephalitis and wasting	• Pathology • Serology: AGID, ELISA • RT-PCR
MALIGNANT OEDEMA	*Clostridium septicum, C. novyi* type A	Entry is usually through wounds, signs appearing 12–48 hours after infection. There is a high fever, toxaemia, weakness, often lameness and a soft doughy swelling at site of infection. Course short (one to two days) and is invariably fatal	• History of trauma and clinical signs • FA on smears from affected tissue (recently dead animals, as *C. septicum* is a rapid post mortem invader) • PCR
MYCOPLASMAL ARTHRITIS (mainly in goats)	*Mycoplasma capricolum* and some other spp.	Infection in goats causes septicaemia with resulting pneumonia and arthritis. It is manifested in three- to eight-week-old kids by lameness, recumbency, diarrhoea and fever. *M. capricolum* is also a cause of arthritis in sheep	• Isolation of the mycoplasmas from joint fluid. Transport medium is desirable • PCR

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Musculoskeletal system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
OSTEOMYELITIS	*Actinomyces bovis* or *Corynebacterium pseudotuberculosis*	Non-specific infection by haematogenous route following omphalitis, castration or docking wounds. Not common in sheep	• Clinical and radiographic examination • Isolation of the pathogen • PCR
OVINE INTERDIGITAL DERMATITIS ('SCALD')	*Fusobacterium necrophorum* and *Trueperella pyogenes*	One or all four feet can be affected. In mild cases, the interdigital skin is reddened, swollen and often covered by a moist film of necrotic material. In severe cases the skin is necrotic and eroded so that sensitive subcutaneous tissues are exposed. Lameness is present	• Clinical signs • Gram-stained smear of necrotic material • PCR
PROLIFERATIVE DERMATITIS ('STRAWBERRY FOOT ROT')	*Dermatophilus congolensis*	Small heaped-up scabs appear from coronet to knee or hock. They enlarge to 3–5 cm and become thick and wart-like. If scabs are removed, a bleeding, fleshy mass (resembling a strawberry) is seen surrounded by an ulcer. Later the ulcer becomes deeper with pus formation. No pruritis or lameness occurs unless lesions extend to interdigital space. Reported from Scotland and Australia	• Direct microscopy on scab material, using Giemsa or Gram stain • Isolation of pathogen if necessary • PCR
TETANUS	*Clostridium tetani*	Infection can be via umbilicus, uterus after dystocia or docking and castration wounds. The clinical signs are similar to those in other species. Course of disease is three to four days and mortality in young animals is high	• History and clinical signs • Gram-stained smear of necrotic material from wounds
Respiratory system: sheep (goats)			
CAPRINE ARTHRITIS-ENCEPHALITIS (CAE)	Caprine arthritis-encephalitis virus (*Lentivirus; Retroviridae*)	'Slow' viral disease, transmitted in colostrum or milk. Syndromes include: • Encephalitis in kids one to four months old • Arthritis and indurative mastitis in adults • Encephalitis and interstitial pneumonia in adults (least common) Only a small proportion of infected goats develop clinical disease	• Serology: AGID, ELISA • Virus isolation: buffy coat • RT-PCR
CONTAGIOUS CAPRINE PLEUROPNEUMONIA (CCPP)	*Mycoplasma capricolum* subsp. *capripneumoniae*	A peracute, acute or chronic disease confined to goats. Similar disease to CBPP but the tendency to form necrotic sequestra (chronic form) is uncommon. Lesions may slowly resolve in surviving animals	• PM findings • Isolation and identification of the mycoplasmas • PCR

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Respiratory system: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
LUNG ABSCESSES	*Trueperella pyogenes* or *Corynebacterium pseudotuberculosis*	May not cause clinical signs, frequently only discovered after death	• Gram-stained smear • Culture and identification • PCR
MAEDI-VISNA (PROGRESSIVE PNEUMONIA OF SHEEP)	Maedi/visna virus (*Lentivirus; Retroviridae*)	'Slow' viral disease that is transmitted in milk, colostrum or by aerosol. Long incubation period so rarely seen in sheep less than two years old and is most common in animals over four years old. Maedi is the respiratory form and lungs are firm and heavy and do not collapse when thorax is opened	• Gross pathology and histopathology • Serology: ELISA, AGID • RT-PCR
PNEUMONIA COMPLEX	Complex involves one or more of the following pathogens: Parainfluenza virus 3 (PI3), *Mycoplasma ovipneumoniae, Mannheimia haemolytica, Pasteurella multocida, Chlamydophila (Chlamydia) pecorum*	Viruses and mycoplasmas cause a relatively mild infection but serious disease with fatalities can occur if pasteurellae are involved as secondary invaders	• Isolation and identification of pathogens • Serology: PI3 (paired serum samples), HAI • PCR-based techniques for viruses and mycoplasmas
PULMONARY ADENOMATOSIS/ JAAGSIEKTE ('DRIVING SICKNESS')	Jaagsiekte sheep retrovirus (*Betaretrovirus; Retroviridae*)	'Slow' virus disease with a long incubation period. Signs occur in three- to four-year-old sheep. Lesions occur in the lungs and range from small nodules to extensive solid areas that are grey and flat with sharp demarcation. Copious froth in air passages is a characteristic finding	• Clinical signs: 'wheelbarrow' test (elevation of hind limbs) • Histopathology • Detection of virus in lung washings/exudate: ELISA, RT-PCR, RIA
SEPTICAEMIC MYCOPLASMOSIS	*Mycoplasma mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum*	Clinical signs include septicaemia, pneumonia, polyarthritis and conjunctivitis	• PM findings • Isolation and identification of the *Mycoplasma* • PCR
Skin: sheep (goats)			
ACTINOBACILLOSIS (OVINE)	*Actinobaccillus lignieresii*	Purulent disease of skin, lymph nodes, lungs and soft tissues of the head and neck in sheep. Most common in head region involving the jaw area	• Microscopy on granules in exudate • Culture: aerobic

Continued

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Skin: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
BORDER DISEASE	Border disease virus *(Pestivirus; Flaviviridae)*	Dam infected in pregnancy: Early pregnancy: foetal death Later in pregnancy: congenital defects that involve skin, skeketon and/or CNS. 'Hairy shakers' have excessively hairy and pigmented fleeces, muscle tremors and ataxia. Nervous signs are due to defective myelination of nerve fibres. Some lambs can recover in three to four months but they may be immunotolerant carriers (V+ve/Ab–ve)	• Virus isolation: blood clots or tissue • FA: frozen sections • RT-PCR
CASEOUS LYMPHADENITIS	*Corynebacterium pseudotuberculosis*	Superficial form: abscessation and rupture of one or more superficial lymph nodes Internal form: characteristic abscesses in internal lymph nodes and occasionally in organs such as the lungs. The caseous lesions can bear a resemblance to those of tuberculosis. The internal form is often subclinical and lesions are not discovered until slaughter	• Clinical signs in superficial form • Characteristic encapsulated, laminated abscesses containing greenish pus • Isolation and identification of the pathogen from pus samples • PCR
CONTAGIOUS PUSTULAR DERMATITIS (ORF)	Orf virus (*Parapoxvirus; Poxviridae*)	Primary lesions on skin of lips and muzzle. In lambs extension can occur to buccal cavity and in adults to other non-woolled areas such as udder and vulva. Initial papules develop into thick crusts, often friable and liable to bleeding if scabs removed	• Clinical signs • EM • PCR • Histopathology • Virus isolation
DERMATOPHILOSIS (STREPTOTHRICOSIS, MYCOTIC DERMATITIS)	*Dermatophilus congolensis*	***Lumpy wool:*** when woolled areas of body are involved. Scab material bound to wool fibres. Crusts usually on dorsal surface due to moisture from rain ***Strawberry foot rot:*** lesions occur on distal parts of limbs	• Direct microscopy on scab • Culture if microscopic results are equivocal • PCR
FACIAL ECZEMA	Sporidesmin in spores of *Pithomyces chartarum*	Mycotoxicosis with bile duct obstruction, liver damage, jaundice and photosensitization (non-excretion of phylloerythrin). Photosensitization seen on non-woolled areas of face and ears. There is erythema and oedema of affected skin. Livers are initially enlarged and icteric and later atrophied and fibrotic	• Spore count on pasture • Gross and histopathology on livers
FOOT-AND-MOUTH DISEASE	Foot-and-mouth disease virus (*Aphthovirus; Picornaviridae*)	Major sign is sudden and acute lameness in several sheep due to interdigital vesicular lesions. Rarely lesions on muzzle or in buccal cavity	• Viral antigen detection: ELISA, CFT (vesicular fluid or epithelial material) • Virus isolation • RT-PCR • Serology: ELISA, CFT, VN

Table 69.2 Principal infectious diseases of sheep (goats)—cont'd

Skin: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
RINGWORM	*Trichophyton verrucosum, Microsporum gypseum* or rarely other dermatophytes	Ringworm is rare in sheep and is usually on non-woolled areas of head or scrotum. High initial dose may be needed, such as occurs when lambs are housed in sheds previously inhabited by infected calves	• Microscopy: skin scraping in 10% KOH to demonstrate arthrospores • Culture and identification
SCRAPIE	Prion	Sheep and occasionally goats can be affected. Incubation period usually two to three years. There is pruritis, fine tremors of head and neck, nibbling movements of lips, weaving gait and staring eyes. Loss of weight and wool loss from rubbing occurs and eventually paralysis and death	• Histopathology: astrocytosis, vacuolation of neurons, confirmation by immunohistochemistry • EM: scrapie-associated fibrils in brain tissue • PrP^{Sc} detection: immunoblotting, ELISA
SHEEPPOX (GOATPOX)	Sheeppox/Goatpox virus (*Capripoxvirus; Poxviridae*)	Acute, severe, generalized disease with mortality rate 5–50%. Widespread skin lesions as raised, circular plaques with red borders. Later necrosis and dark, hard scab formation occurs eventually leaving star-shaped scars and alopecia. Lungs and intestines are involved. Transmission: direct, aerosol or mechanical via biting insects	• EM on lesions • ELISA, AGID for viral Ag • Histopathology • Virus isolation • PCR
STAPHYLOCOCCAL DERMATITIS	*Staphylococcus aureus*	Pustular lesions forming scabs. Predisposing factors usually present, such as insufficient trough space in concrete feeding troughs or thorny plants in vegetation. Lesions usually periorbital or on muzzle but can occur on other non-woolled areas	• Direct microscopy • Culture and identification
ULCERATIVE DERMATOSIS	Unclassified virus	Characterized by destruction of epidermal and subcutaneous tissues with development of raw granulating ulcers on skin of lips, nares, feet, legs and male and female external genitalia. Two fairly distinct syndromes affecting (1) external genitalia or (2) other non-woolled areas. In males the glans penis is affected and the preputial orifice can become blocked. Transmission occurs at breeding time. Morbidity rate 15–20% (up to 60%) but mortality low	• Clinical signs • Histopathology • Differential diagnosis from orf

Table 69.3 Principal infectious diseases of pigs

Buccal cavity: pigs

Disease	Agent(s)	Comments	Diagnosis
NECROTIC STOMATITIS	*Fusobacterium necrophorum*	Necrotic lesions on tongue or in buccal cavity. The predisposing cause is usually trauma	• Clinical signs • Gram- or DCF-stained smear from necrotic material
VESICULAR DISEASES (Foot-and-mouth disease; Vesicular stomatitis; Swine vesicular disease; Vesicular exanthema of swine)	*Aphthovirus* (*Picornaviridae*); *Vesiculovirus* (*Rhabdoviridae*); *Enterovirus* (*Picornaviridae*); *Calicivirus* (*Caliciviridae*)	Most constant sign in these vesicular diseases affecting pigs is sudden and acute lameness. The lameness is due to vesicular lesions in the interdigital cleft and lesions may or may not be present on the snout and in the buccal cavity	• Clinical signs • Specimens: vesicular fluid and epithelial flap • Demonstration of viral antigen: CFT, ELISA, VN • Virus isolation • RT-PCR • Serology: CFT, ELISA, VN
Gastrointestinal tract: pigs			
AFRICAN SWINE FEVER	(*Asfivirus; Asfarviridae*)	In acute form there is fever, depression, erythema of skin, abortion in sows and dysentery. Mortality high. Post mortem findings include 'blackberry-jam spleen', 'blood-clot mesenteric lymph nodes', haemorrhages in many organs and oedema of gall bladder. In subacute disease the clinical signs and pathology are less marked with a mortality rate of about 5%. Vector: *Ornithodoros* ticks	• FA for antigen: frozen sections • Serology: IFA, ELISA • Virus isolation • PCR
CLASSICAL SWINE FEVER (HOG CHOLERA)	Classical swine fever virus (*Pestivirus; Flaviviridae*)	Fever, depression, erythema of skin and nervous signs early in disease. Diarrhoea can be bloody and submucosal haemorrhages widespread ('turkey-egg kidney', 'strawberry lymph nodes'). Spleen is not enlarged but often has infarcts and button ulcers occur in the colon. Pregnant sows abort. Virus strains vary in virulence. Mortality can be high but strains of reduced virulence now appear to be present in Europe	• FA or AGID for viral antigen detection in tissues • RT-PCR • Virus isolation • Serology: VN, ELISA
CLOSTRIDIAL ENTEROTOXAEMIA	*Clostridium perfringens* type C	Very young piglets one to four days old. Claret red diarrhoea is characteristic. Necrosis, haemorrhage and gas bubbles occur in the wall of small intestine. There is blunting of villi. Mortality high and death of whole litter is common	• Pathology • Gram-stained smear of mucosa of small intestine (recently dead pig): large numbers of clostridia are suggestive of the disease • ELISA for toxin • PCR for enterotoxin genes

Table 69.3 Principal infectious diseases of pigs—cont'd

Gastrointestinal tract: pigs

Disease	Agent(s)	Comments	Diagnosis
COLIBACILLOSIS	*Escherichia coli*	***Neonatal diarrhoea:*** profuse watery diarrhoea and dehydration in one- to three-day-old piglets. Death of whole litter can occur. There is gastric distention, watery contents in small intestine but villous atrophy is minimal ***Weanling enteritis:*** greyish diarrhoea with no blood occurs. There is anorexia, mild fever and loss of condition. Mortality less than 10% but signs persist for some time. Common cause of post weaning diarrhoeas	• Isolation of *E. coli* • Check for enterotoxigenicity: fimbrial antigens, enterotoxin or genes encoding enterotoxins
OEDEMA DISEASE	*Escherichia coli* serotypes that produce the oedema disease Shiga toxin, Stx2e (neurotoxin)	The toxin is absorbed from the gut and damages vascular endothelium. Usually seen in well-grown pigs and they are often found dead. If alive, oedema of forehead, eyelids and larynx (hoarse squeal) is observed. Diarrhoea and nervous signs such as ataxia and convulsions may occur. Oedema of stomach wall is the pathognomonic post mortem finding	• Clinical signs • Gross pathology and histopathology • Isolation of *E. coli* (often haemolytic) • Detection of toxin or genes encoding toxin
PORCINE EPIDEMIC DIARRHOEA	Porcine epidemic diarrhoea virus (*Alphacoronavirus; Coronaviridae*)	Present in some European countries. Signs are most common in weaners and adults. Watery diarrhoea occurs with recovery after about one week. Mortality 1–3%	• Clinical signs • FA technique on cryostat sections of small intestine • Viral antigen detection in faeces: ELISA • RT-PCR • Serology: IFA or ELISA
PROLIFERATIVE HAEMORRHAGIC ENTEROPATHY (INTESTINAL ADENOMATOSIS)	*Lawsonia intracellularis*	Young adults show cutaneous pallor, weakness and black tarry faeces. High morbidity but mortality about 6%. There is proliferation of small intestine mucosa with blood clots in lumen. Anaemia may result. Pigs six to 20 weeks old, if infected, can be subclinical or have watery diarrhoea with mucus and blood. Spontaneous recovery in most but chronic wasting in 1–2%. Organisms are intracellular so faecal specimens are of no diagnostic use	• Stanied smears of mucosa • Histopathology: intracellular *Lawsonia* organisms • Isolation is difficult: specialized laboratories • PCR
ROTAVIRUS INFECTION	*Rotavirus* (*Reoviridae*)	Most common in sucking piglets about two weeks old. Watery diarrhoea of short duration (three to five days) is seen. Vomiting if it occurs is less dramatic than in transmissible gastroenteritis. Mortality rare if uncomplicated	• Detection of virus in faeces: EM, ELISA • Virus isolation

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Gastrointestinal tract: pigs

Disease	Agent(s)	Comments	Diagnosis
SALMONELLOSIS	*Salmonella* serotypes. *Salmonella* Choleraesuis (hog cholera bacillus) is still common in some countries. *Salmonella* Typhimurium	Disease can occur in any age group. In septicaemic form there is fever, depression and erythema of skin. Systemic involvement occurs with necrotic foci in liver, petechiated kidneys and swollen mesenteric lymph nodes. Mortality can be high	• Isolation of *Salmonella* species • PCR • Differential diagnosis from swine fever and African swine fever
SWINE DYSENTERY	*Brachyspira hyodysenteriae*	Diarrhoeal syndrome with blood, mucus and necrotic debris in faeces. Only large intestine is involved and the pigs are afebrile. Usually all pigs in a pen are affected. Pigs become gaunt and dehydrated and mortality is 25% in untreated cases	• Clinical signs and appearance of faeces • Histopathology: *Brachyspira* in crypts (silver stain) • FA: mucosal smear • Isolation of pathogen • PCR
POSTWEANING MULTISYSTEMIC WASTING SYNDROME (PMWS)	Associated with porcine circovirus 2 infection (*Circovirus; Circoviridae*)	Multiple aetiology condition of intensively reared pigs. Progressive weight loss, diarrhoea, dyspnoea	• Histopathology: lymphoid tissues, immunohistochemistry • PCR • Serology: IFA, ELISA, immunoperoxidase monolayer assay
TRANSMISSIBLE GASTROENTERITIS (TGE)	*Alphacoronavirus 1* (*Alphacoronavirus; Coronaviridae*)	Profuse watery greenish-grey diarrhoea. Vomiting is a prominent sign. Severe villous atrophy but there is no blood in faeces and the pigs are afebrile. Mortality due to dehydration can be 100% in piglets less than seven days old. All age groups can be affected in a non-immune herd	• Pathology: wash and examine jejunum and ileum, paper thin walls • FA: mucosal impression smears or frozen sections • Detection of virus in faeces: ELISA • RT-PCR • Virus isolation • Serology: VN, ELISA
Liver: pigs			
LISTERIOSIS (SEPTICAEMIC/ VISCERAL)	*Listeria monocytogenes*	Infection can occur in young pigs. Usual course one to three days with fever, depression and death. Necrotic foci in liver and other body organs	• Gram-stained smears from lesions • Culture of pathogen • PCR
LIVER LESIONS (BACTERIAL)	A variety of bacteria including: *Erysipelothrix rhusiopathiae*, streptococci or staphylococci	Non-specific foci of infection can occur in the liver and other body organs following a bacteraemia or septicaemia, in pigs and other domestic animals	• Gram-stained smears from lesions • Culture of pathogen

Table 69.3 Principal infectious diseases of pigs—cont'd

Liver: pigs

Disease	Agent(s)	Comments	Diagnosis
PSEUDOTUBERCULOSIS	*Yersinia pseudotuberculosis*	Disease most common in guinea pigs, cage birds, captive deer and pigs. In classical infections there is emaciation, diarrhoea and death in three to four weeks. Abscesses in liver, spleen and other organs. Pigs may be infected by eating dead wild birds	• Gram-stained smears from lesions • Culture of pathogen • PCR
Genital system: pigs			
AFRICAN SWINE FEVER	African swine fever virus (*Asfivirus; Asfarviridae*)	Endemic in Africa and Sardinia. Acute disease: fever, erythema of skin, diarrhoea, and abortion in pregnant sows Biological vector: *Ornithodoros* ticks	• FA for antigen: frozen sections • Serology: IFA, ELISA • Virus isolation • PCR
AUJESZKY'S DISEASE	Porcine herpesvirus 1 (*Varicellovirus*; *Herpesviridae*)	Transmission by contact or veneral. In susceptible herd: • Neonates: severe generalized disease and death • Young adults: respiratory disease • Pregnant sows: SMEDI syndrome in 50% of sows • Other animals (abnormal hosts) pseudorabies ('maditch')	• Virus isolation • FA: frozen sections or impression smears • Serology: ELISA, VN
BRUCELLOSIS (PORCINE)	*Brucella suis* (*Brucella abortus*)	*B. suis* is the main cause of brucellosis in pigs but sporadic abortion can occur with *B. abortus*. *B. suis* causes abortions in 50–80% of pregnant sows in primary outbreaks, and reduction of fertility and orchitis in boars	• Direct microscopy: MZN-stained smears • Isolation • PCR • Serology: ELISA, agglutination tests or CFT
CLASSICAL SWINE FEVER (HOG CHOLERA)	Classical swine fever virus (*Pestivirus; Flaviviridae*)	A mild fever may be the only sign in sows but: • Abortions, stillbirths and mummification • Congential abnormalities in piglets • Immunotolerant piglets born that excrete virus	• FA or AGID for viral antigen detection in tissues • RT-PCR • Virus isolation • Serology: VN, ELISA
PICORNAVIRUS INFECTION (PORCINE)	Porcine teschovirus 1, 3, 6 (*Teschovirus; Picornaviridae*) and Porcine sapelovirus (*Sapelovirus; Picronaviridae*)	Viruses frequently isolated from normal pigs but also from aborted and stillborn foetuses. Disease-producing status is uncertain. The term SMEDI viruses was given to these viruses but porcine parvovirus now considered to be more important in the SMEDI syndrome	• Serology: VN (precolostral sera) • Virus isolation

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Genital system: pigs

Disease	Agent(s)	Comments	Diagnosis
INCLUSION BODY RHINITIS	Porcine herpesvirus 2 (*Herpesviridae*)	Subclinical disease in herds where endemic. In susceptible herds: • Inclusion body rhinitis in piglets up to 10 weeks of age, after this subclinical infection • Generalized disease in piglets less than two weeks of age when infected in utero. Poor growth rates if they survive • Pregnant sows: sporadic abortions	• Virus isolation • Serology: IFA or ELISA
LEPTOSPIROSIS	*Leptospira interrogans* serovars	Subclinical infections in many pigs. Young pigs can shed leptospires in high concentrations in urine. Abortions in sows late in pregnancy or birth of weak piglets which is often the main sign of the disease. Infertility	Herd basis: • Darkfield microscopy: urine of sow and young pigs on farm • Serology: MAT or CFT Abortions: • PCR or FAT on foetal tissues
MASTRITIS-METRITIS-AGALACTIA (MMA) SYNDROME	Complex aetiology associated with coliform bacteria, hysteria and hormonal imbalance	Seen in young gilts after farrowing. There is depression and anorexia. If untreated the litter will die from starvation	• History and clinical signs • Isolation of *Escherichia coli* or other entero-bacteria from milk and uterine discharges
PARVOVIRUS INFECTION (PORCINE)	Porcine parvovirus (*Parvovirus; Parvoviridae*)	Virus replicates in small intestine. Oral or venereal transmission. On endemic farms subclinical disease occurs. In a susceptible herd or in introduced pigs the following syndromes may be evident: • Infection at mating: early embryonic death and return to service • Sows infected in pregnancy: SMEDI syndrome. Occasional congenital abnormalities in piglets • Low fertility in boars: virus in semen	• FA: frozen sections • Haemagglutination: detects viral haemagglutinin in foetal tissues • Virus isolation • PCR • Serology: ELISA or HAI
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) ('BLUE-EARED' DISEASE)	Porcine reproductive and respiratory syndrome virus (*Arterivirus; Arteriviridae*)	Recorded in USA in 1987, Canada in 1988 and parts of Europe in 1991. Clinical signs variable and course of disease on a property is one to three months Sows: fever, laboured breathing, 'blue-ear' in 1%, SMEDI syndrome Piglets: death, weak piglets, increased susceptibility to secondary infections and haemorrhage easily. About 55% survive to weaning Boars: temporary infertility in affected animals Growers: fever, respiratory signs, 'blue-ear' in a small percentage, increase in pre-valence of diseases endemic on property	• Clinical signs • Serological tests • RT-PCR • Virus isolation

Table 69.3 Principal infectious diseases of pigs—cont'd

Urinary system: pigs

Disease	Agent(s)	Comments	Diagnosis
AFRICAN SWINE FEVER	African swine fever virus (*Asfivirus; Asfarviridae*)	Petechial and ecchymotic haemorrhages in kidneys and other body organs in acute disease. Immune-complex glomerulonephritis in chronic disease	• FA for antigen: frozen sections • Serology: IFA, ELISA • Virus isolation • PCR
CLASSICAL SWINE FEVER (HOG CHOLERA)	Classical swine fever virus (*Pestivirus; Flaviviridae*)	Petechial haemorrhages through kidneys ('turkey-egg kidneys') and elsewhere	• FA or AGID for viral antigen detection in tissues • RT-PCR • Virus isolation • Serology: VN, ELISA
PYELONEPHRITIS (PORCINE)	*Actinobaculum suis*	Disease of sows. Boars are healthy carriers and transmit the infection venereally. Disease precipitated by pregnancy and parturition. Depression, anorexia, passage of blood-stained urine and pain during micturition are features of the disease	• Clinical signs • Culture from deposit of centrifuged urine (anaerobic)
SWINE ERYSIPELAS (KIDNEY INFARCTS)	*Erysipelothrix rhusiopathiae*	Virulent strains cause vascular damage, thrombus formation and metastatic emboli in various body organs, including kidneys, leading to infarcts	• History of swine erysipelas in herd • Isolation of the pathogen from lesions (chronic condition, may be unsuccessful) • PCR
'WHITE-SPOTTED KIDNEY'	*Leptospira interrogans* serovar Pomona, *Escherichia coli* or other bacteria	Usually not discovered until slaughter. If leptospirosis, clinically normal young pigs on farm may be excreting leptospires in urine	• Midstream urine from live pigs. Darkfield or PCR for demonstration of leptospires • Attempted isolation of bacteria from lesions

Eyes and ears: pigs

'BLUE-EARED' DISEASE (PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME)	Porcine reproductive and respiratory syndrome virus (*Arterivirus; Arteriviridae*)	Cyanosis of the ears occurs in a small percentage of pigs in the porcine reproductive and respiratory syndrome	• Clinical signs • Serology • RT-PCR
CONJUNCTIVITIS (Classical swine fever; African swine fever; Atrophic rhinitis)	Classical swine fever virus, African swine fever virus, *Bordetella bronchiseptica*	Conjunctivitis is one sign in the disease syndromes	• Isolation of pathogen • Demonstration of viral antigen or nucleic acid in tissues • Serology to detect Ab for viral diseases
GLASSER'S DISEASE	*Haemophilus parasuis*	Essentially a polyserositis of pigs up to four months of age. Cyanosis and oedematous thickening of the ears is considered almost pathognomonic	• Gross and histopathology • Isolation of pathogen • PCR

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Eyes and ears: pigs

Disease	Agent(s)	Comments	Diagnosis
NECROTIC EAR SYNDROME OF PIGS	Multifactorial aetiology, may involve secondary bacterial invasion	Condition is characterized by unilateral or bilateral necrosis of the pinnae of the ears, occurring sporadically in weaned or growing pigs. A septicaemia with septic arthritis and death are not uncommon sequelae	• Clinical signs • Isolation of secondary bacterial invader, frequently staphylococci and/or streptococci
OTITIS EXTERNA	Mixed non-specific flora	Otitis externa occurs in pigs but is much less common than in the dog. *Sarcoptes* mites may cause ear mange in pigs	• Clinical signs • Isolation of pathogen(s)
Nervous system: pigs			
AFRICAN SWINE FEVER	African swine fever virus (*Asfivirus; Asfarviridae*)	Clinically similar to swine fever but nervous signs much less marked, often limited to weakness and incoordination of hind limbs. Histologically an encephalitis, similar to that in swine fever, can be present	• FA for antigen: frozen sections • Serology: IFA, ELISA • Virus isolation • PCR
BOTULISM	*Clostridium botulinum*	Reports of botulism in pigs are rare. Signs include staggering, limb weakness, vomiting and pupillary dilation. Terminally there is recumbency with flaccid paralysis and death from respiratory failure	• History: feedstuffs • Demonstration of toxin in serum by mouse inoculation
CLASSICAL SWINE FEVER (HOG CHOLERA)	Classical swine fever virus (*Pestivirus; Flaviviridae*)	Severe, contagious and generalized disease. CNS signs are usually seen early in the disease, these include high-stepping, incoordination, paddling of limbs and convulsions. Other signs are high fever, erythema of the skin, burrowing into bedding and piling on top of each other, vomiting, diarrhoea, swaying of hind limbs and muscle tremor. Course of disease is five to seven days. A form of the disease has been recorded where CNS signs predominate such as tetanic then clonic convulsions with loud squealing, the mortality approaches 100%. Occasionally BVD virus can cause signs almost identical to swine fever but usually confined to one litter. Virus strains causing subacute disease, with less severe clinical signs, are now common in Europe	• FA or AGID for viral antigen detection in tissues • RT-PCR • Virus isolation • Serology: VN, ELISA

Table 69.3 Principal infectious diseases of pigs—cont'd

Nervous system: pigs

Disease	Agent(s)	Comments	Diagnosis
CONGENITAL CNS SIGNS	Classical swine fever virus (*Pestivirus; Flaviviridae*)	Infection of pregnant sow: abortions, stillbirths, mummification and weak piglets (carriers). Congenital abnormalities: myoclonia congenita (congenital trembles) associated with cerebellar hypoplasia. Survivors may be immunotolerant carriers (virus +ve/Ab –ve)	• Virus isolation • RT-PCR • Serology: Ab titre in sow
ENCEPHALOMYOCARDITIS	Encephalomyocarditis virus (*Cardiovirus; Picornaviridae*)	Natural hosts are rodents that excrete virus in faeces. Outbreaks of disease in pigs are associated with rodent plagues. Clinical signs in young pigs include depression, trembling and incoordination, but pigs are usually found dead. Mortality over 50%. The ventral myocardium often has multiple, discrete, pale areas due to necrosis and lymphocytic infiltration	• Pathology • Virus isolation: TC or mouse inoculation • RT-PCR • Serology: VN, HAI
GLASSER'S DISEASE	*Haemophilus parasuis*	Meningitis can occur as part of the polyserositis in pigs up to four months of age	• Gross and histopathology • Isolation of pathogen • PCR
OEDEMA DISEASE	Strains of *Escherichia coli* producing oedema disease toxin, Stx2e	Predisposed to by changes in diet and occurs in well-grown, thrifty weaner and growing pigs. Characterized by oedema of eyelids, face and stomach wall, a hoarse squeal, stiffness, incoordination, blindness and complete flaccid paralysis. Course is six to 36 hours or pig may be found dead. Encephalomalacia occurs	• Gross pathology • Isolation of haemolytic *E. coli* • PCR for genes encoding Stx2e
RABIES	Rabies virus (*Lyssavirus; Rhabdoviridae*)	Clinical signs in pigs are very variable. May show excitement and a tendency to attack or dullness and incoordination. There can be twitching of the snout, rapid chewing movements, excessive salivation, walking backwards and clonic convulsions. Terminally there is paralysis and death	• FA technique for viral Ag in brain • RT-PCR • Histopathology: Negri bodies in brain
STREPTOCOCCAL MENINGITIS	*Streptococcus suis* type 2	Epidemic outbreaks of meningitis and septicaemia can occur in five- to 10-week-old pigs. Depression, tremors, incoordination, blindness, opisthotonos, convulsions, paddling, fever, nystagmus and paralysis. There is thickening and congestion of the meninges, oedema of the brain and excess, purulent CSF	• CSF examination • Isolation and identification of *S. suis* type 2 • PCR

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Nervous system: pigs

Disease	Agent(s)	Comments	Diagnosis
TALFAN/TESCHEN	*Porcine teschovirus 1* (*Teschovirus*; *Picornaviridae*)	***Teschen*** (porcine enteroviral encephalomyelitis): virulent form of the disease but limited to Eastern Europe and Madagascar: paralysis, coma and death occurs ***Talfan***: milder form and has a worldwide distribution. Young pigs are affected and there is ataxia and paresis, usually of the hind legs. Pigs are alert with normal appetite and recovery can occur. No gross lesions are seen. Microscopically: lesions in grey matter of brain stem, cerebellum and spinal cord. Non-suppurative encephalitis	• Histopathology: immunohistochemistry • Virus isolation from brain tissue • RT-PCR • Serology: VN, ELISA
TETANUS	*Clostridium tetani*	A high incidence of tetanus can occur after castration in a group of young pigs. Incubation period one to three weeks. Stiffness, muscle tremor, tail held stiffly, difficulty in eating or swallowing are seen. Later, hyperaesthesia occurs and mild stimuli will cause recumbency with tetanic spasms and opisthotonos	• History: castrations • Clinical signs • Gram-stained smear from wound
VOMITING AND WASTING DISEASE	Betacoronavirus 1 (*Betacoronavirus; Coronaviridae*)	Most infections are inapparent, but susceptible pigs four days to three weeks old may vomit, become anorexic, depressed, emaciated and a few develop CNS signs such as hyperaesthesia, jerking movements, posterior paralysis, lateral recumbency and death. No gross lesions are seen. Non-suppurative inflammation of brain stem and upper spinal cord occurs	• Virus isolation • RT-PCR • Serology: VN, HAI
Musculoskeletal system: pigs			
ACTINOBACILLOSIS (PORCINE)	*Actinobacillus suis*	A fatal, acute septicaemia occurs in piglets aged one to eight weeks, but the bacterium has been associated with arthritis, pneumonia and subcutaneous abscesses in older pigs	• Isolation and identification of *A. suis* from joints and other tissues • PCR
AFRICAN SWINE FEVER (CHRONIC)	African swine fever virus (*Asfivirus; Asfarviridae*)	Acute or peracute disease can be followed by the chronic form, or this may be the only syndrome seen in endemic areas. The chronic form is characterized by cutaneous ulcers, arthritis, pneumonia, pleuritis and pericarditis. The pigs are intermittently febrile, become emaciated with soft oedematous swellings over joints and under mandible. They may be persistently infected for life	• Acute disease: PCR or FA on frozen sections • Chronic disease: serology: IFA, ELISA

Table 69.3 Principal infectious diseases of pigs—cont'd

Musculoskeletal system: pigs

Disease	Agent(s)	Comments	Diagnosis
ATROPHIC RHINITIS (AR)	*Bordetella bronchiseptica* +/– AR+ *Pasteurella multocida*	Atrophy of the turbinate bones, especially in piglets infected under three weeks of age. Signs include lacrimation, sneezing, and later twisting of snout	• Clinical signs • Examination of snouts at slaughter • Isolation of pathogen • PCR
BOTULISM	*Clostridium botulinum*	Rare in pigs. Signs include staggering, vomiting, pupillary dilation, flaccid paralysis and recumbency	• History of possible source of toxin • Clinical signs • Demonstration of toxin in serum
FOOTROT (WHITE LINE DISEASE)	*Fusobacterium necrophorum, Trueperella pyogenes,* streptococci and spirochaetes	Usually follows trauma to sole or wall of claw (rough abrasive floor). There is heat and pain when only moderate pressure is applied to the claw. Necrosis can extend between the sole and sensitive laminae with severe lameness	• History of rough concrete floor • Clinical signs • Gram-stained smear of necrotic material or exudates
GLASSER'S DISEASE (PORCINE POLYSEROSITIS)	*Haemophilus parasuis*	Outbreaks of disease in young pigs (three to 16 weeks old). Manifested by acute polyarthritis, pleurisy, pericarditis and peritonitis. Usually occurs after stress such as chilling, transportation or weaning. There is a high fever, anorexia, dyspnoea and lameness with joints swollen and painful. Some pigs may die two to five days after first signs. Survivors often develop chronic arthritis	• History of stress • Clinical signs • Isolation of pathogen from joint fluid or affected tissues at PM • PCR • Gross and histopathology
JOINT-ILL	Streptococci, staphylococci, *Escherichia coli* or *Trueperella pyogenes*	Usually an umbilical infection soon after birth leads to a bacteraemia with localization of the bacterial pathogen in joints and other sites	• History of umbilical infection • Isolation of pathogen from aspirated joint fluid
MALIGNANT OEDEMA	*Clostridium septicum* and other gas gangrene clostridia	Soft, doughy swelling with marked local erythema and pain usually restricted to axillae, limbs and throat. The lesions are oedematous but there is usually no emphysema. Pigs are febrile, depressed, stiff and lame. Death often occurs within one to two days of first signs	• Clinical signs • FA on tissue or exudates • PCR • Isolation of pathogen if necessary
MYCOPLASMAL POLYARTHRITIS	*Mycoplasma hyosynoviae*	Production of arthritis in growing pigs (12–24 weeks old). Sudden onset of acute lameness in one or more limbs but swelling of affected joints is minimal. Pigs are usually afebrile and recovery occurs in three to 10 days. A few animals may become permanently recumbent	• Clinical signs and age group affected • Isolation of pathogen from joints • PCR

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Musculoskeletal system: pigs

Disease	Agent(s)	Comments	Diagnosis
MYCOPLASMAL POLYSEROSITIS	*Mycoplasma hyorhinis*	Produces arthritis and polyserositis in one- to eight-week-old pigs. Transient fever, dyspnoea and acute lameness with moderate swelling in affected joints. Spontaneous recovery may occur in one to two weeks but often the affected pigs become unthrifty with chronic lameness	• Clinical signs and young age group • Isolation of pathogen from joints or serous surfaces
NECROTIC RHINITIS (BULLNOSE)	*Fusobacterium necrophorum*	Associated with trauma to nasal cavity such as placing a ring in a pig's nose. Lesions develop as a necrotic cellulitis of soft tissues of nose and face but may spread to involve bone	• History of injury and clinical signs • Gram-stained smear of necrotic material • Isolation of pathogen if necessary
OSTEOMYELITIS	Miscellaneous bacteria	Non-specific, sporadic infection, often associated with tail-biting in pigs	• Isolation of pathogen
STREPTOCOCCAL MENINGITIS AND ARTHRITIS	*Streptococcus suis* type 2	Outbreaks often occur in cold weather in intensively reared pigs aged four to 12 weeks. They show fever, incoordination, tremors, paddling movements, paralysis, convulsions and death. Polyarthritis occurs as part of a general septicaemia	• History of predisposing causes and clinical signs • Isolation of *S. suis* from tissues • PCR
SWINE ERYSIPELAS (POLYARTHRITIS)	*Erysipelothrix rhusiopathiae*	Acute forms of disease: septicaemia and urticarial (diamond disease) forms. Chronic forms: polyarthritis and vegetative endocarditis. A synovitis occurs at first in the joints with a non-purulent effusion and then degenerative changes in the subendochondral bone, cartilages and ligaments	• History of erysipelas endemic on farm • Isolation of pathogen from joints • PCR
TETANUS	*Clostridium tetani*	Usually occurs in young pigs following castration. The signs are similar to those in other animals and the mortality rate can be high	• History of castration and clinical signs • Gram-stained smear of necrotic tissue from wound
VESICULAR DISEASES	Foot-and-mouth disease (*Aphthovirus*), swine vesicular disease (*Enterovirus*), vesicular stomatitis (*Vesiculovirus*), vesicular exanthema of swine *(Vesivirus)*	Clinical signs in pigs are similar in all four diseases. Sudden lameness in a number of pigs in a group, and often, all four feet affected. The acute lameness is due to vesicular lesions in the interdigital cleft. Pigs less consistently develop vesicles on snout and in buccal cavity	• History and clinical signs • Specimens: vesicular fluid or epithelial flap • Ag detection: ELISA, CFT or VN • RT-PCR • Virus isolation • Serology: ELISA, VN or CFT

Table 69.3 Principal infectious diseases of pigs—cont'd

Respiratory system: pigs

Disease	Agent(s)	Comments	Diagnosis
AFRICAN SWINE FEVER (CHRONIC)	African swine fever virus (*Asfivirus; Asfarviridae*)	The chronic disease, in endemic areas, is characterized by cutaneous ulcers, pneumonia, pericarditis, pleuritis and arthritis. Vector: *Ornithodoros* ticks	• FA for antigen in frozen sections • PCR • Serology: IFA or ELISA
ATROPHIC RHINITIS (AR)	*Bordetella bronchiseptica* ± AR^+ strains of *Pasteurella multocida*	Signs at three to eight weeks of age include lacrimation, sneezing and later twisting of snout may be seen. A primary or secondary pneumonia may occur. Disease most severe if young piglets under three weeks of age are infected	• Examination of snouts of pigs after slaughter • Culture and identification of pathogens • PCR
AUJESZKY'S DISEASE	Porcine herpesvirus 1 *(Varicellovirus; Herpesviridae*)	Generalized disease with encephalitis occurs in piglets. Respiratory signs are most common in weaned and growing pigs with sneezing, coughing and oculonasal discharge. Pregnant sows may abort	• Virus isolation • FA for antigen: frozen sections or brain/pharyngeal impression smears • PCR • Serology: VN, ELISA
GLASSER'S DISEASE	*Haemophilus parasuis*	Affects non-immune pigs usually up to four months of age. There is fever and polyserositis with serofibrinous pleurisy, pericarditis, septic arthritis and meningitis	• Gross and histopathology • Isolation of pathogen • PCR
INCLUSION BODY RHINITIS	Porcine herpesvirus 2 (*Herpesviridae*)	Rhinitis in pigs up to 10 weeks of age. Sneezing, coughing and oculonasal discharges are evident. The virus may occasionally cause foetal death and abortion	• Histopathology: large basophilic IBs in mucous glands of turbinates or exfoliated cells in nasal secretions • Serology: IFA or ELISA • Virus isolation
MYCOPLASMAL PNEUMONIA ('VIRUS PNEUMONIA' OR 'ENZOOTIC PNEUMONIA')	*Mycoplasma hyopneumoniae* ± *Haemophilus parasuis*	All ages can be infected but lung lesions most common at eight to 16 weeks old. Endemic in herds with sporadic flare-ups of disease. Pigs cough if roused. Mild disease on its own, but severe with secondary bacterial invaders	• Gross pathology: grey-purple lung lesions in cranial and middle lobes • Histopathology • Culture and identification • PCR
NECROTIC RHINITIS ('BULL-NOSE')	*Fusobacterium necrophorum*	Predisposing causes include trauma to oral or nasal mucosa. There is swelling and deformity of facial area with snuffling, sneezing and a foul-smelling nasal discharge. Generally only one or two pigs in a group are affected at any one time	• Swelling of face and foul-smelling necrotic tissue • Gram-stained smear • Isolation: anaerobic

Continued

Table 69.3 Principal infectious diseases of pigs—cont'd

Respiratory system: pigs

Disease	Agent(s)	Comments	Diagnosis
PASTEURELLOSIS	*Pasteurella multocida* ± *Mycoplasma* spp.	The condition is a bronchopneumonia, sometimes with pleuritis and pericarditis. Primary pasteurellosis is usually seen in pigs over one year old. Disease can become chronic with polyarthritis and chronic thoracic lesions	• Gross pathology • Isolation and identification of pathogens • PCR
PLEUROPNEUMONIA	*Actinobacillus pleuropneumoniae*	The disease is severe and contagious and usually seen in young pigs less than six months old. Rapid spread and mortality can be high if untreated. Severe respiratory distress, fever, and bloody, frothy nasal and oral discharges can be seen. Explosive outbreaks occur in non-immune herds but it is a chronic disease when endemic	• PM: pleuritic adhesions, lungs dark and swollen. Bloody fluid oozes from cut surfaces • Isolation and identification • PCR • Serology: ELISA (herd basis)
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME ('BLUE-EARED' DISEASE)	Porcine reproductive and respiratory syndrome virus (*Arterivirus; Arteriviridae*)	Respiratory disease occurs in sows, weaners and growers in this novel disease. However, the main economic losses are in disruption of the breeding programme and deaths of piglets	• Clinical findings • Differentiate from other respiratory diseases • Serology • RT-PCR
SWINE INFLUENZA	Influenza A virus (*Influenzavirus A; Orthomyxoviridae*)	The disease is highly contagious. Stress such as cold conditions is an important predisposing cause, and infections often occur in autumn and winter. Plum-coloured lesions can be seen in apical and intermediate lobes of lungs. The condition is accompanied by coughing, fever and muscular weakness. More severe where secondary infections such as *Haemophilus parasuis* occur	• Viral Ag: FA, ELISA • RT-PCR • Isolation of virus early in disease (nasal secretions during febrile period) • Serology: HAI test, ELISA
Skin: pigs			
ANTHRAX	*Bacillus anthracis*	Subacute infection is the most common form in pigs and characterized by localized, subcutaneous, oedematous swelling of ventral neck, thorax and shoulders. Swelling is secondary to a septicaemia. Many pigs make a gradual recovery but some die from asphyxia due to swelling of throat region or from toxaemia	• Polychrome methylene blue or Giemsa-stained smears on peritoneal fluid • Culture • PCR
CONTAGIOUS PYODERMA IN SUCKING PIGS	Streptococci or staphylococci	Pathogens enter through minor abrasions such as bites. Vesicles and pustules occur that rupture forming scabs. Involvement of hair follicles is common and can lead to acne and deeper, extensive lesions	• Gram-stained smears • Culture

Table 69.3 Principal infectious diseases of pigs—cont'd

Skin: pigs

Disease	Agent(s)	Comments	Diagnosis
EXUDATIVE EPIDERMITIS (GREASY PIG DISEASE)	*Staphylococcus hyicus*	Seen in sucking pigs and those recently weaned with high mortality in piglets under 10 days of age. There is marked cutaneous erythema with seborrhoea and greasy exudate. Severe dehydration and weakness occurs in young pigs	• Culture of *S. hyicus*
PITYRIASIS ROSEA	Unknown. Familial susceptibility suspected	Lesions very similar to those of ringworm. Seen in young pigs after weaning and several pigs in a litter can be affected. There are minimal systemic signs with spontaneous recovery in four to eight weeks. Lesions are small red flat plaques enlargening to 2 cm or more, surrounded by a ring of erythema and coalescing to cover ventral abdomen.	• Differential diagnosis from ringworm
RINGWORM	*Microsporum nanum*	Occurs in fattening and mature pigs with high morbidity within a pen. The lesions are round, brownish with scabs and crusts. Usually there is no systemic reaction or pruritis (differentiate from pityriasis rosea)	• Microscopy: visualization of arthrospores around hairs in 10% KOH • Culture: colony and macroconidia
SWINE ERYSIPELAS (URTICARIAL FORM)	*Erysipelothrix rhusiopathiae*	Usually seen in young adults. Small red spots develop into characteristic diamond lesions that are raised with an erythematous edge. Lesions coalesce and the skin may eventually slough. There is accompanying fever and systemic illness. Untreated cases can develop into the chronic forms of vegetative endocarditis or polyarthritis	• Characteristic skin lesions with systemic illness • Isolation of organism • PCR
SWINEPOX	Swinepox virus (*Suipoxvirus; Poxviridae*)	Mild infection in young pigs transmitted by contact and pig lice. Papules, vesicles and circular red-brown scabs occur on belly, axillae and elsewhere on the body. Pigs continue to eat well with recovery in three weeks. Congenital infections can occur with lesions on the foetus Vector: *Haematopinus suis* (mechanical transmission)	• EM on scab • Histopathology on biopsy • FA: frozen sections • Virus isolation • Serology: AGID (screening test)
VESICULAR DISEASES	Foot-and-mouth disease (*Aphthovirus*); Vesicular stomatitis (*Vesiculovirus*); Swine vesicular disease (*Enterovirus*); Vesicular exanthema of swine *(Calicivirus)*	Main and cardinal clinical sign is acute lameness in several pigs in a group due to vesicles between the claws. Vesicular lesions on snout or in buccal cavity are inconsistent	• Detection of viral antigen: CFT, ELISA, VN • RT-PCR • Virus isolation • Serology: CFT, ELISA, VN

Table 69.4 Principal infectious diseases of horses

Buccal cavity: horses

Disease	Agent(s)	Comments	Diagnosis
ACTINOBACILLOSIS	*Actinobacillus linieresii*	Causes tumorous abscesses of the tongue, usually referred to as wooden tongue. It is seen primarily in cattle but also in sheep, horses, pigs and dogs	• Clinical signs • Microscopy and culture if exudate is present
GLOSSOPLEGIA **STRANGLES** **BOTULISM** **EQUINE ENCEPHALOMYELITIDES**	 *Streptococcus equi* subsp. *equi* *Clostridium botulinum* *Alphaviruses* (*Togaviridae*)	Glossoplegia of central origin may accompany or follow such diseases as strangles, botulism or the equine encephalomyelitides. The unilaterally affected tongue is deviated towards the unaffected side and the bilaterally affected tongue is limp and often protrudes from the mouth	• Diagnosis of the specific condition involved
PHARYNGEAL PARALYSIS **BOTULISM** **GUTTURAL POUCH INFECTION** **RABIES**	 *Clostridium botulinum* *Aspergillus fumigatus* *Lyssavirus* (*Rhabdoviridae*)	Paralysis due to *Aspergillus fumigatus* infection and erosion of the guttural pouch wall is relatively common. This condition is also seen in intoxications in the horse such as botulism and chronic lead poisoning	• Differentiate from oesophageal obstruction or foreign bodies wedged between the teeth
VESICULAR STOMATITIS	*Vesiculovirus (Rhabdoviridae)*	The principal lesions in the horse are on the dorsum of the tongue and lips. There is a mild fever and champing of jaws and drooling of saliva. If foot lesions occur there is hyperaemia and ulceration of the coronary band	• Demonstration of viral Ag in vesicular fluid by CFT or ELISA • Virus isolation • RT-PCR • Serology: CFT for rise in Ab titre

Gastrointestinal tract: horses

ADENOVIRUS INFECTION	Equine adenovirus A (*Mastadenovirus, Adenoviridae*)	Subclinical infection in normal foals but Arab foals with combined immunodeficiency disease succumb to adenovirus pneumonia at about two months of age and an enteric syndrome can sometimes be a feature of the disease	• Virus isolation: nasal and ocular discharges • IN inclusions in cells of lacrimal secretions • FA: virus in tissues
CHRONIC DIARRHOEAS (UNDIFFERENTIATED)	Aetiology: miscellaneous	Course five to eight weeks but up to one year. There is weight loss and faeces are porridge-like to fluid in consistency. The appetite is maintained. Prognosis often poor. Possible causes include: • Large strongyle larval migration • Granulomatous enteritis (tissue strongylosis?) • Avian tuberculosis • Chronic *Rhodococcus equi* infection • Chronic salmonellosis • *Eimeria* infections • Allergy to components in food • Neoplasia • Tetracycline- or lincomycin-induced diarrhoea	• Clinical and laboratory diagnosis to eliminate or confirm each of the possible causes

Table 69.4 Principal infectious diseases of horses—cont'd

Gastrointestinal tract: horses

Disease	Agent(s)	Comments	Diagnosis
CLOSTRIDIA-ASSOCIATED ENTEROCOLITIS	*Clostridium perfringens, Clostridium difficile.* Alteration of normal flora, may be associated with administration of certain anti-microbial drugs	Large doses and sometimes normal doses (parenteral route) of the tetracyclines, tylosin and lincomycin. Sudden onset of diarrhoea three to four days after antibiotic administration. Course short and mortality rate high. Post mortem findings include oedema of large intestinal wall, colitis and typhilitis	• History and clinical signs • Gross pathology • Isolation of organism and PCR for toxin-encoding genes • Demonstration of toxin (ELISA) • Differentiate from colitis-X
CLOSTRIDIAL ENTEROTOXAEMIA	*Clostridium perfringens* types B and C	Not common but is a serious disease that occurs in first few days of life. Severe depression, abdominal pain, diarrhoea/dysentery and foals can die in a few hours. Usually afebrile as the disease is a toxaemia. Haemorrhagic enteritis seen on post mortem examination	• Gram-smear on mucosal scraping of small intestine from a recently dead foal (large numbers of clostridia) • Toxin demonstration: neutralization test in mice or ELISA
COLISEPTICAEMIA	*Escherichia coli*	Failure to ingest adequate colostrum is a prime determinant for disease. Signs similar to those in calves. May account for 25% of septicaemias in foals	• Isolation of *E. coli*
COLITIS-X	Uncertain (possibly *Clostridium difficile*)	Adult horses may die within 24 hours of first signs. Profuse diarrhoea may or may not occur. Horses surviving for less than three to four hours often have normal faeces. Usually there is a history of stress. Depression, sweating, abdominal pain, skin cold and clammy, rapid pulse. Post mortem findings: oedema seen early and later haemorrhagic necrosis of walls of caecum and colon. Described in USA, Canada and Australia	• Clinical signs and post mortem findings • Differentiate from salmonellosis, clostridial disease and antimicrobial-associated diarrhoea
***LAWSONIA* INFECTION**	*Lawsonia intracellularis*	Proliferative enteropathy in three- to seven-month-old foals with diarrrhoea, weight loss and ventral oedema	• Ultrasound examination • PCR • Histopathology • Serology
NEONATAL SEPTICAEMIAS	Group C streptococci, *E. coli, Salmonella* spp. or *Klebsiella* spp.	In neonatal septicaemias death can be rapid. If the foal survives for a period then there is often diarrhoea in the terminal part of the illness	• Isolation and identification of pathogen

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Gastrointestinal tract: horses

Disease	Agent(s)	Comments	Diagnosis
POTOMAC HORSE FEVER	*Neorickettsia risticii*	Disease is sporadic and seasonal. Not common in young horses. Mortality 5–30%. Fever, paralytic ileus, and profuse watery diarrhoea (can be projectile). Post mortem: congestion, haemorrhage and mucosal erosion especially in caecum and colon, swollen mesenteric lymph nodes and subcutaneous oedema of abdominal wall. Vector: fluke. Reservoir: snail. Aquatic insects also involved in transmission cycle	• Serology: ELISA and FA • PCR • Differentiate from salmonellosis and colitis-X
ROTAVIRUS INFECTION	*Rotavirus* (*Reoviridae*)	Foals 5 to 35 days old in high-population-density groups are at risk. There is profuse watery diarrhoea and dehydration is serious in neonates but recovery is uneventful in older foals. Villous atrophy occurs and villi return to normal about seven days after onset of the diarrhoea	• Electron microscopy • Viral antigen detection: latex agglutination, ELISA • Virus isolation and identification
SALMONELLOSIS	*Salmonella* serotypes	***Neonatal foals*** (up to four months of age): usually the septicaemic form occurs. Predisposing factors include overcrowding and build-up of salmonellae in foaling paddocks. Rapid onset with fever, anorexia and profuse diarrhoea if animals survive longer than 24–48 hours ***Adult horses:*** up to 50% can be subclinical excretors of salmonellae. Clinical disease is predisposed to by stress (transportation, surgery, anaesthesia). There is high fever, complete anorexia, profuse diarrhoea, faeces may contain blood and have a foetid smell	• Culture for salmonellae • PCR
SLEEPY FOAL DISEASE	*Actinobacillus equuli*	Foal may be infected in utero or via umbilicus. Can be an acute septicaemia and sudden death but if foal survives 24 hours then suppurative lesions occur in kidneys, joints and intestines. Illness seen a few hours after birth to three days of age. Fever, prostration, anorexia, diarrhea and death usually within 24 hours of clinical signs	• Blood culture from live foal, culture of lesions from dead animals • Cervical swab from mare (dam may be a carrier) • PCR
SUPPURATIVE BRONCHOPNEUMONIA	*Rhodococcus equi*	***Foals one to four months old:*** suppurative bronchopneumonia with gradual onset of signs. There is fever, weakness and anorexia. Diarrhoea may occur due to granulomatous colitis and mesenteric lymphadenitis from the foal swallowing infected sputum	• Radiography • Isolation of pathogen from transtracheal aspirates or BAL • PCR

Table 69.4 Principal infectious diseases of horses—cont'd

Liver: horses

Disease	Agent(s)	Comments	Diagnosis
EQUINE HERPESVIRUS ABORTION (foetal infection)	Equine herpesvirus 1 and sometimes equine herpesvirus 4 (*Varicellovirus; Herpesviridae*)	Small necrotic foci in the liver of aborted foetuses	• Pathology: IN inclusions in liver • FA on frozen sections of liver • Virus isolation • PCR
EQUINE INFECTIOUS ANAEMIA	Equine infectious anaemia virus (*Lentivirus, Retroviridae*)	Virus replication in the liver may result in acute hepatic necrosis	• Serology: AGID (Coggins test), ELISA
TYZZER'S DISEASE	*Clostridium piliforme*	Acute, fatal disease of laboratory rodents but also reported in wild and domestic animals. Infection in one- to six-week-old foals. Death can be sudden. The liver is enlarged, pale and mottled. Gastric or duodenal ulceration may also occur	• Gross pathology • Histopathology: Giemsa or Warthin-Starry to demonstrate pathogen within hepatocytes • PCR

Genital system: horses

CONTAGIOUS EQUINE METRITIS (CEM)	*Taylorella equigenitalis*	Specific and highly contagious, uterine infection of horses and donkeys. Stallion is the carrier in prepuce or penis (urethral fossa) and shows no clinical signs. Infection transmitted by coitus or via veterinary instruments. Mares have a profuse mucopurulent discharge two to six days after service or can be subclinical carriers. Low conception rate due to failure of egg implantation or early abortions	• Specimens (see Chapter 27) • Culture from swabs placed in Amies transport medium + charcoal • PCR • Serology: CF Abs detected three to seven weeks postinfection
BACTERIAL METRITIS, PLACENTITIS AND ABORTION ('DIRTY MARE SYNDROME')	Group C streptococci, *Klebsiella pneumoniae, Actinobacillus equuli, Pseudomonas aeruginosa, E. coli* or opportunistic fungi	Vaginal discharge, often purulent, of uterine origin. Seen particularly at oestrus and when mare squats to urinate. Hind legs can be streaked with discharge in severe cases. Some of these pathogens may be spread by coitus. Occasionally the presence in the uterus of these organisms can cause a systemic reaction with fever, anorexia, depression and laminitis. If the infection becomes chronic there may be permanent infertility. Ascending placentitis is a major cause of late-term abortion and premature birth	• Specimens: Cervical swabs introduced through plastic sleeves. Swabs placed in transport medium, especially for streptococci • Culture and identification

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Genital system: horses

Disease	Agent(s)	Comments	Diagnosis
EQUINE COITAL EXANTHEMA	Equine herpesvirus 3 (*Varicellovirus; Herpesviridae*)	Acute but mild disease characterized by pustular and ulcerative lesions on vaginal mucosa, penis, prepuce and perineal region in the mare. Healing in 14 days with white depigmented permanent spots around vulva identifying potential carriers. No systemic signs or abortions. Decreased libido may occur in stallion with disruption of breeding schedule. Virus spread by coitus. Lesions seen four to eight days after service	• EM: epithelial cells from the margins of the lesions • Virus isolation • PCR • Serology: VN, ELISA (epidemiological studies only)
EQUINE VIRAL ARTERITIS (EVA)	Equine arteritis virus (*Arterivirus; Arteriviridae*)	Mild or severe febrile disease with conjunctivitis, nasal discharge, palpebral oedema and dependent oedema of legs, udder and scrotum Abortion in mares during the respiratory disease. Virus shed in nasal secretions for eight to 10 days. Persistent infection in one-third of stallions with venereal transmission	• FA: frozen sections • Virus isolation from foetal lung and spleen • RT-PCR • Serology: CFT, VN, AGID, ELISA and IFA
EQUINE HERPESVIRUS ABORTION	Equine herpesvirus 1 and sometimes EHV 4 (*Varicellovirus; Herpesviridae*)	Respiratory disease in foals and young adults. Abortions in mares one to four months after the outbreak of respiratory disease. Occasionally generalized disease in foals	• Histopathology of aborted foetus: pulmonary oedema, hepatic necrosis and petechiation of mucosae (IN inclusions in liver cells) • FA: frozen sections • Virus isolation • PCR
LEPTOSPIROSIS	*Leptospira interrogans* serovars	Serovars Pomona, Hardjo, Bratislava and Icterohaemorrhagiae have been isolated from aborted foetuses. Mild, transient illness in mare with anorexia, low-grade fever and depression. Abortion occurs some weeks after the fever and periodic ophthalmia may be seen some months afterwards	• Leptospiral abortion in horses often goes undiagnosed. In an infected group of horses about 30% will have positive antibody titres to leptospira • PCR or FAT on foetal tissues
SALMONELLOSIS	*Salmonella* Abortus-equi	This *Salmonella* serotype is now rare. Specific disease of equidae characterized by abortion in mares, testicular lesions in stallions and septicaemia and polyarthritis in foals	• Isolation and identification of the *Salmonella* organism
SCIRRHOUS CORD	*Staphylococcus aureus* (botryomycosis) or *Actinomyces* species	Infection of the stump of the spermatic cord seen a few weeks after castration. May be accompanied by fever, toxaemia and lameness. The lesion is a mass of fibrous tissue interspersed with small abscess cavities and sinus tracts	• Specimens: biopsy or surgically removed tissue • Gram-stained smear • Culture

Table 69.4 Principal infectious diseases of horses—cont'd

Urinary system: horses

Disease	Agent(s)	Comments	Diagnosis
CYSTITIS	Streptococci, *Escherichia coli, Staphylococcus aureus, Proteus* species or *Klebsiella pneumoniae*	Frequent urination and voiding of small volumes of urine. Fever, inappetence and pain on urination may occur. Urine is cloudy and contains inflammatory cells and bacteria. The condition is often accompanied by urethritis	• Clinical signs • Isolation of pathogen from mid-stream urine
EQUINE INFECTIOUS ANAEMIA	Equine infectious anaemia virus (*Lentivirus; Retroviridae*)	Immune-complex glomerulonephritis can occur in chronic disease. This may lead to renal failure and death	• History: endemic area • Clinical signs • Viral antigen in spleen: FA or ELISA • PCR (proviral DNA) and RT-PCR • Serology: AGID (Coggins test) or ELISA
SLEEPY FOAL DISEASE	*Actinobacillus equuli*	Neonatal septicaemia. If foals survive for two to three days, abscesses form throughout kidneys. Joints may also be affected	• Clinical signs • Isolation and identification of pathogen from lesions • PCR • Histopathology

Eyes and ears: horses

CONJUNCTIVITIS		Conjunctivitis may occur as part of a more generalized disease	
EQUINE VIRAL ARTERITIS	Equine arteritis virus (*Arterivirus; Arteriviridae*)	Incubation period one to eight days followed by fever, leukopenia, lacrimation, conjunctivitis, nasal discharge and depression. Photophobia and oedema of eyelids, conjunctiva, legs and ventral body wall can occur. Abortion in pregnant mares	• Virus isolation • RT-PCR • Serology: CFT, VN, AGID, ELISA and IFA
AFRICAN HORSE SICKNESS	African horse sickness virus (*Orbivirus; Reoviridae*)	In the cardiac form, conjunctivitis, oedema of eyelids and subcutaneous oedema of localized areas of head and neck with bulging of supraorbital fossa occurs. Dependent oedema appears terminally, secondary to cardiac insufficiency	• History: endemic area • Clinical signs • Virus isolation: TC or i/c inoculation of two- to six-day-old mice • RT-PCR • Serology: VN, ELISA, CFT, AGID
EQUINE SARCOID	Papillomavirus (*Papillomaviridae*)	Eyelids are a comparatively common site for sarcoids	• History and signs • Histopathology on biopsy
KERATOMYCOSIS	*Aspergillus fumigatus*	Infection of the cornea can occur following antibacterial and steroid treatment, suggesting immunosuppression and impaired colonization resistance as predisposing causes	• Clinical signs and history • Isolation of pathogen

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Eyes and ears: horses

Disease	Agent(s)	Comments	Diagnosis
PERIODIC OPHTHALMIA ('MOON BLINDNESS')	*Leptospira interrogans* serovars (especially Pomona)	Strong presumptive evidence for leptospires as the aetiological agent. Recurrent episodes of inflammation of one or both eyes occurs, the iris and uveal tract are affected. The sequel may be blindness. Probably an immune-complex disease, as condition occurs several months after initial infection	• Clinical signs • Serology: MAT, ELISA • PCR

Nervous system: horses

ABSCESSES IN SPINAL CORD	*Staphylococcus aureus* and streptococci	Pressure on spinal cord and nervous signs will depend on the site of the abscess	• Pathology • Isolation of pathogen
BOTULISM	*Clostridium botulinum* types B, C	Onset three to 17 days after ingestion of feed containing the botulinum toxin. Signs include muscle tremor then stumbling, knuckling and ataxia with inability to lift the head. Animals in sternal recumbency. In some cases the tongue is paralysed with drooling of saliva and inability to chew. Death from respiratory failure one to four days after first signs	• History of suspect feed • Differentiate from other causes of motor paralysis • Acute cases: demonstration of toxin in serum by mouse inoculation
EQUINE ENCEPHALOMYELITIDES	*Western equine encephalitis virus* (WEE), *Eastern equine encephalitis virus* (EEE) and *Venezuelan equine encephalitis virus* (VEE) (*Alphavirus; Togaviridae)*	WEE and EEE have a wild bird/mosquito cycle with the horse (and man) as accidental hosts. Non-contagious as there is minimal viraemia in horses. Fever, drowsiness, paralysis of lips and pharynx, head-pressing, 'sawhorse' stance, incoordination, general paralysis and death may occur. Mortality 30% for WEE and 90% for EEE VEE has a swamp rodent/mosquito cycle. The disease is contagious as horses often have a significant viraemia. Mortality: peracute 80% and acute 50%	• History: seasonal • Histopathology • Serology: CFT, ELISA, VN or HAI • Virus isolation from brain • RT-PCR
GUTTURAL POUCH INFECTION	*Aspergillus fumigatus* and/or Group C streptococci	Enlarged guttural pouch causes pressure on seventh cranial nerve. Horse is unable to prehend or chew and there are inappropriate movements of lips	• Clinical signs • Isolation of pathogen from biopsy
HERPESVIRUS PARALYSIS	Equine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	A myeloencephalopathy that can accompany outbreaks of respiratory disease and abortions. Sudden onset of signs varying from mild ataxia to severe paralysis. Urine dribbling is a feature. The CSF is yellow and abnormal. The condition is seen in foals and adults. Vaccines do not always protect against this neurological form of the disease	• History of other herpesvirus clinical presentations • Isolation of virus from brain • PCR • Serology: HAI, CFT. VN (rise in antibody titres in in-contact horses)

Table 69.4 Principal infectious diseases of horses—cont'd

Nervous system: horses

Disease	Agent(s)	Comments	Diagnosis
MENINGITIS (BACTERIAL)	*Streptococcus equi* subsp. *equi*	Uncommon sequel to strangles with excitation, hyperaesthesia, rigidity of neck and terminal paralysis	• Pathology • Isolation of pathogen • PCR
RABIES	Rabies virus (*Lyssavirus; Rhabdoviridae*)	An ascending paralysis with hypersalivation, incoordination followed by paralysis and recumbency in two to four days. Death within one week. Occasionally horses can show extreme agitation and become aggressive. Self-inflicted injuries may also occur	• FA technique for Ag in brain • RT-PCR • Histopathology for Negri bodies
RYE GRASS STAGGERS (MYCOTOXICOSIS)	*Acremonium loliae*	Ingestion of toxic metabolic products of fungus growing in rye grass pastures. Can affect cattle, sheep, horses and deer. Tremor, hypersensitivity, drunken gait and posterior paralysis occur. Animals recover if removed from affected pasture	• Recovery on removal from affected pasture • Histopathology: degeneration of Purkinje fibres in long-standing cases
SHAKER FOAL SYNDROME (TOXICO-INFECTIOUS BOTULISM)	*Clostridium botulinum* type B	Reported from UK and USA. Sporadic disease in three- to eight-week-old foals of either sex and in all breeds. There is a sudden onset of severe muscular weakness and foals become prostrate but are bright and alert. If foals are lifted, or try to rise, there is severe muscle tremor. Death occurs within 72 hours from respiratory failure	• As for botulism • In toxico-infectious botulism it may be possible to isolate *C. botulinum* from the tissues
TETANUS	*Clostridium tetani*	Incubation period one to three weeks. Signs include prolapse of third eyelid, anxious expression, pricked ears, flared nostrils, paralysis of jaw muscles, urinary retention, stiffness of muscles with 'sawhorse' posture, tail held high, difficulty in walking with a tendency to fall and become recumbent. Tetanic convulsions are triggered by touch or sound and respiratory arrest may occur during a tetanic spasm. Death in five to 10 days. The longer the incubation period the better the prognosis and mild cases can recover	• History: vaccination? • Clinical signs • Gram-stained smear from deep wound (if present)
WEST NILE VIRUS	West Nile virus (*Flavivirus; Flaviviridae*)	Transmitted by mosquitoes. Small percentage of infected horses develop neurological signs, high mortality	• Viral antigen detection • RT-PCR • Serology: ELISA, plague reduction neutralization test
BORNA DISEASE	Borna disease virus (*Bornaviridae*)	Sporadic disease with most cases recorded in Central Europe. Somnolence, ataxia, pharyngeal paralysis and high mortality	• Histopathology of brain: Joest-Degen bodies • RT-PCR • Serology: ELISA

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Nervous system: horses

Disease	Agent(s)	Comments	Diagnosis
LOUPING ILL	Louping ill virus (*Flavivirus; Flaviviridae*)	Mainly an encephalomyelitis of sheep but rarely the horse can be infected. Signs include fever, abnormality of gait, convulsions and paralysis. Horses can develop a sufficiently high viraemia to infect blood-sucking insects. Vector: ticks. Reservoir: grouse	• History: endemic area • Histopathology: brain and brain stem • Serology: HAI and VN • Virus isolation: brain
VERTEBRAL OSTEOMYELITIS	*Salmonella* serotype	Specific syndrome if cervical vertebrae four to six are affected with stumbling gait and stiffness. There is a reluctance to bend the neck so that the animals often have difficulty in grazing. Atrophy of cervical muscles occurs	• Pathology • Isolation of pathogen
Musculoskeletal system: horses			
BOTULISM	*Clostridium botulinum*	Onset three to 17 days after ingestion of food containing toxin. Signs include muscle tremor, stumbling, knuckling, ataxia and inability to raise the head. Animals go down in sternal recumbency. Death from respiratory failure one to four days after first clinical signs	• History of food that might have contained toxin • Clinical signs • Demonstration of toxin in serum
FISTULOUS WITHERS AND POLL EVIL	*Actinomyces* species or *Brucella abortus*	Bursitis. Trauma predisposes to the condition, including an ill-fitting saddle or blow to the poll	• Gram- or MZN-stained smears from aspirate or exudates • Isolation of pathogen • Serology for *Brucella abortus*
JOINT ILL (POLYARTHRITIS)	*Escherichia coli, Actinobacillus equuli, Salmonella* serotype or Group C streptococci	Young foal infected via the umbilicus or following a bacteraemia or septicaemia with localization in the joints and in other tissues	• Clinical signs • Isolation of pathogen from aspirated joint fluid
LYMPHANGITIS	Aetiology includes: *Sporothrix schenckii* (sporothricosis), *Corynebacterium pseudotuber-culosis* (ulcerative lymphangitis), *Histoplasma farciminosum* (African farcy) or *Burkholderia mallei* (farcy)	Lameness may be present in any of these infections that cause lymphangitis, depending on the severity of the condition	• Direct microscopy • Culture • FA technique: *H. farciminosum* • Serology: farcy (CFT)

Table 69.4 Principal infectious diseases of horses—cont'd

Musculoskeletal system: horses

Disease	Agent(s)	Comments	Diagnosis
OSTEOMYELITIS	*Salmonella* serotype and other bacteria	Infection introduced by haematogenous route or by trauma. Lameness and local swelling are the major signs if limb bones are affected. Foals with polyarthritis may have osteomyelitis of the bones adjacent to the affected joints. Infection of vertebrae usually causes nervous signs	• Clinical signs and radiography • Isolation of pathogen from biopsy or tissue
TETANUS	*Clostridium tetani*	Entry of spores usually through puncture wounds in the hooves or penetrating wounds of muscle. Incubation period one to three weeks. Muscle stiffness is followed by prolapse of third eyelid, anxious expression, erect ears, dilated nostrils and an exaggerated response to normal stimuli. Later, muscle tetany increases and horse adopts a 'sawhorse' position. The tail may be deviated to one side. In a fatal infection there are tetanic convulsions and death from respiratory failure five to 10 days after first signs	• Vaccination history • Clinical signs • Gram-stained smear from wound for 'drumstick' sporing form
THRUSH OF THE FROG	*Fusobacterium necrophorum*	Degeneration of the frog with secondary bacterial infection. Predisposing factors include dirty, wet conditions and failure to clean the hooves regularly. The affected region is moist and contains a black, thick discharge with a foul odour. The condition is more common in the hind feet	• Clinical signs • Gram-stained smear of exudate
Respiratory system: horses			
ADENOVIRUS INFECTION	Equine adenovirus A (*Adenoviridae*)	Usually mild URT signs or asymptomatic in most young horses but Arab foals with severe combined immunodeficiency disease suffer a generalized disease with pneumonia that is usually fatal	• Virus isolation: nasal and ocular discharges • IN inclusions in cells from lacrimal secretions • FA: virus in tissues • Serology: VN and HAI; rising titres
AFRICAN HORSE SICKNESS	African horse sickness virus (*Orbivirus; Reoviridae*)	Severe pulmonary form: fever, pulmonary oedema, hydrothorax with frothy fluid from nares in moribund horses. Mortality is over 95%	• Virus isolation: TC or i/c inoculation of two- to six-day-old mice • RT-PCR • Serology: VN, ELISA, CFT, AGID

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Respiratory system: horses

Disease	Agent(s)	Comments	Diagnosis
CHRONIC OBSTRUCTIVE PULMONARY DISEASE	Spores of thermophilic actinomycete *Saccharopolyspora rectivirgula* and dust mites in mouldy hay (hypersensitivity reaction)	Poor performance of thoroughbreds at exercise. There are degrees of coughing, dyspnoea and double respiratory effort	• Intradermal skin tests: test positive for dust mites and/or *Saccharopolyspora rectivirgula*
EQUINE INFECTIOUS ANAEMIA (EIA)	Equine infectious anaemia virus (*Lentivirus; Retroviridae*)	In acute primary infections there are fever, ocular and nasal discharges, subcutaneous oedema of ventral abdomen and legs, widespread serosal and mucosal haemorrhages, splenomegaly and hepatomegaly. Relapses are common but intermittent and last three to five days with fever, depression, anaemia, and ventral oedema. Emaciation and incoordination are progressive	• Histopathology • Serology: Coggin's AGID or ELISA • Ag detection in leukocytes by FA • PCR (proviral DNA) and RT-PCR
EQUINE INFLUENZA ('THE COUGH')	*Influenza A virus* (A/equi/1/H7N7 and A/equi/2/H3N8) (*Influenzavirus A; Orthomyxoviridae*)	Usually seen in young horses under two years of age. Short incubation period of one to three days. Characterized by a persistent, strong, dry cough, nasal discharge, fever and weakness. In severe cases the virus causes myocarditis and pneumonia. A/equi/2 is the more pneumotropic of the two subtypes	• Virus isolation: nasopharyngeal swab in first few days of illness (transport medium essential) • RT-PCR • Serology: HAI, VN. Paired sera for four-fold rise in Ab titre
EQUINE VIRAL ARTERITIS (EVA)	Equine arteritis virus (*Arterivirus; Arteriviridae*)	The virus has a predilection for arteries. Fever, respiratory signs, oedema of legs and leucopenia occur. Abortion in 50–80% of mares is concurrent with clinical signs	• Histopathology: lesions in arteries • FA: frozen sections • Virus isolation from nasal cavity and conjunctival sac • RT-PCR • Serology: CFT, VN, AGID, ELISA and IFA
EQUINE RHINOPNEUMONITIS	Equine herpesvirus 4, occasionally equine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Latent carrier state often exists with infection inapparent in older horses. Respiratory disease occurs in foals and young adults. Mares may abort one to four months after an inapparent infection	• Virus isolation early in disease • PCR: capable of differentiating EHV-4 from EHV-1 • Serology: CFT, VN, ELISA

Table 69.4 Principal infectious diseases of horses—cont'd

Respiratory system: horses

Disease	Agent(s)	Comments	Diagnosis
GLANDERS (FARCY)	*Burkholderia mallei*	Glanders has been eradicated from many countries and is now comparatively rare. It is a highly contagious disease of equidae although other animal species and man are susceptible. An acute form can occur with fever, respiratory signs, septicaemia and death in a few days. The chronic pulmonary form is a debilitating disease with tubercle-like nodules in the nasal cavity or lungs. Farcy is the chronic cutaneous form with ulcerating nodules along lymphatic vessels	• Mallein test • Isolation and identification of *B. mallei* • Serology: CFT, indirect HAI
GUTTURAL POUCH INFECTION	*Aspergillus fumigatus* and/or Group C streptococci	Nasal discharge, swelling or displacement of parotid gland, dysphagia and profuse epistaxis unrelated to exercise and occasionally unilateral facial paralysis	• Endoscopic examination: fungal plaques • Radiography: fluid in pouch • Biopsy: isolation and identification of pathogen
NASAL POLYPS OR NASAL GRANULOMAS	Usually caused by one of the following: *Aspergillus fumigatus, Cryptococcus neoformans, Rhinosporidium seeberi* or *Conidibolus coronatus*	Lesions cause interference with breathing and a nasal discharge, often unilateral, occurs	• Demonstration of fungal elements microscopically and culturally from biopsy
PNEUMONIA (BACTERIAL)	Group C streptococci, *Actinobacillus equuli* or *Bordetella bronchiseptica*	Predisposing causes include a comparatively mild viral respiratory infection and/or stress. A secondary bacterial pneumonia can often be severe or fatal	• Isolation and identification of secondary invaders
RESPIRATORY INFECTIONS (MINOR VIRAL)	Equine rhinitis A and B viruses (*Picornaviridae*); Equine herpesvirus 2 (*Herpesviridae*)	Mild URT infection in young horses with nasal discharge and cough unless complicated by secondary bacterial invaders	• Isolation of viruses • PCR and RT-PCR • Serology
STRANGLES	*Streptococcus equi* subsp. *equi*	Young horses are at greatest risk. There is fever, cough, purulent oculonasal discharge and abscesses (especially in submaxillary and pharyngeal lymph nodes). In bastard strangles, abscesses can develop in lungs and other body organs	• Isolation and identification of *S. equi.* subsp. *equi* • PCR

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Disease	Agent(s)	Comments	Diagnosis
Respiratory system: horses			
SUPPURATIVE BRONCHOPNEUMONIA	*Rhodococcus equi*	Foals one to four months old are affected. Infection is usually by inhalation leading to bronchopneumonia	• Radiography • Isolation and identification of *R. equi* from transtracheal aspirates or BAL • PCR
Skin: horses			
CONTAGIOUS ACNE OF HORSES	*Corynebacterium pseudotuberculosis*	Uncommon. Development of pustules (1.0–2.5 cm) particularly in harness area. Pustules rupture and exude greenish pus and a crust forms. There is no pruritis but lesions can be painful to the touch. Healing in one week but there may be successive crops of pustules. Transmission via harness and grooming gear	• Gram-stained smear from pus • Culture • PCR
DERMATOPHILOSIS (STREPTOTHRICOSIS)	*Dermatophilus congolensis*	Exudative dermatitis with extensive scab formation. Clumps of hairs come away with scab leaving an ovoid bleeding surface. Affected area of skin is rough and lumpy	• Gram- or Giemsa-stained smears from scab • Culture if necessary under 10% CO_2 • PCR
EQUINE COITAL EXANTHEMA	Equine herpesvirus 3 (*Varicellovirus; Herpesviridae*)	Acute but mild disease characterized by pustular and ulcerative lesions on vaginal mucosa and perineal region in mares and on penis and prepuce of stallions. Healing occurs in about 14 days with white depigmented spots marking the site of lesions, that are particularly noticeable around vulval area in mares. The spots remain for life and identify potential carriers. It is a venereal disease and lesions are seen four to eight days after coitus. No systemic signs or abortions occur	• EM • Virus isolation • PCR
EQUINE PAPILLOMATOSIS (EQUINE WARTS)	Equine papillomavirus (*Zetapapillomavirus; Papillomaviridae*)	Warts or papillomas are usually seen in young horses under three years of age. They are self-limiting and will regress in a few months. Warts are comparatively small. Individual growths often around the lips or on the face	• Histopathology • EM
EQUINE SARCOID	Papillomavirus (*Papillomaviridae*)	All ages are susceptible. Sarcoids can persist for life, do not regress, can become large and tend to have a multiple base	• History and signs • Histopathology: biopsy
GREASY HEEL (SEBORRHEA)	Attributed to a poxvirus. Parasites (chorioptic mange) or secondary bacteria may complicate the infection	Often on hind legs of horses and predisposed to by unsanitary conditions but can occur in well-managed horses. Thickening and greasiness of skin on back of pastern to coronary band. It can spread if not treated	• Gram-stained smear • Examination for mange mites • Culture for secondary bacteria

Table 69.4 Principal infectious diseases of horses—cont'd

Skin: horses

Disease	Agent(s)	Comments	Diagnosis
HORSEPOX	Vaccinia virus, Uasin Gishu disease virus *(Orthopoxvirus; Poxviridae)*	Historically horsepox was associated with vaccinia virus infections acquired from recently vaccinated humans and took two forms: **'Grease' form:** pustules and scabs on back of pastern with pain, lameness and a slight systemic reaction **Buccal form:** pustules on inside of lips that can spread over entire buccal mucosa In Africa a type of horsepox called Uasin Gishu disease has been known for many years	• Electron microscopy
LYMPHANGITIS			
Ulcerative lymphangitis	*Corynebacterium pseudotuberculosis*	Infection of skin wounds occurs with invasion of lymphatic vessels and abscesses along their course. Lesions on lower limbs with minimal lymph node involvement. Abscesses exude greenish pus and ulcerate. Heal in one to two weeks but fresh crops can occur for up to 12 months	• Direct microscopy (exudate): pleomorphic Gram +ve rods • Culture and identification • PCR
Sporotrichosis	*Sporothrix schenckii*	Fungus ubiquitous but infections are sporadic. Raised cutaneous nodules occur along the lymphatics of lower limbs. Nodules ulcerate and discharge pale yellow pus. Infection mild and localized	• Direct microscopy (exudate): cigar-shaped yeast • Culture: dimorphic fungus
Farcy	*Burkholderia mallei*	Now a rare condition. Subcutaneous nodules occur along lymphatics that ulcerate and discharge a sticky, honey-like pus. Ulcers heal in star-shaped scars and the lymphatics are fibrous and thickened. Lesions often present in hock area but can extend over body. Local lymph nodes are involved, there is debility and pneumonic lesions may also be present	• Direct microscopy (exudate): Gram –ve rods • Culture • Serology: CFT
Epizootic Lymphangitis (African Farcy)	*Histoplasma farciminosum*	Moveable nodules along superficial lymph vessels. The nodules ulcerate and discharge a thick, yellow, oily pus. There is cording of lymphatics and the local lymph nodes are swollen and hard. Lesions may also occur in lungs and nasal mucosa. It is considered a chronic and incurable disease	• Direct microscopy (exudate): oval, double-contoured yeast • FA technique
RINGWORM	*Microsporum gypseum, Trichophyton equinum* or *Microsporum canis*	Lesions (3 cm) with raised hair, alopecia and fine scab. May be sore to the touch and itchy. Regrowth of hair often with loss of pigmentation in 25–30 days *M. gypseum* (from soil): back and sides of horse *T. equinum* (from harness and grooming gear): in girth strap area or widespread over body *M. canis*: stable cats may be affected	• Direct microscopy on scab and hairs in 10% KOH for arthrospores • Culture on Sabouraud agar • Identification: colonial morphology and macroconidia

Continued

Table 69.4 Principal infectious diseases of horses—cont'd

Skin: horses

Disease	Agent(s)	Comments	Diagnosis
STAPHYLOCOCCAL DERMATITIS	*Staphylococcus aureus,* occasionally *Staphylococcus intermedius*	Quite common but sporadic. Lesions often under harness suggesting transmission by contact with contaminated harness. Small (5 mm) nodules then pustules appear that are painful to the touch and horse may not tolerate the harness	• Gram-stained smear • Culture
VESICULAR STOMATITIS	*Vesiculovirus* (*Rhabdoviridae*)	Disease once common in US military horses but now mostly seen in cattle and pigs. Vesicular lesions in horses usually on lips and tongue but can occur on udder of mares and prepuce of stallions. Vesicles rupture with rapid healing	• Demonstration of viral Ag in vesicular fluid by CFT or ELISA • Virus isolation • RT-PCR • Serology: CFT for rise in Ab titre

Table 69.5 Principal infectious diseases of dogs

Buccal cavity: dogs

Disease	Agent(s)	Comments	Diagnosis
CANINE ORAL PAPILLOMATOSIS	*Canine oral papillomavirus (Lambdapapilloma-virus; Papillomaviridae*)	Usually occurs in dogs under one year old. The condition can spread to all young dogs in kennels. Warts first appear on the lips but extend into the buccal cavity. Spread of lesions occurs for four to six weeks then there is spontaneous regression over several months	• Clinical signs • Histopathology: biopsy
LEPTOSPIROSIS (CANINE)	*Leptospira interrogans* (many serovars, Canicola and Icterohaemorrhagiae becoming less common owing to vaccination)	In the icteric form, oral mucous membranes may first show irregular haemorrhagic patches which later become dry and necrotic and slough in sections. A tenacious salivary secretion is present around the gums, at times blood-tinged	• Clinical signs • Darkfield microscopy on urine • PCR • Culture from urine difficult • Serology: agglutination tests, ELISA
MYCOTIC STOMATITIS	*Candida albicans*	The condition often follows prolonged antibiotic therapy in pups. It is a specific type of ulcerative stomatitis characterized by ulcers and soft, white, slightly elevated patches on the oral mucosa. The periphery is reddened and the lesions may coalesce	• History of antibiotic therapy • Clinical signs • Isolation of pathogen
TONSILLITIS	Beta-haemolytic streptococci	Comparatively common in dogs and may occur as a primary disease or secondary to infections in buccal cavity or pharynx. There may be fever, a short, soft cough followed by retching and expulsion of small amounts of mucus	• Clinical signs • Isolation of pathogen
ULCERO-MEMBRANOUS STOMATITIS (TRENCH MOUTH)	Commensals in mouth: spirochaetes and fusiform bacteria	The condition is usually secondary to other infections or deficiency diseases. At first there is reddening and swelling of gingival margins, that bleed easily. The lesions progress to ulceration and necrosis of gingivo-alveolar tissues with the formation of pseudomembranes	• History of predisposing causes • Clinical signs • Gram of DCF-smear of affected tissue
Gastrointestinal tract: dogs			
CANINE CORONAVIRUS INFECTION	Canine coronavirus (*Alphacoronavirus; Coronaviridae*)	Highly contagious enteric disease of dogs marked by vomiting and diarrhoea. Dogs, foxes and coyotes are susceptible. The virus replicates in cats without clinical signs. Faecal–oral transmission. Dogs can excrete the virus for over two weeks. Signs similar to, but milder than, canine parvovirus infection. There is no leukopenia, faeces are liquid, may contain blood and mucus and have a foetid odour. Atrophy of villi with deepening of crypts occurs	• EM: faeces • Virus isolation • RT-PCR • Serology: VN, IFA

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Gastrointestinal tract: dogs

Disease	Agent(s)	Comments	Diagnosis
CANINE HAEMORRHAGIC GASTROENTERITIS	Aetiology uncertain, enteropathogenic *Escherichia coli*, *Clostridium perfringens* or *Clostridium difficile* may be involved	Toy breeds particularly susceptible. An acute onset of intestinal haemorrhage occurs in mature dogs with collapse, bloody diarrhoea, rapid course and death in untreated cases. Elevated packed cell volume (over 70% in severe cases)	• Clinical findings • Haematology: elevated packed cell volume • Haemoglobin concentration • PCR for pathogens
CANINE HERPESVIRUS INFECTION IN PUPS	Canine herpesvirus 1 (*Varicellovirus*; *Herpesviridae*)	Acute generalized disease and death in neonates less than one week old. Soft yellow-green faeces, abdominal pain, anorexia and incessant crying occur. Pups are infected in utero, via birth canal or by aerosol after birth. Usually the whole litter is affected. Post mortem findings include haemorrhages in kidneys, liver and other organs. Bitch only aborts or has an affected litter once, as immunity is acquired. Venereal transmission may occur	• Pathology: IN inclusions in lungs, liver and kidneys • FA: frozen sections • Virus isolation • PCR • Serology: VN, IFA
CANINE HISTOPLASMOSIS	*Histoplasma capsulatum*	Sporadic occurrence worldwide and endemic in central USA. Chronic respiratory disease (lungs primary site) and ulceration of intestines with diarrhoea. The disease may be suspected in dogs with intractable cough, diarrhoea, emaciation and a lack of response to antibiotics	• Histopathology: small yeast in macrophages • Culture at 25° and 37°C • Serology: CFT
CANINE PARVOVIRUS INFECTION	Canine parvovirus (*Parvovirus; Parvoviridae*)	Stable virus spread in minute particles of faeces on fomites such as human shoes. There is no extended carrier state but virus is excreted in faeces for five to eight weeks after illness. Targets are lymphoid tissue (immunodepression), bone marrow (leukopenia) and small intestine (necrosis + secondary Gram –ve bacterial overgrowth giving diarrhoea, vomiting and endotoxaemia). Infection in utero or in pups under two weeks old can lead to generalized disease and death. Pups infected when three to eight weeks old suffer myocarditis (rare now as immunity has built up in dog population)	• EM on faeces in acute phase • Haematology: leucopenia • Histopathology • Virus isolation • Detection of viral antigen in faeces or tissues: FA, ELISA, HA • PCR • Serology: HAI, VN, ELISA

Table 69.5 Principal infectious diseases of dogs—cont'd

Gastrointestinal tract: dogs

Disease	Agent(s)	Comments	Diagnosis
COLITIS (BACTERIAL)	*Salmonella* serotypes or *Campylobacter jejuni* may be involved	**Acute:** vomiting, haemorrhagic mucoid diarrhoea causing dehydration **Chronic:** slow onset with tenesmus and scant watery blood-stained, mucoid faeces. Insidious weight loss, anaemia and a gaunt-look. Appetite is normal. Colon thick-walled, rubbery and lumen narrow. Mesenteric lymph nodes enlarged and firm	• Lab examinations for bacteria or parasites • Histopathology on colon biopsy • Differentiate from parasites or foreign bodies
ENTERIC CAMPYLOBACTERIOSIS	*Campylobacter jejuni*	Causes severe diarrhoea in young animals and is most common in urban dogs. Faeces are watery, contain mucus and can be bile-streaked. Dog may be febrile, partially anorexic and occasional vomiting may occur. Diarrhoea usually lasts three to seven days but intermittent diarrhoea may last two weeks to two months	• Isolation from faeces • Phase contrast on fresh faeces: large numbers of motile bacteria in acute phase of disease • PCR
ENTERITIS	Feature of: canine distemper, infectious canine hepatitis, canine parvovirus infection, canine cornavirus infection, salmonellosis, *Escherichia coli* infections in neonates	Diarrhoea is the outstanding sign. Can be accompanied by vomiting when anterior duodenum or stomach is involved and by tenesmus when inflammation extends into the colon. Faeces are liquid, foul-smelling and may be dark-green or black (bleeding in small intestine) or blood-streaked (haemorrhage in large intestine). Fever occurs if the cause is an infectious agent. Abdominal pain is a feature and dogs may stretch out on a cool floor or adopt a praying position	• Clinical examination • Lab examination for bacteria • Differentiate from protozoan infections, poisons (especially heavy metals) or helminths
PROTOTHECOSIS	*Prototheca zopfii, P. wickerhamii* (colourless algae)	Protothecosis in the dog is usually a widely disseminated disease. Bloody diarrhoea (intermittent or protracted) is the most common syndrome with weight loss and debility. CNS involvement (about 40% of cases) can lead to depression, ataxia, circling or paresis. Eye involvement is common. Chronic skin lesions, characterized by ulcers with crusty exudates on trunk or extremities are seen in some cases	• Histopathology (PAS or silver stains) • Direct microscopy (CSF, urine) • Culture and identification

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Disease	Agent(s)	Comments	Diagnosis
Gastrointestinal tract: dogs			
SALMON POISONING	*Neorickettsia helminthoeca*	The disease occurs along the Californian and Alaskan coasts. Rickettsiae are transmitted through various stages of a fluke in a snail–fish–dog cycle. The dog becomes infected by ingestion of salmon containing encysted metacercariae. Signs occur five to nine days after ingestion and last seven to 10 days before death (90% of untreated cases). Fever, depression, anorexia, vomiting, bloody diarrhoea, dehydration, weight loss, lymphadenopathy, nasal and conjunctival discharge occur and the disease can resemble canine distemper	• Fluke ova in faeces is suggestive • Giemsa-stained smears to visualize intracellular rickettsiae in aspirates from lymph nodes • PCR
Liver: dogs			
INFECTIOUS CANINE HEPATITIS	Canine adenovirus 1 (*Mastadenovirus; Adenoviridae*)	Common only in unvaccinated populations of dogs. Pups are most susceptible shortly after weaning and can become moribund within a few hours of first clinical signs. In the acute hepatic form there is severe hepatitis, oedema of the gallbladder, multifocal vasculitis and haemorrhage. All *Canidae* are susceptible. CNS signs are not common except in the fox (fox encephalitis). Glomerulonephritis is a possible sequel	• Clinical signs • Pathology: IN inclusions in many tissues • FA on frozen sections • Virus isolation • PCR • Serology: VN, HAI
LEPTOSPIROSIS (CANINE)	*Leptospira interrogans* (many serovars, Canicola and Icterohaemorrhagiae becoming less common owing to vaccination)	The pathogens replicate in liver and kidneys where they produce degenerative changes. Icterus occurs due to liver damage and destruction of red cells. Serovar Icterohaemorrhagiae often causes the acute haemorrhagic or icteric forms and Canicola is associated with the icteric and uraemic syndromes	• Darkfield microscopy on recently collected urine • PCR • Isolation from urine or kidneys is difficult • Serology: agglutination tests, ELISA
Genital system: dogs			
BALANOPOSTHITIS	Miscellaneous bacteria	Comparatively common in dogs as the preputial cavity offers an ideal environment for bacterial growth. Mucopurulent preputial discharge is seen but systemic signs only occur if deeper tissues of glans penis are invaded	• Bacterial culture and identification

Table 69.5 Principal infectious diseases of dogs—cont'd

Genital system: dogs

Disease	Agent(s)	Comments	Diagnosis
BRUCELLOSIS (CANINE)	*Brucella canis* (*B. abortus, B. suis* or *B. melitensis* rarely)	Epidemics of abortion can occur in breeding kennels. Transmission: congenital, venereal and by ingestion. Abortions in last trimester, stillbirths or infertility are observed. Prolonged discharge often follows abortion and males may be sterile following an infection	• MZN-stained smears on aborted foetuses, discharge or semen • Culture for brucellae • PCR • Serology: agglutination test
CANINE HERPESVIRUS INFECTION	Canine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Transmission: in utero, via birth canal, contact with saliva, nasal secretions or urine and venereal. • Bitch: occasional abortions, stillbirths or vesicular vaginitis • Generalized disease in neonatal pups and death within 24 hours: one cause of 'fading puppy syndrome'	• Pathology in pups: focal necrosis and petechiation occur in most organs. IN inclusions in lung, liver and kidney lesions • Virus isolation • PCR • FA: frozen sections • Serology: VN, IFA
CANINE PARVOVIRUS INFECTION	Canine parvovirus (*Parvovirus; Parvoviridae*)	Parvoviruses are known to cross the placental barrier. As modified live virus vaccine causes a viraemia it is unwise to use modified live vaccines in pregnant bitches as this might result in foetal infection or abortion	• History of vaccination of pregnant bitch • Virus isolation • PCR • Serology: HAI, VN, ELISA
'FADING PUPPY SYNDROME'	Canine herpesvirus 1 (CHV 1) Bacterial septicaemias often caused by *Escherichia coli* and streptococci	The pups usually appear normal at birth but may acquire infection from dam's vagina or the environment soon after birth. There are sequential deaths in the litter, usually within one week of parturition	• Clinical signs • Histopathology • Bacteria: culture from vaginal discharge and from pups • CHV 1: FA, virus isolation, PCR and serology
METRITIS	*Escherichia coli*, streptococci or staphylococci	Usually infection occurs at time of parturition. Acute illness with fever, depression, purulent and foul-smelling discharge. Bitch may neglect the pups	• Clinical signs and examination • Radiography to check for retained foetus • Guarded swab from cervix for culture of bacteria
ORCHITIS EPIDIDYMITIS	*Escherichia coli*, streptococci, staphylococci, or *Brucella canis*	Predisposing causes: trauma or concomitant posthitis or cystitis. There is a stilted gait or stance and licking of scrotum occurs. Swelling of scrotum with pain and fever are common findings	• Physical examination • Urinalysis and bacterial culture • Serology or PCR for *Brucella canis*

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Genital system: dogs

Disease	Agent(s)	Comments	Diagnosis
PROSTATIC ABSCESS	Miscellaneous bacteria	Abnormal gait or stance, pain, haematuria and sometimes bulging of the perineum can be present. Chronic peritonitis occurs if abscess drains into abdominal cavity. Abscess can be secondary to a urinary tract infection or may be blood-borne	• Rectal palpation and radiography • Rectal massage and collection of semen or exudate for culture
PYOMETRA	*Escherichia coli*, streptococci, staphylococci, *Pseudomonas* or *Proteus* spp.	Excess progesterone levels can result in endometrial growth and accumulation of secretions with subsequent bacterial infection. High progesterone levels also inhibit the leucocyte response. Exogenous oestrogen ('mismating shots') tend to stimulate progesterone and increase the risk of pyometra. Clinical signs depend on patency of cervix. Closed cervix: very serious with depression, anorexia, toxaemia, vomiting, polydipsia and polyuria. Partially open cervix: discharge continues for four to eight weeks after oestrus with less severe clinical signs	• Physical examination • Radiography • Elevated WBC count • Culture of discharge or of uterine contents if surgically removed
VAGINITIS	Bacteria such as *Escherichia coli, Brucella canis* or streptococci	Vaginal discharge occurs but usually no systemic signs. Vaginitis may have developed secondarily to conformational abnormalities or vaginal foreign bodies	• Guarded swabs from anterior of vagina for culture. Significant if heavy growth of not more than two potential pathogens

Urinary system: dogs

CANINE HERPESVIRUS IN PUPS	Canine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	One cause of the 'fading puppy syndrome': pups usually die within one to two days of first clinical signs. Focal renal haemorrhages are characteristic, although ecchymoses can also occur in other abdominal organs	• Clinical signs • Pathology: intranuclear inclusions • FA on frozen sections • Isolation of virus • PCR • Serology: VN or ELISA, IFA for a high titre in adult dogs
CATARRHAL CYSTITIS (CANINE DISTEMPER)	Canine distemper virus, *Morbillivirus (Paramyxoviridae)*	This may be associated with canine distemper. Inclusion bodies are present in cells of the urinary bladder	• Clinical signs • Histopathology for tissue changes and IBs in bladder tissue • FA on conjunctival smears or frozen tissue sections of bladder, lung and intestine • Serology: VN, IFA

Table 69.5 Principal infectious diseases of dogs—cont'd

Urinary system: dogs

Disease	Agent(s)	Comments	Diagnosis
CYSTITIS AND URETHRITIS	*Escherichia coli*, staphylococci, *Proteus* species, *Klebsiella pneumoniae*	Frequent urination, haematuria and dysuria occur. Bladder may be thickened and tender when palpated. There may be a predisposing cause if recurring infections occur	• Clinical signs • Direct microscopy on urine collected by cystocentesis or midstream urine. Gram-stained smear on one drop of urine. One bacterial cell or more per oil-immersion field is suggestive of a bacteriuria • Bacterial count on urine. Clinical bacteriuria suggested by 10^5 bacteria/mL or more • Isolation of pathogen from urine
INFECTIOUS CANINE HEPATITIS	Canine adenovirus 1 (*Mastadenovirus*; *Adenoviridae*)	Immune-complex glomerulonephritis can occur in the chronic phase of the disease. Prolonged shedding of virus in urine can occur	• History and clinical signs • Histopathology for tissue changes and IBs in liver and kidney • FA technique on frozen tissues • Serology: HAI
LEPTOSPIROSIS (CANINE)	*Leptospira interrogans*, many serovars, Canicola and Icterohaemorrhagiae becoming less common owing to vaccination	Acute or chronic interstitial nephritis may be present. A chronic condition with progressive renal failure and uraemia can occur one to three years after an acute episode of disease	• Darkfield microscopy and FA technique on urinary deposits • PCR • Isolation of leptospires from urine is difficult • Histopathology if death occurs • Serology: agglutination tests, ELISA
PYELONEPHRITIS	*Escherichia coli*, staphylococci, *Proteus* species, *Klebsiella pneumoniae*	Systemic signs such as fever, anorexia, depression and vomiting may be present. Kidneys are painful when palpated. Chronic disease may lead to uraemia	• Investigation as for cystitis or urethritis

Eyes and ears: dogs

BLINDNESS			• Diagnosis of the generalized infection
Canine distemper	*Morbillivirus (Paramyxoviridae)*	Rarely retinal or optic nerve damage	
Protothecosis	*Prototheca zopfii* *P. wickerhamii*	Rare disseminated algal infection but blindness occurs in over 50% of cases	
Toxoplasmosis	*Toxoplasma gondii*	Blindness (retinochoroiditis) has been recorded in some infections	
Cryptococcosis	*Cryptococcus neoformans*	Eye involvement leading to chorioretinitis occurs but is more common in the cat	

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Disease	Agent(s)	Comments	Diagnosis
Eyes and ears: dogs			
'BLUE-EYE' (INFECTIOUS CANINE HEPATITIS)	Canine adenovirus 1 (sometimes canine adenovirus 2 (*Mastadenovirus, Adenoviridae*))	Transient opacity of the cornea can occur in the acute disease, as the only sign in subclinical disease and after vaccination with attenuated vaccine. It is an immune complex phenomenon resulting in oedema and anterior uveitis. Afghan hounds are particularly susceptible to blue eye. The sequel can be glaucoma. CAV 2 virus can also cause blue eye but rarely	• Clinical signs and history of disease or vaccination • Serology: HAI, VN
CONJUNCTIVITIS (NON-SPECIFIC)	Staphylococci, streptococci, *Bordetella bronchiseptica*	Predisposing causes include irritants, allergens or a concurrent viral infection such as canine distemper or 'kennel cough'. Purulent discharge indicates a secondary bacterial component	• Clinical signs • Isolation of a potential pathogen in heavy, pure, culture
OTITIS EXTERNA	One or more of the following are present: Staphylococci, Streptococci, *Proteus* spp., *Pseudomonas aeruginosa, Aspergillus fumigatus, Malassezia pachydermatis*	Predisposing causes include faulty drainage (pendulous ears), foreign bodies, ear mites (*Otodectes cynotis*) or polyps in ear canal, constant wetting or hot, humid weather. Signs include violent shaking of head, scratching and rubbing ears, often with haematoma formation in pinna, dark purulent discharge and swelling and inflammation of the mucosa of ear canal	• Clinical signs • Isolation of one or more pathogens from exudates • Examination for ear mites and foreign bodies
OTITIS MEDIA AND OTITIS INTERNA	Non-specific, similar to aetiology of otitis externa	An inflammation of tympanic cavity as an extension of otitis externa or due to penetration of ear drum by a foreign body. Occurs in all species but most common in dog, cat, rabbit and pig. Otitis media can lead to otitis interna and may result in ataxia and deafness	• Clinical signs • Radiography (sclerotic changes in bone of tympanic bulla) • Isolation of pathogens from discharge
Nervous system: dogs			
ASPERGILLOSIS	*Aspergillus fumigatus*	In untreated nasal granulomas, *A. fumigatus* can erode cribriform plate and head tilt, ataxia or seizures may occur	Histopathology and culture on biopsy or tissues after death
BOTULISM	*Clostridium botulinum*	Natural botulism is rare in dogs. Incubation period varies from hours to six days. There is progressive symmetrical ascending weakness from rear to forelimbs although the tail-wag is maintained. Eventually quadriplegia occurs. There is normal pain response but lack of withdrawal reflex. Pupillary dilation is characteristic. A diminished jaw tone is present. The course in dogs that recover is 24 hours to 14 days	• Demonstration of toxin in dog's serum (mouse neutralization test) or in suspect food (ELISA)

Table 69.5 Principal infectious diseases of dogs—cont'd

Nervous system: dogs

Disease	Agent(s)	Comments	Diagnosis
CANINE DISTEMPER	*Canine distemper virus (Morbillivirus; Paramyxoviridae)*	CNS signs seen one to three weeks after recovery from the generalized disease but also after mild ('kennel cough' syndrome) or inapparent infections. Signs include one or more of the following: myoclonus, ataxia, hyperaesthesia, cervical rigidity and seizures: petit mal ('chewing gum' syndrome) or grand mal convulsions	• Histopathology • FA technique for Ag in brain • History: typical distemper signs? • PCR • Serology: VN, ELISA, IFA
CANINE EHRLICHIOSIS	*Ehrlichia canis*	• **Acute**: fever, enlarged spleen and lymph nodes, mucopurulent nasal discharge and loss of stamina. CNS signs referable to inflammation and bleeding into meninges and include hyperaesthesia, twitching of muscles and cranial nerve deficits. Most animals recover • **Chronic: Tropical canine pancytopenia;** an immunologically mediated sequel to the acute form. Mortality high and epistaxis, petechiation of skin and mucous membranes, haematuria, wasting, cerebellar ataxia, glomerulonephritis and renal failure occur Vector: *Rhipicephalus sanguineus*	• Giemsa or FA on blood smears for *E. canis* (present in monocytes) • PCR • Haematology • Serology: IFA
CANINE HERPESVIRUS INFECTION	Canine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	• Adult dogs: mild respiratory signs or subclinical. Venereal transmission can occur with minor vesicular lesions on genitalia. After infection the bitch develops an immunity so only the initial litter is affected • Neonates: infected in utero or shortly after birth (ingestion or inhalation). 'Fading puppy syndrome' (pups less than two weeks old): dullness, depression, incessant crying, soft faeces and abdominal pain. Terminal opisthotonos and seizures can occur but are not always apparent. A meningoencephalitis is present. The few pups that survive may show persistent CNS signs such as blindness and ataxia	• Virus isolation from adrenals, kidneys of pups or external genitalia of adults • FA: frozen sections • PCR • Histopathology • Serology: VN, IFA

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Nervous system: dogs

Disease	Agent(s)	Comments	Diagnosis
CRYPTOCOCCOSIS	*Cryptococcus neoformans*	CNS and eyes are most commonly affected in dog. Head-tilt, nystagmus, facial paralysis, ataxia, circling, neck pain, dilated pupils, seizures, and general paresis can occur. Skin lesions are seen in about 25% of cases. In some areas pigeon faeces can be a source of the yeast	• Gram or culture on CSF, exudates, aqueous or vitreous humour of eye • Histopathology
MENINGITIS OR BRAIN ABSCESSES	*Staphylococcus pseudintermedius*, streptococci, *Pasteurella multocida, Nocardia asteroides*, disseminated fungal pathogens	Entry of pathogens: • Haematogenous: may enter subarachnoid space from extracranial foci. The outcome depends on virulence and underlying damage to CNS defences • Local: from paranasal sinuses or tympanic bullae. Signs depend on site and severity of infection. Seizures can be caused by excessively high fever, forebrain oedema and increased intracranial pressure	• CSF evaluation: cytology biochemistry; Gram-stained smears; culture • Histopathology
OLD-DOG ENCEPHALITIS	Associated with canine distemper virus but pathophysiology is uncertain	Occurs in older adult dogs. There may not be a history of previous generalized canine distemper. Marked by encephalitic signs of ataxia, compulsive movements such as head-pressing, or continual pacing and an incoordinated high-stepping gait. Canine distemper viral antigen can be demonstrated in brain by FA technique. Convulsions and neuromuscular twitching do not seem to occur in the old-dog encephalitis syndrome	• History: canine distemper earlier in life • FA technique on brain tissue for Ag
PROTOTHECOSIS	*Prototheca zopfii* or *P. wickerhamii* (colourless algae)	Usually a disseminated disease with bloody diarrhoea, weight loss, debility and blindness. Internal lesions are widespread as granular foci. In about 40% of the generalized cases nervous signs are seen with ataxia, incoordination or paresis. Necrotic foci occur in the brain with numerous protothecal cells present in the lesions	• Clinical signs: bloody diarrhoea and blindness • Histopathology: *Prototheca* spp. present in lesions • Culture: will grow on agar media
PSEUDORABIES	Porcine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Disease hyperacute and fatal in dogs, with a course of 48 hours or less. Intense pruritis most commonly occurs in the head region and self-mutilation results. Signs include hypersalivation, hyperaesthesia, convulsions, changes in behaviour and deficits in cranial nerve function such as head tilt, paresis of facial muscles and voice change	• Virus isolation from brain or spleen (not always successful in dogs) • FA on brain stem • PCR • History of association with pigs or eating raw offal • Serology: VN

Table 69.5 Principal infectious diseases of dogs—cont'd

Nervous system: dogs

Disease	Agent(s)	Comments	Diagnosis
RABIES	Rabies virus (*Lyssavirus; Rhabdoviridae*)	Incubation period averages two to 12 weeks but can be up to six months. Virus is excreted in saliva one to 14 days before clinical signs appear. Stages of disease are: • Prodromal (two to three days): changes in temperament • Furious stage (one to seven days): dogs are aggressive, hyperactive and lose all fear of humans • Paralytic (dumb) stage within 10 days of first signs: dropped jaw, excessive salivation, choking noises and finally, generalized paralysis, coma and death	• Clinical signs • FA on brain tissue • Histopathology: Negri bodies in brain • RT-PCR • Serology: VN, ELISA
TETANUS	*Clostridium tetani*	Although tetanus occurs in dogs and cats they are comparatively resistant to toxin with incubation period of up to three weeks. Localized tetanus can occur with stiffness of a muscle or of a limb. This may progress to generalized tetanus. Dogs tend to have a worried facial expression, erect ears and show stiffness when walking. Mild stimuli can lead to tetanic spasms and these continue until death occurs from respiratory arrest. *C. tetani* spores enter via wounds or following surgery	• History of wound or surgery • Differentiate from strychnine poisoning • Gram-stained smear of necrotic tissue if wound is present
TOXOPLASMOSIS	*Toxoplasma gondii*	Disseminated toxoplasmosis can occur in normal dogs that ingest high numbers of oocysts or bradyzoites or in neonates and immunocompromised animals. There is an affinity for lymphoid tissue, lungs, liver, heart and skeletal muscle. Fever, jaundice and muscle stiffness (myositis) are seen. CNS signs occur in chronic postnatal infections with cerebral inflammation, eye lesions, hind leg paresis and cerebellar ataxia. The disease is often associated with canine distemper due to immunosuppression by the virus	• Histopathology • Serology: IHA, IFA, ELISA
Musculoskeletal system: dogs			
ARTHRITIS	Staphylococci, streptococci	Non-specific infection of one or more joints, either following a bacteraemia or traumatic injury	• Clinical signs • Culture of joint aspirates

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Musculoskeletal system: dogs

Disease	Agent(s)	Comments	Diagnosis
COCCIDIOIDOMYCOSIS	*Coccidioides immitis*	Primary lesions in dogs are invariably in lungs. Dogs with disseminated disease have a chronic cough, cachexia, lameness, enlarged joints and fever. The fungus can cause an osteomyelitis	• History of visit to endemic area • Radiography • Clinical signs • Direct microscopy on aspirates or exudates for spherules • Culture should only be attempted using a biohazard cabinet
LYME DISEASE	*Borrelia burgdorferi* sensu lato	Fever and arthritis involving the limb joints are the main signs. Lameness is recurrent and may progress to a chronic arthritis. Vector: *Ixodes* ticks	• History: endemic area • Clinical signs • Serology: ELISA or IFA • Demonstration of pathogen in joint fluid by PCR or culture (difficult)
NASAL GRANULOMA	*Aspergillus fumigatus*	Lesion usually in region of ventral maxilloturbinate bone. Unilateral nasal discharge, serous to mucopurulent and epistaxis occurs. The mucosa and underlying bone may be necrotic with loss of bone definition in radiographs	• Radiography • Clinical signs • Culture from biopsy
OSTEOMYELITIS/ DISCOSPONDYLITIS	Aetiology includes: *Staphylococcus pseudintermedius, Brucella canis, Nocardia* spp. and streptococci	Comparatively common condition in young to middle-aged adult dogs, especially males and those of heavy breeds. Condition is often due to blood-borne bacterial emboli. Signs range from hyperaesthesia to severe paresis/paralysis	• Clinical signs • Radiography • Culture of bacterial pathogen
ROCKY MOUNTAIN SPOTTED FEVER	*Rickettsia rickettsii*	Acute illness with high fever, lymphadenopathy, oedema of limbs and sheath and scrotum of males. Haemorrhages may be seen in ocular, oral, genital mucosae and on non-pigmented skin. Vector: ticks	• History (endemic area) • Clinical signs • FA on skin biopsy • PCR • Serology: IFA, ELISA
TETANUS	*Clostridium tetani*	Localized (ascending) tetanus can occur in dogs with stiffness in one or more limbs. This may progress to generalized tetanus with a stiff gait, erect ears, worried expression and tetanic convulsions	• History of a wound or surgery • Differentiate from strychnine poisoning • Gram-stained smear of necrotic material if a wound is present
***TOXOPLASMA* MYOSITIS**	*Toxoplasma gondii*	A condition that can occur in neonatal pups. There is muscle wasting, stiffness of limbs and progressive paresis or tetraparesis	• Clinical signs • Serological tests: IHA, IFA, ELISA

Table 69.5 Principal infectious diseases of dogs—cont'd

Respiratory system: dogs

Disease	Agent(s)	Comments	Diagnosis
ACTINOMYCOSIS (CANINE)	*Actinomyces viscosus*	Clinical signs and lesions indistinguishable from canine nocardiosis. Granulomatous skin lesions and/or bloody fluid and granulomas in thoracic cavity	• Gram and MZN direct smears (*A. viscosus* MZN–ve and *N. asteroides* MZN+ve) • Culture • Histopathology
CANINE DISTEMPER	*Canine distemper virus (Morbillivirus; Paramyxoviridae)*	Strains vary greatly in virulence. Clinical signs reflect the predilection sites: respiratory tract, intestinal tract, CNS and skin. *B. bronchiseptica* often responsible for purulent oculonasal discharges	• Intracytoplasmic IBs in smears from conjunctiva • Pathology • FA: impression smears from urinary bladder epithelium, cerebellum or lymph nodes • RT-PCR • Serology: VN, IFA, ELISA (rising titres)
CHRONIC RHINITIS	*Aspergillus fumigatus*	Chronic nasal discharge (often unilateral), sneezing and possibly epistaxis	• Radiography: turbinate bone destruction • Endoscopy: fungal plaques • Culture from biopsy, not just from nasal discharge • Histopathology on biopsy
'KENNEL COUGH' (CANINE INFECTIOUS TRACHEOBRONCHITIS)	Major agents: Canine parainfluenza 2, canine distemper virus, canine adenovirus 2, *Bordetella bronchiseptica*	The condition is a mild upper respiratory tract infection that is self-limiting. Coughing usually occurs for a few days but longer if complicated (laryngotracheitis) by secondary bacterial invaders. Most commonly seen in kennels where a number of young dogs are kept together	• Virus isolation early in the disease • PCR for viruses • Culture: check on primary or secondary bacterial pathogens • Serology (rising Ab titre)
MYCOTIC INFECTIONS		Sporadic, chronic diseases where the primary site of infection, in dogs, is usually the respiratory tract. Dissemination may occur	• Direct microscopy • Histopathology: PAS stain • Culture: 25°C and 37°C for the dimorphic fungi
Cryptococcosis	*Cryptococcus neoformans*	Chronic URT signs and possible CNS involvement	• Serology
Histoplasmosis	*Histoplasma capsulatum*	Chronic respiratory and intestinal signs	
North American blastomycosis	*Blastomyces dermatitidis*	Primary respiratory involvement and dissemination to the skin	
Coccidioido-mycoses	*Coccidioides immitis*	Chronic cough and dissemination to bones and other organs	

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Respiratory system: dogs

Disease	Agent(s)	Comments	Diagnosis
NOCARDIOSIS (CANINE)	*Nocardia asteroides*	Indistinguishable from canine actinomycosis on clinical grounds. Granulomatous skin lesions and/or bloody fluid and granulomas in thoracic cavity	• Gram and MZN direct smears (*A. viscosus* MZN–/*N. asteroides* MZN+) • Culture • Histopathology
INFLUENZA	Influenza A virus (*Influenzavirus A; Orthomyxoviridae*)	Outbreaks in greyhounds associated with isolates closely related to H3N8 equine isolates	• Virus isolation • RT-PCR • Serology
TUBERCULOSIS (CANINE)	*Mycobacterium tuberculosis*, *M. bovis*	Dogs are susceptible to both mycobacteria. Lymph node lesions can resemble carcinomas, while lung and liver lesions are exudative. Grey-white areas of bronchopneumonia often occur with cavitation. Fluid in thorax	• Direct ZN-stained smear • Culture • Molecular typing of mycobacteria • PCR
Skin: dogs			
ALGAL PATHOGENS **Protothecosis**	*Prototheca zopfii* or *P. wickerhamii* (colourless algae)	Cutaneous lesions can occur as part of a disseminated disease (bloody diarrhoea, CNS signs and eye involvement). The skin lesions are chronic and characterized by ulcers with crusty exudates in the skin of the trunk and extremities	• Histopathology on biopsy • Direct microscopy • Culture and identification
BACTERIAL INFECTIONS (PRIMARY)		Occur in normal skin and a single pathogen is usually isolated. There is a characteristic lesion or pattern of lesions. Antimicrobial therapy is usually successful	
Cutaneous canine actinomycosis	*Actinomyces viscosus*	Identical syndromes to canine nocardiosis. Thoracic form is more common than granulomatous skin lesions	• Clinical signs • Microscopy: Gram/MZN (*Nocardia* MZN +ve and *A. viscosus* MZN –ve) • Culture
Cutaneous canine nocardiosis	*Nocardia asteroides*	• Cutaneous form: granulomatous skin lesions with discharging sinuses • Thoracic form: lung involvement and accumulation of purulent, bloody fluid in thorax	• Clinical signs • Microscopy: Gram/MZN (*Nocardia* MZN +ve and *A. viscosus* MZN –ve) • Culture
Dermatophilosis (streptothricosis)	*Dermatophilus congolensis*	Rare in dogs but natural infections have been described. Systemic signs are minimal. Lesions occur in hairy regions of body. There is dry adherent scab formation (entrapped in hair) and removal of scabs leaves an ulcerated area	• Gram or Giemsa-stained smear from scab • Culture if necessary • PCR

Table 69.5 Principal infectious diseases of dogs—cont'd

Skin: dogs

Disease	Agent(s)	Comments	Diagnosis
Folliculitis	Bacterial and fungal pathogens or *Demodex* spp.	Infection of hair follicles may be localized or generalized. Most common in short-haired dogs. The characteristic lesion is a pustule or papule surrounding a hair shaft **Furunculosis:** A more severe form of folliculitis in which rupture of the follicle causes spread of infection into the dermal tissues **Carbuncle:** A localized area in which there is multiple furunculosis and consequent sinus formation. The lesion is circumscribed with hair loss. There is thickening of the skin and numerous sinus openings from which there is a purulent discharge	• Direct microscopy on aspirated fluid if possible or from exudates • Culture and identification
Staphylococcal pustular dermatitis	*Staphylococcus pseudintermedius*	Generalized pustular dermatitis with multiple pustular lesions over the entire body of either puppies or adults	• Culture of aspirated fluid from pustules
BACTERIAL INFECTIONS (SECONDARY)		Occur in damaged skin and a mixed bacterial flora is usually isolated. The primary skin disorder and/or predisposing cause must be resolved before antimicrobial therapy will be effective	
Pyodermas (Classification can be based on age, depth of infection or anatomical site of the lesion)	*Staphylococcus pseudintermedius* or *S. aureus* are commonly involved	**Juvenile pyoderma** is seen in pups under four months of age. Most common in short-coated breeds, particularly Labradors, Bassets and Beagles. The initial lesion may be allergic in type. There is oedema of the area followed by a deep pyoderma often of head and lips. It is accompanied by a marked cervical and submandibular lymphadenitis. Prognosis is poor. In cases that recover, healing of the lesions is usually accompanied by permanent, disfiguring, hyperpigmented and hairless scarring of the affected areas **Acute moist pyoderma** ('Hot spot' or pyotraumatic dermatitis): a superficial pyoderma secondary to self-inflicted trauma in response to pruritis (flea infestation or some mange infestations). There is hair loss, erythema, exudation, excoriation and crusting	• Investigation of underlying causes including parasitic infestation • Direct microscopy on aspirates or exudates • Culture of aspirate from intact pustules • Histopathology on a punch biopsy SPECIMENS • Aspirate from intact pustules with fine hypodermic needle (ideal specimen). Surface should first be disinfected with 70% ethyl alcohol • Surface swab is a less useful specimen • Punch biopsy for histopatholgy

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Skin: dogs

Disease	Agent(s)	Comments	Diagnosis
Pyodermas (*continued*)		**General deep pyoderma** is seen in adult dogs. There is involvement of an extensive area of the trunk with numerous discharging openings interconnected by sinus tracks, that run through the dermis over much of the affected skin. The condition may be secondary to demodectic mange **Interdigital pyoderma** has a multifactorial aetiology, often demodectic mange + secondary bacterial infection. Most common in the front feet with swelling, erythema, hyperpigmentation of skin, discharging sinus openings and crusting **Nasal pyoderma** occurs on bridge of nose and is most common in long-nosed dogs, especially in dogs that tend to root around in the ground. There are erosions, exudation and crusting of skin **Callus pyoderma:** infection of a callus or pressure point that tends to occur in heavy breeds of dog **Pyoderma in moist areas** such as **lip fold pyoderma** (lower longitudinal lip folds), **facial fold** (between nose and eyes of brachycephalic breeds) and **tail fold pyoderma** (tailhead area) **Canine acne:** pustule plus secondary bacterial infection, usually in the chin region. Predisposing cause can be a hormonal disturbance (Cushing's syndrome). In younger animals the condition may resolve at puberty or can persist for life. There is often swelling and erythema of the chin and bacteria invade the sebaceous and apocrine glands	• Investigation of underlying causes including parasitic infestation • Direct microscopy on aspirates or exudates • Culture of aspirate from intact pustules • Histopathology on a punch biopsy SPECIMENS • Aspirate from intact pustules with fine hypodermic needle (ideal specimen). Surface should first be disinfected with 70% ethyl alcohol • Surface swab is a less useful specimen • Punch biopsy for histopathology.
Interdigital cyst (interdigital granuloma)	*Staphylococcus pseudintermedius* and others	Primarily a foreign body granuloma with the foreign body being fragments of implanted hair shaft. Secondary bacterial infection may occur. There is an inherited predisposition in certain breeds or individuals	• Histopathology on punch biopsy • Microscopy and culture from deep tissue

Table 69.5 Principal infectious diseases of dogs—cont'd

Skin: dogs

Disease	Agent(s)	Comments	Diagnosis
Staphylococcal scalded skin syndrome (SSSS)	*Staphylococcus pseudintermedius*	A generalized dermatitis with raised, reddish lesions. Within 24–48 hours the superficial skin layers separate from the underlying epidermis and there is a characteristic slipping or wrinkling of the superficial skin layer. This separated epidermal layer sloughs leaving an ulcerated area. Systemic reaction may or may not be present	• Blood culture at acute stage of the disease (no bacteria in epidermal bullae) • Histopathology on a biopsy
FUNGAL INFECTIONS			
Canine cryptococcosis	*Cryptococcus neoformans*	Uncommon in dogs. Infection occurs by inhalation (high titre of yeast in pigeon faeces). Primary lesion in lungs with dissemination to the CNS and eyes. About 25% of infected dogs have skin lesions: ulceration of nose, lips, nail beds and buccal cavity	• Clinical signs • Microscopy: yeast with a thick capsule • Culture at 37°C • Histopathology
Canine sporotrichosis	*Sporothrix schenckii*	Uncommon in dogs. It is usually a cutaneous infection with multiple, nodular, ulcerated lesions that form crusts and alopecia occurs. Limited to skin and usually involves head, trunk or limbs. Dissemination of infection is rare. The fungus is a saprophyte in soil rich in organic matter or on the bark of trees and wooden posts	• Microscopic examination of exudate: cigar-shaped yeast • Culture: 25 and 37°C. Dimorphic fungus
North American blastomycosis	*Blastomyces dermatitidis*	Primary lesion in dogs is almost always in the lungs. Soil organism in certain areas of Africa, eastern USA and in Central America. Skin lesions seen in about 40% of dogs with disseminated disease. They are granulomatous with sinuses and exudates	• Clinical signs • Radiography for lung lesions • Microscopy: thick-walled yeast budding on a broad base • Culture at 25°C and 37°C
Ringworm	*Microsporum canis*	Classical ring-shaped localized lesions (most common); chronic generalized infection; infection of nail beds (onychomycosis); or kerions	SPECIMENS: plucked hairs and skin scrapings from edge of lesion. • Clinical signs
	Microsporum gypseum	Uncommon in dogs (more common in horses). Recorded infecting noses of dogs fond of rooting in soil or in dogs with an immunodeficiency. Geophilic fungus	• Hairs in 10% KOH for arthrospores • Culture: Sabouraud's agar at 27°C for up to three weeks
	Trichophyton mentagrophytes var. *mentagrophyes*	Often acquired from rodents. Lesions can be: • Localized on muzzles and front paws and legs • Generalized infection involving much of the body	• Identification: colonial morphology and macroconidia

Continued

Table 69.5 Principal infectious diseases of dogs—cont'd

Skin: dogs

Disease	Agent(s)	Comments	Diagnosis
Ringworm (*continued*)	*Trichophyton metagrophytes* var. *erinacei*	Seen on muzzles, front paws and legs of dogs that worry hedgehogs. Spines of hedgehogs break skin and aid establish infection	
VIRAL INFERCTIONS			
Canine distemper	Canine distemper virus (*Morbillivirus; Paramyxoviridae*)	**Hard pad:** hyperkeratosis of footpads and often nose. Strong correlation with the development of nervous signs. If the dog survives, the thickened skin of the footpads sloughs but the nose lesions are usually permanent **Rash on abdomen in pups:** these pups rarely develop the nervous signs of canine distemper	• Clinical examination of skin lesions • History: previous signs of generalized canine distemper • Serology: VN, IFA
Canine papillomatosis	Canine papillomaviruses (*Papillomaviridae*)	**Canine oral papillomatosis:** most common form and seen in young dogs under two years of age. Appears to be contagious within a group of dogs but is self-limiting and dogs are not infected a second time. There are white cauliflower-like growths in buccal cavity and on lips where they can be pigmented. Surgical interference is only needed if there is difficulty in eating. Autogenous vaccines are said to be effective **Ocular and facial papillomatosis:** not common and can involve cornea, conjunctiva and eyelid margins. Recorded in dogs six months to four years of age. These warts may be caused by the oral papillomavirus **Cutaneous papillomatosis:** occurs in mature or older dogs and the papillomas do not regress spontaneously. Lesions are present anywhere on body and are thought to be caused by a different and distinct papillomavirus	• Gross appearance of lesions • Histopathology: cryosurgery biopsy • EM for viral particles

Table 69.6 Principal infectious diseases of cats

Buccal cavity: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE CALICIVIRUS INFECTION	Feline calicivirus (*Vesivirus*; *Caliciviridae*)	Ulcerative stomatitis is a constant finding in this respiratory disease. Ulcers occur on the tongue and elsewhere in the buccal cavity. URT signs are usually present and occasionally those of pneumonia	• Clinical signs • Virus isolation and identification • RT-PCR • Serology: VN, IFA, ELISA
FELINE VIRAL RHINOTRACHEITIS	Feline herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Ulceration of tongue and buccal cavity mucosa is a less constant sign compared to calicivirus infection. The disease is severe in neonates and often causes a milder URT infection in older kittens and cats	• Histopathology or FA on conjunctival scrapings early in disease • Virus isolation • PCR • Serology: VN, ELISA
GINGIVITIS (SECONDARY)		A gingivitis or ulceromembranous stomatitis in cats may be secondary to a deficiency, dental calculus or a chronic debilitating disease particularly due to feline immunodeficiency virus or feline leukaemia virus infection. Reddening and swelling of the gingival margins is followed by ulceration and necrosis of gingivo-alveolar tissue. There is a foul odour and brown slimy saliva. Lesions are caused by bacteria, normally commensals in the buccal cavity	• Investigation of primary disease • Clinical signs • Gram-stained smear of necrotic debris for bacteria
Feline immunodeficiency virus disease	Feline immunodeficiency virus (*Lentivirus; Retroviridae*)		
Feline leukaemia	Feline leukaemia virus (*Gammaretrovirus; Retroviridae*)		
MYCOTIC STOMATITIS	*Candida albicans*	Usually seen in kittens and often follows a prolonged course of antibiotic therapy. Characterized by ulceration and elevated, soft, white patches on the oral mucosa. The periphery of the lesions is usually reddened and the affected patches may coalesce	• History of antibiotic therapy • Clinical signs • Isolation of pathogen

Gastrointestinal tract: cats

ENTERITIS/COLITIS (BACTERIAL)	Diverse aetiology including Gram –ve bacteria and occasionally fungi	Non-specific diarrhoea can be predisposed to by an immature natural flora (neonates), or changes to the indigenous flora (prolonged antibacterial therapy) with subsequent overgrowth of opportunistic enteric bacteria	• Clinical signs • Isolation of the pathogen in heavy culture is suggestive
FELINE IMMUNODEFICIENCY VIRUS DISEASE	Feline immunodeficiency virus (*Lentivirus; Retroviridae*)	Infected cats tend to suffer intermittently or persistently from intestinal, respiratory, urinary tract or ear and eye infections	• Serology: ELISA or IFA

Continued

Table 69.6 Principal infectious diseases of cats—cont'd

Gastrointestinal tract: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE INFECTIOUS PERITONITIS (FIP)	Feline coronavirus (*Alphacoronavirus; Coronaviridae*)	Clinical signs are most common in six-month- to two-year-old cats. Three syndromes are recognized: • **Effusive ('wet') FIP**: characterized by an accumulation of fibrin-rich fluid in the abdominal cavity with progressive, painless, enlargement of the abdomen. The course is one to eight months and the prognosis is poor • **Non-effusive ('dry') FIP**: insidious onset with clinical signs reflective of pyogranulomatous involvement of many organs including lungs, liver, pancreas, brain and eyes. Weight loss, depression, anaemia and fever are constant signs. Course often protracted (over one year) • Combination of 'wet' and 'dry' forms: this is less common	• Clinical signs • Pathology • Serology: IFA, ELISA
FELINE LEUKAEMIA	Feline leukaemia virus (*Gammaretrovirus; Retroviridae*)	• A panleukopenia-like syndrome as a form of the non-neoplastic associated diseases can occur with secondary diarrhoea • A feline leukaemia virus-induced enteritis, as a distinct syndrome, has been described. Vomiting and diarrhoea are the predominant signs • Alimentary lymphoma can occur in older cats. Clinical signs include vomiting, diarrhoea and weight loss	• Detection of viral antigen: ELISA (serum) and FA (blood smears) • PCR for provirus in tissues and RT-PCR for viral RNA in saliva, plasma or faeces
FELINE PANLEUKOPENIA	Feline panleukopenia virus (*Parvovirus; Parvoviridae*)	Most infections are subclinical. Virus is shed from all body secretions during active infection and for six weeks after recovery. No extended carrier state occurs but virus is viable at room temperature for one year. Main targets are lymphoid tissue (immunodepression), small intestine (necrosis plus secondary Gram –ve bacterial overgrowth with vomiting, diarrhoea and endotoxaemia) and bone marrow (leukopenia). Fever and oculonasal discharges present. If infected in utero or before two to three weeks of age, cerebellar hypoplasia resulting in ataxia can occur	• Pathology • Detection of virus in faeces in acute cases: EM, ELISA, HA • Virus isolation • PCR • Serology: HAI, VN, ELISA
HISTOPLASMOSIS (FELINE)	*Histoplasma capsulatum*	More common in dogs than in cats. Chronic intractable cough, diarrhoea and emaciation	• Histopathology: small yeast in macrophages

Table 69.6 Principal infectious diseases of cats—cont'd

Liver: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE INFECTIOUS PERITONITIS (FIP)	Feline coronavirus (*Alphacoronavirus; Coronaviridae*)	Granulomatous lesions in the liver may occur in the non-effusive ('dry') form of FIP. Signs include progressive jaundice and anorexia	• Gross pathology • Histopathology • Serology: ELISA, IFA
Genital system: cats			
FELINE INFECTIOUS PERITONITIS	Feline coronavirus (*Alphacoronavirus; Coronaviridae*)	'Wet' form of disease in males: abdominal distention with viscid yellow fluid (peritonitis) and enlarged scrotum due to inflammation of the tunica vaginalis	• Clinical signs • Histopathology: pyogranuloma • Serology: IFA
FELINE LEUKAEMIA	Feline leukaemia virus (*Gammaretrovirus; Retroviridae)*	Foetal resorption or abortion (reproductive failure) can be a part of the non-neoplastic disease syndrome associated with the virus	• FA on blood smears and ELISA on serum to detect virus in the queen
FELINE PANLEUKOPENIA	Feline panleukopenia virus (*Parvovirus; Parvoviridae*)	Result of infection: • In utero infection: death of embryos and abortion. Queen may not show systemic signs of illness • Infection of foetus in last two weeks of gestation or in first two weeks of life: cerebellar ataxia • Kittens over three weeks old or susceptible adults: feline panleukopenia	• Pathology • Detection of virus in foetal tissues: FA, ELISA • Virus isolation • PCR • Serology: HAI, VN, ELISA
FELINE RHINOTRACHEITIS	Feline herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Syndromes include: • Abortions in queens • Kittens and susceptible young adults: severe URT infection with destruction of turbinates in some cases • Corneal ulceration in some adult cats Infection in cats over six months of age is usually mild or inapparent	• Histopathology on foetal tissue • Virus isolation • PCR • Serology for Ab in dam: VN, ELISA
KITTEN MORTALITY COMPLEX	Aetiology includes: feline leukaemia virus, feline parvovirus and bacterial septicaemias in neonates	The complex has been divided into two main syndromes: • Reproductive failure: repeat breeding, abortions and stillbirths • Fading kitten syndrome: deaths of neonates	• Clinical signs • Isolation of bacterial pathogens • Serology for viral pathogens
PYOMETRA	Miscellaneous bacteria	Pathophysiology similar to that in bitch except that queens are induced ovulators requiring coitus before progesterone secretion occurs. This results in a lower frequency of pyometra in queens	• Clinical signs • Radiography to check for retained foetus • Guarded swab from cervix for culture
VAGINITIS	Miscellaneous bacteria	No discharge may be obvious because of the fastidious nature of cats with excessive licking of the vulva. Otherwise as for bitches	• Guarded swab from anterior of vagina for culture

Continued

Table 69.6 Principal infectious diseases of cats—cont'd

Urinary system: cats

Disease	Agent(s)	Comments	Diagnosis
CYSTITIS AND URETHRITIS	*Escherichia coli*, *Staphylococcus* species, *Proteus* species or *Klebsiella pneumoniae*	Similar to the condition in dogs but it is comparatively uncommon in cats. Signs include voiding of small amounts of urine, dysuria and urine that is cloudy and may contain blood	• Clinical signs • Isolation of pathogen in pure heavy growth from urine
FELINE IMMUNODEFICIENCY VIRUS INFECTION	Feline immunodeficiency virus (*Lentivirus; Retroviridae*)	Recurrent, bacterial urinary tract infections can be associated with the immunosuppression induced by this virus	• Serology: ELISA, IFA • Isolation of the secondary bacterial pathogens
FELINE INFECTIOUS PERITONITIS	Feline coronavirus (*Alphacoronavirus; Coronaviridae*)	Pyogranulomatous lesions are commonly present in kidneys of cats with generalized, non-effusive FIP. Clinical signs of renal insufficiency may result	• Histopathology • Serology: IFA, ELISA
FELINE LEUKAEMIA	Feline leukaemia virus (*Gammaretrovirus; Retroviridae*)	Persistent high levels of circulating viral antigens can lead to the formation of immune complexes with resultant glomerulonephritis	• Detection of viral antigen: ELISA (serum) and FA (blood smears) • PCR for provirus in tissues and RT-PCR for viral RNA in saliva, plasma or faeces
Eyes and ears: cats			
FELINE PNEUMONITIS	*Chlamydophila (Chlamydia) felis*	Lesions are confined principally to the upper respiratory tract and conjunctival membranes. Affected mucosa is reddened, swollen and covered with exudate. Signs include fever, inappetence, oculonasal discharges, sneezing and coughing. Course lasts about two weeks with occasional relapses	• Clinical signs • Conjunctival scrapings for inclusions • Culture: TC or inoculation of yolk sac of fertile eggs • PCR
FELINE VIRAL RHINOTRACHEITIS	Feline herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Severe URT infection in neonates with a purulent conjunctivitis, necrosis and resorption of turbinate bones and fever. Milder infection in older cats but corneal ulceration can occur in adults and pregnant queens may abort. The lungs are not usually involved but occasional ulcers in buccal cavity occur	• Histopathology (IBs early) • Virus isolation • PCR • Serology: VN, ELISA
MYCOPLASMAL CONJUNCTIVITIS	*Mycoplasma felis*	Conjunctivitis with hypertrophy of conjunctival mucosa giving a red, velvet appearance	• Clinical signs • Isolation and identification of *Mycoplasma* • PCR

Table 69.6 Principal infectious diseases of cats—cont'd

Eyes and ears: cats

Disease	Agent(s)	Comments	Diagnosis
OCULAR ABNORMALITIES		Local eye abnormalities can be due to trauma with secondary bacterial invasion. However, the eye can often be involved in systemic diseases. Occasionally the systemic manifestation remains occult	• Diagnosis of the primary disease in all cases
Cryptococcosis	*Cryptococcus neoformans*	Blindness as part of CNS syndrome	
Feline immunodeficiency virus disease	Feline immunodeficiency virus (*Lentivirus; Retroviridae*)	Anisocoria has been observed. More commonly there is a chronic ocular discharge as part of a persistent URT infection	
Feline infectious peritonitis (FIP)	Feline coronavirus	Ocular signs, most common in non-effusive FIP, are usually bilateral and present as an anterior uveitis with keratic precipitates ('mutton fat' deposits)	
Feline leukaemia (FeL)	Feline leukaemia virus (*Gammaretrovirus; Retroviridae*)	Anterior uveitis is the most common ocular syndrome in FeL. Paradoxical pupil movements can occur in healthy FeLV-infected cats. These are irregular periods of anisocoria, bilateral mydriasis or miosis without evidence of intraocular inflammation. Most of these cats eventually develop systemic signs of FeL	
Toxoplasmosis	*Toxoplasma gondii*	Ocular disease is more commonly seen in cats than in dogs and is often a posterior retino-choroiditis that can result in retinal detachment	
OTITIS EXTERNA, MEDIA AND INTERNA	Non-specific bacteria and fungi	Similar to conditions in dogs but are less common in cats	

Nervous system: cats

CONGENITAL CNS LESIONS	Feline panleukopenia virus (*Parvovirus; Parvoviridae*)	Due to infection in utero or when neonate is under two to three weeks of age. Cerebellar hypoplasia occurs with consequent ataxia. The debility is permanent	• History of illness in dam (often subclinical) • Clinical signs in kitten • Pathology • PCR
CRYPTOCOCCOSIS	*Cryptococcus neoformans*	Forms in cat: • Respiratory: nasal granulomas and chronic nasal discharge • Cutaneous: firm nodules on face and head that ulcerate (30% of cases) • CNS and ocular: seizures, ataxia and paresis. There is a dilated, unresponsive pupil and blindness due to retinal detachment or optic neuritis Pigeon faeces can be a source of infection	• Radiography to determine if nasal granuloma present • Gram-stained smear of exudates: budding yeast with thick capsule • Histopathology on biopsy material: capsulated yeast present in tissue

Continued

Table 69.6 Principal infectious diseases of cats—cont'd

Nervous system: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE IMMUNODEFICIENCY VIRUS (FIV) INFECTION	Feline immunodeficiency virus (*Lentivirus; Retroviridae*)	Neurological signs, including psychotic behaviour, dementia, facial twitching and seizures, can occur in infected cats. These signs are thought to be due to a direct effect of FIV involvement of the brain	• Serology: ELISA, IFA
FELINE INFECTIOUS PERITONITIS (FIP)	Feline coronavirus (*Alphacoronavirus; Coronaviridae*)	Neurological sign may be seen in non-effusive FIP. Most frequent signs are posterior paresis or ataxia progressing to tetraparesis	• Pathology • Serology: IFA, ELISA
FELINE LEUKAEMIA/ SARCOMA COMPLEX	Feline leukaemia virus (*Gammaretrovirus*; *Retroviridae*)	Tumours, as part of the neoplastic syndrome of FeLV or feline sarcoma virus activity, can cause CNS signs such as hind leg paralysis due to spinal cord compression	• Detection of viral antigen: ELISA (serum) and FA (blood smears) • PCR for provirus in tissues and RT-PCR for viral RNA in saliva, plasma or faeces
FELINE SPONGIFORM ENCEPHALOPATHY	Prion	A novel disease of cats with neurological signs similar to those seen in cattle with BSE. Reported in five- to 12-year-old cats. The main signs include ataxia, particularly of the hind limbs, becoming progressively worse and leading to a crouching stance, changes in temperament to timidity or aggression, hyperaesthesia, head-nodding, hypersalivation unrelated to feeding and abnormal grooming. Appetite and bodily condition remain normal	• Clinical signs • Electron microscopy on frontal lobe preparations for scrapie-associated fibrils • Histopathology for neuronal vacuolation • Immunochemistry for PrP-related proteins
MENINGITIS (BACTERIAL)	Aetiology includes: *Staphylococcus aureus* and anaerobic bacteria	Signs include fever, hyperaesthesia, neck rigidity and ataxia	• CSF analysis and culture
PSEUDORABIES	Porcine herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Cats with close direct or indirect contact with pigs or rats are at risk. The excitement stage is preceded by a period of sluggishness. There is salivation, persistent mewing and the cat resists being caught. Paresis or paralysis of the limbs may occur before death. The course is often rapid and pruritis may not be seen. Recovery in a few cases has been reported	• History: association with pigs and eating raw offal (pig or rat) • Histopathology • FA technique on brain for Ag • Virus isolation, (not always successful) • PCR

Table 69.6 Principal infectious diseases of cats—cont'd

Nervous system: cats

Disease	Agent(s)	Comments	Diagnosis
RABIES	Rabies virus (*Lyssavirus; Rhabdoviridae*)	Cats often develop the furious form and attack suddenly, biting and scratching viciously at any moving object. The paralytic form follows on about the fifth day of illness with general paralysis and death. Mandibular and laryngeal paralysis is less common in the cat than in the dog	• FA on brain tissue • Histopathology: Negri bodies in neurons (not always reliable in cats) • Virus isolation: i/c inoculation of mice or TC • RT-PCR
TETANUS	*Clostridium tetani*	Cats are comparatively resistant to the toxin and incubation period is often up to three weeks. A deep and obvious wound is usually present in cats. Localized tetanus in one limb is not uncommon. Generalized tetanus is characterized by stiff legs and an outstretched tail. In fatal cases tetanic spasms leading to respiratory arrest occur	• Clinical signs • Gram-stained smear from deep in wound
TOXOPLASMOSIS	*Toxoplasma gondii*	Cats can act as definitive and intermediate host. They are usually healthy carriers but can develop either acute or chronic disease. Lesions in eye or CNS may occur. Signs include depression or hyperexcitability, tremors, seizures, paresis or paralysis	• Histopathology • Serology: IHA, IFA, ELISA
Musculoskeletal system: cats			
ARTHRITIS (SUPPURATIVE)	Miscellaneous bacteria including *Pasteurella* spp., streptococci and anaerobic bacteria	Suppurative arthritis may occur as a sequel to a bite wound. The joint is swollen, hot and painful. The cat is often febrile	• Culture from aspirated joint fluid
CHRONIC PROGRESSIVE POLYARTHRITIS	Associated with feline syncytium-forming virus (FeSFV) *(Retroviridae)*	This condition is thought to be an immune-mediated disease. It affects males between 18 months and five years of age. Lymphadenopathy, swollen joints and a stiff gait are constant findings. Feline leukaemia or feline immunodeficiency viruses may potentiate the ability of FeSFV to cause disease	• Clinical signs • Analysis of joint fluid • Serology: IFA, AGID
FELINE CALICIVIRUS INFECTION	Feline calicivirus (*Vesivirus; Caliciviridae)*	An atypical syndrome in six- to 12-week-old kittens is attributable to this virus. There is transient muscle stiffness and joint pain. The affected kitten may resent being handled	• History of respiratory syndrome in other cats • Clinical signs • Serology: VN, IFA, ELISA

Continued

Table 69.6 Principal infectious diseases of cats—cont'd

Musculoskeletal system: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE VIRAL RHINOTRACHEITIS	Feline herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Necrosis of turbinate bones can occur in severe infections in neonates. Signs include foul-smelling nasal discharge, conjunctivitis and bronchopneumonia	• Histopathology (IBs) or FA (virus) on conjunctival scrapings early in disease • Virus isolation from ocular or pharyngeal swabs • PCR • Serology: VN, ELISA
OSTEOMYELITIS	Miscellaneous bacteria including staphylococci, streptococci, Gram –ve and anaerobic bacteria	Occurs most commonly as a sequel to open fractures or surgical repair of closed fractures. In acute cases there is heat, swelling and pain and a serous or purulent discharge from the wound. Chronic signs include soft tissue swelling, lameness and draining sinuses	• Culture and identification of bacterial pathogen
TETANUS	*Clostridium tetani*	Localized tetanus occurs comparatively commonly in cats because of their relative resistance to the toxin. Increased stiffness of a muscle or limb may be first noticed close to a wound site. Later the stiffness spreads leading to the characteristic stiff limbs and outstretched tail of generalized tetanus	• Clinical signs • Gram-stained smear from deep in wound
Respiratory system: cats			
CRYPTOCOCCOSIS (FELINE)	*Cryptococcus neoformans*	The main systems affected are URT, CNS (incoordination and blindness) and skin. The respiratory syndrome is the most common in cats. The signs are sneezing, snuffling and a unilateral or bilateral nasal discharge. These signs are related to granulomas in the nasal cavity. There is often a hard, subcutaneous swelling over the bridge of the nose ('Roman-nose' appearance). The regional lymph nodes are usually enlarged	• Microscopy: India ink (thick capsule) • Histopathology: PAS stain • Culture: Sabouraud agar
FELINE CALICIVIRUS INFECTION	Feline calicivirus (*Vesivirus; Caliciviridae*)	Continous shedding of virus and carrier state probably for life. Ulcers in buccal cavity are pathognomonic. Other signs include pneumonia in susceptible cats, transient stiffness and joint pains in six- to 12-week-old kittens and queens may abort. Some strains of virus cause an URT infection and ulcers only	• Clinical signs • Virus isolation and identification • RT-PCR • Serology: VN, IFA, ELISA
FELINE IMMUNODEFICIENCY VIRUS DISEASE	Feline immunodeficiency virus (*Lentivirus; Retroviridae)*	Recurrent or chronic respiratory and intestinal infections are common syndromes. Other conditions include gingivitis, stomatitis, emaciation, chronic skin disease, cystitis, lymphadenopathy, and neurological abnormalities	• Serology: ELISA and IFA

Table 69.6 Principal infectious diseases of cats—cont'd

Respiratory system: cats

Disease	Agent(s)	Comments	Diagnosis
FELINE INFECTIOUS ANAEMIA (HAEMOBARTONELLOSIS)	*Mycoplasma haemofelis (Haemobartonella felis)*	• Acute cases: fever, anaemia, jaundice, anorexia, weakness and splenomegaly occur • Chronic cases: these present with anaemia, weakness, depression and emaciation Dyspnoea is often seen, varying in severity with the degree of anaemia	• Clinical signs • Haematology • Blood smears stained with Giemsa or Wright's stains (erythrocytic bodies) • PCR
FELINE INFECTIOUS PERITONITIS (FIP)	Feline coronavirus *(Alphacoronavirus; Coronaviridae)*	Dyspnoea may occur in effusive FIP due to accumulation of fluid in the abdomen. A persistent cough, without noticeable dyspnoea, can be due to a pyogranulomatous pneumonia in non-effusive FIP	• Clinical signs • Pathology • Serology: IFA, ELISA
FELINE PNEUMONITIS	*Chlamydophila (Chlamydia) felis*	Signs include purulent conjunctivitis, rhinitis with sneezing and coughing. Chronic infections with relapses are common	• Giemsa or MZN staining of smears from conjunctival scrapings • FA technique (conjunctival smears) • Culture: TC (McCoy cells) or yolk sac of fertile eggs • PCR
FELINE VIRAL RHINOTRACHEITIS	Feline herpesvirus 1 (*Varicellovirus; Herpesviridae*)	Latent infections occur. Signs include purulent conjunctivitis, corneal ulcerations (adults), necrosis and resorption of nasal turbinates (neonates) and abortion in pregnant queens. Ulcers in buccal cavity are less common than in feline calicivirus infection	• Viral isolation from nasal, ocular or pharyngeal swabs • Histopathology: IBs early in disease • PCR • Serology: VN and ELISA (rising Ab titres)
MYCOPLASMAL CONJUNCTIVITIS	*Mycoplasma felis*	Conjunctivitis with hypertrophy of conjunctival surface giving a deep-red, velvet appearance	• Isolation and serological identification of the *Mycoplasma* • PCR

Skin: cats

ALGAL INFECTIONS **Protothecosis**	*Prototheca zopfii* or *P. wickerhamii*	Only the cutaneous form has been described in cats. Soft dermal mass on forehead or legs extending into underlying tissue. *Prototheca* in all stages of reproduction compose a large proportion of the granulomatous lesion	• Histopathology on biopsy for *Prototheca* in tissues • Culture: growth on agar media

Continued

Table 69.6 Principal infectious diseases of cats—cont'd

Skin: cats

Disease	Agent(s)	Comments	Diagnosis
BACTERIAL INFECTIONS (PRIMARY)		A single pathogen is usually isolated. A characteristic lesion or pattern of lesions is present	
Feline Leprosy	*Mycobacterium lepraemurium*	Infection probably acquired from rodents infected with the rat leprosy bacillus. The lesions consist of rapidly growing, painless, cutaneous nodules that are freely mobile. The lesions usually ulcerate and healing is slow	• ZN-stained smear from lesions: large numbers of acid-fast rods • No growth in vitro • PCR
***Mycobacterium smegmatis* infection**	*Mycobacterium smegmatis*	This organism is usually a saprophyte. Infection has only been reported in cats. They appear to be exceptionally susceptible to atypical mycobacterial infections. There is thickening of the skin with numerous sinus openings some of which exude a purulent discharge. The skin of the ventral abdomen is most commonly affected	• ZN-stained smear • Histopathology on biopsy • Culture on Lowenstein–Jensen agar • PCR
Nocardiosis (Feline)	*Nocardia asteroides*	Infection is less common in the cat than in the dog. Nasal granulomas or pleuritis with pleural effusion can occur. Chronic, non-healing fistulous tracts associated with an underlying osteomyelitis have been described	• MZN and Gram-stained smears on pleural effusion • Culture: aerobic at 37°C for up to four days
Plague	*Yersinia pestis*	The disease is endemic in rodents in areas such as southwest USA and is transmitted by fleas. Acute disease in cats is characterized by high fever, lymphadenopathy and abscessation of cervical and submandibular lymph nodes. Mortality is about 50%. Infected cats and their fleas pose a health hazard for humans	Specimens: exudate from abscesses to public health service laboratory • Identification of organism in exudates by FA • Culture and identification of pathogens • PCR
BACTERIAL INFECTIONS (SECONDARY)		Mixed bacterial flora is often isolated. The primary skin disorder or predisposing cause(s) must be resolved before antimicrobial therapy will be effective	
Cat bite abscesses (Cat fights)	*Pasteurella dagmatis* and other buccal cavity flora	One of the most common bacterial skin infections in cats (especially in tom cats). Many of these bacteria are part of the normal flora of the buccal cavity. Predilection sites for abscesses are root of tail, hindlegs, head and neck. The abscess may not be noticed until it ruptures. Abscesses in some sites (back of neck), where there is inadequate drainage may develop into chronic granulomas	• Clinical signs • Gram-stained smears on exudate • Culture

Table 69.6 Principal infectious diseases of cats—cont'd

Skin: cats

Disease	Agent(s)	Comments	Diagnosis
BACTERIAL INFECTIONS (SECONDARY) *(continued)*			
Pyoderma	Most common *Staphylococcus aureus* or *S. pseudintermedius*	Similar to the conditions in dogs but not as common ***Acute moist pyoderma*** (pyotraumatic dermatitis) is secondary to self-inflicted trauma in response to pruritis (flea or mite infestations) ***Feline acne*** occurs on the point of the chin and results from comedone formation and subsquent bacterial infection involving sebaceous and apocrine glands	• Investigation of underlying causes • Direct microscopy on aspirates or exudates • Histopathology on punch biopsy • Bacterial culture of aspirates from intact pustules
FUNGAL INFECTIONS			
Cryptococcosis (Feline)	*Cryptococcus neoformans*	Comparatively common infection in the cat. Systems affected are the upper respiratory tract, CNS and skin. In the cutaneous form, the skin of head and neck are most frequently affected. Multiple, firm nodules occur that tend to ulcerate with a raw, granular surface	• Clinical signs • Direct microscopy: yeast with large capsule • Culture at 37°C. Mucoid, yeast-like colonies
Ringworm	*Microsporum canis*, other dermatophytes rare	Syndromes: ***Subclinical:*** in adults a slight hair loss may be the only sign of infection ***Classical:*** most common in young kittens, characteristic round lesions ***Chronic generalized:*** almost total loss of hair with erythema and accumulations of sebum on skin surface	• Specimens: plucked hair and skin scrapings from the edge of the lesion • Wood's lamp for *M. canis* • Hairs in 10% KOH for arthrospores • Culture and identification
Sporotrichosis (Feline)	*Sporothrix schenckii*	Cutaneous lesions include shallow ulcers, granulomas, nodules and abscesses with draining sinuses. Disseminated disease is uncommon and may be related to immunodeficiency	• Cigar-shaped yeast in exudates • Culture: dimorphic fungus
NON-INFECTIOUS			
Pre-auricular hypotrichosis		Bilateral bald patches in front of the pinnae that are a natural condition in some cats. The skin is normal	• Differentiate from other causes of hypotrichosis or alopecia
VIRAL PATHOGENS			
Feline poxvirus infection	Cowpox virus in cats *(Orthopoxvirus; Poxviridae)*	Distribution limited to parts of Europe. A rodent reservoir is suspected. Initial lesion is at the site of a bite wound. A viraemia occurs with secondary and multiple skin lesions. The lesions are 3–15 mm, red, hairless or ulcerated and scabbed. They develop over several weeks. Lesions heal in four to five weeks with localized alopecia. The disease is mild unless the cat is immunosuppressed	• EM • Histopathology: biopsy • Virus isolation: TC or CAM of fertile eggs • PCR • Serology: VN
Feline sarcoma virus infection	Feline sarcoma virus *(Gammaretrovirus; Retroviridae)*	Fibrosarcomas induced by this virus are usually rapidly growing causing multiple cutaneous or subcutaneous nodules that are locally invasive. Metastasis to other sites, such as lungs, often occurs	• Clinical signs • Pathology

Table 69.7 Principal infectious diseases of domestic birds

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
AVIAN SPIROCHAETOSIS	*Borrelia anserina*	Gallinaceous birds and water fowl. Occurs in tropical and temperate regions. Fever, leg weakness, listlessness, greenish diarrhoea and eventually complete paralysis can occur. PM: swollen spleen with haemorrhages. Heart and liver may be enlarged Vector: fowl ticks	• Darkfield or Giemsa-stained blood smears from birds with a bacteraemia • Serology: AGID • PCR
AVIAN TUBERCULOSIS	*Mycobacterium avium* subsp. *avium*	Many avian species Chronic, granulomatous disease with a prolonged course. Infected birds with advanced lesions can excrete organisms in faeces. Progressive emaciation may be only sign. Granulomas in many body organs. In chickens, lesions are most common in liver, spleen, bone marrow and gut. Lesions do not become calcified. This bacterium can sensitize cattle to the tuberculin and johnin skin tests. Very resistant bacterium: can survive in soil for up to four years	• Direct ZN-stained smears from lesions. Large numbers of acid-fast bacteria present in avian tuberculosis • Isolation: Lowenstein–Jensen medium • PCR • Histopathology
COLIBACILLOSIS/ COLISEPTICAEMIA	*Escherichia coli*	Chickens and turkeys. Common invader especially after respiratory pathogens such as infectious bronchitis virus, mycoplasmas and Newcastle disease virus ***Colisepticaemia:*** often follows a primary viral infection: pericarditis, perihepatitis and air sacculitis. Fibrinous exudates covering liver and other organs. Salpingitis and synovitis of hock joints commonly found in broiler birds ***Coligranuloma*** (Hjarre's disease): focal granulomas in caeca and elsewhere. It can appear very similar to avian tuberculosis ***Egg peritonitis:*** *E. coli* is isolated from most cases. Yolk mixed with exudate in abdominal cavity. Death due to a septicaemia ***Acute salpingitis:*** affected oviducts in laying birds are very enlarged and occupy much of abdominal cavity. *E. coli* usually isolated ***Omphalitis*** ('mushy-chick disease'): yolk sac of newly hatched chicks infected. Distended abdomen	• Isolation of *E. coli* from lesions in heavy, pure culture • Identification by biochemical tests or molecular methods • Histopathology

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
FOWL CHOLERA	*Pasteurella multocida* (specific serotypes)	Chickens, turkeys, ducks, geese, swans and other birds are susceptible ***Acute:*** sudden death or fever, depression, anorexia, ruffled feathers, diarrhoea, increased respiratory rate and rales. Haemorrhages throughout body organs and increased fluid in all cavities. Necrotic foci in liver ***Chronic:*** swollen wattles and fibrinosuppurative exudate in sternal bursas and joints. Conjunctivitis and pharyngitis may occur	• Isolation on blood agar • Serotyping • Histopathology • Direct Giemsa-stained smear of blood in acute cases • PCR
FOWL PLAGUE	Influenza A virus, virulent subtypes H5 or H7 *(Influenzavirus A; Orthomyxoviridae*)	Many avian species are susceptible. Generalized disease with high mortality. Diarrhoea, respiratory signs, cyanosis and oedema of wattles, head and comb, blood-stained oral and nasal discharges occur and there can be CNS involvement. Haemorrhages are present throughout body organs	• Isolation in fertile eggs (allantoic cavity) • Identification of virus: HAI • RT-PCR • Serology: VN, ELISA, AGID
INFECTIOUS BURSAL DISEASE (GUMBORO DISEASE)	Infectious bursal disease virus (*Avibirnavirus*; *Birnaviridae*)	Chickens are the only important natural host. Transmission faecal–oral and possibly via egg ***Chicks 0–2 weeks old:*** no clinical signs but 50% incidence of immunodeficiency ***Chicks 3–6 weeks old:*** depression, ruffled feathers, anorexia, diarrhoea, trembling and dehydration. Morbidity 100% and mortality 20–30%. Bursa enlarged (up to five times), striped and oedematous, but atrophied at time of death. All recovered chicks are immunodeficient and prone to other infections with poor vaccination takes ***Adults:*** unaffected but seroconvert and protect chicks, via antibodies in the egg, through critical period. As flock immunity develops little clinical disease is seen. Very resistant virus, hard to eliminate from a poultry farm	• Ag detection: FA, EM or AGID using bursal tissue • Isolation on CAM of fertile eggs • RT-PCR • Histopathology

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
LYMPHOID LEUKOSIS	Avian leukosis virus (*Alpharetrovirus; Retroviridae*)	Part of avian leukosis-sarcoma complex. Peak mortality in six- to nine-month-old birds. Egg transmission. Viruses can cause various types of tumours but lymphoid leukosis is most common. Transformation occurs in bursa four to eight weeks after infection. Birds infected via egg or a few days after hatching are immunotolerant and persistently viraemic. About 3–20% develop disease depending on genetic susceptibility. Infiltration of lymphoid cells into liver, spleen, kidneys and bursa. Birds depressed with pale comb prior to death. No vaccine available	• Pathology • Virus detection: PCR, ELISA • Serology: VN, ELISA, IFA
MAREK'S DISEASE	Gallid herpesvirus 2 *(Mardivirus; Herpesviridae*)	Chickens are the only important natural host. Peak mortality in two- to five-month-old birds. Replicates in feather follicles. No egg transmission. Four forms of the disease occur: • ***Visceral form***: this is becoming the most common and must be differentiated from lymphoid leukosis. Diffuse or focal infiltration of liver, spleen, kidney, gonads, lungs and heart by lymphoblastic cells. Bursa not usually affected • ***Classical neural form:*** infiltration of the peripheral nerves: paralysis of legs and/or wings • ***Ocular form:*** grey iris and eccentric pupil • ***Cutaneous form:*** nodular tumors at feather follicle sites Vaccine available	• Pathology • Virus isolation • PCR
NEWCASTLE DISEASE	Newcastle disease virus (*Avulavirus*; *Paramyxoviridae*)	Many avian species are susceptible. Often introduced into countries via cage birds. Many strains: lentogenic (mild), mesogenic (moderate virulence), velogenic (virulent). ***Viscerotropic form:*** respiratory signs, diarrhoea, swelling and oedema around the eyes ***Neurotropic form:*** CNS signs of paresis of legs and wings, torticollis and tremors. Rapid spread with depression and fever. Mortality can be 100% with velogenic strains. Haemorrhages may occur in larynx and throughout intestines. Necrotic foci found in intestines. Stable virus, survives several months in particles of faeces. Transmission: aerosols and transovarially. Virus is present in all exudates and body tissues	• Isolation in allantoic cavity of fertile eggs • Identification of virus: HAI • RT-PCR • Serology: HAI, ELISA

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
PSITTACOSIS/ ORNITHOSIS	*Chlamydophila (Chlamydia) psittaci*	Wild and domestic birds Disease is uncommon in food birds but is more common in parrots, pigeons and non-psittacine cage birds. Clinical disease often activated by stress such as egg-laying or transportation in small cages. Nasal discharge, diarrhoea, dullness, weakness, inappetence and loss of weight occurs. Generalized disease with enlargement of liver and spleen, pericarditis, air-sacculitis, perihepatitis and peritonitis. Chlamydiae are excreted in respiratory discharges and faeces. Transmission: aerosols and airborne particles	• Impression smears: FA, Giemsa or MZN stains • Isolation in yolk sac of fertile eggs or in McCoy cell line • PCR
STAPHYLOCOCCAL INFECTIONS	*Staphylococcus aureus*	Many avian species ***Synovitis:*** in growing birds. Large quantity of purulent exudate especially in hock joint. May be associated with a reovirus infection ***Osteomyelitis:*** lameness, erosion of cartilage over tibiotarsal condyles. Occurs in growing birds ***Septicaemia:*** sporadic infection in adult birds. Hepatic lesions and vegetative endocarditis (streptococci can cause similar lesions) ***Omphalitis*** ('mushy-chick disease'): can be caused by *E. coli* or staphylococci ***'Bumble-foot':*** infection distorting one or both feet (staphylococci or *Mycobacterium avium* subsp. *avium*). Most common in free-range chickens and in birds of prey	• Direct Gram-stained smear • Isolation from lesions and identification using biochemical or molecular methods • Histopathology
NECROTIC ENTERITIS	Associated with *Clostridium perfringens* types A or C	Chickens and other birds Seen most commonly in broiler birds three weeks old onwards. Mucosal surface of small intestine is pale due to necrosis of tips of villi. Later the mucosa fissures and sloughs irregularly. Most of the upper small intestine is involved. It is an acute enterotoxaemic disease with sudden deaths and explosive overall mortality. Seen especially in birds on litter (build-up of spores). Peak incidence at three to 12 weeks old. Diarrhoea may be seen but death usually occurs in a few hours	• Gram-stained scraping of small intestine mucosa: large numbers of Gram +ve rods • Clinical and PM findings • PCR • Histopathology

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
ROTAVIRUS INFECTION	Avian rotavirus (*Reoviridae*)	Chickens and turkeys Characterized by enteritis and diarrhoea in young birds. Appetite is poor and dehydration occurs rapidly with mortality up to 50%. Transmission is faecal–oral. No egg transmission reported. Dehydrated carcase and watery contents in gut may be seen. Subclinical infections can occur	• Electron microscopy on clarified faeces • Isolation in embryo liver cells: CPE • Virus detection: ELISA, RT-PCR
SALMONELLOSIS	*Salmonella* serotypes	***Fowl typhoid:*** Now rare in many parts of the world, *Salmonella* Gallinarum. Egg transmitted but most common in growing and adult birds, although it can cause illness in chicks similar to that caused by *S.* Pullorum. In adults, mortality can be high. There is enteritis and enlarged and congested liver and spleen ***Pullorum disease*** (bacillary white diarrhoea): Now rare in many parts of the world, *Salmonella* Pullorum. Seen in first few days of life up to two to three weeks of age. Transmission by egg and contact. Chicks huddle, appear sleepy, anorexic and have white pasting around vent. They die or become carriers. Focal necrosis of liver or spleen occurs, nodules in lungs and heart, cheesy material in caeca and synovitis can be prominent ***Fowl paratyphoid:*** infection with many other *Salmonella* serotypes. Most infections subclinical but high mortality in chicks a few weeks old can occur with stress factors increasing susceptibility. Enlarged liver with or without focal areas of necrosis occurs. Egg transmission with serotype Enteritidis	• Culture: lesions and heart blood • Serotyping of isolates • Biochemical tests or molecular methods to distinguish between *S.* Pullorum and *S.* Gallinarum • Histopathology
THRUSH OF THE CROP	*Candida albicans*	Many bird species affected Lesions are most common in crop: thickened mucosa with white raised diphtheritic membrane ('terry-towelling' effect). Mouth and oesophagus can also be affected. Birds are depressed and become emaciated. Condition commonly seen after extensive use of antibiotics	• Direct microscopy: Gram or 10% KOH • Isolation and identification

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Generalized diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
ULCERATIVE ENTERITIS (QUAIL DISEASE)	*Clostridium colinum*	Quail, chickens and turkeys Bobwhite quail most susceptible but can occur in five- to seven-week-old Leghorn chickens or in turkey poults. Outbreaks tend to follow coccidiosis or infectious bursal disease. Explosive outbreaks in quail with 100% deaths and in chickens with mortality of 10% without obvious illness. Faeces streaked with urates and lesions vary from enteritis to ulceration of gut (ulcers of up to 3 mm). Yellow foci occur in liver. Faecal–oral transmission. Survivors become carriers of the bacterium	• Gram-stained smears of blood, liver or spleen of septicaemic birds • Gross and histopathology • Anaerobic isolation of *C. colinum* • PCR
Respiratory system: poultry			
ASPERGILLOSIS	*Aspergillus fumigatus*	Chickens, turkeys and other birds such as penguins in zoos ***Brooder pneumonia:*** chicks are infected by spores during hatching. Seen at two days of age. Gasping, anorexia, huddling together and increased thirst. From 10–50% of chicks can be affected. Caseous focal lesions are seen mainly in lungs but can occur in other organs ***Generalized aspergillosis:*** all ages from one week of age. Infected via inhalation of spore-laden dust. Sporadic disease in older birds. Yellowish nodules occur in air sacs, lungs and in other body organs, including the brain	• Isolation from lesions on Sabouraud agar • Identification: 1. Colonial morphology 2. Fruiting heads • Histopathology: PAS or silver stains
AVIAN INFLUENZA	Influenza A virus, many subtypes (*Influenzavirus A, Orthomyxoviridae*)	Many avian species affected ***Mild form:*** mild respiratory signs and a slight drop in egg production ***Fowl plague:*** virulent subtypes with rapid spread, generalized disease and a high mortality in a short space of time. There is diarrhoea, respiratory signs, cyanosis of the face and swelling of the face and wattles. Haemorrhages through body organs and CNS can be involved. Mortality 40–100%. Can be introduced into a country by migrating birds	• Isolation in allantoic cavity of fertile eggs • Identification by HAI • RT-PCR • Serology: ELISA, VN, AGID

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Respiratory system: poultry

Disease	Agent(s)	Comments	Diagnosis
CHRONIC RESPIRATORY DISEASE (CRD) (CHICKENS) AND INFECTIOUS SINUSITIS (TURKEYS)	*Mycoplasma gallisepticum*	Chickens and turkeys ***CRD:*** mild respiratory disease but severe with concurrent pathogens. Sneezing, coughing, rales, and nasal discharge. Morbidity high but mortality usually low ***Infectious sinusitis*** in turkeys: swollen face, as sticky exudate fills infraorbital sinuses. Egg transmission and transmission by aerosol and on fomites (human clothes)	• Isolation on *Mycoplasma* medium • Identification of microcolonies by FA or growth-inhibition tests • PCR • Serology: slide agglutination (stained Ag) or HAI
FOWL CORYZA	*Avibacterium paragallinarum*	Chickens, guinea fowl, turkeys and pheasants Chickens can be infected from four weeks old and susceptibility increases with age. Mild to subacute disease on its own: depression, nasal discharge and swollen face because of oedema and filling of infraorbital sinuses with exudate. Occasionally the oedema extends to the wattles Transmission: contact, aerosol and via water	• Isolation on chocolate agar under 10% CO_2 • Identification using colonial morphology and biochemical or molecular tests • PCR
FOWL POX	Fowlpox virus (*Avipoxvirus; Poxviridae*)	Chickens and turkeys ***'Dry' form:*** scabby lesions on unfeathered parts of head, especially on combs and around eyes ***'Wet' form:*** respiratory disease: caseous and diphtheritic lesions in larynx and trachea. Slow spread by pecking at lesions but epidemic if mosquitoes are plentiful	• Isolation on CAM of fertile eggs • Virus identified by FA, EM or neutralization tests • PCR • Serology: AGID, VN, ELISA
INFECTIOUS BRONCHITIS (IB)	Avian coronavirus (*Gammacoronavirus*; *Coronaviridae*)	Chickens ***Young birds*** less than six weeks old: severe respiratory disease. Can be complicated with *Escherichia coli* and mycoplasmas ***Broilers*** over six weeks old: immune complex glomerulonephritis ***Adults:*** mild or inapparent URT infection unless complicated by mycoplasmas, *E. coli* or other secondary invaders. Main signs: • Drop in egg production (20–25%) • Misshapen and soft-shelled eggs Not egg-transmitted	• FA: tracheal scraping • Isolation: allantoic cavity of fertile eggs. Dwarfing of chick embryos characteristic. Virus identification by VN test • RT-PCR • Serology: VN, ELISA, gel diffusion
INFECTIOUS LARYNGOTRACHETIS (ILT)	Gallid herpesvirus 1 (*Iltovirus; Herpesviridae*)	Chickens and pheasants Acute, highly contagious disease of most importance in laying birds. Dyspnoea, high-pitched squawk on expiration, coughing up blood. Caseous plugs and haemorrhages found in trachea and bronchi. Rapid drop in egg production that returns to normal on recovery. Mortality varies but can reach 50%	• Viral antigen detection: ELISA, AGID • Isolation: CAM of fertile eggs or in TC • PCR • Serology: VN, AGID, ELISA

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Respiratory system: poultry

Disease	Agent(s)	Comments	Diagnosis
INFECTIOUS SYNOVITIS	*Mycoplasma synoviae*	Chickens and turkeys • Mild respiratory disease with some strains • Synovitis: chicken four to six weeks and turkeys 10–12 weeks of age. Swelling of hocks and foot pads. Yellow viscid exudate in bursa of keel and hock and wing joints • Air-sacculitis especially if infected concurrently with infectious bronchitis virus Egg transmission and via fomites (human clothes)	• Isolation on *Mycoplasma* medium • Identification by FA, by growth-inhibition tests or by molecular methods • PCR • Serology: slide agglutination test (stained antigen) or HAI test
NEWCASTLE DISEASE	Newcastle disease virus (*Avulavirus; Paramyxoviridae*)	Many avian species susceptible. Respiratory signs of gasping and coughing may accompany but often precede other signs	• Isolation in allantoic cavity of fertile eggs • Identification of virus: HAI • RT-PCR • Serology: HAI, ELISA
Skin: poultry			
FAVUS (AVIAN RINGWORM)	*Trichophyton gallinae*	Gallinaceous birds Not common but can affect chickens and turkeys. White patchy overgrowths of comb and wattles that develop into thick white crusts (resembles fowlpox). In severe infections the feather follicles can be invaded with systemic signs of illness	• Direct microscopy of scab in 10% KOH • Isolation on Sabouraud agar
NECROTIC DERMATITIS	*Clostridium septicum* (*C. perfringens*, staphylococci and streptococci can also be present)	Gangrenous necrosis with wet, inflamed skin. Sudden onset and birds rarely seen ill but death rate high. Most common in four- to 16-week-old chickens and occasionally in turkeys. Often predisposed to by infectious bursal disease or a previous staphylococcal infection. Mortality 10–60% and affected birds die in eight to 24 hours	• Clinical signs • Histopathology • FA for *C. septicum* • PCR
FOWL POX	Fowlpox virus (*Avipoxvirus; Poxviridae*)	Chickens and turkeys In the 'dry' form of the disease, scabby lesions occur on unfeathered parts of the head, especially on the comb and around the eyes	• Clinical signs • EM • Isolation on CAM of fertile eggs • PCR

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Nervous system: poultry

Disease	Agent(s)	Comments	Diagnosis
BOTULISM (LIMBERNECK)	*Clostridium botulinum,* type C most common	Poultry and wild water fowl Growth of the bacterium occurs in suitable foodstuffs and invertebrate carcases (anaerobic) with production of toxin. Not all birds develop the classical 'limberneck' (paralysis of neck) but this is seen in swans, geese and ducks. A constant finding is the flaccid paralysis of legs and wings and death from respiratory paralysis. Some mildly affected birds recover if fed and kept away from the source of the neurotoxin. No specific lesions on necropsy ***Toxico-infectious botulism:*** reported in broiler flocks with 10% mortality. Alteration in normal gut flora may predispose to this form of the disease	• Clinical signs • Demonstration of toxin in serum of acutely ill birds by mouse-neutralization tests
EPIDEMIC TREMOR (AVIAN ENCEPHALOMYELITIS)	Avian encephalomyelitis virus (*Tremovirus; Picornaviridae*)	Chicks, ducks, pheasants, turkeys and Japanese quail Most common in seven- to 10-day-old chicks. Ataxia and fine tremors of head and neck, paralysis and death. Any birds that recover are brain-damaged. Mortality 50% when virus first enters flock. Once endemic the chicks are protected for 21 days after hatching by maternal antibodies in eggs. Subclinical in adults except for a transient drop in egg production when first infected. Spread by faecal–oral route and via eggs for one month after infection	• Clinical signs • Histopathology • Isolation in yolk sac of fertile eggs • Detection of virus: RT-PCR, FA
LISTERIOSIS	*Listeria monocytogenes*	Chickens, turkeys, geese, pigeons and canaries. Septicaemia often with encephalitis in young birds. Sudden death is common but wasting before death may occur. Myocardial necrosis, pericarditis, focal hepatic necrosis and encephalitis (tremors, torticollis and circling) can be present. Listeriae can survive in a chicken flock for four years or more without active disease	• PM: massive necrosis of myocardium and focal hepatic necrosis • Isolation from heart, liver or brain • Identification by biochemical or molecular tests • PCR
MAREK'S DISEASE	Gallid herpesvirus 2 (*Mardivirus*; *Herpesviridae*)	In the classical neural form of Marek's disease there is infiltration of peripheral nerves by lymphoblastic cells with resulting paralysis of legs and/or wings	• Pathology • Virus isolation • PCR
NEWCASTLE DISEASE	Newcastle disease virus (neurotropic strains) (*Avulavirus*; *Paramyxoviridae*)	Nervous signs include drooping wings, leg paresis, twisting of head and neck ('star-gazing'), depression and total paralysis. Clonic spasms may be seen in moribund birds. CNS signs may accompany, but often follow, respiratory signs	• Isolation in allantoic cavity of fertile eggs • Identification of virus: HAI • RT-PCR • Serology: HAI, ELISA

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Miscellaneous diseases: poultry

Disease	Agent(s)	Comments	Diagnosis
AVIAN INFECTIOUS HEPATITIS	*Campylobacter jejuni*	Variety of birds and mammals can be infected. Typically the infection is subclinical and the bacterium is excreted in faeces. In clinical disease in chicken there is a major decline in egg production and severely affected birds lose weight, have shrivelled combs and are listless. There may be sudden deaths in acute cases. Only a few birds are affected at any one time. At PM there are haemorrhagic and necrotic lesions in the liver, ascites, hydropericardium, enlarged pale kidneys and a catarrhal enteritis. In younger birds heart lesions are more severe and constant	• Direct microscopy on bile • Isolation from bile or faeces • PCR
CHICKEN ANAEMIA VIRUS INFECTION (CAV)	Chicken anaemia virus (*Gyrovirus; Circoviridae*)	CAV not known to infect birds other than chickens. Chicks under one week of age, without maternal antibodies, develop disease. Characterized by anorexia, lethargy, anaemia, atrophy or hypoplasia of lymphoid organs, haemorrhages and increased mortality rates	• Clinical signs • Gross and histopathology • PCR • Serology: VN, IFA, ELISA
EGG DROP SYNDROME	Duck adenovirus A (*Atadenovirus*; *Adenoviridae*)	Natural hosts of virus are ducks and geese. Introduced to an unnatural host (chicken) probably via Marek's disease vaccine grown in duck tissue culture cells. Egg transmission occurs in chicken. The virus is latent in chicks until puberty and then they excrete virus in faeces with the production of antibodies. Lateral spread is slow. If virus enters a laying flock without immunity, loss of pigment in brown eggs is the first sign followed by soft-shelled or shell-less eggs. Production drops by 10–40% but there is no effect on hatchability or on fertility. Repeated episodes of infection can occur with no specific signs of illness in the birds	• Serological screening of flocks that are producing defective eggs: VN and HAI • Isolation difficult because of lack of signs and intermittent excretion. Fertile duck eggs or tissue culture may be used for isolation • Virus detection: PCR, ELISA, FA
VIRAL ARTHRITIS	Avian orthoreovirus (*Orthoreovirus; Reoviridae*)	Chickens and turkeys Reovirus ubiquitous in flocks. Some strains can cause viraemia and localize in joints: arthritis, tendonitis and synovitis. Egg transmitted. Any respiratory or intestinal signs are of short duration. The arthritic form is most common in four- to eight-week-old broilers, the birds have a stilted gait, and rupture of the gastrocnemius can be associated with this infection. Morbidity rate 5–50%	• Isolation in yolk sac of fertile eggs or in fowl cell lines • RT-PCR • Serology (many birds positive early in the infection): gel-diffusion

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Infectious diseases: turkeys

Disease	Agent(s)	Comments	Diagnosis
ARIZONA INFECTION (PARACOLON INFECTION)	*Salmonella enterica* subsp. *arizonae*	Disease most common in turkey poults. Bacterium can be isolated from other birds, mammals and reptiles. Signs and lesions generally similar to those caused by some other *Salmonella* spp. Faecal and egg transmission occurs. The infection tends to persist in a flock. Acute disease most common in three- to four-week-old age group. Birds are listless, develop diarrhoea and corneal opacity with blindness is common. Nervous signs, such as torticollis, ataxia, leg paralysis and convulsions can occur due to localization of salmonellae in the brain. Mortality variable, being highest in young birds	• Isolation on selective media • Serotyping of isolates • PCR • Histopathology
BLUE COMB (CORONAVIRUS ENTERITIS OF TURKEYS)	Avian coronavirus (*Gammacoronavirus; Coronaviridae*)	Acute, highly contagious disease. Sudden onset with marked depression, anorexia, diarrhoea and weight loss. Morbidity and mortality approach 100% in young poults. Weight loss in adult and growing birds may be economically more important. Poults chirp constantly and appear to be cold. The intestines are distended and contents watery. Diarrhoea is profuse in older birds. Cyanosis of head in common ('blue comb'). There is a drop in egg production and catarrhal enteritis with haemorrhages in viscera. Variable morbidity/mortality in adults	• Pathology • Virus isolation • RT-PCR
HAEMORRHAGIC ENTERITIS OF TURKEYS	Turkey adenovirus A (*Siadenovirus*; *Adenoviridae*)	Infection of the spleen in turkeys over four weeks old with secondary intestinal haemorrhage. Acute onset with bloody droppings. Mortality is usually 5–10% but is occasionally higher. Short course (two weeks) but often complicated and prolonged by a concurrent *Escherichia coli* infection. The spleen is enlarged and mottled or can be shrunken and pale. Haemorrhages from tips of villi are characteristic	• Histopathology • Detection of virus: AGID, PCR • Serology: AGID
***MYCOPLASMA* MELEAGRIDIS DISEASE**	*Mycoplasma meleagridis*	Syndromes include: • Air-sacculitis in one-day-old poults • Reduced hatchability (egg transmission and venereal) • Respiratory rales in three- to eight-week-old birds • Hock joints and cervical vertebrae affected in growing birds	• Rapid plate agglutination tests for antibodies • Isolation and identification of *Mycoplasma* • PCR

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Infectious diseases: turkeys

Disease	Agent(s)	Comments	Diagnosis
PSEUDOTUBERCULOSIS	*Yersinia pseudotuber-culosis*	The bacterium can infect many mammalian and avian species. Cage birds and turkey poults are particularly susceptible. Acute septicaemia and death within 24 hours (differentiate from listeriosis and salmonellosis in cage birds). Chronic disease with wasting, diarrhoea and local necrotic lesions in internal organs can occur but is less common	• Isolation on BA and MacConkey agar • Identify by biochemical or molecular tests • PCR
RETICULOENDOTHELIOSIS	Reticuloendotheliosis virus *(Gammaretrovirus; Retroviridae)*	Turkeys, ducks, pheasants and quail. Chickens have antibodies to virus. Non-defective virus strains cause: • Acute neoplasia of lymphoreticular system • Runting syndrome characterized by abnormal feathering and atrophy of thymus and bursa • Chronic lymphomas 1. Bursal lymphoma 2. Non-bursal lymphoma Replication defective strain can cause acute reticulum cell neoplasia in chickens and turkeys. This is rare in the field	• Gross and histopathology • RT-PCR • Serology: VN, ELISA
TURKEY CORYZA	*Bordetella avium*	Resistant bacterium and remains viable in contaminated litter for long periods. Spread by aerosol and in drinking water. Disease is most pronounced in poults but broiler and layer chickens are also susceptible. There is mucus from nares, swelling of submaxillary region, mouth breathing and sneezing. Morbidity 100% but mortality low if disease is uncomplicated. However, concurrent *Escherichia coli* infection is common	• Isolation and identification • PCR
TURKEY ERYSIPELAS	*Erysipelothrix rhusiopathiae*	Mainly turkeys, less common in chickens, ducks and geese Source of infection: uncooked meat or fish scraps. Disease most common in growing birds. They become listless, anorexic and have greenish diarrhoea. A red-purple swelling of caruncle and face is suggestive of this disease. Mortality can reach 40%. Lesions: diffuse haemorrhages in abdominal, pectoral and femoral muscles and subserosal haemorrhages occur in viscera. Liver and spleen are enlarged and may have infarcts	• Isolation on BA • Identification by biochemical or molecular tests • PCR

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Infectious diseases: turkeys

Disease	Agent(s)	Comments	Diagnosis
TURKEY RHINOTRACHEITIS	Avian metapneumovirus (*Metapneumovirus; Paramyxoviridae*)	Turkeys and chickens are affected. Mild respiratory signs may precede a drop in egg production with a high level of white-shelled eggs, reduced hatchability and fertility. Signs last about five to six weeks. Poults may show severe respiratory signs with swelling of the infraorbital sinuses and submandibular oedema	• Isolation in allantoic cavity of fertile eggs, early in disease • RT-PCR • Serology: ELISA, VN
TURKEY X DISEASE (AFLATOXICOSIS)	*Aspergillus flavus*	Peanut meal or cereals are most commonly implicated. Depression, anorexia, reduced growth rate, loss of condition, bruising, decreased egg production, fertility and hatchability occur. Duckling and turkey poults are particularly susceptible. Ataxia, convulsions and opisthotonos can be seen. PM: ascites, visceral oedema and liver is pale with widespread necrosis	• Histopathology • Demonstration of aflatoxin in feedstuff
VIRAL HEPATITIS OF TURKEYS	Novel, unassigned picornavirus (*Picornaviridae*)	Usually subclinical unless birds are stressed. In poults under six weeks of age the morbidity can be 100% and mortality 10–15%. In adults there is decreased production, fertility and hatchability. PM: focal necrosis of liver with haemorrhages and congestion superimposed on the degenerative changes. Generally the morbidity and mortality vary according to degree of stress	• Pathology • Virus isolation • RT-PCR
Infectious diseases: ducks			
DUCK PLAGUE	Anatid herpesvirus 1 (*Herpesviridae*)	Duck, geese and swans Sudden deaths of birds in good condition, sometimes still sitting on eggs. If seen before death: photophobia, anorexia, extreme thirst, ataxia, nasal discharge and bloody diarrhoea are noticed. High mortality. Haemorrhages and necrosis occur in internal organs	• Pathology: intranuclear IBs • FA technique • Virus isolation • PCR
NEW DUCK DISEASE	*Riemerella anatipestifer*	Contagious and widely distributed disease. Primarily affects young ducks two to seven weeks old but waterfowl, chickens and turkeys can be affected. Ocular and nasal discharges, tremor of head and neck and incoordination are seen. Fibrinous exudate occurs in all body cavities and a fibrinous meningitis can be present	• Isolation on BA under 5–10% CO_2 • Molecular identification • PCR

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Disease	Agent(s)	Comments	Diagnosis
Infectious diseases: ducks			
DUCK VIRUS HEPATITIS	Duck hepatitis A virus (*Avihepatovirus; Picornaviridae*)	Highly contagious with a short course, high mortality and characteristic liver lesions. Liver is pale red, slightly enlarged and covered by haemorrhagic foci up to 1 cm in diameter. Spleen and kidneys may be enlarged. Disease seen in ducklings under seven weeks old. Mortality 95%. Adults are infected but disease is subclinical. Waterfowl may be carriers of the virus	• Pathology • Virus detection: FA, RT-PCR • Virus isolation
Infectious diseases: geese			
GOOSE PARVOVIRUS INFECTION (GOOSE VIRAL HEPATITIS, DERZSY'S DISEASE)	Goose parvovirus (*Dependovirus; Parvoviridae*)	Highly contagious disease of young geese under four weeks of age and Muscovy ducklings • Goslings under one week of age: anorexia, prostration and death in two to five days. Survivors exhibit runting, loss of neck down and marked ascites • Older goslings: anorexia, weakness, nasal and ocular discharge and diarrhoea Lesions include serofibrinous pericarditis, perihepatitis and excess fluid in abdominal cavity	• Gross and histopathology • Isolation of virus in TC or fertile goose eggs (allantoic cavity). IBs in TC cells • PCR • Serology: VN, AGID
Infectious diseases: pigeons			
PARAMYXOVIRUS INFECTION OF PIGEONS	Avian paramyxovirus (APMV-1 serogroup) (*Avulavirus; Paramyxoviridae*)	Pigeons can be infected with both classical Newcastle disease virus strains and with pigeon strains. Severe and rapidly spreading disease: anorexia, diarrhoea, conjunctivitis and paralysis of wings and legs. Pigeon APMV-1 is lentogenic for chickens	• Virus isolation • RT-PCR
PARATYPHOID ('DROPPED WING')	*Salmonella* Typhimurium	As well as enteric disease, arthritis of the wing joints occurs quite commonly in young pigeons	• Isolation from synovial fluid in acute cases • PCR
PIGEON HERPESVIRUS INFECTION	Columbid herpesvirus 1 (*Mardivirus; Herpesviridae*)	Most adult birds are latent, asymptomatic carriers. In acute disease, young susceptible birds show conjunctivitis, blocked nares and caruncles turn from white to yellow-grey. Chronic disease with sinusitis and dyspnoea occurs with secondary bacterial invasion	• Histopathology: IBs in URT and liver cells • Isolation in TC and identification of virus by FA technique or PCR

Continued

Table 69.7 Principal infectious diseases of domestic birds—cont'd

Infectious diseases: pigeons

Disease	Agent(s)	Comments	Diagnosis
ADENOVIRUS INFECTION	Fowl adenoviruses (*Aviadenovirus; Adenoviridae*)	Two clinical forms described: type 1, vomiting, watery diarrhoea and weight loss; type 2, necrotizing hepatitis	• Pathology: intranuclear inclusion bodies • PCR
TRICHOMONIASIS	*Trichomonas gallinae*	Most common in pigeons but outbreaks have occurred in chickens and turkeys. Pigeon parents can infect squabs via pigeon milk. Rapid course: small yellowish lesions on oral mucosa that grow and spread and can completely block the oesophagus. Death often occurs in eight to 10 days. Some pigeons are healthy carriers of the organism in their throats	• Microscopic examination of wet mount for *Trichomonas* sp.

Appendix 1

Reagents and stains

BIOCHEMICAL TEST REAGENTS

Decarboxylase Broth Base (Falkow's)

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Distilled water	1.0 L
Bromocresol purple (0.2% solution)	10.0 mL
L-arginine hydrochloride	5.0 g
or L-lysine hydrochloride	5.0 g
or L-ornithine hydrochloride	5.0 g

The solids are dissolved in the distilled water and the indicator solution is added. The pH is adjusted to 6.7 and the medium dispensed in 2–5 mL quantities in small bottles or tubes. The broth is sterilized at 115°C for 10 minutes.

Lead Acetate Paper Strips for Hydrogen Sulphide Detection

Filter paper strips (approximately 5 × mm) are immersed in saturated lead acetate solution and dried in an oven at 70°C. These are suspended above a suitable medium and held in place by the cap or cotton wool plug.

Kovac's Reagent for the Detection of Indole

p-dimethylamino-benzaldehyde	20.0 g
Iso-amyl alcohol	300.0 mL
Concentrated hydrochloric acid	100.0 mL

The aldehyde is dissolved in the alcohol with gentle heat. The acid is added after cooling. Store in a dark bottle at 4°C.

Methyl Red Reagent

Methyl red	0.1 g
95% ethyl alcohol	300.0 mL
Distilled water	200.0 mL

The methyl red is disssolved in the alcohol and the solution is diluted to a total volume of 500 mL with distilled water.

Nitrate Reduction Test

Nitrate broth (5 mL) is inoculated with the test bacterium and incubated at 37°C for 24 hours (rarely, an incubation period of five days is necessary). This broth can be obtained commercially and contains 1 g potassium nitrate per litre.

Reagents A and B (test for nitrite)

Reagent A: 5 g alpha-naphthylamine in 1 L 5N acetic acid

Reagent B: 8 g sulphanilic acid in 1 L 5N acetic acid.

To obtain 5N acetic acid, add two parts glacial acetic acid to five parts distilled water. Five drops of each reagent (A and B) are added to the nitrate broth. The test is shaken and read after one to two minutes. A colourless reaction indicates that no nitrite is present in the broth. Red colouration means that the nitrate in the test broth has been reduced to nitrite.

A pinch of zinc powder can be added to a test where a broth has remained colourless. If the broth then becomes a red colour, this means that nitrate has now been

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converted to nitrite and the test bacterium did not reduce the nitrate in the broth. If, however, the broth remains colourless, this indicates that the nitrate in the broth was reduced to nitrogen gas by the test bacterium.

Oxalic Acid Test Papers for Indole Production (SIM Medium)

A piece of filter paper is soaked in saturated oxalic acid solution. The paper is dried and cut into strips approximately 10 × 50 mm. The strips are suspended over the medium and held in place with the cap. Pink colouration indicates a positive reaction.

Potassium Nitrate Paper Strips for Nitrate Reduction Test

Filter paper strips (10 × 50 mm) are soaked in 40% potassium nitrate solution. The strips are dried and autoclaved at 115°C for 10 minutes.

A strip is placed on the surface of a blood agar plate. The test bacterium is then stab inoculated into the agar about 20 mm from the paper strip. The plate is incubated at 37°C and examined after four and 24 hours incubation. A positive test result is indicated by a wide zone of browning of the medium between the colony of the test bacterium and the strip. A negative reaction is denoted either by no reaction or by a very narrow zone of browning around the stab inoculation.

Test Reagent for Sodium Hippurate Hydrolysis (Acid Ferric Choride Solution)

Ferric chloride	12.0 g
Concentrated hydrochloric acid	2.5 mL
Distilled water	100.0 L

The acid is diluted with 75 mL of water and the ferric chloride is dissolved in the fluid by warming. The volume is adjusted to 100 mL with distilled water.

ANTIGEN PREPARATION FOR LANCEFIELD GROUPING BY THE RING PRECIPITATION TEST

Hot HCl Extraction

- A pure culture of the *Streptococcus* species is grown in 25 mL of Todd–Hewitt broth at 37°C for 24–48 hours.
- Centrifuge the broth to concentrate the cells. Discard the supernatant.
- Add 1 mL of the stock HCl–saline mixture (1 ml conc. HC1 + 99 mL of N saline) and resuspend the cells.
- Place in a boiling water bath for 15 minutes and allow to cool.
- Add one drop of phenol red indicator and neutralize with N/10 NaOH until the suspension is a pale pink colour.
- Centrifuge and use the supernatant as the antigen for the test.

Autoclave Extraction

- A pure culture of the *Streptococcus* species is grown in 25 mL of Todd–Hewitt broth at 37°C for 24–48 hours.
- Centrifuge the broth to concentrate the cells. Discard the supernatant.
- Add 0.5 mL of 85% NaC1 solution to the cells and shake to resuspend.
- Autoclave the suspension for 15 minutes at 121°C.
- Cool and centrifuge. Decant the supernatant into a clean tube for use as antigen in the test.

Ring Precipitation Test for Lancefield Grouping

To economize on commercial antisera use a capillary tube of outside diameter 1.2–1.5 mm. Autoclave the capillary tubes and store in a sterile container. The antisera chosen to test against the antigen extract will depend on the animal species and lesion from which the *Streptococcus* was isolated. For example, the majority of the streptococci isolated from horses are Lancefield Group C.

- Dip a sterile capillary tube into the antiserum until a column of about 1 cm long has been drawn into the tube. Plunge the lower end of the capillary tube into plasticine stuck on a microscope slide so that the tube is held upright.
- With a finely drawn glass Pasteur pipette carefully layer the antigen solution on top of the antiserum, taking care that there are no air bubbles and that no mixing of the antiserum and antigen occurs.
- Examine the tube in bright light against a dark background. A white ring of precipitate should appear, in a positive reaction, in five to 30 minutes. Precipitate formation after 30 minutes should be disregarded.

MICROBIOLOGICAL STAINING SOLUTIONS AND PROCEDURES

Preparation of Staining Solutions

The staining solutions can be prepared from dye powders but some of the staining reagents can be bought as

solutions, such as strong carbol fuchsin solution and the reagents for the Gram stain.

The methanol (methyl alcohol), ethanol (ethyl alcohol), acetic acid and concentrated hydrochloric acid used in the formulae should be of Analar grade. After a staining solution has been prepared it should be filtered into a clean container before use. Commercially prepared solutions may occasionally require filtering if a deposit forms.

Blue-Black Ink (for Staining Fungal Elements)

10% potassium hydroxide (KOH)	1 part
Permanent blue-black ink	2 parts
Wetting agent solution	1 part

Wetting agent solution:

Sodium benzoate	0.15 g
Dioctyl sodium sulphosuccinate	0.85 g
Distilled water	100.0 mL

The fungi take up the dye (ink) selectively and the specimen is also satisfactorily cleared by the 10% KOH.

Calcofluor White (for Fungal Elements)

Preparation of solutions

- Stock solution: 1% (w/v) calcofluor white H2R (Polysciences, Inc.) or fluorescent brightener 28 (Sigma Chemical Co.). Dissolve 1.0 g powder in 100 mL of distilled water with gentle heat.
- Working solution: 0.1% calcofluor white with 0.05% Evans blue dye for a counter-stain. For use add one drop of 10% KOH and one drop of calcofluor white working solution to the specimen on a microscope slide.

Uses for calcofluor white

- Hair and skin samples: one drop of 10% KOH and one drop calcofluor white working solution (0.1%) is used. The preparation is examined microscopically under excitation light of 350 nm.
- Exudates and fluids: one drop of calcofluor white (0.1%) is emulsified with the specimen on a microscope slide and covered with a coverslip. The preparation is examined microscopically under excitation light of 350 nm.
- Histopathological sections: either paraffin-embedded or frozen sections can be used. Four or five drops of calcofluor white (0.1%) are placed on unstained sections and allowed to stand for 1 minute. The preparation is rinsed with tap water and counter-stained with 0.05% Evans blue for one minute to minimize background fluorescence. The preparation is examined microscopically under an excitation light of 350 nm.

Castaneda's Technique (for Chlamydial Elementary Bodies)

- Impression smears are air-dried and fixed in mordant solution (formalin 100 mL and glacial acetic acid, 7.5 mL).
- Wash in tap water.
- The smear is stained for 10 minutes in formol blue solution that consists of 90 mL 0.15M phosphate buffer, pH 7.0, 10 mL Unna's blue (1% azure II in methyl alcohol) and 5 mL formalin.
- Wash thoroughly in tap water.
- The stained smear is then differentiated for 5–10 seconds in 0.25% aqueous safranine.

Chlamydial elementary bodies stain blue against a reddish background.

Dienes' Stain (for Mycoplasmal Microcolonies)

Methylene blue	2.4 g
Maltose	10.0 g
Azure II	1.25 g
Sodium chloride	0.25 g
Distilled water	100.0 mL

Dissolve all the ingredients in the distilled water.

Giemsa Stain

Giemsa-stain reagents

Giemsa powder	1.0 g
Glycerol	66.0 mL
Absolute methanol	66.0 mL

The Giemsa powder is dissolved in the glycerol at 55–60°C for about two hours. Methanol is added and mixed thoroughly.

Buffer for Giemsa stain (pH 7.0)

Sodim phosphate, Na_2HPO_4 (anhydrous) M/15 solution (9.47 g/L) **or** $Na_2HPO_4.2H_2O$, M/15 solution (11.87 g/litre)	61.1 mL
Potassium phosphate, KH_2PO_4 (anhydrous) M/15 solution (9.08 g/litre)	38.9 mL
Distilled water	900.0 mL

The solution from the above formula is mixed with the buffer solution before use (one volume of stain to nine volumes of buffer).

Gram Stain Reagents

Crystal violet

Crystal violet	2.0 g
Ethanol 95% (vol/vol)	20.0 mL
Ammonium oxalate	0.8 g
Distilled water	80.0 mL

The crystal violet is first dissolved in the ethanol, then the ammonium oxalate is dissolved in the distilled water. The two solutions are added together. To aid the dissolving process, both mixtures are agitated in a bath of hot water.

Gram's iodine (mordant)

Iodine crystals	1.0 g
Potassium iodide	2.0 g
Distilled water	200.0 mL

The iodine crystals and the potassium iodide are ground together in a mortar and the distilled water is added slowly. If necessary the mixture can be agitated in a bath of hot water to aid dissolving.

Decolourizer

Ethanol 95% (vol/vol)

Dilute carbol fuchsin (counter-stain)

Concentrated carbol fuchsin (see ZN stain)	10.0 mL
Distilled water	90.0 mL

India Ink (for demonstrating capsules)

India ink (Pelican brand)	1.0 mL
Formalin (40%)	9.0 mL

The solutions are mixed together for use.

Lactophenol Cotton Blue Stain (for staining fungal elements in wet preparations)

Phenol crystals	20.0 g
Glycerin	40.0 mL
Lactic acid	20.0 mL
Distilled water	20.0 mL

The ingredients are dissolved by gently heating over a steam bath. When in solution, 0.05 g of cotton blue dye is added. Mix the solution thoroughly.

Macchiavello's Method (for staining chlamydial elementary bodies)

Basic fuchsin solution

Basic fuchsin (88–90% dye content)	0.25 g
Distilled water buffered at pH 7.4 with 4.0 mL of 0.15 M phosphate buffer	100.0 mL

Filter through coarse filter paper directly onto the slide.

Staining method

- The prepared smear is air-dried and gently heat-fixed.
- The slide is flooded with basic fuchsin for four to six minutes.
- The stain is poured off and the slide washed with tap water.
- The slide is dipped in 0.5% citric acid solution and immediately removed and washed with tap water.
- The preparation is counter-stained for 10 seconds with 1% aqueous methylene blue and then washed with tap water and air-dried.

The chlamydial elementary bodies stain red and the background is blue.

The methylene blue staining procedure for chlamydiae

- McCoy cell coverslip cultures are fixed in two changes of methanol for three to five minutes.
- Methanol is replaced with an aqueous solution of methylene blue (5 g/L) and the preparation stained for no longer than five to seven minutes.
- The stain is removed and the cultures are washed in tap water.
- The preparations are washed for 15 seconds in 0.025% H_2SO_4 followed rapidly by a rinse with tap water.
- Dehydration is carried out in acetone for 15 seconds and this is followed by clearing in xylene for 15 seconds and mounting in DPX. If the slides are examined under darkfield microscopy, the chlamydial inclusions will appear as refractile yellow-green bodies surrounded by a halo.

Modified Ziehl–Neelsen Stain Reagents

Dilute carbol fuchsin

Same formula as for the Gram stain.

Acetic acid (decolourizer)

Concentrated acetic acid	1.0 mL
Distilled water	200.0 mL

Methylene blue (counter-stain)

Methylene blue	8.0 g
Ethanol 95% (v/v)	300.0 mL
Distilled water	1300.0 mL
Potassium hydroxide	0.13 g

If using Loeffler's methylene blue, the potassium hydroxide can be omitted.

Nigrosin Staining Solution (for Demonstrating Capsules)

Nigrosin (granular)	10.0 g
Formalin 10%	100.0 mL
or 1 : 10,000 merthiolate	100.0 mL

The solution is placed in a bath of boiling water for 30 minutes and then any solvent lost by evaporation is replaced. The solution should be filtered twice through double filter paper (Whatman No. 1).

Silver Stain for Flagella (West et al. 1977)

Solution A

Saturated aqueous aluminium phosphate	25.0 mL
5% aqueous ferric chloride	5.0 mL
10% aqueous tannic acid	10.0 mL

Solution B

1. A solution of 100 mL of 5% silver nitrate is prepared.
2. Two to five drops of concentrated ammonium hydroxide are added to 90 mL of the silver nitrate solution. A brown precipitate forms which dissolves as more base is added. Adding the ammonium hydroxide is discontinued just at the point of clearing.
3. The procedure is then reversed and the remaining silver nitrate solution is added, one drop at a time, until the stain solution is cloudy.
4. The solution is stored in a dark bottle at room temperature. It is stable for several months.

Staining procedure

A loopful of culture is mixed with 3–5 mL of distilled water. One drop of this bacterial suspension is placed on a clean, grease-free, microscope slide and allowed to air-dry. The smear is flooded with solution A and left for four minutes. The slide is rinsed with distilled water and then flooded with solution B. The slide is heated gently using a low bunsen flame held under the staining rack. Steam should issue from the slide but the stain must not be allowed to boil. This heating is continued for four minutes, then the preparation is rinsed with distilled water and dried in a slanted position.

Victoria Blue Stain (for *Brachyspira Hyodysenteriae*)

Victoria blue 4-R	0.5 g
Ethyl alcohol (absolute)	5.0 mL
Distilled water	95.0 mL

The dye is dissolved in the alcohol and then the distilled water is added and the solution mixed well.

Staining method

A smear of faeces or colonic mucosal scrapings is made on a microscope slide. It is fixed in 10% formalin for 15 minutes and air-dried. The smear is flooded with the 0.5% solution of Victoria blue 4-R and stained for five minutes. The slide is washed under a running tap, air-dried and examined microscopically under the oil-immersion objective.

Ziehl-Neelsen Stain Reagents

Concentrated carbol fuchsin

Basic fuchsin	1.0 g
Ethanol 95% (v/v)	10.0 mL
Phenol	5.0 g
Distilled water	100.0 mL

The basic fuchsin is dissolved in the ethanol, the phenol is then dissolved in the distilled water and the two solutions mixed together. The solution is allowed to stand for a few days and then filtered into a clean container.

Acid-alcohol (decolourizer)

Concentrated hydrochloric acid	8.0 mL
Ethanol 95% (v/v)	97.0 mL

The acid should be added to the alcohol.

Methylene blue (counter-stain)

Same formula as in the modified Ziehl–Neelsen stain.

Brilliant green (alkaline)

Can be used as an alternative counter-stain.

Brilliant green	1.0 g
Sodium hydroxide 0.01%	100.0 mL

REFERENCE

West, M., Burdash, N.M., Freimuth, F., 1977. Simplified silver-plating stain for flagella. Journal of Clinical Microbiology 6, 414–419.

Appendix 2

Culture and transport media

CULTURE MEDIA

Selective Medium for *Brucella abortus*

Columbia agar	42.5 g
Dextrose	1.0 g
Distilled water	1.0 L

The medium is autoclaved at 121°C for 15 minutes and cooled to 50°C.

Sterile horse serum (5%) and the antibiotic supplement are then added.

Antibiotic supplement

Polymyxin B	6.25 units/mL of medium
Bacitracin	25.0 units/mL
Nalidixic acid	5.0 µg/mL
Actidione	00.0 µg/mL

Selective Medium for *Brucella ovis*

Mixture A

GC agar base (BBL)	7.2 g
Distilled water	100.0 mL

Mixture B

Bovine haemoglobin powder	1.0 g
Distilled water	100.0 mL

Mixture A is heated in a boiling water bath to dissolve the agar. The ingredients of B are mixed to obtain a solution of haemoglobin. Each solution is sterilized by autoclaving at 121°C for 15 minutes and then allowed to cool to 50°C. The contents of a vial of VCN inhibitor (BBL) is dissolved in 10 mL of sterile water and added to mixture A. Mixture B is then added to A and mixed well but foaming should be avoided. The medium is poured into Petri dishes. The final concentration of antibiotics per mL of medium is: vancomycin 15 µg/mL, colistin 37.5 µg/mL and nystatin 62.5 units/mL.

Chocolate Agar

Columbia blood agar base	39.0 g
Distilled water	1.0 L

The blood agar base is boiled to dissolve the base prior to autoclaving at 121°C for 15 minutes. After cooling to 50°C, 70 mL of sterile defibrinated sheep blood is added and the mixture is heated to 80°C in a water bath with constant gentle mixing until it turns brown. The medium is cooled to 50°C and poured into Petri dishes.

Clostridium chauvoei Blood Agar (Batty and Walker)

Broth base	94.0 mL
Liver extract (Oxoid)	3.0 g
Glucose	1.0 g
New Zealand agar	1.6 g
Defibrinated sheep blood	5.0 mL

The broth base, liver extract, glucose and agar are mixed together and the mixture autoclaved at 115°C for 10 minutes. The medium is cooled to 50°C and the blood added. The blood is mixed gently with the medium and poured into Petri dishes.

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Clostridium novyi (types B and C) and *C. haemolyticum* Medium (Moore)

Basal medium

Neopeptone (Difco)	1.0 g
Yeast extract (Difco)	0.5 g
Proteolysed liver	0.5 g
Glucose	1.0 g
New Zealand agar	2.0 g
*Salts solution	0.5 mL
Distilled water	100.0 mL

Salts solution (g/litre):

$MgSO_4.7H_2O$	40.0 g
MnSO4	2.0 g
$FeC1_3$ (anhydrous)	0.4 g
HC1 (concentrated)	0.5 mL

The neopeptone, yeast extract, liver extract and glucose are dissolved in 50 mL of water. The salt solution is added and the pH adjusted to 7.6–7.8. The agar is dissolved in 50 mL of water and the two solutions are combined. This basal medium is dispensed in 18 mL volumes in 28 mL screw-capped bottles and is sterilized by autoclaving at 115°C for 10 minutes. It is cooled and stored at 4°C.

Preparation of the reducing solution

Cysteine HC1	120.0 mg
Dithiothreitol	120.0 mg
Glutamine	60.0 mg
Distilled water	10.0 mL

The ingredients are dissolved in the water and the pH adjusted to 7.6–7.8. The solution is sterilized by filtration and must be freshly prepared before use.

Complete medium

An 18 mL volume of basal medium is melted and cooled to 50°C. Defibrinated horse blood (2 mL) and reducing solution (0.15 mL) are added to, and mixed gently with the basal medium. The plates should be poured immediately.

Milk Agar for Casein Digestion

Skim milk	500.0 mL
Nutrient agar (double-strength)	500.0 mL

The skim milk is sterilized at 115°C for 10 minutes and then cooled to 50°C. Sterilized, double-strength nutrient agar is cooled to 50°C and added to the skim milk. The skim milk and agar are mixed gently and poured into Petri dishes. (*Corynebacterium renale* is used as the positive control.)

*Salts solution (g/L)

Niger Seed (Birdseed) Agar (Staib Agar)

Pulverized *Guizotia abyssinicia* seed	50.0 g
Agar	15.0 g
Glucose	1.0 g
Creatinine	1.0 g
KH_2PO_4	1.0 g

The seed is added to 100 mL of distilled water and ground in a blender. The mixture is boiled for half an hour and then strained through gauze to separate the seed from the extract. The volume of the seed extract is adjusted to 1000 mL with distilled water. The agar and other ingredients are added and the mixture boiled gently until the agar is dissolved. The medium is autoclaved at 110°C for 20 minutes, cooled to 50°C and then poured into Petri dishes.

PLET Agar (for *Bacillus anthracis*)

Heart infusion agar (Difco)	25.0 g
EDTA	0.3 g
Thallous acetate	0.04 g
Deionized distilled water	1000.0 mL

The ingredients are mixed and dissolved completely. The medium is sterilized by autoclaving at 121°C for 15 minutes and then cooled to 56°C. A filter-sterilized solution of polymyxin B (30,000 units) and lysozyme (300,000 units) are added to the medium and mixed well before being poured into Petri dishes.

Smith–Baskerville Medium for *Bordetella bronchiseptica* (Smith & Baskerville 1979)

Bacto peptone	20.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	857.0 mL

The basal medium is autoclaved at 121°C for 15 minutes and then cooled to 55°C. The following supplementary solutions are mixed together and added to the cooled agar medium.

Antimicrobial supplement (given as the final concentration in the medium)

Gentamicin	0.5 µg/mL
Penicillin	20.0 µg/mL
Furaltadone	20.0 µg/mL

Carbohydrate supplement (filter-sterilized)

Glucose (10%)	100.0 mL
Lactose (10%)	100.0 mL

Indicator solution (filter-sterilized)

Bromothymol blue (0.2%)	40.0 mL

The bromothymol blue is first prepared as a 2.0% stock solution:

Bromothymol blue	1.0 g
0.1 N NaOH	25.0 mL
Distilled water	475.0 mL

Taylorella equigenitalis Medium (Timoney et al. 1982)

Chocolate agar is prepared with Eugon agar (BBL) and 5% horse blood.

The chocolate agar is supplemented with:

5% lysed horse blood

Trimethoprim	1 µg/mL
Clindamycin	5 µg/mL
Amphotericin B	5 µg/mL

The addition of Isovitalex (BBL), 1 mL reconstituted per 100 mL of medium, is optional. The shelf life of the medium is four weeks at 4°C.

Tween 80 Medium for the Hydrolysis of Tween 80

Blood agar base (Oxoid)	40.0 g
Distilled water	1000.0 mL
10% $CaC1_2$ (filtered)	10.0 mL
Tween 80	10.0 mL

The blood agar base is added to the distilled water and allowed to soak for 10 minutes. The mixture is heated gently to dissolve the agar and the other ingredients are added. The medium is autoclaved at 121°C for 20 minutes, cooled and poured into Petri dishes. (*Corynebacterium cystitidis* is used as the positive control.)

Skirrow Medium

Suggested as a medium suitable for both *Campylobacter fetus* and *Brucella* species by Terzolo et al. (1991).

Blood agar base No. 2 (Oxoid CM 271)	1000 mL
Lysed, defibrinated horse blood	50 mL
Vancomycin	10 mg
Polymyxin B	2500 units
Trimethoprim	5 mg

The autoclaved blood agar base is cooled to 50°C and the other ingredients are added to the sterile molten agar medium.

Stonebrinks Medium for *Mycobacterium bovis*

Ingredients

Salt mixture

Sodium pyruvate	5.0 g
Potassium dihydrogen orthophosphate	2.0 g
Distilled water	300.0 mL

Dye mixture

Crystal violet	100.0 mg
Malachite green	800.0 mg
Distilled water	100.0 mL

Hens' eggs

Twenty fresh eggs from hens that have not received antimicrobial agents either therapeutically or prophylactically.

Preparation of medium

- The salt solution is added to the distilled water and mixed until completely dissolved. The pH is adjusted to 6.5 using a solution of sodium phosphate, dibasic (Na_2HPO_4). The solution is placed in a two-litre flask.
- The dyes are added to the distilled water and stirred until completely dissolved. The solution is placed in a 100 mL bottle.
- Both solutions are sterilized by autoclaving at 121°C for 15 minutes.
- The eggs are washed in soapy water, rinsed in tap water and dried. They are then placed in a container and covered with 75% isopropyl alcohol for 30 minutes. The eggs are air-dried on sterile cotton wool in a cabinet with ultraviolet strip lighting. When dry, the eggs are cracked on the edge of a sterile beaker and the contents placed in a sterile blender jar. The contents of the eggs are homogenized gently in a blender.
- The dye mixture is added first to the sterile salt solution and then the homogenized egg mixture is added and the mixtures are combined thoroughly.
- The mixture is dispensed in 12 mL amounts into universal bottles.
- The bottles are slanted and the contents inspissated at 80°C until the medium has solidified (usually 40–60 minutes).
- The slants are cooled for 30 minutes and then incubated at 37°C for 24–48 hours to facilitate the absorption of moisture into the medium.

Ureaplasmas: Hayflick's Medium (Modified)

Broth medium

Mycoplasma broth base (BBL)	2.1 g
Distilled water	66.0 mL
Horse serum	20.0 mL
Yeast extract 25% (w/v)	10.0 mL
Urea 25% (w/v)	1.0 mL
Phenol red 0.5%	0.2 mL
Penicillin solution	500 units/mL

Final pH should be adjusted to 6.0 ± 0.2.

Agar medium

For solid agar medium add 1.0 g Ionagar (No. 2) and omit the urea and phenol red.

Vitamin K-Haemin Supplement for Nonsporing Anaerobes

Stock haemin solution

50 mg haemin is dissolved in 1 mL 1 N NaOH.
100 mL of distilled water is added and the solution is autoclaved at 121°C for 15 minutes.

Stock vitamin K solution

100 mg menadione (vitamin K) is dissolved in 20 mL of 95% ethanol and the solution is sterilized by filtration.

For addition to media

Sterile menadione solution	1.0 mL
Sterile haemin solution	100.0 mL

1 mL of the vitamin K-haemin solution is added to 100 mL of sterile medium.

TRANSPORT MEDIA

Transport Medium and Transportation Procedures for Anaerobes

Modified Cary–Blair medium

Cary–Blair medium (BBL)	2.5 g
1% solution of $CaC1_2$	1.8 mL
Resazurin solution 0.1% w/v	0.1 mL
L-cysteine HC1	0.1 g
Distilled water	198.0 mL

All the ingredients, except the cysteine, are heated in a flask with glass beads until the agar has dissolved. The mixture is cooled in an oxygen-free atmosphere with carbon dioxide. The cysteine is then added and the pH adjusted to 8.4. The medium is dispensed in 10 mL aliquots in 16 × 125 mm tubes and stoppered with rubber bungs. The filling of the tubes must be carried out under oxygen-free nitrogen gas or in an anaerobic chamber. The tubes are sterilized by steaming for 15 minutes. Any pink colouration in the tubes indicates that the contents are not anaerobic.

Oxygen-free swabs

These oxygen-free swabs are sterilized in a tube filled with oxygen-free nitrogen gas. When taking specimens, the swabs are removed from the tube, used quickly and then pushed deep into a pre-reduced, anaerobically sterilized tube of modified Cary–Blair medium (prepared as above).

Transport Medium for *Campylobacter fetus* (Clark & Dufty 1978)

Fresh bovine serum containing per mL:

5-fluorouracil	300 µg
Polymyxin B sulphate	100 units
Brilliant green	50 µg
Nalidixic acid	3 µg
Cycloheximide	100 µg

The mixture is dispensed in 10 mL aliquots into 30 mL vaccine bottles and the rubber stoppers inserted. The bottles are placed in a boiling water bath for two minutes to allow the serum to solidify. The medium is stirred with a sterile glass rod when cool. An 18-gauge hypodermic needle is inserted through the rubber stoppers and the bottles are placed in an anaerobic jar under an atmosphere of 2.5% oxygen, 10% carbon dioxide and 87.5% nitrogen. The needles must be removed immediately after opening the jar. The medium should be stored at 4°C for at least one week before use. The transport medium has a shelf life of up to three months at 4°C.

Transport Medium for Chlamydiae (Spencer & Johnson 1983)

Make up the sucrose-phosphate-glutamate (SPG) solution in distilled water as follows:

Sucrose	74.6 g/L
KH_2PO_4	0.512 g/L
K_2HPO_4	1.237 g/L
L-glutamic acid	0.721 g/L
Phenol red	0.015 g/L

The pH can be adjusted to 7.0 with KOH. The SPG solution is sterilized by autoclaving.

Aseptically add:

Vancomycin	100.0 µg/mL
Nystatin	50.0 µg/mL
Streptomycin	100.0 µg/mL
Gentamicin	50.0 µg/mL
Foetal calf serum	10%

The medium is dispensed aseptically in 4 mL amounts and stored at −20°C.

Mycoplasmal Transport or Culture Medium

Transport medium

PPLO broth (Difco)	16.8 g
Distilled water	800.0 mL
Horse serum	200.0 mL
50% yeast extract	10.0 mL
DNA (Sigma)	0.02 g
Penicillin	2000 units/mL medium
Thallium acetate	1 : 100,000

Swabs can be placed into 2 mL aliquots of this medium.

Culture medium

The medium is also suitable as a broth culture medium for *Mycoplasma* species. To convert the broth medium to an agar culture medium, substitute 28 g PPLO agar (Difco) for the 16.8 g of PPLO broth base.

Transport Medium for Viral Specimens

The medium is essentially an isotonic solution containing protein, a buffer to control the pH and antimicrobial agents to control contaminating bacteria and fungi.

Hank's Balanced Salt Solution	1000.0 mL
Sodium bicarbonate	8.0 g
Bovine albumin	10.0 g
Phenol red (0.4%)	5.0 mL
Penicillin	500 units/mL of medium
Streptomycin sulphate	500 µg/mL
Nystatin	50 units/mL

The transport medium is sterilized by filtration through a 0.22 µm membrane filter. It can be dispensed into sterile bijoux bottles in 4 mL amounts. The shelf life is approximately three weeks at 4°C.

REFERENCES

Clark, B.L., Dufty, J.H., 1978. Isolation of *Campylobacter fetus* from bulls. Australian Veterinary Journal 54, 262–263.

Smith, I.M., Baskerville, A.J., 1979. A selective medium facilitating the isolation and recognition of *Bordetella bronchiseptica* in pigs. Research in Veterinary Science 27, 187–192.

Spencer, W.N., Johnson, F.W.A., 1983. Simple transport medium for the isolation of *Chlamydia psittaci* from clinical material. Veterinary Record 113, 535–536.

Terzolo, H.R., Paolicchi, F.A., Moreira, A.R., et al., 1991. Skirrow agar for simultaneous isolation of *Brucella* and *Campylobacter* species. Veterinary Record 129, 531–532.

Timoney, P.J., Shin, S.J., Jacobson, R.H., 1982. Improved selective medium for isolation of the contagious equine metritis organism. Veterinary Record 111, 107–108.

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