# Mycoplasma Diseases of Ruminants

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**Robin Nicholas**

**Roger Ayling**

**Laura McAuliffe**


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CABI Head Office
Nosworthy Way
Wallingford
Oxfordshire OX10 8DE
UK

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: cabi@cabi.org
Website: www.cabi.org

CABI North American Office
875 Massachusetts Avenue
7th Floor
Cambridge, MA 02139
USA

Tel: +1 617 395 4056
Fax: +1 617 354 6875
E-mail: cabi-nao@cabi.org



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# Contents

The colour plate section can be found following p.118.

# Contributors

The following made a major contribution to parts of this book:

The Mycoplasma Group at the Veterinary Laboratories Agency, in particular Joanna Lawes, Katie Parham and Colin Churchward (identification and molecular typing)

Guido Ruggero Loria, Istituto Zooprofilattico Sperimentale, Palermo, Italy (contagious agalactia, eye infections)

Rosário Gonçalves, Laboratório Nacional Investigaçao Veterinaria, Estrada di Benfica, Lisbon, Portugal (immunoblotting, contagious bovine pleuropneumonia and atypical pneumonia)

Otto J.B. Hubschle, Chief Veterinary Officer, Windhoek, Namibia (contagious bovine pleuropneumonia)

Umit Ozdemir, Pendik Veterinary Control and Research Institute, Pendik, Istanbul, Turkey (contagious caprine pleuropneumonia)

Massimo Scacchia, Istituto Zooprofilattico Sperimentale, Teramo, Italy (contagious bovine pleuropneumonia and caprine respiratory diseases)

José Regalla, formerly of the Laboratório Nacional di Investigaçao Veterimaria, Estrada di Benfica, Lisbon, Portugal (contagious bovine pleuropneumonia)

Mark Fielder, Kingston University, Penrhyn Road, Kingston, Surrey, UK (atypical pneumonia)

Former PhD students: Bilal Houshaymi, Waleed Al-Momani, L. Ali Khan, Masoud Shahram, Paola de Santis and Yi Ching Lin of King's College London; and Hiren Patel of Kingston University (many aspects)

And the late Roger Miles of King's College London (many aspects)

# Preface

Mycoplasma diseases of livestock such as contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP) and contagious agalactia still pose major problems for animal health authorities worldwide. CBPP remains one of the biggest impediments to cattle farming in many countries in sub-Saharan Africa. New regions and new animal species have been infected by CCPP since the turn of the century and little effective control exists for contagious agalactia, which is endemic in sheep and goat populations in countries surrounding the Mediterranean and in the Middle East, this despite a major research effort stimulated by the European Union following the unexpected reappearance of CBPP in Italy in the early 1990s. Sadly, and probably as a result of the eradication of CBPP from Europe at the end of the 20th century, many groups active in mycoplasma research a decade ago have had to develop research interests elsewhere because of financial constraints. Consequently, the development of improved vaccines and better treatments for mycoplasma diseases in developing countries now move more slowly than ever. However, many significant improvements have been seen as a result of research into these degenerate bacteria, mainly in the area of diagnosis, with the availability of exquisite new molecular tools to detect minute quantities of these pathogens, some of them highly fastidious or unculturable and often in mixed bacterial culture, directly from clinical samples. Epidemiological fingerprinting tools are now available to identify the likely origins of outbreaks as well as providing fascinating insights into the evolution of these organisms. Fundamental research on biofilm production has also shown for the first time how these fragile organisms can withstand hostile environments and antimicrobials. The last decade has also shed light on other mycoplasmas, most importantly *Mycoplasma bovis*, which causes a whole range of clinical conditions, including calf pneumonia, mastitis, arthritis, eye diseases and, more recently, brain diseases. The increasing interest shown by the major pharmaceutical companies, evident with the production of new antimicrobials and the funding of vaccine research, is an indication of the financial

rewards that can be made for developing control measures against these endemic and often untreatable diseases of livestock.

Despite the introduction of 16S rDNA sequence data to help describe new and unclassified species, mycoplasma taxonomy moves agonisingly slowly. It seems likely that the unclassified M. ovine/caprine serogroup 11 will soon be merged into the species *M. bovigenitalium*. Attempts to reclassify the mycoplasmas of the *mycoides* cluster will, no doubt, grind on for many more years to come. A great deal of genetic, biochemical and immunological evidence has accumulated to enable the merging of the two caprine pathogens, *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* large colony (LC) into a single subspecies or even species. However, official acceptance of this recommendation is unlikely to take place before publication of this book, although several authors have begun using the name *M. mycoides* subsp. *capri* to describe both mycoplasmas. Consequently, readers will come across both names, often used synonymously and we apologise for any confusion this may cause.

This book aims to encapsulate research and development carried out on mycoplasma diseases of sheep, goats and cattle over the last decade by laboratories worldwide but focusing on that performed by our group at the Veterinary Laboratories Agency (Weybridge, UK) and its many international collaborators, in particular those at the Istituti Zooprofilattico Sperimentale in Sicily and Abruzzo e Molise (Teramo) and the University of Milan in Italy; the Central Veterinary Laboratory in Namibia; the Pendik Veterinary Control and Research Institute near Istanbul, Turkey; the Laboratorio Nacional de Investigacao Veterinaria, Lisbon, Portugal; the University of Missouri, Columbia, USA; the Department of Primary Industries, New South Wales, Australia; and, closer to home, King's College and Imperial College London.

# Acknowledgements

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Personal thanks go to: Chris Nicholas for proofreading the manuscript and providing a lay view of the work, Ashley Nicholas for translating old German texts and to Mrs Kathy Holley for her encouragement. Final thanks to Federigo Santini for showing us our first case of CBPP.

# I Methods

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# 1 Isolation and Growth of Mycoplasmas from Ruminants

Mycoplasmas, or more correctly mollicutes, the bacterial class which incorporates all the degenerate, wall-less bacteria, including mycoplasmas, acholeplasmas, ureaplasmas, spiroplasmas, entomoplasmas, mesoplasmas, phytoplasmas and the recently reclassified eperythrozoans and haemobartonellas, are characterized by their small genome size (0.58–1.38 Mbp), a low G+C content (23–40 mol%) of the genome and a permanent lack of a cell wall. Over 200 species have so far been described. According to the recent taxonomy of prokaryotes, the mollicutes belong to the phylum Firmicutes, which contains the Gram-positive bacteria and also comprises the *Bacilli* and *Clostridia*, from which the mollicutes have derived by a process of degenerative evolution. The terms mycoplasmas and molllicutes are used synonymously throughout this book.

## Growth and Nutritional Requirements of Mycoplasmas

The difficulty of culturing mycoplasmas *in vitro* is a major obstacle to research and laboratory diagnosis of these fastidious organisms, and it is highly likely that many more mycoplasmas exist in nature but have not yet been isolated, despite great efforts over many years (Razin *et al.*, 1998) including the introduction of PCR; in addition, many isolated mycoplasmas still grow very poorly even on the best mycoplasma medium (Razin, 1994).

The limited capability of mycoplasmas to synthesize macromolecules essential for growth reflects their evolutionary development, which has resulted in the small size of the mycoplasma genome. To overcome these deficiencies, complex media are used for their cultivation. The medium is usually based on beef heart infusion, peptone, yeast extract and serum with various supplements (Razin, 1991). Mycoplasmas are completely dependent on the host for exogenous fatty acids and require amino acids, nucleic acid precursors, lipid precursor molecules and vitamins. The medium must contain sterol such as cholesterol, which may be

replaced by other sterols such as cholestanol or ergosteroll (Rodwell and Mitchell, 1979). Glycerol oxidation is very important for the synthesis of glycophospholipid and glycerides, which is consequently important for lipid synthesis.

Glucose is the main source of energy in fermentative mycoplasmas, as well as a source of carbon for the synthesis of other sugars and polysaccharides. The fermentative mycoplasmas can also use maltose, trehalose, starch and glycogen (Razin and Freundt, 1984); pyruvate can replace glucose in non-fermentative mycoplasmas such as *Mycoplasma bovis* and *M. agalactiae* for energy production (Miles *et al.*, 1988).

Peptones provide the media with different polypeptides, di-peptides and amino acids (Miles, 1992). A novel medium, called TSB-1, that is devoid of ruminant peptone and which may improve isolation of animal mycoplasmas from tissues and increase growth yields for antigen and vaccine production, has been reported (Khan *et al.*, 2005; Patel *et al.*, 2008); the use of vegetable peptones also reduces the risk of contamination of vaccines with agents causing the transmissible spongiform encephalopathies. Different types of animal sera (calf, horse, porcine) are used at 5–20% as a source of essential lipids. Other nutrients are provided by the sera, including sugars, urea and inorganic ions. There is a considerable difference in the nutritional properties of different animal sera, which depends on their lipid concentration. Animal sera are usually inactivated by heating in a water bath at 56°C for 30 min to reduce the complement component of the serum, which can cause cell lysis. Continual efforts have been made to replace the serum component with albumin, fatty acids and cholesterol supplemented with serum albumin to neutralize free fatty acid toxicity (Razin, 1978), but few of these efforts have been successful.

Beef heart infusion and yeast extract provide a variety of nutrients, including nucleotides, vitamins and mineral salts. Fresh yeast extracts are superior to commercial dehydrated extracts because they contain labile components which are destroyed during commercial processing. The addition of organic components including DNA and NADH (a coenzyme present in animal tissue and yeast extracts) may enhance the growth of different types of mycoplasmas by lowering the oxidation–reduction potential of the media and making them more suitable for the growth of anaerobic or microaerophilic organisms (Miles, 1992). Energy sources are provided through the inclusion of glucose, pyruvate, arginine or urea.

Mycoplasmas lack a cell wall and therefore they are more susceptible to cell lysis in hypo-osmotic media than other cell-walled bacteria, so they need sodium chloride to increase medium tonicity. They also require an osmotic pressure of 7–14 atmospheres for optimal growth. For most mycoplasmas growth is best at pH 7–8 (Rodwell and Mitchell, 1979) and typical media have a pH of 7.6. The growth of mycoplasmas is sensitive to any change in pH; a decrease in pH to less than 6.5 due to sugar fermentation causes a limit to growth and consequently leads to cell death; an increase in pH above 8.0 may also lead to cell death. Mycoplasma media should be well buffered because of the narrow range of pH values for mycoplasma growth. The buffers mostly used are phosphate buffer and N-2 hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Miles, 1992).

Oxygen plays an important role in mycoplasma growth. The need for oxygen depends on the strain; some mycoplasmas prefer to grow in anaerobic conditions

while others prefer microaerophilic conditions. Gentle aeration may increase growth rates, which may be due to the fact that this increases the rate of oxidation and thus the production of ATP during the metabolism of glucose or other carbohydrate (Miles and Agbanyim, 1998), while excessive aeration may reduce the culture viability.

Although the numerous nutritional requirements of mollicutes dictate the need for complex growth media, the notion that the richer the medium the better may be wrong. It appears that the lack of growth of mycoplasmas in a rich medium is, in some cases, not the result of the lack of specific nutrients but rather is due to the presence of a component toxic to mycoplasma. The growth inhibitors found in the complex media are mostly components of the peptone and yeast extract.

While DNA amplification techniques are being used with ever-increasing frequency for the detection and identification of mycoplasmas, the isolation of a mycoplasma by conventional techniques is still required by most national and international authorities, particularly where diseases of great importance are concerned, such as contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP). Furthermore, live mycoplasmas are required in antibiotic sensitivity tests, molecular typing, vaccines and for use as antigens in diagnostic procedures. Consequently, the use of a high-yielding, and ideally selective, medium is still essential.

## Mycoplasma Culture

To date over 30 mollicute species have been isolated from small and large ruminants; however, this chapter will concentrate on methods for sampling, transporting and isolating the small number of mycoplasmas which have been shown to be pathogenic, in particular those that cause respiratory disease such as *M. mycoides* subsp. *mycoides* SC, *M. capricolum* subsp. *capripneumoniae* and *M. bovis*, the causes of CBPP, CCPP and calf pneumonia, respectively, and those that cause contagious agalactia, in particular *M. agalactiae* and *M. mycoides* subsp. *capri*.

With the exception of *M. c. capripneumoniae* and *M. dispar*, a cause of respiratory disease in calves, the majority of pathogenic mycoplasmas are not intrinsically difficult to grow and most general-purpose mycoplasma media (Eaton's, Friis modified, Chanock's or Hayflick's) will suffice. A highly productive medium, called PRM, was developed specifically for *M. m. mycoides* SC by Rice and Miles at King's College London (Table 1.1) and is equally useful for other fermenting mycoplasmas (Nicholas *et al.*, 2000). In response to the need to remove ruminant proteins from media because of the risk of TSE (transmissible spongiform encephalopathies), Khan *et al.* (2005) developed a medium containing vegetable proteins for *M. bovis*, and Patel *et al.* (2008) developed a similar medium for *M. ovipneumoniae*. Both media showed a similar, if not increased, production of antigens compared with media containing ruminant proteins. Despite this, most laboratories use two media for primary isolation, in order to maximize isolation. What may complicate isolation, however, is bacterial contamination, the heavy presence of antibiotics

**Table 1.1.** Composition of PRM medium (pH 7.6).

| Component | Concentration ($l^{-1}$) |
|---|---|
| Peptone (g) | 20 |
| Yeast extract (g) | 5 |
| Fresh yeast extract (ml) (Freundt, 1983) | 100 |
| Glucose (g) | 5 |
| Sodium pyruvate (g) | 2 |
| Glycerol (g) | 5 |
| Sodium chloride (g) | 5 |
| HEPES (mmol) | 30–120 |
| Heat-inactivated porcine serum (ml) | 100 |

in the clinical samples and/or where there is overgrowth by less important but rapidly growing mollicutes, notably acholeplasmas. Various strategies are available to offset these problems, such as the use of antibacterial agents like the toxic thallium acetate in addition to the usual range of non-mycoplasmastatic antibiotics. The use of selective inhibitors such as the mycoplasma-resistant nisin (Abu-Amero *et al.*, 1996) promises important advances in the development of selective media. Not only is the compound capable of suppressing acholeplasmas but it would also contribute to controlling cell-walled bacteria.

Since the development of a medium which would support the growth of *M. c. capripneumoniae* for the first time in 1976 by MacOwan and Minette (1976), several others (Bölske, 1988; Thiaucourt *et al.*, 1996) have been reported, including a commercially available one (Mycoplasma Experience, UK), which appear suitable for most strains. As with all media the quality of the components is crucial.

*M. dispar* is a particularly fastidious and slow-growing mycoplasma, especially on solid media. It is easily overgrown by *M. bovirhinis*, a commonly occurring mycoplasma of little significance which is often present in the same samples. A selective medium has been reported which suppresses *M. bovirhinis* while promoting the growth of *M. dispar* (Friis, 1979).

## Sample Collection and Transport

Table 1.2 shows the range of samples that can be taken for the isolation of the pathogenic mycoplasmas. The normal bacteriological procedures apply to sample taking. To ensure optimal recovery, fresh samples of milk and synovial fluid must be taken. Lung lavage techniques have been advocated for detecting invading mycoplasmas in the lower respiratory tract (Thomas *et al.*, 2002).

### Nose, eye or ear swabs

A sterile cotton swab is pre-wetted in transport medium then inserted deep into the nasal passage or ear canal; the surface of the conjunctiva may also be gently

**Table 1.2.** Isolation of mycoplasmas from ruminants.

| Species | Growth *in vitro* | Host | Samples from live animal | Samples from dead animal |
|---|---|---|---|---|
| *M. m. mycoides* SC | Good | Cattle | Nasal swabs, nasal discharge, bronchoalveolar washings, pleural fluid | Lung lesions, pleural fluid, bronchopulmonary lymph nodes |
| *M. c. capripneumoniae* | Fastidious | Goats | Nasal and ear swabs | Lung lesions, pleural fluid, mediastinal lymph nodes |
| *M. agalactiae* | Good | Sheep/goats | Milk, joint fluid, ocular swabs, nasal and ear swabs | Udder and associated lymph nodes, joint fluid, lung lesions, brain |
| *M. bovis* | Good | Cattle | Nasal swabs, ocular swabs, joint fluid, milk, bronchoalveolar washings, pleural fluid, semen | Lung lesions, joint fluid, udder, brain |
| *M. m. capri*/ LC | Good | Goats | Milk, joint fluid, ocular swabs, nasal swabs | Udder and associated lymph nodes, joint fluid, lung lesions |
| *M. c. capricolum* | Good | Goats/sheep | Milk, joint fluid, ocular swabs, nasal swabs | Udder and associated lymph nodes, joint fluid, lung lesions |
| *M. dispar* | Fastidious | Cattle | Nasal swabs, bronchoalveolar washings, pleural fluid | Lung lesions |
| *M. bovigenitalium** | Good | Cattle/sheep | Vulvo-vaginal swabs, discharge from reproductive system, milk, semen | Endometrium and other reproductive sites |
| *M. conjunctivae* | Fastidious | Sheep, goats, wild small ruminants | Eye swabs | Eye swabs |
| *M. putrefaciens* | Good | Goats | Milk | Udder and associated lymph nodes |
| *M. ovipneumoniae* | Varies | Sheep/goats | Nasal swabs, nasal discharge, bronchoalveolar washings, pleural fluid | Lung lesions, pleural fluid, bronchopulmonary lymph nodes |
| *M. canis* | Good | Cattle | Nasal swabs, nasal discharge, bronchoalveolar washings, pleural fluid | Lung lesions, pleural fluid, bronchopulmonary lymph nodes |
| *Ureaplasma* spp | Requires specialized medium containing urea | Cattle, sheep | Semen, vulvo-vaginal swabs/washes/ discharges, nasal swabs | Lung lesions, pleural fluid, bronchopulmonary lymph nodes |

* includes *M.* ovine/caprine group 11

swabbed. The swab is then placed into the transport medium. The swab is agitated briskly in the medium and then discarded. Some workers advocate leaving the swab in the transport medium, which enables the swab to be used to streak the solid medium, which can lead to the direct isolation of mycoplasma colonies. However, in our experience gross contamination often results.

### Lung samples

Lesions are located and the exterior of the organ is sterilized by searing with a hot instrument, flaming or boiling if possible. Small pieces of tissue from the interface between diseased and healthy tissue are aseptically removed. Where possible a fresh set of sterile instruments should be used for each tissue. Each piece of 1–3 $cm^3$ tissue is placed in a separate sterile screw-capped jar containing transport medium. Where lesions are encapsulated, samples from the internal surface should be taken or scraped using a scalpel blade and placed in transport medium.

### Milk samples

The tip of the animal's teat should be cleansed. The initial stream of milk should be discarded. A sterile tube should be filled with the next stream of milk and the milk allowed to stand. Two drops of the milk layer should be placed into a broth using a Pasteur pipette. If there is clotting, a portion of the clot should be used in preference to the supernatant liquid. The whole milk should be used if no layer develops. Bulk or individual milk which appears normal (and is therefore likely to contain only small numbers of mycoplasmas) can be incubated whole after the addition of ampicillin to between 1 and 10 mg/ml, and subcultured after 1 or 2 days. This procedure increases the levels of contamination with ampicillin-resistant organisms but will occasionally result in isolation from milk which proves negative by the standard procedure. Smears may be made for polychrome methylene blue or fluorescent antibody staining.

### Pleural fluid

Pleural fluid is the sample of choice for the diagnosis of CBPP and CCPP but is only present in animals in the acute phase of the disease (Provost *et al.*, 1987; Nicholas and Baker, 1998). Mycoplasmas can be isolated from this sample in pure culture and in high numbers. Animals in the acute phase of disease are identified by clinical signs and euthanazed by humane means; the carcass should be positioned carefully for post-mortem examination (raising the carcass vertically should be avoided). Alternatively, it is possible for a veterinary surgeon to take a few ml of pleural fluid from the acutely affected live animal by puncturing the thoracic cavity in its sloping part between the 7th and 8th ribs. The chest should be opened and at least 10 ml of straw-coloured pleural fluid removed aseptically.

### Transport

Samples should be sent to the laboratory as quickly as possible, preferably the same day, keeping the samples cool (about 4°C). If microbiological examination cannot be performed immediately, samples and whole, or parts of, organs should be stored deep frozen, where mycoplasmas will remain viable for up to several months. For international transport, where freezing during transport is not possible, samples should be lyophilized. Freeze-drying lung homogenates from CCPP-affected goats before transport overseas has proved highly successful with the recovery of *M. c. capripneumoniae* (Houshaymi *et al.,* 2000). Many countries require a special import licence to be obtained in advance for any biological material, especially for tissues which contain animal pathogens, including mycoplasmas.

## Isolation of Mycoplasmas from Samples

### Isolation on solid and liquid media

Tenfold dilutions ($10^{-1}$ to $10^{-6}$) are made of liquid sample (pleural fluid, nasal exudate, synovial fluid, etc.) or lung homogenate in appropriate medium. Tissue samples are best chopped with scissors then shaken vigorously or pulverized in medium (10% w/v). Dilution of the samples has a number of benefits: first, it reduces the effects of mycoplasmacidal substances, including antibiotics released by the tissues; and second, it reduces bacterial contamination and the overgrowth by less important but more exuberant mollicutes. A few drops of each sample are deposited and spread on the solid medium and a 10% (v/v) inoculum dispensed into liquid medium. In addition, a direct impression should be made on the solid medium with the cut surface of a lung lesion or lymph node without spreading it. Swabs (if available) are streaked directly on to solid medium. The broths (optimally with gentle shaking) and plates are incubated at 37°C in a humidified atmosphere with 5% $CO_2$.

The broths are examined daily for signs of growth or changes of pH, indicated by a colour change in the medium. Broths should be examined against a good, even light. Bacterial contamination will be seen as gross turbidity evident within 24 h. Mycoplasma growth will appear between 3 and 5 days and is usually seen as a very fine cloudiness, usually described as 'opalescence'; it may be necessary to compare with an uninoculated broth to see the growth, particularly in the case of *M. c. capripneumoniae*. *M. m. mycoides* grows well and usually produces 'whirls' from the bottom of the tube when shaken. If film appears on the surface of the liquid medium which also has an orange colour, *M. bovis* should be suspected if the isolate is from cattle and *M. agalactiae* if from small ruminants. The plates are inspected after 2–3 days under 35× magnification, or 100× if colonies are small, for the typical 'fried egg' appearance. Mycoplasmas grow into the agar, which makes the use of a loop for subculturing unsatisfactory. All members of the *M. mycoides* cluster except *M. c. capripneumoniae* grow within 3 days, producing colonies of between 1 and 3 mm. *M. ovipneumoniae* can be suspected when isolated from small ruminant lung when the colonies are centreless and do not stick

to the agar surface. In general, however, colonies may vary greatly in size in a single culture. The form may also vary in different cultures of the same strain, for example in having small or large centres or a granular or smooth appearance. Occasionally colonies or a pure culture on the same plate will differ in appearance, perhaps because of age. It follows then that colonial morphology is relatively valueless as a typing aid.

## Subculturing

For most serological identification tests, it is necessary to grow the mycoplasma on solid medium. Broths should be subcultured immediately growth is apparent or pH change is seen. Some mycoplasmas, notably *M. m. mycoides*, will die rapidly at a pH much below 7, and in the case of *M. m. mycoides*, which produces acid rapidly in the medium, subculture at 3-day intervals is likely to result in loss of the strain. Suspected samples are subcultured three times before rejecting the material as negative. Previous subcultures should be held so that at the end the primary broth will have been incubated for 3 weeks. Plates are not normally incubated for more than a week. For broth to broth subculturing, 10% (v/v) inoculum should be placed into the new broth using a Pasteur pipette. For broth to plate, the plate is put on a level surface and a single drop (about 25 µl) of incubated broth is carefully streaked across the agar. The drop is allowed to soak in before the plate is moved. For plate to broth subculturing, a block with colonies on is cut out with a sterile spatula and dropped into the broth. It is possible to pick up

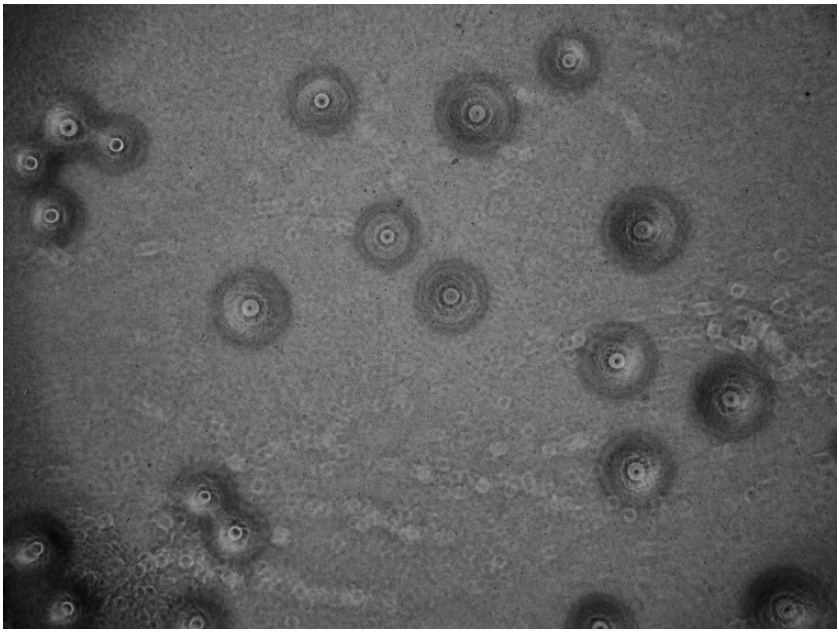

**Fig. 1.1.** Colonies of *Mycoplasma bovirhinis* (×10).

a single colony by taking out a plug with a Pasteur pipette or a syringe and hypodermic needle, or just a needle. For plate to plate subculturing, a block with colonies is cut out and placed face down on the new plate, and is then carefully slid about on the surface.

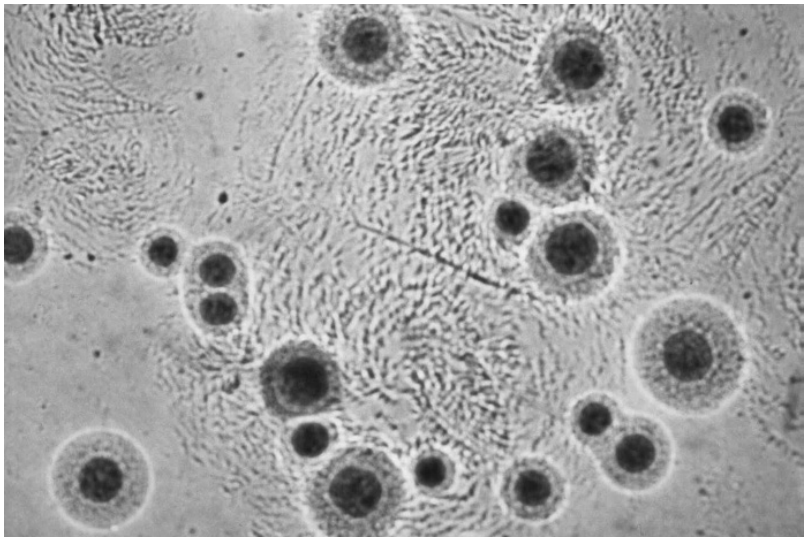

**Fig. 1.2.** Colonies of *Mycoplasma agalactiae* (×15).

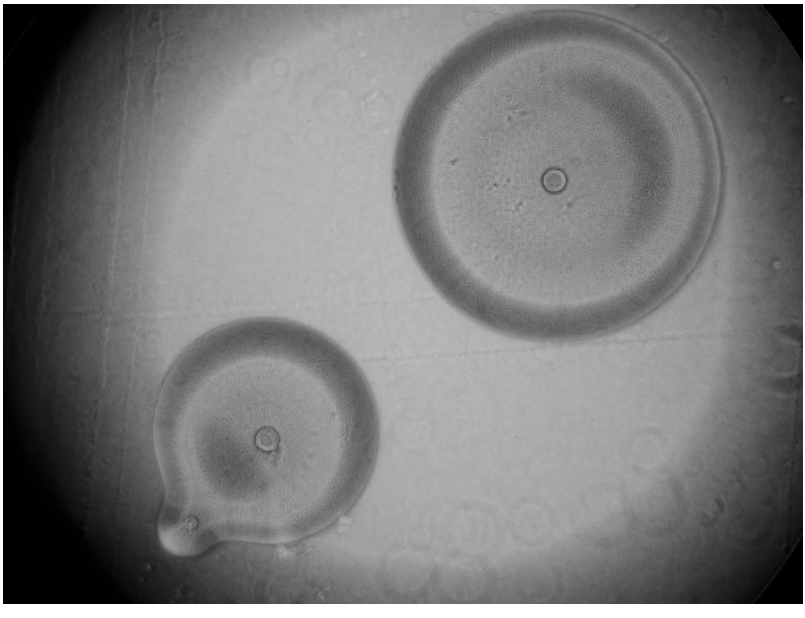

**Fig. 1.3.** Colonies of *Mycoplasma mycoides* subsp. *capri* (×25).

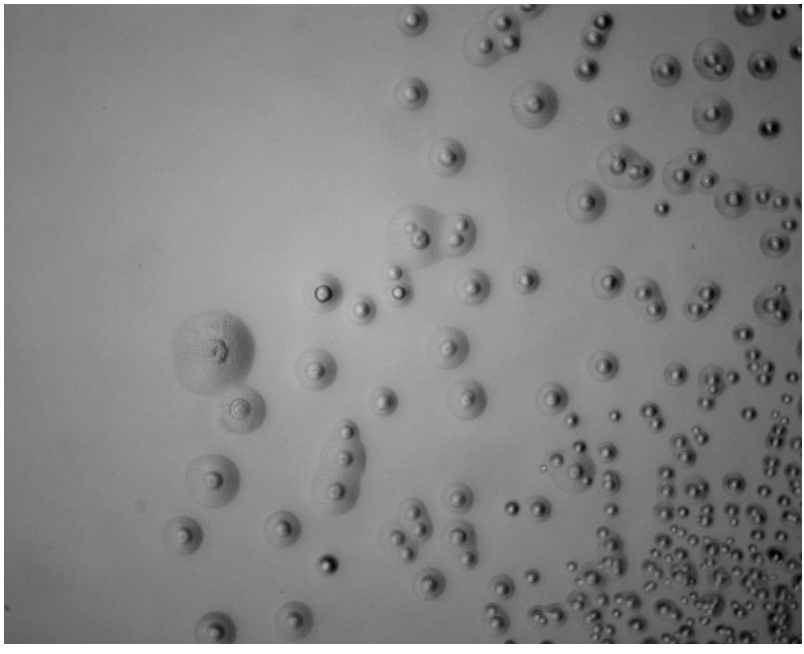

**Fig. 1.4.** Colonies of *Mycoplasma bovis* (×5).

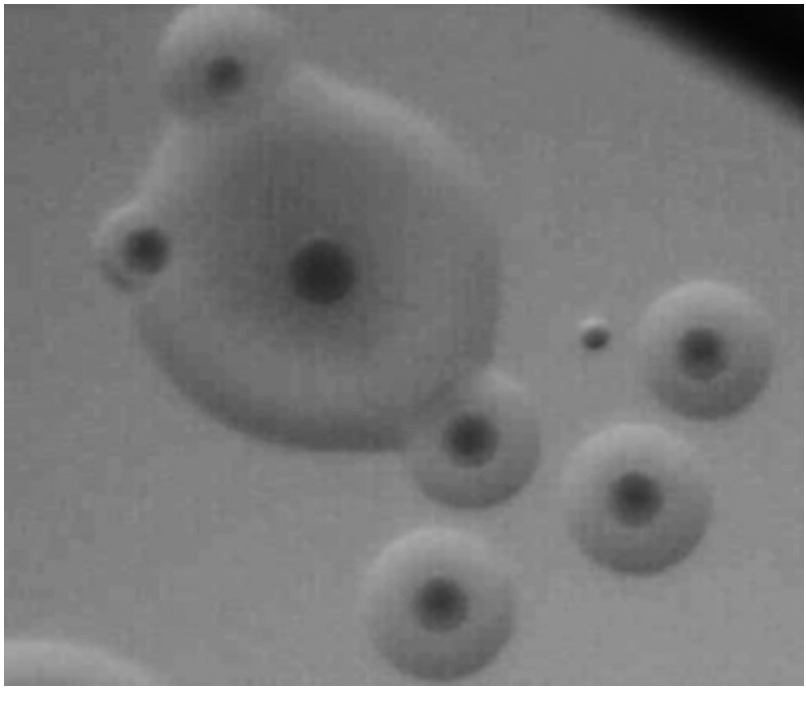

**Fig. 1.5.** Colonies of *Mycoplasma capricolum* subsp. *capripneumoniae* (×40).

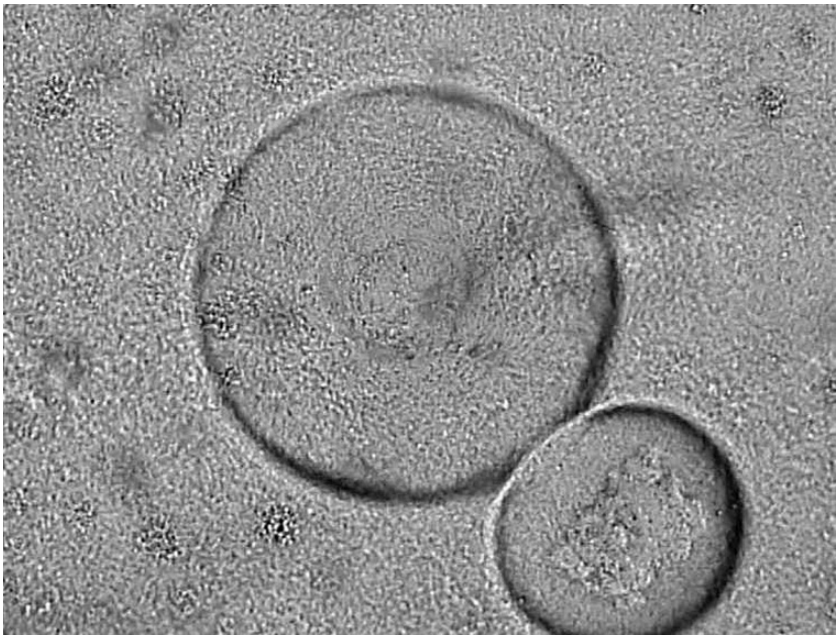

**Fig. 1.6.** Colonies of *Mycoplasma ovipneumoniae* (×50).

Once isolated the mycoplasmas can be identified by conventional biochemical and immunological methods (Poveda and Nicholas, 1998) or increasingly by molecular methods such as polymerase chain reaction (PCR) assays, which can be found in Chapters 2 and 3 of this book.

Figures 1.1–1.6 show colony morphology of several important mycoplasmas of ruminants.

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# 2 Detection and Differentiation of *Mycoplasma* Species using PCR/ Denaturing Gradient Gel Electrophoresis

## Introduction

Mycoplasmas cause a wide range of disease in both humans and animals and are commonly associated with pneumonia, arthritis, conjunctivitis, infertility and abortion. Specific diagnosis of mycoplasma infections is often difficult due to the limitations of current diagnostic tests together with the similarity in the diseases that they cause. Mycoplasmas are highly fastidious, typically taking weeks to culture, and many serological tests are non-specific and insensitive. More recently PCR has been used to detect a number of *Mycoplasma* species. However, with over 125 mycoplasmas currently recognized it is not feasible to develop PCR tests for each species and there is a pressing need for a single, generic test that can both detect and differentiate mycoplasmas. Denaturing gradient gel electrophoresis (DGGE) can theoretically detect single base mutations in DNA (Fisher and Lerman, 1983; Lerman and Beldjord, 1999). The method is based on the prevention of migration of DNA fragments following strand separation caused by chemical denaturants. DGGE has been used extensively for diversity analysis in microbial ecology (Muyzer, 1999) but has not been widely used for the identification and differentiation of pathogenic bacteria except for the detection and identification of *Listeria* species (Schmolker *et al*., 1998) and for the molecular typing of *Staphylococcus aureus* (Gürtler *et al.,* 2001) and *Campylobacter* species (Nielsen *et al*., 2000). Previously we demonstrated the ability of DGGE using universal primers for the V3 region of 16S rDNA to detect and differentiate 27 mycoplasmas of veterinary importance (McAuliffe *et al*., 2003). The development of mycoplasma-specific primers has enabled the application of this method directly to clinical material such as swabs and tissue samples. In addition, we have also extended the scope of the DGGE method to include human, equine, sea mammal, canine and feline *Mycoplasma* species and a variety of field isolates (McAuliffe *et al.,* 2005). The generic nature of the test may lead to the detection of mycoplasma infections

that would be difficult to identify using traditional culture techniques. The applicability of this method to mixed infections is also described.

## Design of Mycoplasma-specific Primers

A specific reverse primer for mollicutes was designed using PRIMROSE (Ashelford *et al.*, 2002). A reverse primer, R543 5′-ACC TAT GTA TTA CCG CG, for *Mycoplasma* species was designed by aligning 102 *Mycoplasma* species. The forward primer of Muyzer, GC341, was used as described below (Muyzer *et al.*, 1993). A 340 bp PCR product was generated with all 72 mycoplasmas tested. The mollicute-specific reverse primer was tested against a range of other bacterial pathogens to ensure specificity. A gradient thermocycler (Biorad, iCycler) was used to test a range of annealing temperatures to ensure specificity. For all future experiments an annealing temperature of 56°C was used.

## DNA Extraction and 16S PCR

Mycoplasma DNA was extracted from a 1ml aliquot of stationary-phase culture using the Genelute genomic DNA kit according to the manufacturer's instructions (Sigma, Poole, UK). DNA was extracted from swabs by swirling the swab in 1 ml of PBS, removing the swab and then using the Genelute kit as described above. DNA was extracted from tissue samples by removing a 1 $cm^2$ portion of tissue using sterile instruments, placing it in 1ml of PBS, homogenizing to produce a suspension and extracting DNA using a Sigma tissue kit according to the manufacturer's instructions. Amplification of the V3 region of the 16S RNA gene was performed according to the method of Muyzer with minor modifications (Muyzer *et al.*, 1993) using the universal bacterial primer GC-341F 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG and the mollicute-specific primer R543. For the PCR, 1 µl of lysate was added as a template to 49 µl of a reaction mixture containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM of each deoxynucleoside triphosphate and 0.5 U of Taqgold (Applied Biosystems). The cycling conditions were: denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 56°C for 45 s and 72°C for 1 min, a final extension step of 72°C for 10 min and samples were kept at 4°C until analysis. Aliquots were checked for correct amplification by electrophoresis on a 2% agarose gel followed by visualization with ethidium bromide under UV illumination.

## Denaturing Gradient Gel Electrophoresis

DGGE was performed using the Ingeny PhorU 2 × 2 apparatus (GRI Molecular Biology, Essex, UK). Samples (20 µl) were loaded on to 10% polyacyrlamide/bis (37.5:1) gels with denaturing gradients from 30 to 60% (where 100% is 7 M urea and 40% (v/v) deionized formamide) in 1 × TAE electrophoresis buffer (Severn

Biotech Ltd, Worcestershire, UK). Electrophoresis was performed at 100 V at a temperature of 60°C for 18 h. Gels were then stained with SBYR Gold (Cambridge BioScience, Cambridgeshire, UK) in 1 × TAE for 30 min at room temperature and visualized under UV illumination.

## Mycoplasma-specific Primers

Members of mycoplasma, acholeplasma and ureaplasma groups could be amplified using the mycoplasma-specific primer R543 and the universal primer GC341; however, members of the related haemoplasma group could not. All 72 *Mycoplasma* species tested produced a PCR product of approximately 340 bp. These products were subjected to DGGE in groups according to host animal. In the majority of cases only one band was seen, indicating that there was no interspecific variation in the amplified sequence. The presence of multiple bands indicated that more than one 16S rDNA operon was present and that there were some sequence differences between the copies. The migration of the bands in the gels is a function of the melting behaviour of the amplicons in the chemical gradient used. A faint background band was sometimes seen on the DGGE gels, which is probably due to a degree of primer–dimer formation and should as such be considered an artefact. The background band was easily differentiated from bands generated from 16S operons as it was faint in intensity, had an irregular shape and was not straight.

## Applicability of DGGE Directly to Clinical Samples

In order to test the practicality of DGGE in the clinical laboratory DNA extraction was performed directly on swabs and tissue samples received for veterinary diagnostic investigations. In total 202 clinical samples were analysed, of which 89 were found to be positive for mycoplasma infection. Mycoplasma DNA was successfully amplified for DGGE from a wide variety of diagnostic samples, including nasal, eye, ear and foot swabs, lung tissue, milk, brain tissue, synovial joint fluid and tissue from an aborted bovine fetus. In order to test the robustness of the procedure on samples that had undergone long-term storage, DGGE was used on bovine lung samples obtained from outbreaks of contagious bovine pleuropneumonia in Botswana and Tanzania that had been frozen at –80°C for approximately 9 years. DGGE identified *M. mycoides* SC in eight out of nine samples; culture of the lung samples also yielded *M. mycoides* SC in eight out of nine samples.

## Use of DGGE to Detect Mixed Cultures

DGGE using mycoplasma-specific primers was particularly useful for detecting mixed cultures. As shown in Fig. 2.1, analysis of a number of bovine diagnostic samples demonstrated that a mixed infection of *M. bovirhinis*/*M. alkalescens* could be detected easily. In addition, analysis of small ruminant clinical samples showed

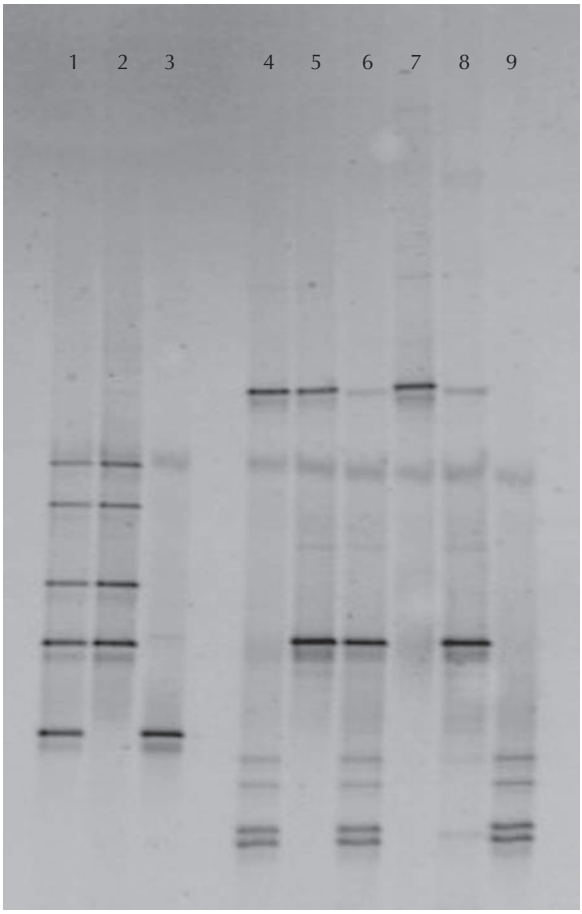


**Fig. 2.1.** Use of DGGE to detect mixed mycoplasma infections in cattle and sheep. Lane 1, *M. bovirhinis* and *M. alkalescens* mixed field strains; lane 2, *M. bovirhinis* NCTC; lane 3, *M. alkalescens* NCTC; lane 4, *M. ovipneumoniae* and *M. arginini* mixed field strains; lane 5, *M. ovipneumoniae* and *M. conjunctivae* mixed field strains; lane 6, *M. conjunctivae* and *M. arginini* mixed field strains; lane 7, *M. ovipneumoniae* NCTC; lane 8, *M. conjunctivae* NCTC; lane 9, *M. arginini* NCTC.

that mixed infections of *M. ovipneumoniae*/*M. arginini*, *M. ovipneumoniae*/*M. conjunctivae* and *M. conjunctivae*/*M. arginini* could be detected.

## DGGE of Human *Mycoplasma* Species

All 12 human *Mycoplasma* species tested could be differentiated using DGGE (Fig. 2.2). *M. primatum* and *M. fermentans* had a similar migration pattern.

## DGGE of Avian *Mycoplasma* Species

Seventeen avian mycoplasmas could be easily distinguished using DGGE (summarized in Table 2.1). Perhaps most importantly, DGGE could distinguish the four avian *Mycoplasma* species of major economic importance, *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*. However, *M. iowae* and *M. glycophilum* gave

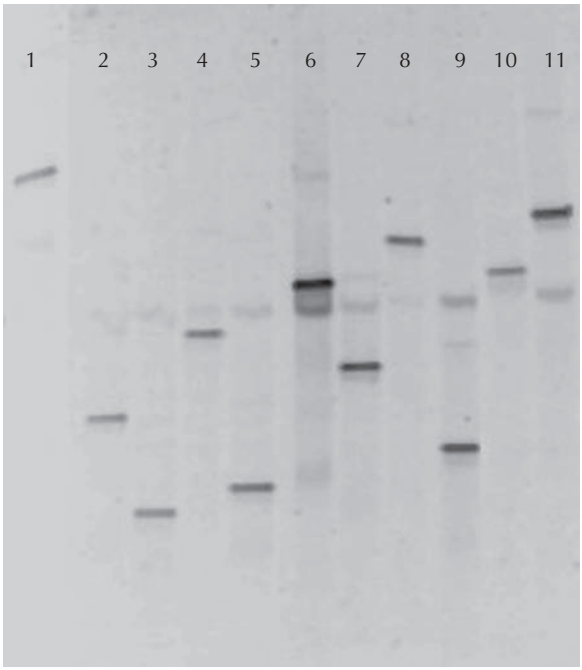


**Fig. 2.2.** DGGE of human *Mycoplasma* species. Lane 1, *M. pneumoniae*; lane 2, *M. hominis*; lane 3, *M. faucium*; lane 4, *M. buccale*; lane 5, *M. arthritidis*; lane 6, *M. spermatophilum*; lane 7, *M. salivarum*; lane 8, *M. primatum*; lane 9, *M. orale*; lane 10, *Mycoplasma genitalium*; lane 11, *M. fermentans*.

similar profiles, but when their full-length 16S sequences were compared, using a two-way BLAST alignment, only 80% similarity was found (AF412981 *M. glycophilum* and M24293 *M. iowae*). Two pigeon *Mycoplasma* species could not be differentiated using DGGE. *M. columborale* and *M. columbinasale* could not be distinguished and gave the same profile by DGGE. However, analysis of the 16S 23S intergenic spacer (IGS) regions for *M. columborale* and *M. columbinasale* (AY796061 and AY796062, respectively) indicated that the species were not highly similar, with only 84% congruence. BLAST of shorter IGS on *M. columbinasale* AJ780986 indicated only 99/122 (81%) similarity, with gaps of 12/122 (9%).

## Marine Isolates

The sea mammal *Mycoplasma* species *M. phocirhinis*, *M. phocicerebrale* and *M. phocidae* were easily distinguished using DGGE (Fig. 2.3). Interestingly, a feline mycoplasma, *M. gateae*, gave an identical profile to *M. phocicerebrale* (lane 4). Comparison of DNA 16S 23S IGS sequences for *M. gateae* and *M. phocicerebrale* (AF443609 and AY766092, respectively) revealed a high degree of similarity between the two sequences, with similarity of 97% and gaps of only 1% as determined using a two-way BLAST alignment (bl2seq, NCBI). Comparison of full-length 16S sequences also revealed congruence between the sequences, with 98% similarity and no gaps (U15796 and AF304323 for *M. gateae* and *M. phocicerebrale*, respectively).

**Table 2.1.** *Mycoplasma* strains, host and summary results.

| Species | Strain designation | Host species | Specific detection by DGGE/comments |
|---|---|---|---|
| *M. anatis* | NCTC 10156 | Avian | + |
| *M. cloacale* | NCTC 10199 | Avian | + |
| *M. columbinasale* | NCTC 10184 | Avian | – same as *M. columborale* |
| *M. columbinum* | NCTC 10178 | Avian | + |
| *M. columborale* | NCTC 10179 | Avian | – same as *M. columbinasale* |
| *M. gallinaceum* | NCTC 10183 | Avian | + |
| *M. gallinarum* | NCTC 10120 | Avian | + |
| *M. gallisepticum* | PG31T | Avian | + |
| *M. gallopavonis* | NCTC 10186 | Avian | + |
| *M. glycophilum* | NCTC 10194 | Avian | + similar to *M. iowae* |
| *M. imitans* | NCTC 11733 | Avian | + |
| *M. iners* | NCTC 10165 | Avian | + |
| *M. iowae* | NCTC 10185 | Avian | + similar to *M. glycophilum* |
| *M. lipofaciens* | NCTC 10191 | Avian | + |
| *M. meleagridis* | NCTC 10153 | Avian | + |
| *M. pullorum* | NCTC 10187 | Avian | + |
| *M. synoviae* | NCTC 10124 | Avian | + |
| *M. alkalescens* | NCTC 10135 | Bovine | + |
| *M. alvi* | NCTC 10157 | Bovine | + |
| *M. bovigenitalium* | NCTC 10122 | Bovine | – same as *M.* ovine/caprine serogroup 11 but both are likely to be assigned to the same species |
| *Mycoplasma* species bovine group 7 | NCTC 10133 | Bovine | + |
| *M. bovirhinis* | NCTC 10118 | Bovine | + |
| *M. bovis* | NCTC 10131 | Bovine | + similar to *M. verecundum* and *M. canadense* |
| *M. bovoculi* | NCTC 10141 | Bovine | + |
| *M. californicum* | NCTC 10189 | Bovine | + |
| *M. canadense* | NCTC 10152 | Bovine | + similar to *M. verecundum* and *M. bovis* |
| *M. canis* | NCTC 10146 | Bovine/ canine | + different from all bovine species but same as *M. edwardii* |
| *M. dispar* | NCTC 10125 | Bovine | + |
| *M. mycoides* subsp. *mycoides* small colony type | PG1 | Bovine | + |
| *M. verecundum* | NCTC 10145 | Bovine | + similar to *M. canadense* and *M. bovis* |
| *M. cynos* | NCTC 10142 | Canine | + |
| *M. edwardii* | NCTC 10132 | Canine | – same as *M. canis* |
| *M. maculosum* | NCTC 10168 | Canine | – same as *M. leopharyngis* |
| *M. opalescens* | NCTC 10149 | Canine | + |
| *M. spumans* | NCTC 10169 | Canine | + |
| *M. equigenitalium* | ATCC 29869 | Equine | + |

(*Continued*)

**Table 2.1.** *Continued.*

| Species | Strain designation | Host species | Specific detection by DGGE/comments |
|---|---|---|---|
| *M. equirhinis* | NCTC 10148 | Equine | + |
| *M. fastidiosum* | NCTC 10180 | Equine | + |
| *M. felis* | NCTC 10160 | Equine | + |
| *M. subdolum* | NCTC 10175 | Equine | + |
| *M. arthriditis* | NCTC 10162 | Human | + |
| *M. buccale* | NCTC 10136 | Human | + |
| *M. faucium* | NCTC 10174 | Human | + |
| *M. fermentans* | NCTC 10117 | Human | + similar to *M. primatum* |
| *M. genitalium* | NCTC 10195 | Human | + |
| *M. hominis* | NCTC 10111 | Human | + |
| *M. lipophilum* | NCTC 10173 | Human | + |
| *M. orale* | NCTC 10112 | Human | + |
| *M. pneumoniae* | NCTC 10119 | Human | + |
| *M. primatum* | NCTC 10163 | Human | + similar to *M. fermentans* |
| *M. salivarum* | NCTC 10113 | Human | + |
| *M. spermatophilum* | NCTC 11720 | Human | + |
| *M. flocculare* | NCTC 10143 | Porcine | + |
| *M. hyopneumoniae* | NCTC 10110 | Porcine | + |
| *M. hyorhinis* | NCTC 10130 | Porcine | + |
| *M. hyosynoviae* | NCTC 10167 | Porcine | + |
| *M. gateae* | NCTC 10161 | Sea mammal/ feline | – same as *M. phocicerebrale* |
| *M. phocicerebrale* | NCTC 11721 | Sea mammal | – same as *M. gateae* |
| *M. phocidae* | Strain 105 | Sea mammal | + |
| *M. phocirhinis* | NCTC 11722 | Sea mammal | + |
| *M. agalactiae* | NCTC 10123 | Small ruminant | + |
| *M. arginini* | NCTC 10129 | Small ruminant | + |
| *M. capricolum* subsp. *capricolum* | NCTC 10154 | Small ruminant | + |
| *M. capricolum* subsp. *capripneumoniae* | NCTC 10192 | Small ruminant | + |
| *M. conjunctivae* | NCTC 10147 | Small ruminant | + |
| *M. cottewii* | NCTC 11732 | Small ruminant | – same as *M. yeatsii* |
| *M. mycoides* subsp. *capri* | NCTC 10137 | Small ruminant | – indistinguishable from *M. m. m.* LC but both are likely to be the same species |
| *M. mycoides* subsp. *mycoides* LC | F30 | Small ruminant | – indistinguishable from *M. m. capri* but both are likely to be the same species |
| *M. ovipneumoniae* | NCTC 10151 | Small ruminant | + |
| *Mycoplasma* ovine/caprine serogroup 11 | Strain 2D | Small ruminant | – same as *M. bovigenitalium* but both are likely to be assigned to the same species |
| *M. putrefaciens* | NCTC 10155 | Small ruminant | + |
| *M. yeatsii* | NCTC 11730 | Small ruminant | – same as *M. cottewii* |

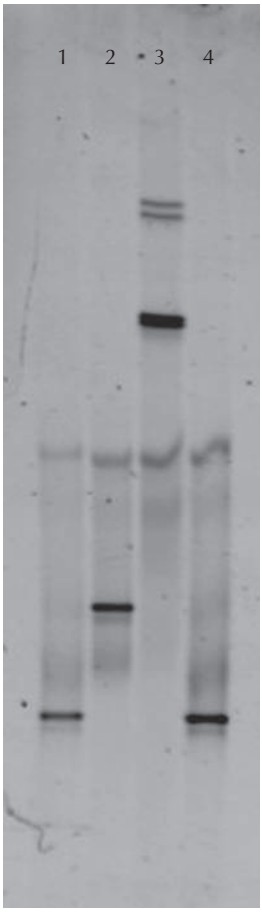


**Fig. 2.3.** DGGE of sea mammal *Mycoplasma* species. Lane 1, *M. phocicerebrale*; lane 2, *M. phocirhinis*; lane 3, *M. phocidae*; lane 4, *M. gateae*.

## Bovine *Mycoplasma* Species

DGGE could differentiate all 13 bovine *Mycoplasma* species tested (as summarized in Table 2.1). A similar migration pattern was seen in three bovine species, *M. verecundum*, *M. canadense* and *M. bovis*. However, careful analysis showed that there was a small difference in the distance of migration between the three species. *M. bovis* produced a different profile from that of the small ruminant mycoplasma *M. agalactiae*, which can be difficult to distinguish from *M. bovis* by normal culture and serological tests. Significantly, *M. m. m.* SC, the causative agent of CBPP, was easily distinguished from all other *Mycoplasma* species tested and had a characteristic pattern of four bands. *M. m. m.* SC was also easily distinguished from all other members of the closely related *M. mycoides* cluster.

## Small Ruminant *Mycoplasma* Species

Twelve small ruminant *Mycoplasma* species were analysed using DGGE (summarized in Table 2.1). All species gave easily distinguishable profiles except for the closely related *M. mycoides* subsp. *mycoides* large colony and *M. mycoides* subsp. *capri*, which were identical; similarly, *M. cottewii* and *M. yeatsii* could not be differentiated. Analysis of full-length 16S sequences and 16S 23S spacer sequences of *M. m. m.* LC and *M. m. capri* showed a very high degree of similarity (>99%) between the species, in line with previous studies that have suggested that the two species should be amalgamated into a single species (Pettersson *et al.*, 1996). Similarly *M. yeatsii* and *M. cottewii* were also at least 99% similar when both full-length 16S and 16S 23S IGS were compared. Significantly, a number of members of the closely related *M. mycoides* cluster could be differentiated, and *M. putrefaciens* gave a unique profile.

## Canine *Mycoplasma* Species

The canine *Mycoplasma* species, *M. spumans*, *M. opalescens*, *M. cynos* and *M. maculosum* were easily distinguished using DGGE. However *M. canis* and *M. edwardii* gave highly similar profiles using DGGE; given the high 16S sequence homology between these two species (98% with no gaps, U73903 and AF412972), this is not unexpected. Interestingly, when *M. maculosum* was compared with a number of feline isolates, it gave an identical profile to the lion mycoplasma *M. leopharyngis*. Comparison of 16S and 16S 23S IGS sequences for *M. maculosum* and *M. leopharingis* also indicated that the species are identical.

## Equine *Mycoplasma* Species

The four main *Mycoplasma* species found in horses, *M. subdolum*, *M. fastidiosum*, *M. equirhinis* and *M. equigenitalium*, were all easily distinguishable using DGGE (Fig. 2.4). In addition, the feline *Mycoplasm*a species, *M. felis*, which has been associated with respiratory disease in horses, was also easy to distinguish from the other equine-associated mycoplasmas using DGGE.

## Porcine *Mycoplasma* Species

The four main porcine *Mycoplasma* species, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*, were easily distinguished using DGGE (Fig. 2.5).

## Discussion

DGGE analysis has enabled the detection and differentiation of 67 *Mycoplasma* species. For at least 40 of these *Mycoplasma* species there has not previously been a DNA-based diagnostic test available and many have only been identifiable through lengthy culture or serological tests. Previously we demonstrated that

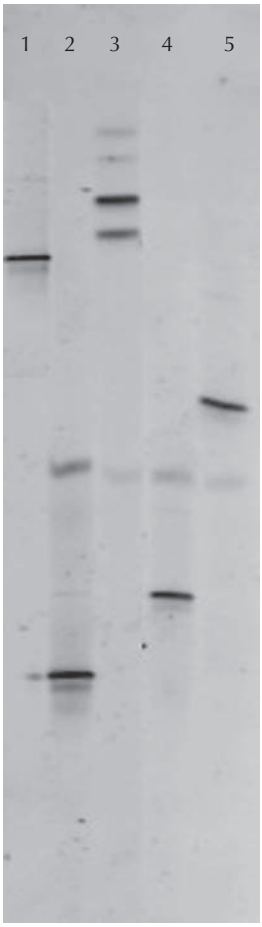


**Fig. 2.4.** DGGE of equine *Mycoplasma* species. Lane 1, *M. fastidiosum*; lane 2, *M. subdolum*; lane 3, *M. felis*; lane 4, *M. equirhinis*; lane 5, *M. equigenitalium*.

DGGE could be used to differentiate 27 *Mycoplasma* species of veterinary importance (McAuliffe *et al.*, 2003). This has now been extended to include 67 *Mycoplasma* species and presents significant improvements to the technique, including the use of mycoplasma-specific primers. Whereas DGGE using universal primers required a media-enrichment step to ensure that only mollicute DNA was amplified (McAuliffe *et al.*, 2003), with the advent of mollicute-specific primers, DGGE can be applied directly to clinical material. As a result of this, mycoplasma infections can now be diagnosed in less than 24 h compared with 1 to 2 weeks for traditional culture. The use of mycoplasma-specific primers has also enabled the detection of mixed cultures, which would have been difficult to detect by conventional methods, as less fastidious species would be outgrown.

DGGE may prove to be particularly useful for human mycoplasmas and is the first generic test capable of differentiating 12 species. Previously a multiplex PCR has been used to differentiate genital *Mycoplasma* species (Stellrecht *et al.*, 2004) and a reverse line blotting procedure has been used to differentiate five human mollicute pathogens (Wang *et al.*, 2004), but there has not been a single generic

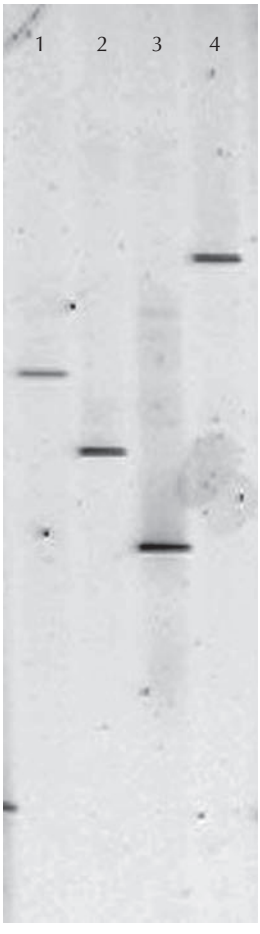


**Fig. 2.5.** DGGE of porcine mycoplasmas. Lane: 1, *M. flocculare*; lane 2, *M. hyopneumoniae*; lane 3, *M. hyosynoviae*; lane 4, *M. hyorhinis*.

test for other human *Mycoplasma* species. Significantly *M. genitalium* and *M. pneumoniae* can be differentiated easily by DGGE, thus demonstrating the specificity of the technique as there is 98% similarity between the two species based on 16S homology (Jensen *et al*., 2003).

A number of mycoplasmas could not be differentiated using DGGE and gave identical profiles. For example, *M. mycoides* subsp. *capri* and *M. mycoides* LC were indistinguishable, indicating that there was no variation in the 16S rDNA sequence over the V3 region amplified. This may provide further support for the notion that *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* are in fact the same species (Pettersson *et al*., 1996). Some unexpected isolates also gave identical profiles by DGGE, for example the feline mycoplasma *M. gateae* and the sea mammal species *M. phocicerebrale.* These results were also supported by comparison of full-length 16S and 16S 23S IGS sequences for the isolates, which also indicated a very high degree of similarity between the species. If these species are closely related it is difficult to explain how they could have been transmitted between two very different hosts, cats and seals, which seem unlikely to have come

into close contact with one another. Similarly, the canine mycoplasma *M. maculosum* showed a high degree of similarity to the lion mycoplasma *M. leopharingis* by 16S and 16S 23S IGS analysis and gave identical DGGE profiles. Previous studies have also highlighted the high degree of similarity in the 16S sequence and identical biochemical characteristics of these species (Pettersson *et al.*, 2001).

Two canine *Mycoplasma* species, *M. canis* and *M. edwardii*, gave indistinguishable DGGE profiles. This is not unexpected as previous analysis of full-length 16S sequences and 16S 23S IGS sequences found that the species are highly similar (Chalker and Brownlie, 2004). Interestingly, *M. cynos* could be differentiated from all other canine *Mycoplasma* species whereas previous studies based on sequence analysis have shown it grouped closely with *M. canis* and *M. edwardii* (Chalker and Brownlie, 2004).

Two species, *M. columbinum* and *M. columbinasale*, could not be distinguished, although previous studies have indicated that they are less than 97% similar by 16S sequence (Pettersson *et al.,* 2001). Even when cultures were obtained from several different collections, the two isolates gave identical profiles. It is likely that the species were previously identified using serological tests and emphasizes the need for DNA sequencing of historical isolates in collections to ensure that they are correctly identified. Nevertheless, whether species should be designated based on serological or molecular methods is still a contentious issue within mollicute taxonomy. Recently, denaturing HPLC analysis has been used to detect and type bacterial pathogens (Domann *et al.*, 2003; Hurtle *et al.*, 2003) and could theoretically be used as an alternative to DGGE to target single nucleotide polymorphisms in the V3 region of 16S rDNA of *Mycoplasma* species. However, dHPLC would require expensive, specialized equipment and more laborious standardization and interpretation compared with DGGE.

In conclusion, DGGE enables the rapid detection and differentiation of *Mycoplasma* species and can be used to diagnose infections either directly from tissues or from cultured isolates. It is capable of detecting mixed cultures or even new mollicute species and is suitable for routine use in the diagnostic laboratory. In the event of the detection of a new or unusual profile then the product can be identified by 16S rDNA sequencing; however, if there is no matched sequence in the database then the isolate will need to be characterized by conventional methods.

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# 3 Detection of *Mycoplasma* Species Using Polymerase Chain Reaction (PCR)

## Introduction

Although a number of novel generic techniques such as denaturing gradient gel electrophoresis (DGGE) (McAuliffe *et al.*, 2003a, 2005), amplified rDNA restriction analysis (ARDRA) (Stakenborg *et al.*, 2005) and reverse line blot (Wang *et al.*, 2004) have been developed which enable the identification of *Mycoplasma* species, PCR remains the most useful, simple and rapid method of detecting specific mycoplasmas. With the advent of real-time PCR it is now possible not only to achieve results quickly but also to quantify the number of organisms present. New, small, portable PCR and real-time PCR systems have also been developed, which means that detection in the field using these methods is now possible.

PCR is one of the most sensitive of the existing rapid methods to detect infectious pathogens in clinical specimens. PCR is particularly useful for pathogens such as mycoplasmas which are difficult to culture *in vitro* or require a long cultivation period. However, the application of PCR to clinical specimens has many potential pitfalls due to the susceptibility of PCR to inhibitors, contamination and experimental conditions. Moreover, it is known that the sensitivity and specificity of a PCR assay is dependent on many variables including target genes, primer sequences, PCR techniques, DNA extraction procedures and PCR product detection methods.

Previously PCRs were frequently designed based solely on the 16S rDNA gene owing to a lack of sequenced mycoplasma genes. However, with the increased number of complete genome sequences, PCRs are now being developed on a number of different gene targets. The specificity and sensitivity of PCR can vary enormously depending on the chosen gene target and is a factor worthy of consideration when designing PCRs.

Many PCRs have been developed which enable the specific detection of a number of ruminant mycoplasmas including members of the closely related *Mycoplasmas mycoides* cluster: *M. mycoides* SC, *M. mycoides* LC, *M. c. capricolum*,

*M. c. capripneumoniae*, *M.* species bovine group 7, *M. putrefaciens*, *M. agalactiae*, *M. bovis*, *M. ovipneumoniae*, *M. conjunctivae*, *M. fermentans*, *M. dispar*, *M. canis* and others (Table 3.1). PCRs are, however, still lacking for a number of important mycoplasma species, including *M. canadense*, *M. verecundum* and *M. californicum*. Real-time PCR has only been developed to date for a selected few species, including *M. mycoides* subsp. *mycoides* SC, *M. agalactiae* and *M. conjunctivae* (as summarized in Table 3.2).

PCR has been particularly useful for the detection and differentiation of members of the *M. mycoides* cluster as these species are difficult or impossible to differentiate using traditional culture and serological techniques. The causative agent of CBPP, *M. mycoides* SC, was among the first species for which PCRs were developed. Initial tests for *M. mycoides* SC were based on the Cap21 gene fragments, and although useful as a screening test to rule out the presence of exotic mycoplasmas

**Table 3.1.** PCRs available for ruminant *Mycoplasma* species.

| *Mycoplasma* species | Gene target | Reference(s) |
|---|---|---|
| *M. mycoides* SC | IS1296/8.8kb deletion region<br>Cap21 | Miles *et al.*, 2006<br>Bashiruddin, 1998 |
| *M. mycoides* SC/LC/*capri* | Cap21 | Bashiruddin, 1998 |
| *Mycoides* cluster | Cap21<br>Glk | Bashiruddin, 1998<br>Woubit *et al.*, 2007 |
| *M.* sp. bovine group 7 | gts | Frey *et al.*, 1998 |
| *M. c. capricolum* | LppA | Monnerat *et al.*, 1999 |
| *M. c. capripneumoniae* | Adi | Woubit *et al.*, 2004 |
| *M. agalactiae* | urvC | Subramaniam *et al.*, 1998 |
| *M. bovis* | uvrC | Subramaniam *et al.*, 1998 |
| *M. ovipneumoniae* | 16S | McAuliffe *et al.*, 2003b |
| *M. bovigenitalium* | 16S | Parham *et al.*, unpublished results |
| *M. conjunctivae* | 16S, lppS | Giacometti *et al.*, 1999; Belloy *et al.*, 2003 |
| *M. putrefaciens* | 16S | Unpublished<br>Peyraud *et al.*, 2003 |
| *M. canis* | 16S | Unpublished |
| *M. dispar* | 16S | Miles *et al.*, 2004 |
| *M. fermentans* | IS1550 | Afshar *et al.*, 2007 |

**Table 3.2.** Real-time PCR for ruminant *Mycoplasma* species.

| *Mycoplasma* species | Gene target | Reference |
|---|---|---|
| *M. mycoides* SC | 16S rRNA and hypothetical lipoproteins | Gorton *et al.*, 2005 |
| *M. agalactiae* | P81 | Lorusso *et al.*, 2007 |
| *M. bovis* | 16S | Cai *et al.*, 2005 |
| *M. conjunctivae* | lppS | Vilei *et al.*, 2007 |

in areas free of CBPP, a second time-consuming enzyme-digestion step was necessary to differentiate *M. mycoides* SC from the closely related small ruminant pathogen *M. mycoides* LC. More recently, PCRs based on the insertion sequence IS1296 have enabled not only the specific detection of *M. mycoides* SC but also the differentiation of African/Australian strains from the European strains (Miles *et al.*, 2006). The development of Taqman real-time PCR for *M. mycoides* SC shows great promise and will also enable the quantification of the number of organisms present (Gorton *et al.*, 2005).

Specific PCRs have been developed for other members of the *M. mycoides* cluster, such as *M. c. capricolum*, *M. c. capripneumoniae* and *M.* species bovine group 7 (as summarized in Table 3.2).

PCRs have also been developed for the important ruminant pathogens *M. bovis* and *M. agalactiae*. These mycoplasmas are closely related and can be difficult to distinguish using 16S rDNA alone as they only differ by a few bases. Currently used PCRs for both *M. bovis* and *M. agalactiae* are based on the *uvr*C gene. Recently it has been shown that a number of unusual *M. bovis* strains, which have proved to be *M. bovis* using serology and 16S rDNA sequencing, are negative on the *urv*C PCR (McAuliffe *et al.*, 2008, unpublished results). A real-time test has been developed for *M. bovis* and shows improved sensitivity compared with conventional PCR (Lorusso *et al.*, 2007). *M. agalactiae* was also positive using this method but could be distinguished as it had a different melt peak. Real-time PCR has also been developed as a diagnostic test for *M. agalactiae* based on the p81 lipoprotein gene; this PCR was highly sensitive, capable of detecting $10^1$ copies in a 10 µl template from milk (Lorusso *et al.*, 2007).

## Limitations of PCR

PCR only enables the detection of a single *Mycoplasma* species and will not enable diagnosis of new or unusual *Mycoplasma* species or those species in unusual hosts. For this reason we find that generic tests such as PCR/DGGE (see Chapter 2) are advantageous as they enable the detection of mixed cultures; it is not uncommon to find between two and six different *Mycoplasma* species present in clinical samples, particularly in birds, that would be missed by conventional single-species PCR.

## Sensitivity and Specificity

Mycoplasma PCRs vary in sensitivity: the *M. mycoides* SC *cap*21 PCR was shown to be relatively sensitive, detecting between 10 and 100 organisms (Bashiruddin *et al.*, 1994), whereas other PCRs for non-ruminant mycoplasmas such as *M. hyopneumoniae* only detect 500 organisms (Baumeister *et al.*, 1998).

The sensitivity of PCR can be increased in some instances by using a nested system. These PCRs are ideally suited to detecting very small numbers of pathogens in clinical samples. The process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains either two nested primers that are internal to the first primer pair or one of the first primers and a

single nested primer. The larger fragment produced by the first reaction is used as the template for the second PCR. The sensitivity and specificity of DNA amplification can be considerably improved by using such nested PCR, sometimes with 1000 times more sensitivity than a standard PCR. Nested PCRs have been used for *M. bovis* (Pinnow *et al.*, 2001), where it had a sensitivity of $5^1$ cfu/ml, greater than culture of traditional PCR.

Both traditional and nested PCR were evaluated for the detection of *M. conjunctivae* and it was found that the detection level was estimated to be 20 cells per swab when the nested PCR procedure was used and $2 \times 10^5$ by the single PCR method (Giacometti *et al.*, 1999).

The sensitivity of PCR has also been enhanced by the use of real-time PCR technology. Real time, developed for *M. bovis*, gave a detection limit of 550 cfu/ml in milk and 650 cfu/25 mg in lung tissue. In validation testing of clinical samples, the relative sensitivity and specificity were 100 and 99.3%, respectively for individual milks and 96.6 and 100%, respectively for the lung tissue. *M. agalactiae* was also positive using this method but could be distinguished as it had a different melt peak. As mentioned above, real-time PCR has also been developed as a diagnostic test for *M. agalactiae* based on the p81 lipoprotein gene (Lorusso *et al.*, 2007).

## False Positive/Negative Results

As PCR is based on amplification of DNA, false positive or false negative results may easily occur. In particular, as a single PCR cycle results in very large numbers of amplifiable molecules, these can potentially contaminate subsequent amplifications of the same target sequence and generate false positive results. Carryover contamination of reagents, pipetting devices, laboratory surfaces or even the clothing and skin of workers can all lead to false positive results. To control carryover contamination, the physical transfer of DNA between amplified samples and between positive and negative experimental controls must be prevented. It is recommended that samples are prepared for PCR in a room or biosafety hood separate from that in which the reactions are performed. Using a pipette tip with an aerosol barrier is also essential for avoiding cross and carryover contamination. Using uracil N-glycosylase (UNG) to cleave the dUTP incorporated in PCR products is considered a powerful protocol to prevent carryover amplicon contamination enzymatically (Longo *et al.*, 1990), particularly in a clinical laboratory that is performing PCR extensively. This is performed by substituting dUTP for dTTP and adding UNG to the master mixture. To protect the dUTP-containing product, UNG must be inactivated chemically or by heat before the PCR product can be analysed further. Therefore, the dUTP protocol requires only two changes in a standard PCR protocol: the substitution of dUTP for dTTP in all reactions and the incubation of all PCR mixtures with UNG prior to temperature cycling. The exposure of laboratory surface, pipettes and racks to UV is also able to destroy contaminating amplicons but is efficient only on amplicons greater than 300 bp size (Espy *et al.*, 1993).

False negative results can also create problems in the diagnostic laboratory. The choice of suitable DNA extraction methods is crucial, as when PCR is applied

directly to DNA extracted from clinical samples the effect of inhibitors must be considered as they may interfere with amplification. Inhibitors include haemoglobin, lactoferrin, CSF, bone marrow aspirates, faeces and the blood anticoagulants heparin and SPS. The effect of inhibitors can be minimized by careful DNA extraction methods, and the addition of surfactants may be particularly useful for extraction from milk samples as used in some *M. bovis* PCR protocols (Pinnow *et al.*, 2001). The use of internal controls may be prudent to ensure that false negative results are not obtained. Inhibitors can be detected either by spiking negative samples with the target DNA by diluting the specimen to minimize the effects of inhibitors or by adding a control template.

## Previously Unpublished PCRs

### *Mycoplasma canis*

This PCR is based on the 16S rDNA gene of *M. canis* and amplifies a 400 bp fragment in both canine and bovine *M. canis* isolates. The primers McaF1 TGA TGA TTA GCT GAT AGT AGA ACT and McaR1 GAT TTG CTT GAC GTC GCC GTT are used at a concentration of 50 pmoles/µl. Total genomic DNA from *M. canis* (NCTC 10146) is used as a positive control. A $MgCl_2$ concentration of 2.0 mM is used and the cycling conditions are as follows: 94°C for 5 min, followed by 33 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by 72°C for 7 min and then 4°C until analysis. For the test to be valid, a band of 400 bp should be present for the positive control and no bands should be present in the negative controls upon agarose gel electrophoresis, staining with ethidium bromide and visualization under UV light.

### *Mycoplasma putrefaciens*

This PCR is based on the 16S rDNA gene of *M. putrefaciens* and amplifies a 340 bp fragment in all isolates. The primers SSF1 GCG GCA TGC CTA ATA CAT GC and SSR1 AGC TGC GGC GCT GAG TTC A are used at a concentration of 50 pmoles/µl. Total genomic DNA from *M. putrefaciens* (NCTC10155) is used as a positive control. A $MgCl_2$ concentration of 2.0 mM is used and the cycling conditions are as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s, followed by 72°C for 7 min and then 4°C until analysis. For the test to be valid, a band of 340 bp should be present for the positive control and no bands should be present in the negative controls upon agarose gel electrophoresis, staining with ethidium bromide and visualization under UV light.

### *Mycoplasma bovigenitalium*

The forward non-specific primer was MbgF (GGC TGT GTG CCT AAT ACA TGC), and the reverse specific primer was MbgR (CCT AGA GTG CTC AAT TG), designed

to give a PCR amplicon size of 1061 bp. The PCR cycling conditions used were 94°C for 4 min followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 48 s, followed by a final extension at 72°C for 7 min.

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# 4 Molecular Typing of *Mycoplasma* Species

## Introduction

Analysis of populations and assessment of strain variability provide important information regarding bacterial pathogens. Molecular epidemiological analysis enables the genotyping of strains and has uses in the tracing and control of disease outbreaks. In many cases pathogenic strains of bacteria have been identified by typing methods as belonging to distinct clonal lineages that demonstrate a unique combination of virulence alleles or genes (Musser, 1996). Therefore typing may enable the detection of certain genotypes that are associated with more severe or unusual disease signs. These isolates, or particularly those genotypes associated with low virulence, may be useful in vaccine development. Recently developed molecular typing methods are capable of distinguishing whether genetic variation is due to recombination or point mutation; they may also have applications for fundamental research as they can enable the definition and further understanding of microbial population structures and dynamics.

Over the past decade, nucleic acid-mediated procedures have largely replaced serology and protein-based methods, and procedures that were once laborious, expensive and complex have now been adapted to the high-throughput working environment of the diagnostic laboratory. Many methods are now available for use in molecular typing studies and the use of PCR has enabled the development of a plethora of rapid typing methods for strain identification. Traditional methods such as pulsed field gel electrophoresis (PFGE) are still a valuable tool for genotyping mycoplasmas, and more recently the increased number of sequenced mycoplasma genomes has meant that methods such as multilocus sequence techniques (MLST) and variable number tandem repeat (VNTR) analysis are gaining popularity and being applied to an increasing range of *Mycoplasma* species. The development of species-specific DNA-based methods means that typing can now, in theory, be directly applied to clinical samples without the need for culture, offering

the possibility in the near future of rapid pen-side typing methods for use in outbreak situations.

The choice of typing technique depends on available equipment, time considerations and cost but also on the need for discrimination power and reproducibility. Ideally, methods of strain differentiation must have high enough discriminatory power to clearly differentiate unrelated strains, as well as to demonstrate the relationship of isolates from individuals infected through the same source. Genotyping methods should also have a high degree of reproducibility, which is particularly important during construction of reliable databases. Furthermore, genotyping methods should be easy to interpret and rapid to perform. It is frequently necessary to have multiple typing methods available for use in genotyping mycoplasmas as studies have shown that different techniques are suited to different *Mycoplasma* species; as some methods may detect little variation between strains, alternative methods may be more suitable and capable of differentiating strains.

## Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) analysis is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: (i) restriction of the DNA and ligation of oligonucleotide adapters; (ii) selective amplification of sets of restriction fragments; and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. AFLP has been applied to *M. bovis* (McAuliffe *et al.*, 2004), *M. gallisepticum* (Boettger and Dohms, 2006), *M. hyosynoviae* (Kokotovic *et al.*, 2002), *M. mycoides* SC (Kusiluka *et al.*, 2001) and others. AFLP is a highly discriminatory technique but requires specialist equipment, including an automated DNA sequencer or separation of DNA fragments. The use of AFLP for molecular epidemiology studies of *M. bovis* is described below.

### AFLP analysis of *M. bovis*

*M. bovis* is a primary cause of bovine pneumonia, arthritis and mastitis and has also been associated with keratoconjunctivitis, otitis, meninigitis, infertility and abortion (Pfützner and Sachse, 1996; Nicholas and Ayling, 2003). *M. bovis* was first isolated in 1961 in the USA from a cow with severe mastitis (Hale *et al.*, 1962). Subsequently, infection has been reported throughout the world, including most European countries (Nicholas and Ayling, 2003). As the prevalence of

*M. bovis* varies widely across the world, there are important trade implications and a pressing need for monitoring cattle for *M. bovis*.

AFLP was carried out on 60 *M. bovis* isolates from the UK. DNA restriction, ligation of AFLP adapters and amplification of the modified fragments was carried out as described previously (Kusiluka *et al.*, 2000). Briefly, the template DNA was simultaneously restricted with 5 U *Bgl*II and 5 U *Mfe*I (New England Biolabs, Ipswich, MA) at 37°C for 2 h in a restriction buffer containing 10 mM Tris-acetate (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate, 5 mM dithiothreitol and 50 ng/µl bovine serum albumin. A 5 µl aliquot of the DNA digest was added to 15 µl of a ligation mixture containing 2 pmol of BglII adapter and 20 pmol MfeI adapter, 1 U of T4 DNA ligase (Amersham Pharmacia Biotech, Bucks, UK), 2 µl of 10 × ligase buffer (supplied with the enzyme) and 8 µl restriction buffer. Ligation reactions were carried out overnight at room temperature.

The modified genomic fragments were amplified by using a Bgl-2F-0 primer, which was labelled at the 5′ end with 6-carboxyfluorescein (FAM), and an unlabelled MfeI-0 primer, as described previously (Kokotovic *et al.*, 1999). The PCR reactions were performed in a 50 µl total volume containing 2 µl (tenfold diluted) ligation product, 0.2 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 65 ng of each primer and 1.5 U Taq polymerase (Gibco BRL, Delhi, India). Cycling conditions included an initial denaturation step of 94°C for 3 min and 25 cycles of denaturation (94°C for 60 s), annealing (56°C for 60 s) and extension (72°C for 90 s). The last cycle also included a final extension step at 72°C for 10 min. The PCR products were detected using an ABI 377 automated DNA sequencer (Perkin Elmer, Waltham, MA) as described previously (Kusiluka *et al.*, 2000).

In total 54 isolates were typed using AFLP and 35 different profiles were obtained. By comparison of AFLP fragments in the size range 50–400 bp, the *M. bovis* isolates could be separated into two distinct groups (A and B) with about 79% similarity between groups (Fig. 4.1). Several bands were detected that were not shared between members of the two groups: fragments of 199, 269 and 337 bp were detected only among the members of group B, whilst fragments of 223, 230, 248 and 387 bp were present only among the members of group A. In addition, all of the members of group A had a fragment of either 254 or 255 bp that was not detected among any of the group B strains.

Distinct differences in heterogeneity were seen between group A and group B. Group B exhibited the greatest genetic diversity, with 93% of isolates having unique profiles compared with 39% of group A isolates. Group B could also be further divided into two subgroups (Ba and Bb). Three fragments (157, 267 and 287 bp) were present exclusively among the strains of subgroup Ba. Interestingly, a single fragment (137 bp) was detected that was present in all subgroup Ba strains and all group A strains but none of the Bb strain.

## Random Amplified Polymorphic DNA Analysis

Random amplified polymorphic DNA (RAPD) typing is a PCR technique which uses short primers with a low annealing temperature to randomly amplify target DNA. The primers are intended to bind multiple times within the genome, and

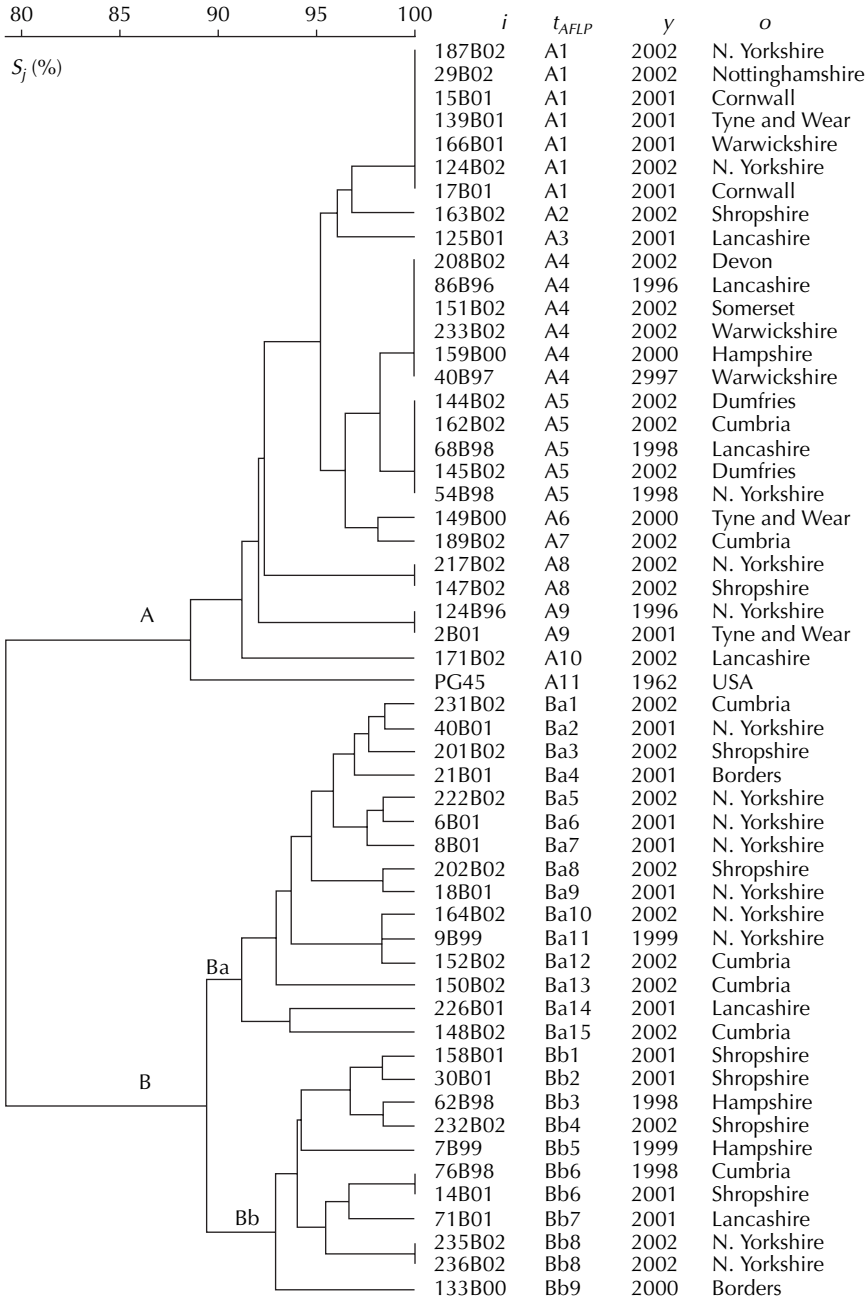


**Fig. 4.1.** Genetic relationship between *M. bovis* strains based on comparison of AFLP profiles (50–400 bp fragment size range) produced by amplification of *Bgl*II and *Mfe*I DNA templates with non-selective primers. The dendrogram was produced with the UPGMA method with Jaccard similarity coefficient (SJ) matrix. *i*, strain designation; $t_{AFLP}$, arbitrarily assigned AFLP type; *y*, year of isolation; *o*, origin of strains.

agarose gel electrophoresis is used to compare the banding pattern of different strains. RAPD is a very rapid typing method and it is possible to obtain results within a matter of a few hours; it also requires little specialist equipment. However, the differing intensity of banding patterns could be difficult to interpret without an automated gel analysis package, such as BioNumerics. Also, RAPD requires very careful sample preparation to ensure reproducibility. The RAPD technique has intrinsic problems of reproducibility because numerous experimental parameters, such as $MgCl_2$ concentration, Taq polymerase concentration and source template DNA concentration and thermocycler programme and model, all affect the reproducibility of the technique in different laboratories and over time in the same laboratory (Tyler *et al.*, 1997). RAPD analysis using a variety of primers has been carried out on *M. bovis* (McAuliffe *et al.*, 2004), *M. ovipneumoniae* (Parham *et al.*, 2006), *M. gallisepticum* (Geary *et al.*, 1994) and *M. hyopneumoniae* (Vicca *et al.*, 2003). The application of RAPD typing to *M. bovis* is described below.

## RAPD of *M. bovis*

The single primer Hum4 5′-ACGGTACACT-3′ (Hotzel *et al.*, 1998) was used for the generation of RAPD profiles. Amplification was performed in a 50 µl total reaction volume containing 100 ng of DNA sample, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each deoxynucleoside triphosphate and 0.5 U of TaqGold (Perkin-Elmer). Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 15 s, 37°C for 60 s and 72°C for 90 s (Byrne *et al.*, 2001). The last cycle included a final elongation at 72°C for 7 min. PCR products were resolved by electrophoresis on 10 cm 2% agarose gels at 60 mA for 1.5 h, stained with ethidium bromide and visualized under UV illumination.

RAPD of *M. bovis* gave similar results to AFLP analysis and indicated that UK *M. bovis* isolates fell into two genetically distinct groups. RAPD analysis using the single primer Hum4 produced between two and nine bands, ranging from approximately 200 to 1600 bp. In total, 30 different RAPD profiles were obtained from 54 analysed *M. bovis* strains. The isolates could be divided into two major groups (A and B) with approximately 49% similarity between the groups, and a third, smaller group (C), which showed about 28% similarity to the two major groups (Fig. 4.2). Isolates were unevenly distributed among the groups, with 54% of isolates in group A, 35% of isolates in group B and 11% of isolates in group C. All group B and C isolates had characteristic bands of approximately 1400 or 2000 bp that were not found in group A isolates (Fig. 4.2). Group C isolates were unusual compared with all other isolates tested as they showed a very simple fingerprint pattern consisting of two or three fragments. The type strain, PG45, clustered within group A and was most closely related to the field isolate 149B00, with about 73% similarity. The technique proved to be reproducible with the same banding pattern obtained for replicate isolates even when DNA extractions were done on separate occasions (results not shown). The only variation between replicate samples was in terms of the intensity of bands rather than the presence or absence of bands.

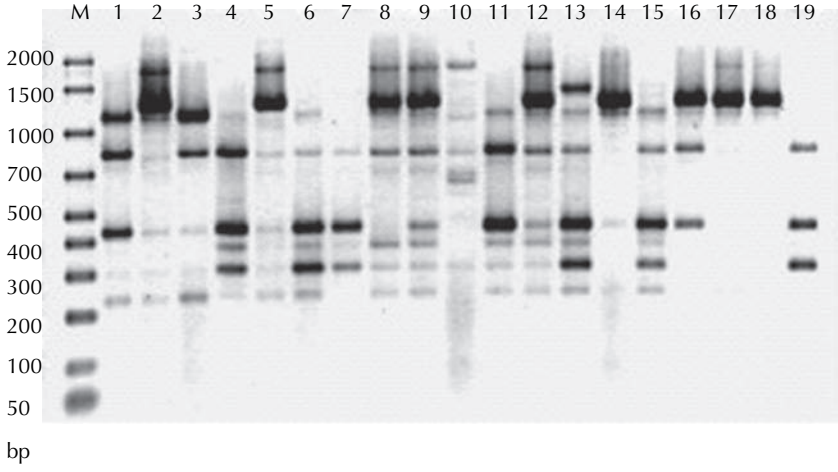


**Fig. 4.2.** Representative profiles produced by RAPD with the primer Hum4. Lane M, Bio-Rad markers; lane 1, profile A6; lane 2, profile B1; lane 3, profile A1; lane 4, profile A7; lane 5, profile B3; lane 6, profile A6; lane 7, profile A2; lane 8, profile B1; lane 9, profile B3; lane 10, profile B11; lane 11, profile A4; lane 12, 30B01 (profile B3); lane 13, 68B98 (profile A5); lane 14, profile B12; lane 15, profile A6; lane 16, profile B13; lane 17, profile C2; lane 18, profile C2; lane 19, profile A14.

## Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is a typing method that is based on the digestion of genomic DNA using restriction enzymes and separation of large fragments of DNA in agarose gels using specialized electrophoresis equipment with a hexagonal array of clamped electrodes. PFGE is often described as the 'gold standard' of typing techniques due to its very reproducible nature. PFGE is laborious, requiring complex sample preparation, and takes in excess of 7 days to achieve results. PFGE has been used successfully on a number of *Mycoplasma* species including *M. bovis* (McAuliffe *et al.*, 2004), *M. ovipneumoniae* (Parham *et al.*, 2006), *M. hyopneumoniae* (Stakenborg *et al.*, 2005) and *M. synoviae* (Dufour-Gesbert *et al.*, 2006). The protocol for PFGE analysis *of M. ovipneumoniae* is given below.

### PFGE of *M. ovipneumoniae*

*M. ovipneumoniae* is the cause of a non-progressive (atypical) pneumonia in sheep (Gilmour *et al.*, 1979). We have used PFGE to analyse 43 strains of *M. ovipneumoniae* collected from samples submitted for routine diagnostic investigation in the UK.

Aliquots (20 ml) of stationary-phase *M. ovipneumoniae* culture (absorbance $A_{600}$ of approximately 0.3) were used for PFGE analysis. Cells were harvested by centrifugation (3500 *g* for 20 min at 4°C), washed three times with Tris-EDTA buffer and resuspended in 300 ml of cold Tris-EDTA buffer. Agarose plugs were

made from a 1:1 mixture of 2% low-melting-point agarose (Bio-rad, Hercules, CA) and the cell suspension. Plugs were incubated in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% lauroyl sarcosine, 1 mg/ml proteinase K) for 48 h at 56°C. Plugs were washed four times with Tris-EDTA buffer for 30 min at 4°C. Slices (2 mm) were cut aseptically from plugs and equilibrated in restriction buffer (Promega, Madison, WI) for 1 h. Subsequently, restriction digestion was performed by using 30 U of *Sma*I (Promega) for 16 h according to the manufacturer's instructions. The fragments were resolved on 1% pulsed field-certified agarose (Biorad) gels using a CHEF-DRIII system (Biorad) at 6 V/cm, with a running time of 20 h at 14°C, included angle of 120°, initial pulse time of 4 s and final pulse time of 40 s. Gels were stained with ethidium bromide (0.5 mg/ml) for 15 min, destained in distilled water for 1 h and photographed under UV light. A lambda ladder PFGE marker (Sigma, Ronkonkoma, NY) was used for fragment size determination.

PFGE analysis of *M. ovipneumoniae* from the UK gave a high degree of genetic heterogeneity, with 40 different PFGE profiles generated from 43 isolates. These results are summarized in Fig. 4.3. There was no link between the genotype and geographical origin of isolates.

## Variable Number Tandem Repeat Analysis

Variable number tandem repeats (VNTRs) are simple repeated DNA sequences that vary in copy number and have been described for a variety of organisms, including *Staphylococcus aureus* (Sabat *et al.*, 2003), *Mycobacterium tuberculosis* (Frothingham and Meeker-O'Connell, 1998), *Borrelia* spp. (Farlow *et al.*, 2002), *Leptospira* spp. (Slack *et al.*, 2005), *Brucella* spp. (Whatmore *et al.*, 2006), *Francisella tularensis* (Farlow *et al.*, 2001) and *Legionella pneumophila* (Pourcel *et al.*, 2003), but have not previously been used to differentiate *Mycoplasma* strains. VNTRs have been shown to give a high level of discriminatory power and can provide information regarding both evolutionary and functional bacterial diversity (van Belkum *et al.*, 1998). With the increasing number of sequenced genomes and the development of VNTR databases, VNTR is an increasingly amenable technique for bacterial typing. VNTR requires little specialized equipment, only thermocyclers and agarose gel electrophoresis, and does not necessitate sequencing, unlike some typing methods such as MLST analysis. Despite their small genome size, many *Mycoplasma* species contain a large proportion of repetitive DNA, therefore the use of typing tools which target repetitive DNA is likely to be most promising.

VNTR analysis has been successfully used for the molecular typing of MmmSC (McAuliffe *et al.*, 2007) and is described in more detail below.

### VNTR analysis of *M. mycoides* subsp. *mycoides* SC

Undoubtedly, the most important mycoplasma disease is contagious bovine pleuropneumonia (CBPP), an Office International des Epizooties (OIE) notifiable disease, which is caused by *M. mycoides* subsp. *mycoides* small colony (MmmSC). Studies to date on the molecular epidemiology of MmmSC are consistent with there

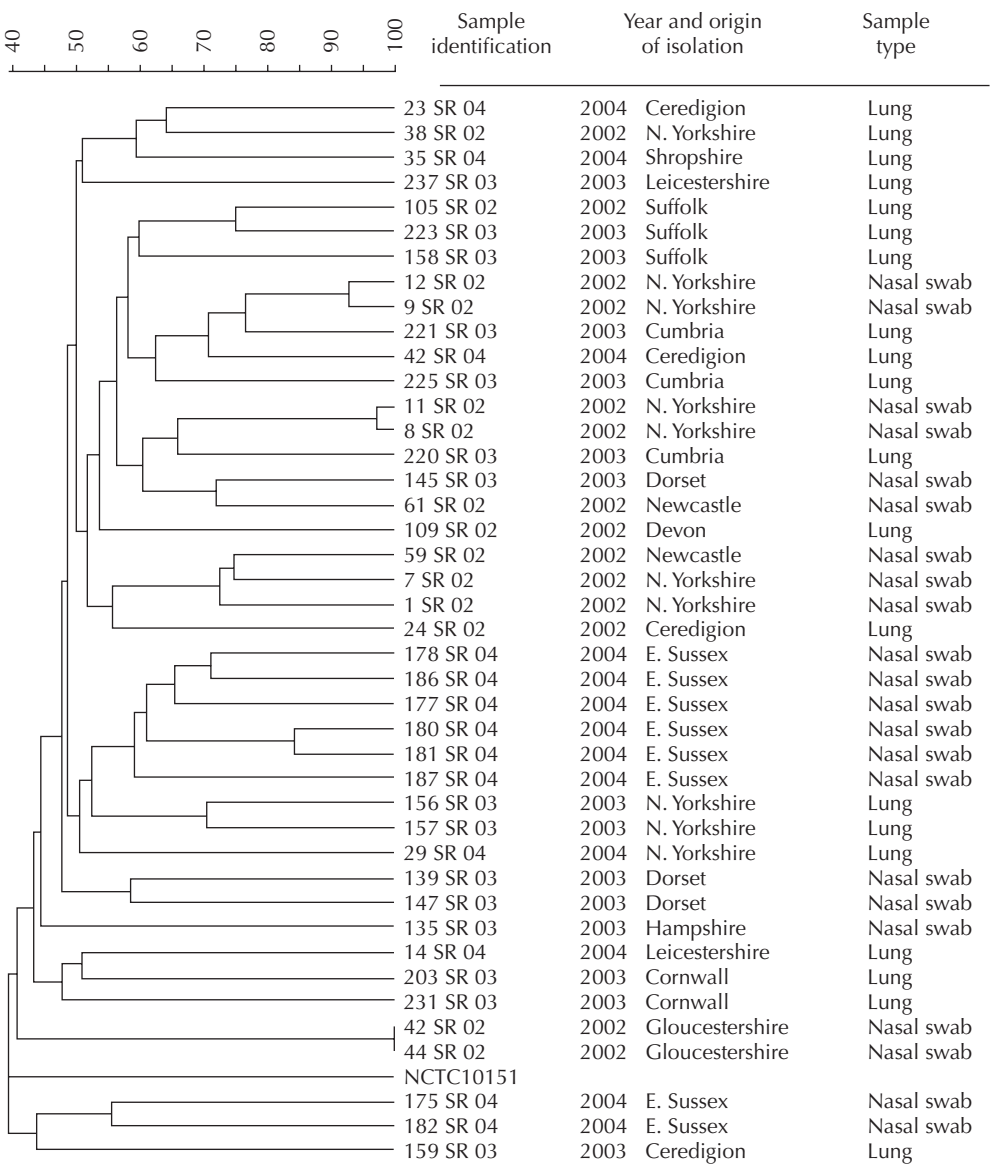


**Fig. 4.3.** Dendrogram illustrating genetic diversity of *M. ovipneumoniae* as determined using PFGE analysis.

being two main subtypes: that isolated from recent European outbreaks (post-1980) and that isolated from African and Australian outbreaks, some of which date back to 1936 (March, 1999; March *et al.*, 2000).

This study has applied VNTR typing to MmmSC and has found that, despite its minimal size, the MmmSC genome has a plethora of VNTRs. We have examined 60 VNTRs throughout the MmmSC genome and found that there was remarkable

genetic homogeneity between strains from different geographical origins, with only three VNTRs exhibiting variation between strains.

Three primer sets were used for VNTR analysis: VNTR 4F AATCAGCGTGATTCAGCTTT, 4R TTGCTTTGATTAACTTGTTGTTTT; 5FTGAACAAAATAAATTACGTGAAATG, 5R AAGTTTAAGATGCAAAACAAACG,; and 42F ATTATGATGATGGAGAGCAA, 42R CATTCTGCTTTTCAAGATGTF.

For the PCR, 1 µl of lysate was added as a template to 49 µl of a reaction mixture containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each deoxynucleoside triphosphate and 0.5 U of Taqgold (Applied Biosystems, Foster City, CA). The cycling conditions were: denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 56°C for 45 s and 72°C for 1 min, a final extension step of 72°C for 10 min and samples were kept at 4°C until analysis. Aliquots were checked for correct amplification by electrophoresis on 2.5% agarose gels followed by visualization with ethidium bromide under UV illumination. Selected VNTRs were subjected to sequence analysis.

VNTR 4 and 5 were located within a similar region of the genome and all were located in a region containing the gene *natA*, encoding the ATP-binding component of a Na+ ABC transporter, MSC_0398 and a hypothetical transmembrane protein MSC_0399. VNTR 5 spans a region at the end of *natA* and an intergenic non-coding region and ends within MSC_0399. VNTR 4 is contained within the gene *natA*. VNTR 42 was positioned within the hypothetical surface-located membrane protein MSC_0523.

VNTR 4 analysis showed that there was intraspecific variation between the strains, with eight different-sized PCR products found. Strains were assigned arbitrary numbers, depending on the size of the PCR product obtained. VNTR 5 gave two different-sized PCR products, all isolates tested giving an identical-sized product with the exception of the African strain Afade, which gave a smaller-sized PCR product. There was no difference in size of PCR product found for VNTR 42. However, it enabled the differentiation of African/Australian and European isolates as it was present in African and Australian strains and absent in European isolates. This VNTR is in the previously identified 8.8 kb region that is known to be deleted in European strains (Vilei *et al.*, 2000). In total 12 different groups were identified by combining the VNTR analysis to give a three-number profile, for example 031. The European strains tested gave four profiles; African/Australian strains were more heterogeneous compared with European strains, with eight different profiles obtained. These results are summarized in Table 4.1.

This study has also enabled the first subgrouping of European strains. Using VNTR analysis four profiles were obtained from the 19 European strains tested. Previous studies have found that European strains are largely homogeneous, although studies of whole cell proteins showed that many Italian strains lacked a 98 kDa band that was present in other European strains (Goncalves *et al.*, 1998). MLST analysis only tested six European strains but found that they fell into three groups, with all European strains being identical except the single Portuguese strain tested and the unusual ancestral strain PO1967 (Lorenzon *et al.*, 2003). Insertion sequence analysis using IS1634 of European strains was homogeneous (Vilei *et al.*, 1999). Similarly, analysis of Vmm showed that European strains were

**Table 4.1.** Summary of VNTR results for representative strains.

| Strain | Origin | VNTR 42 profile | VNTR 4 profile | VNTR 5 profile |
|---|---|---|---|---|
| M545/91 | Portugal 1991 | 0 | 3 | 1 |
| 138/5 | Italy 1992 | 0 | 4 | 1 |
| 197 | Italy 1992 | 0 | 5 | 1 |
| Astercous | Spain 1987 | 0 | 6 | 1 |
| Tan8 | Tanzania 1998 | 1 | 1 | 1 |
| SF0177 | Namibia 2004 | 1 | 2 | 1 |
| Mandingwan | Namibia 2001 | 1 | 4 | 1 |
| Gladysdale | Australia pre-1964 | 1 | 5 | 1 |
| Afade | Chad 1968 | 1 | 5 | 2 |
| IS31 | Tanzania 1998 | 1 | 6 | 1 |
| M375 | Botswana 1996 | 1 | 7 | 1 |
| KH3J | Sudan 1940 | 1 | 8 | 1 |

homogeneous, with the exception of a single strain that demonstrated variation (Persson *et al.*, 2002).

In conclusion, VNTR analysis is capable of subtyping MmmSC strains and it may prove to be an interesting complement to existing typing tools, and owing to its rapid nature and ease of use, it may make an ideal first-line screening tool for isolates.

## Insertion Sequence Analysis

Insertion sequences (IS) are mobile genetic elements of between 0.7 and 2.5 kb and encode only those proteins required for their transposition. IS elements are capable of moving within the genome and generating extensive DNA rearrangements. Mycoplasmas are thought to contain a high density of IS elements, and as such IS analysis is a promising typing technique. IS typing is based on the digestion of genomic DNA using restriction enzymes, the separation of digested DNA using agarose gel electrophoresis, the Southern blotting of DNA fragments to a membrane, probing of the membrane with a labelled insertion sequence probe and detection of probe binding using a colorimetric or chemiluminescent substrate. The different banding pattern exhibited between different strains can then be compared. IS analysis has previously been used for *M. bovis* (Miles *et al.*, 2005), *M. agalactiae* (Pilo *et al.*, 2003) and MmmSC (Vilei *et al.*, 1999; Westberg *et al.*, 2002). The application of IS analysis to *M. bovis* is described below.

### IS analysis of *M. bovis*

IS analysis of *M. bovis* was carried out using the insertion sequences ISMbov2 and ISMbov3. DIG-labelled probes for IsMbov2 and IsMbov3 were amplified using the primers 5im_ismmy1 and 3im_ismmy1 (Westberg *et al.*, 2002) and IS1634F CAGGAAATGTTGCTGATCCA and IS1634R TTGTTTGCTTCCAGCTTT in the

MmmSC PG1 genome. ISMbov2 shows 98% homology to the MmmSC ISMmy1, and ISMbov3 shows 96% homology to IS1634. Production of the DIG-labelled probe was carried out using a Roche PCR Dig probe synthesis kit with amplification conditions of 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 1 min, with a final extension at 68°C for 5 min.

Genomic DNA was extracted from *M. bovis* cultures using a Sigma genelute kit and 3 *u*g was digested using 12U of the enzyme EcorI (Promega) for 18 h at 37°C. The resulting DNA fragments were denatured then separated on 0.8% agarose gels at 40V for 16–18h. Fragments were then transferred to N+ membranes using Southern blotting in 0.4 M NaOH for 16 h. Following transfer, membranes were soaked in 2 × SSC for 30 min and transferred to hybridization tubes and prehybridized at 65°C for 1 h with 10 ml of hybridization buffer containing 5 × SSC, 0.1% (w/v) N lauryl sarcosine, 0.02% (w/v) SDS and 1% (w/v) blocking agent (Roche, Basel, Switzerland). The insertion sequence probe was heated at 95°C for 5 min and rapidly cooled on ice before dilution at 1:250 in 5 ml of hybridization buffer and incubated with the membrane at 65°C for 18 h. Unbound probe was removed by washing once at 65°C with a buffer containing 2 × SSC, 0.1%(w/v) SDS and twice at room temperature with a buffer of 0.1% SSC, 0.1% (w/v) SDS. Anti-DIG antibody was used to detect the bound, labelled probe using a NBT/BCIP substrate (Roche). Hybridization patterns were then analysed using Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium).

Hybridization profiles showed considerable variation between strains: IsMbov 2 was present in between two and 17 copies whereas *Mbov*3 was present in between three and 14 copies (Fig. 4.4). These data supported previous findings using AFLP and RAPD. The presence of such a high density of IS elements within

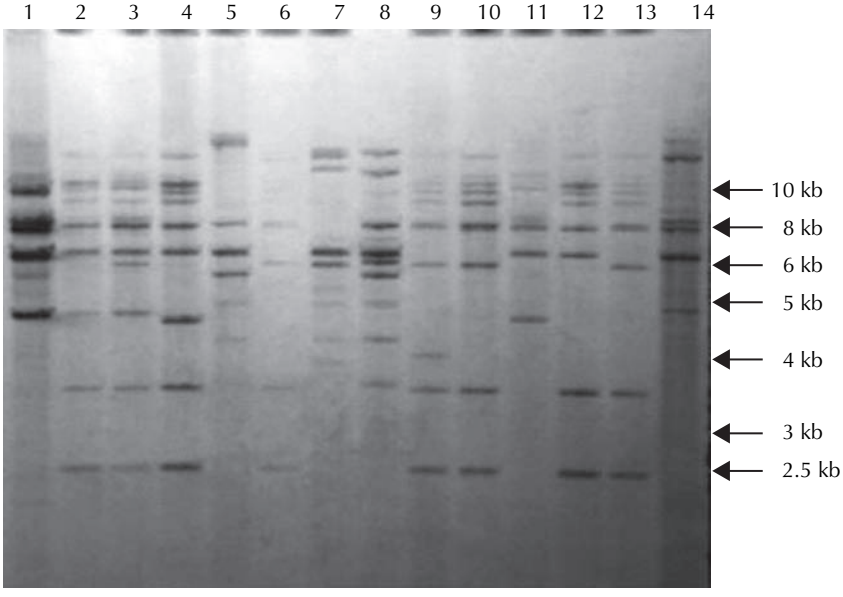


**Fig. 4.4.** Representative blotting results with the 185 bp IS1634 probe.

these small genomes indicates an important function in the stability of populations. It is likely that they have a role in genome rearrangement, causing expression of variable proteins.

## Other Typing Methods Applied to *Mycoplasma* Species

### Gene sequencing approaches

Recently sequencing methods have been introduced as a new approach for studying the molecular epidemiology of bacterial pathogens (Enright and Spratt, 1999). Gene sequencing remains a popular typing method used among a wide range of *Mycoplasma* species, including *M. gallisepticum*, *M. hyopneumoniae*, *M. synoviae*, *M. conjunctivae* and others (summarized in Table 4.2). For *M. gallisepticum* overall, gene targeted sequencing analysis of multiple surface-protein genes was demonstrated to have better discriminatory power than RAPD analysis (Ferguson *et al.*, 2005). Some authors have analysed strains using restriction enzyme analysis without the need for sequencing. 16S sequencing has also proved useful for the genotyping of *M. c. capripneumoniae* isolates (Pettersson *et al.*, 1998) and has recently been applied to strains from outbreaks of CCPP in the Thrace region of Turkey (Churchward *et al.*, unpublished results).

### MLST approaches

Given the rapidly increasing number of sequenced mycoplasma genomes the use of MLST is an increasingly attractive method for typing of *Mycoplasma* species. MLST is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of seven housekeeping genes. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Each isolate of a species is therefore unambiguously characterized by a series of seven integers which correspond to the alleles at the seven housekeeping loci. In MLST, the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site) or by a recombinational replacement (which will often change multiple sites).

MLST is based on the well-established principles of multilocus enzyme electrophoresis but differs in that it assigns alleles at multiple housekeeping loci directly by DNA sequencing rather than indirectly via the electrophoretic mobility of their gene products.

More recently molecular epidemiology studies have used multilocus sequence analysis for the typing of MmmSC (Lorenzon *et al.*, 2003). This approach differed from traditional MLST analysis, as the targets for sequencing were not housekeeping genes but non-coding regions or regions adjacent to insertion sequences

**Table 4.2.** Summary of typing methods applied to *Mycoplasma* species.

| *Mycoplasma* species | Typing method | Reference |
|---|---|---|
| *M. mycoides* SC | VNTR | McAuliffe *et al.*, 2007 |
| | MLSA | Lorenzon *et al.*, 2003 |
| | AFLP / PFGE | Kusiluka *et al.*, 2001 |
| | SDS PAGE | Goncalves *et al.*, 1998 |
| | Immunoblotting | Poumarat and Solsona, 1995 |
| | IS 1296 analysis | Cheng *et al.*, 1995 |
| | IS MyMy analysis | Westberg *et al.*, 2002 |
| | Bgl REA | Vilei and Frey, 2004 |
| *M. mycoides* LC | LppA REA | Monnerat *et al.*, 1999 |
| *M.* sp. bovine group 7 | REA of genomic DNA | Djordjevic *et al.*, 2001 |
| *M. c. capripneumoniae* | 16S rDNA sequencing | Pettersson *et al.*, 1998 |
| | H2 sequencing | Lorenzon *et al.*, 2003 |
| | AFLP/PFGE | Kusiluka *et al.*, 2001 |
| *M. agalactiae* | PFGE/ | Tola *et al.*, 1996 |
| | SDS PAGE | |
| | RAPD/VNTR | McAuliffe *et al.*, 2008 |
| | IS analysis | Pilo *et al.*, 2003 |
| | P40 adhesin analysis | Fleury *et al.*, 2002 |
| | 16S rDNA sequencing | Konigsson *et al.*, 2002 |
| *M. bovis* | AFLP/RAPD/PFGE | McAuliffe *et al.*, 2004 |
| | IS analysis | Parham *et al.*, 2006 |
| | 16S rDNA sequencing | Konigsson *et al.*, 2002 |
| | Vsp analysis | Beier *et al.*, 1998 |
| *M. ovipneumoniae* | RAPD/PFGE | Parham *et al.*, 2006 |
| *M. gallisepticum* | RAPD/PFGE | Ley *et al.*, 1997 |
| | Gene sequencing | Ferguson *et al.*, 2005 |
| | REA of PvpA | Liu *et al.*, 2001 |
| | AFLP | Hong *et al.*, 2005 |
| *M. synoviae* | PFGE/RAPD | Marois *et al.*, 2001 |
| | AFLP | Feberwee *et al.*, 2005 |
| | Sequencing of vlhA | Hong *et al.*, 2004 |
| *M. hyopneumoniae* | AFLP/RAPD | Stakenborg *et al.*, 2006 |
| | PFGE | Stakenborg *et al.*, 2005 |
| | P146 sequencing | Mayor *et al.*, 2008 |
| | VNTR of adhesion genes | de Castro *et al.*, 2006 |
| *M. conjunctivae* | LppS sequencing | Belloy *et al.*, 2003 |
| *M. cynos* | RAPD/PFGE | Mannering *et al.*, 2008 |

as it was thought likely that these regions would harbour greater variation than housekeeping genes. This approach was able to differentiate strains to some extent, with isolates being subdivided into 15 different groups (Lorenzon *et al.*, 2003).

Recently an MLST based on the sequence of five housekeeping genes has been developed for members of the *M. mycoides* cluster (Manso-Silván *et al.*, 2007). This method was initially developed for use in determining phylogeny of the ambiguous *mycoides* cluster but it may also have limited application for use in the

intraspecific genotyping of some species. MmmSC and *M.* species bovine group 7 exhibited very little/no intraspecific variation using this approach but some limited variation was seen between strains of *M. c. capricolum* and *M. mycoides* LC/*M. capri*. MLST approaches have many advantages, the data are entirely free from subjective analysis and it is particularly amenable to intra-laboratory comparison as internet-based databases can be constructed.

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# 5 Antigenic Analysis of Mycoplasmas

## Introduction

Western (immuno) blotting analysis has been applied successfully to the assessment of antibody response for several *Mycoplasma* species such as *M. bovis* (Sachse *et al*., 1992; Poumarat *et al*., 1994), *M. mycoides* subsp. *mycoides* SC (Gonçalves *et al*., 1994; Poumarat and Solsona, 1995; Nicholas *et al*., 1996), *M. agalactiae* (Solsona *et al*., 1996) and several species belonging to other members of the *Mycoplasma mycoides* cluster (Costas *et al*., 1987; Olsson *et al*., 1990). This method allows the analysis of the host humoral immune response based on the electrophoretic profile of the bacterial antigens, thus overcoming problems relating to non-specific binding in other immunoassays. This was a particular problem in Portugal during the CBPP eradication campaign, where false positive results in the complement fixation test often led to the slaughter of healthy cattle. In an effort to overcome this problem, a study was initiated at the Laboratorio Nacional Investigacao Veterinaria in Lisbon to study the immunogenicity of *M. mycoides* SC to identify specific and immunodominant antigens which could be applied to confirmatory diagnostic testing. This led to the development of an immunoblotting test (IBT) for the detection of the antibody response in sera from cattle affected with CBPP (Regalla *et al.*, 1996). A common immunological pattern consisting of five immunodominant antigens with apparent molecular weights of 110, 98, 95, 62/60 and 48 kDa was identified in a preliminary study with sera from cattle experimentally and naturally affected by CBPP.

## Materials and Methods

### Strain, growth conditions and antigen preparation

A log-phase culture of an appropriate *M. mycoides* SC isolate (derived from Europe or Africa depending on the source of test sera), growing in modified Gourlay

medium at 37°C, is harvested by centrifugation at 14,600 *g* for 1 h at 4°C, followed by three washes in phosphate-buffered saline solution (PBS, 0.1M $Na_2HPO_4$, 0.1M $NaH_2PO_4$, 0.15M NaCl, pH 7.2). Washed cells are resuspended in the same buffer and stored at –20°C (Goncalves *et al.*, 1998).

## Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

The protein content of the thawed mycoplasma cells is estimated by an acceptable method. The samples are mixed with lysis buffer (500 mM Tris/HCl, pH 6.8; 4.6% (w/v) SDS; 20% (v/v) glycerol; 10% (v/v) 2-mercaptoethanol and 0.004% bromophenol blue) and boiled for 5 min. The lysates are separated by SDS-PAGE in a 1.5 mm-thick, 4% stacking/5–15% gradient resolving gel and the electrophoresis is run at a constant current of 40 mA. The separated proteins are transferred to 0.45 µm nitrocellulose membranes (NC) at 70 V for 1.5 h.

## Preparation of antigen strips

The NC membranes are dried, labelling the side on which the proteins are blotted. The NC membranes are incubated in blocking buffer (PBS containing 5% skimmed milk, 1M glycine and 1% egg albumin) for 2 h at room temperature (about 22°C). The membranes are then washed three times each for 15 min at room temperature in 0.1% (v/v) Tween 20 in PBS and again for 10 min in PBS alone. The NC membranes are dried and cut into 3 mm-wide strips and labelled. These strips contain the antigen used for immunoblotting and should be used immediately, dried or kept at –20°C until they are needed.

## Quality control of antigen strips

After performing the Western blot, one 20 mm-wide strip from each side of the membrane is cut and stained with Indian ink to verify the entire range of the transferred cellular proteins and molecular weight standards.

After blocking, the membrane is reacted with the positive control serum, one strip from each edge of the membrane, to identify the specific bands of 110, 98, 95, 62/60 and 48 kDa (see also the Figs 5.1 and 5.2 below).

## Test procedure

**1.** Serum samples to be tested are not heat treated. The serum samples, and positive and negative controls, are diluted 1:3 in dilution buffer (PBS with 0.1% skimmed milk and 0.1% egg albumin) and are applied to the antigen-impregrenated side of the strip before incubating at 37°C for 2 h with continuous agitation.

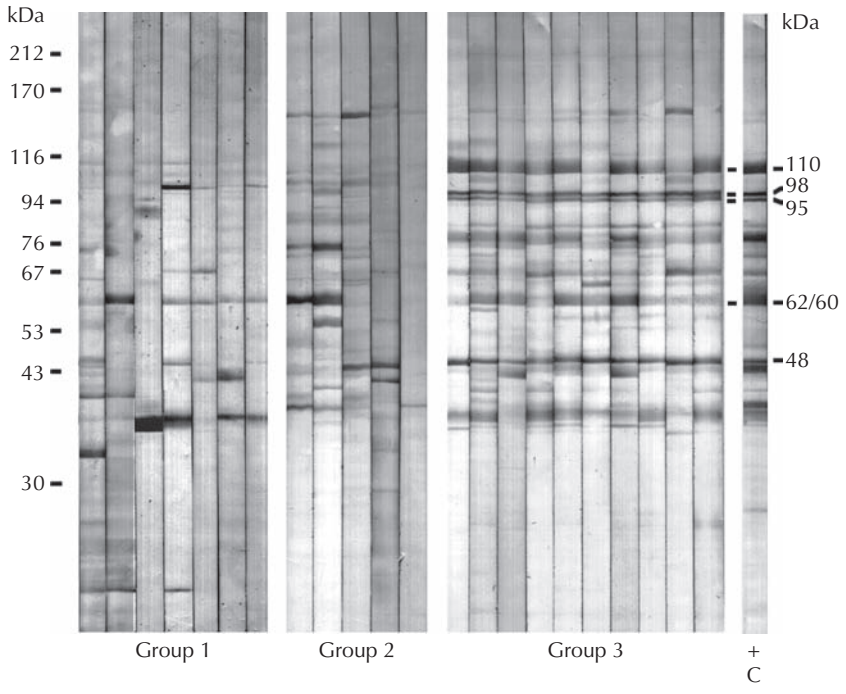


**Fig. 5.1.** Immunoblot analysis of sera from different groups of cattle in Portugal.

**2.** After washing as above, an appropriate dilution of peroxidase-conjugated anti-bovine-IgG (H+L chains) in dilution buffer is applied to the strip and incubated for 1 h at room temperature with continuous agitation.
**3.** After another wash the substrate comprising 30 mg 4-chloro-1-naphtol dissolved in 10 ml methanol mixed with 50 ml PBS and 30 $\mu$l $H_2O_2$ is added and left in the dark with continuous agitation until the protein bands are suitably dark. The reaction is stopped with distilled water.
**4.** After drying the strips the reading is carried out based on the presence of the core IgG immunoblot profile of five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa (Fig. 5.1). Sera showing this core immunological profile are considered positive.

## Sensitivity and Specificity of the IBT

In a field evaluation of sera from CBPP-affected cattle, the IBT detected 92.5% of infected animals, the CFT detected 77.5%, histology detected 36.9% and mycoplasma culture detected only 31%. All sera from nearly 500 cattle from CBPP-free

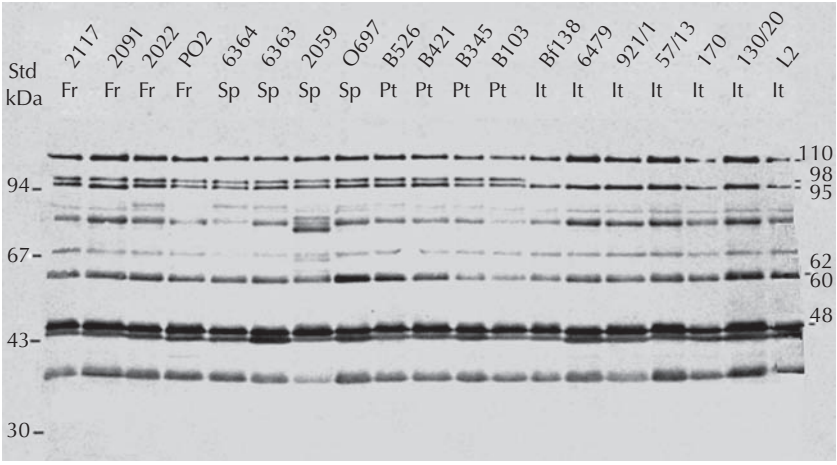


**Fig. 5.2.** Immunoblots of *M. mycoides* SC strains from France, Spain, Portugal and Italy. Seven strains on the right lack 98 kDa protein.

regions, including 177 sera positive by CFT, were negative by IBT (Fig. 5.1). It was concluded that the IBT was highly specific and the most sensitive serological test so far described for the detection of cattle affected by CBPP.

Within the framework of the Portuguese eradication programme, a more comprehensive and longer-term study beginning in 1998 has tested over 2.5 million sera, of which just over 12,000 (0.5%) were positive by CFT. Of these only 64 (0.6%) from 39 herds were positive by IBT and were located in the infected areas prior to 1999, when the last CBPP outbreaks were seen. Following the last outbreaks and up to to 2001, no positive results have been seen in a total of just over 2000 IBT tests, most of which originated from areas previously identified as infected.

## Analysis of *M. mycoides* SC Strains by Immunoblotting

To assess the antigenic homogeneity of *M. mycoides* SC strains, 46 isolates from European countries (Portugal ($n$ = 24), Spain ($n$ = 6), France ($n$ = 4) and Italy ($n$ = 12)) were examined. The study was also extended to ten strains from Africa, three strains from Australia and to the type strain PG1 (Gonçalves *et al.,* 1998). The results showed that African and Australian strains could be differentiated from European strains by a strong reaction at the 72/70 kDa level, suggesting for the first time that these strains have evolved separately and that the resurgence of CBPP in Europe was not the result of reinfection from Africa. A distinct antigenic difference was also seen between some of the European strains concerning the 98 kDa protein, with several Italian and Portuguese strains lacking this protein (Fig. 5.2). Therefore, the results indicated that the European strains are antigenically different, suggesting that the Italian strains are a variant clone with the same geographic origin.

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# 6 Antimicrobial Sensitivity Testing

## Introduction

*Mycoplasma* species are the smallest organisms capable of self-replication (Razin *et al.*, 1998). Their lack of a cell wall is thought to make them vulnerable to changing osmotic pressures, temperature changes and desiccation, so they were not thought to survive in the environment for long. Their genomes are extremely small, making them reliant upon their host to provide their required nutrients and amino acids for survival. Based on this it could be anticipated that mycoplasmas should be extremely vulnerable to host immune defences and antimicrobial treatment. However, many mycoplasmas devote a relatively large percentage of their genomes to mechanisms promoting their survival, including antigenic variation to evade the host. Their lack of a cell wall also means that many antimicrobials, including the penicillins and cephalosporins, that inhibit the cross-linking of amino acid chains in peptidoglycan synthesis are not effective. Mycoplasmas are also resistant to sulphonamides, which inhibit folic acid synthesis, and those aminoglycosides that inhibit microbial respiration (Barragry, 1994). Mycoplasmas should be sensitive to antimicrobials such as: tetracyclines, which interrupt amino acid transfer to growing peptide chains at ribosome complexes; quinolones and fluoroquinolones, which affect DNA gyrase activity; and the macrolides and lincosamides, which inhibit protein synthesis. However, the emergence of strains resistant to these and other potentially effective antimicrobials is well documented (Bébéar, 1996). Some mycoplasmas such as *M. bovis* appear to have the ability to disseminate widely within the host, which must further limit their effective treatment by antimicrobials. In addition, recent work (McAuliffe *et al.*, 2006) has shown that mycoplasmas are able to produce biofilms, which may enable their survival in the environment and within the host, also reducing their susceptibility to antimicrobial treatment.

The genetic basis of antimicrobial resistance has been described for a number of antimicrobial classes in a number of organisms, mainly the major human

pathogens. Resistance by *M. hominis* and *M. pneumoniae* to the macrolides and fluoroquinolones is caused by point mutations in the 23S rDNA gene (Pereyre *et al.*, 2007) and in the DNA gyrase and topoisomerase IV, *Par*C gene (Bébéar *et al.*, 2003), respectively. Undoubtedly, similar mutations are responsible for the antimicrobial resistance but very few studies have been performed. Opportunities therefore exist here to use the information and technologies available from work on other organisms (Fluit *et al.*, 2001) to utilize methods such as PCR, gene sequencing and DNA chip technology to assess and screen for the presence and mechanisms of antimicrobial resistance.

Most antimicrobial studies on mycoplasmas have been carried out *in vitro* and involve determining minimum inhibition concentration (MIC) values for specific isolates against a range of antimicrobials. These may be useful in predicting which antimicrobials are most likely to be effective *in vivo* and for assessing the development of antimicrobial resistance. However, the pharmocokinetics and pharmacodynamics of drugs are complex and are affected by their behaviour within the host, how well the active ingredient is absorbed into the body, where in the body it concentrates and how long it remains at an effective concentration at the site of infection. It has been reported that some compounds have a preference for localizing in the lungs, and this may be enhanced in pneumonic lungs with the macrolide tilmicosin reaching three times the concentration of that reached in non-pneumonic lungs (Reeve-Johnson, 1999). In man, the newer fluoroquinolones have been shown to concentrate in lung tissue at levels many times higher than that observed in serum (Wise and Honeybourne, 1999). Other factors such as drug interactions may alter the effectiveness of treatment; some of the macrolides, specifically erythromycin, may form complexes that affect other drugs (Anadōn and Reeve-Johnson, 1999). It is worth bearing in mind too that adverse reactions to antimicrobials may occur so the manufacturers' instructions should be followed carefully. Some antimicrobials, for example tilmicosin, are not recommended for use in lambs under 15 kg.

## Minimum Inhibitory Concentrations

MICs are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after a suitable incubation period. MICs are used by diagnostic laboratories mainly to confirm resistance but most often as a research tool to determine the *in vitro* activity of new antimicrobials.

## Mycoplasmacidal Activity

While MIC endpoint determination measures the inhibitory effects of antibiotics, it does not assess the killing or cidal effect that the antimicrobials have on the organism, particularly in mycoplasmas. Several methods of determining the minimum mycoplasmacidal concentration (MMC) have been described (Cooper *et al.*, 1993; Hannan, 2000). We have found the easiest method is to subculture all of the wells of an MIC microtitre plate into a fresh microtitre plate, diluting 1 in 20

with fresh broth, i.e. 10 µl of MIC culture into 190 µl of fresh media, which effectively reduces the concentration of the antimicrobials below the MIC level, enabling an MMC value to be obtained after a suitable incubation period. This method may lack some reproducibility owing to the variation in cell concentration but it is generally reliable, giving replicate results within a few dilutions.

Other methods such as that used by Cooper *et al.* (1993) rely on prolonged incubation times, where it is assumed that the antimicrobial activity declines with incubation time, such that where growth is initially inhibited antimicrobial concentrations will fall below MIC values. This approach clearly has limitations in relation to the kinetics of antimicrobial decay, the loss of unstable medium components and the possible growth of resistant mutants, initially present in very low numbers.

## Determination of Breakpoints

Antimicrobial susceptibility testing is a key requirement in enabling veterinarians to have an informed choice about the possible effectiveness of antimicrobials in treating disease. However, in practice, the selection of antimicrobials for treatment is often little more than an educated guess as information on breakpoints for the different antimicrobials for the organisms within the different host species is not available. The provision of MIC data will guide the veterinarian away from selecting the apparently less effective antimicrobials. However, the pharmocokinetics of some antimicrobials, which may concentrate in the lungs or milk for example, may make some antimicrobials suitable for treating some conditions despite having an apparently high MIC. There is therefore a need to determine breakpoints, which have been defined as the relationship between MIC values and effective treatment dose for different antimicrobials against mycoplasmas in different host animals.

## Mutation Prevention Concentration

Concern about the development of antimicrobial resistance has seen interest grow in mutant prevention concentrations (MPC). MPC is used to determine the capacity of an antimicrobial compound to minimize or limit the selection of first-step resistant mutants in a bacterial population. For a given combination of antimicrobial compound and bacterial strain, MPC is defined as the minimum inhibitory concentration (MIC) of the least-susceptible mutant. Thus, bacterial growth in the presence of antimicrobial concentrations greater than the MPC requires the strain to develop two or more spontaneous chromosomal point mutations that each cause resistance. MPC is estimated *in vitro* as the lowest antimicrobial concentration that prevents colony growth when approximately $10^{10}$ cells are applied to agar plates containing the antimicrobial. The choice of $10^{10}$ cells as an inoculum is based on the following considerations:

- An inoculum of $10^{10}$ cells is large enough for mutant subpopulations to be present and for these to be isolated for further testing if required.

- Clinical infections rarely contain more than $10^{10}$ organisms.
- Testing an inoculum greater than $10^{10}$ is logistically difficult.

MPC determinations have used two basic approaches. First, approximately $10^{10}$ cells are plated on single agar plates that differ in drug concentration by two-fold increments. This approach allows large numbers of isolates to be tested, but consecutive plates in the series may yield confluent growth followed by no growth because of the large concentration increment. Furthermore, such a high inoculum density may affect the apparent susceptibility of some strains. Second, cells are applied to multiple agar plates at each antimicrobial concentration such that the total number of cells tested for a given concentration exceeds $10^{10}$. If sufficiently narrow concentration increments are used, isolated colonies can be enumerated to show that their number progressively approaches zero as the antimicrobial concentration increases (when colony number is plotted against antimicrobial concentration, the 'mutant selection curve' becomes steeper as the MPC is approached).

In practice these methods for testing mycoplasmas are difficult because it is not easy to obtain the large number of cells required and the mycoplasma media are not usually compatible with the use of large plates, where moisture and agar disruption is a problem. The MPC is then best carried out using a series of small Petri dishes. Our experience has shown that controls grow well to give individual colonies but the antimicrobials may affect the growth so that individual colonies cannot be defined without prolonged incubation, which may affect the antimicrobial being tested or be the result of a mycoplasmastatic effect rather than a true mutation effect.

## Factors Affecting Antibiotic Susceptibility Testing

### Media

A standard medium does not exist for all mollicutes because of their diverse nutritional requirements. Consequently a recognized medium that gives optimal growth for the organism being tested should be used. Using unsuitable media will result in suboptimal growth, giving falsely low MIC values. The medium used should not contain any other antimicrobials such as the penicillins, which are often used to inhibit bacterial contamination, as these may interact with the antimicrobials being tested.

### Mycoplasma isolates

It is essential that the purity and identification of the isolate being tested is established. The inoculum must be standardized and it is recommended that between $10^3$ and $10^5$ colony-forming units (cfu) per ml is used, ideally in the log phase of growth. We have found that the best way to approximate the concentration of cells for use in MIC tests is to measure the optical density and then confirm the concentration later using a standard dilution count method (Postgate, 1969).

Briefly, mycoplasmas growing in a broth medium are distributed evenly by shaking, and 1 ml of medium containing the cells is placed into an Eppendorf

tube. The tube is then micro-centrifuged at 600 *g* for 3 min. The supernatant is carefully removed and the cells resuspended in 1 ml of PBS. The tube is then centrifuged as before, the supernatant removed, the cells resuspended in 1 ml of PBS and the $OD_{450}$ determined. As a guide an $OD_{450}$ of 0.1 is equivalent to approximately $1 \times 10^8$ cfu per ml. The concentration of the inoculum can then be calculated and adjusted as necessary. 10 µl of this adjusted cell concentration is then dispensed into 190 µl of medium with antibiotic in duplicate and PBS used as a blank. This has been shown to work well for many mycoplasmas but should be evaluated for the species being tested.

## Antimicrobial agents

For results to be comparable between tests it is important that the MIC value is based on the active antimicrobial ingredient. Tests should therefore be carried out on the pure antimicrobial compound for which the concentration of active ingredient is known. Calculations can then be made to adjust the active concentration to 100%. It is always preferable to use fresh stocks of antimicrobials, although some may be stable if stored under the correct conditions (Hannan, 2000). Many antimicrobials are water-soluble and therefore easy to make into solutions; however, dissolving others may require agitation, mild warming or the use of organic solvents, acids or alkali. If acids or alkali are used, the pH of the solution must be readjusted to pH 7.0 before carrying out the MIC test. It is also essential to ensure that the solvents are not inhibitory to the organisms being tested, so appropriate controls must be used. The dilutions used to determine the MIC are dependent on the test requirements. Often doubling dilutions from 64 to 0.03 µg/ml are used, but it may be necessary to obtain concentrations above 64 µg/ml if an end point is required for some of the antimicrobial-resistant organisms. Essentially, antimicrobial dilutions should be prepared according to the current Clinical and Laboratory Standards Institute (CLSI) guidelines (www.clsi.org).

## Incubation conditions

The conditions and times of incubation should be those that enable optimal mycoplasma growth to occur. However, consideration needs to be given to the effect these may have on the antimicrobial being tested, as heat, gases or light may adversely affect their action. In agar dilution assays, drying out can be avoided by using a humidified incubator or by the inclusion of a wet paper towel in the container with the MIC plates. In liquid microtitre MIC assays, it is essential that each well is sealed to prevent exchange of gases between wells, which may result in false colour changes, leading to erroneous MIC end point values.

## Detection of mycoplasma growth in broth

It is essential that adequate controls are used to enable non-specific inhibitory factors which may affect mycoplasma growth to be identified. A number of different

methods for reading the end point have been used. For most ruminant mycoplasmas, we use visible detection of cell growth for determining the MIC value. At the end of the assay, the microtitre plates are centrifuged at 800 *g* for 3 min and then examined using an inverted mirror in a light box so that the growth or absence of growth can be clearly observed at the bottom of each microwell. However, for many organisms where visible growth is hard to detect, pH changes can be recorded. A comparison of MIC end points for *M. mycoides* subsp. *mycoides* small colony type gave no differences in end point values using pH change or cell growth. For non-glucose-fermenting organisms such as *M. bovis*, other indicators of growth have been used, including Tween 80 hydrolysis (Devrieuse and Haesebrouck, 1991), tetrazolium (ter Laak *et al.*, 1993) and alarmaBlue (Rosenbusch *et al.*, 2005).

Care should be taken in the interpretation of end points, particularly when metabolic inhibition or indicator systems are used, as colour changes may be seen even though the cells are not actively growing as metabolism continues albeit at a low rate. Therefore for standardization purposes, isolates for which MIC values have previously been determined should be used in parallel to enable comparisons to be made.

## Other Methods

Antimicrobial sensitivity discs containing a wide range of antimicrobials are used routinely for many bacteria. These can give a comparatively rapid guide to the sensitivity or resistance of an organism to an antimicrobial. However, many variables exist, such as the type of agar used, which can affect antimicrobial diffusion rates. Moreover, the interpretation of the size of the zones of inhibition is rarely precise and does not relate to an MIC value. However, a major advance on disc methods is the E test strips, which use a defined gradient technology to give MIC values by reading the zone of growth inhibition against the marked strips. The methodology requires the use of standard media and inocula. Although the range of antimicrobials offered for use on the E test is growing, currently some veterinary antimicrobials that are used in treating mollicute infections are not readily available. E tests have been used successfully for *M. bovis* (Francoz *et al.*, 2005) and *M. hominis* (Waites *et al.*, 1997, 1999).

An ATP-dependent luminometry method has been described for determining the MICs of highly fastidious species (Robertson and Stemke, 1995; Bébéar and Robertson, 1996), which may be more sensitive than traditional tests. Assunçao *et al.* (2006) described the use of a rapid flow cytometric method using Sybr green-I and propidium iodide stains to determine the MICs for *M. agalactiae,* which compared favourably with a microbroth dilution method; flow cytometry has the additional advantage that it allows the effect of each antibacterial agent to be determined at the single-cell level in real time.

## Standardization

Recent work by a CLSI subcommittee on human mycoplasmas has determined standard test conditions for both agar dilution and microbroth dilution for

*M. pneumoniae*. They also identified a strain which gave reference ranges for quality control against at least five antimicrobials. Similar work needs to be carried out for the key veterinary mycoplasma pathogens.

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# II Diseases

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# 7 Contagious Bovine Pleuropneumonia

*As for 'lung sick' which is a dreadful form of pneumonia, very prevalent in this country, they had all been inoculated against it. This is done by cutting a slit in the tail of an ox, and binding in a piece of the diseased lung of an animal which has died of the sickness. The result is that the ox sickens, takes the disease in a mild form, which causes its tail to drop off, as a rule about a foot from the root, and becomes proof against future attacks. It seems cruel to rob the animal of his tail, especially in a country where there are so many flies, but it is better to sacrifice the tail and keep the ox than to lose both tail and ox, for a tail without an ox is not much good, except to dust with.* (H. Rider Haggard, King Solomon's Mines, 1888)

## Introduction

Contagious bovine pleuropneumonia (CBPP), the only bacterial disease classified as a list A disease by the Office International des Epizooties in 2004, is caused by *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) variant. This mycoplasma was isolated in the late 19th century, and, while eradicated from Europe, still presents immense problems in Africa. Following the eradication of rinderpest from most parts of the African continent, CBPP is now the most important transboundary disease, along with foot and mouth disease, although its effect on the animals is far more severe.

## Geographical Distribution

### Africa

In *World Animal Health in 2006*, the OIE (2008) reported outbreaks of CBPP in 20 countries, with the highest number of cases in Ethiopia, Angola and Cameroon. It is clear that this is not an accurate figure as there were no reports from a number of countries, including Tanzania, Somalia, Congo and Burundi, where CBPP is

believed to be endemic. It is also evident that the number of cases reported by the countries is an underestimate and is highly dependent on the limited resources of many of these countries.

Endemic infection extends throughout the pastoral herds of much of western, central and eastern Africa, with Angola, Zambia and northern Namibia in southern Africa. Newly infected areas in the 1990s include much of Uganda, parts of Kenya, the Democratic Republic of Congo and most of Tanzania, where the disease spread alarmingly, with estimates of losses calculated at US$40 million (Nicholas *et al*., 2007). Rwanda (1994), Burundi (1997), Botswana (1995) and Zambia (1997) were the latest countries re-infected, although Botswana remains free after an extensive slaughter campaign in 1995. After 60 years of freedom, the Caprivi region of Namibia became infected twice from Zambia within a 2-year period. Mortality rates in affected herds ranged from 10 to 90% (Nicholas *et al.*, 2007). It is hoped now that with an improving economic and political climate in Angola control measures will be implemented to prevent the continual spread of disease into the neighbouring countries of Namibia and Zambia.

## Asia

CBPP has been reported in recent times in the Far East but it is now believed to be absent, with India, Myanmar and Pakistan declaring freedom in 1990, 1995 and 1997, respectively. However, as recently as the late 1970s, high incidences of CBPP were being reported in the Brahmaputra Valley districts of Akajan, Sissiborgaon, Dhemaji and Dhakuakhana (Choudhary *et al*., 1987). Little information has emerged from China until the last few years. The disease caused great economic losses to China's cattle industry between the 1950s and 1970s, but following an eradication campaign initially involving mass vaccination, followed by quarantine and slaughter, CBPP was reported to have been eradicated in 1989 (Xin *et al.*, 2007). However, recent serological surveys have reported small numbers of seropositive reactions by both ELISA and CFT in Tibet, Yunnan and Guizhou provinces (Xin *et al.*, 2007); whether these constitute true positives is not clear. Japan, infected in 1910 from Australia, declared freedom in 1941.

Sporadic outbreaks occur in the Middle East, including United Arab Emirates (1990), Kuwait (1991) and Saudi Arabia (1995), largely as a result of importation of cattle from north-east Africa. Suspicions of CBPP in Turkey have not been confirmed by the small surveys performed in the last few years. In the most comprehensive study, just under 1000 animals were examined at abattoirs in eastern Turkey. The testing of blood and tissue samples from cattle showing suspicious lung lesions showed less than 0.5% of sera were positive with no isolation of *M. mycoides* SC from any lesions, strongly suggesting that CBPP was absent or at a very low seroprevalence in Turkey (Çetinkaya *et al.,* 2003).

## Europe

The traditional home of European CBPP in the 18th century was Switzerland, in particular the canton of Suabia, where the disease broke out between 1790

and 1812, which neatly coincided with the Napoleonic military campaigns which criss-crossed Europe and required large herds to feed the invading forces (Salmon, 1896). Although believed to have been present in the 17th and 18th centuries, Britain became re-infected in 1839 through Ireland from Holland, which had become the 'hot-bed of pleuropneumonia' and responsible for an increased spread of CBPP within Europe as trade opened up when the French Wars ended (cited in Fisher, 2003). No real attempt was made to control the disease in Britain in the next 30 years, resulting in 1500 outbreaks being reported in 1870, and 3000 5 years later; a major breakthrough was perhaps inevitably seen in 1878 with the payment of full compensation to owners for slaughtered animals, and the number of outbreaks fell to just over 2000 in 1890 (Hutyra and Marek, 1913). Once responsibility for CBPP control was transferred from local to central government in 1890 there was a final drive for complete eradication and the last case was diagnosed in 1892.

CBPP was largely eradicated from Europe around the end of the 19th century but there was a resurgence after the First World War. The disease reappeared in Eastern Europe, including the Balkans, but was eventually suppressed in Germany (1924), Russia (1928), Georgia (1932) and Poland (1936). As the outbreak of the Second World War a few years later would have prevented any further surveillance for this disease, it is not inconceivable that pockets of disease remained in Eastern Europe; indeed, outbreaks occurred in Moldova until 1946. Furthermore, small outbreaks continued in southern Europe during most decades of the 20th century, mainly in the Iberian Peninsula. A resurgence of CBPP occurred in the early 1980s, with reports of outbreaks in southern France on several occasions between 1980 and 1984, while in Italy the disease reappeared in 1990 after an absence of nearly 100 years but was eliminated 3 years later (Nicholas *et al.*, 2000). The last case recorded in Spain was in 1994. In Portugal the disease reappeared, probably from Spain, in 1983 and became endemic in northern Portugal. With an intensification of the eradication campaign, the number of cases declined rapidly, from nearly 3000 in 1996 to a single case in 1999 (Nicholas *et al.*, 2000). No outbreaks have been reported in the present millennium, although it is still too early to conclude that CBPP has finally disappeared from Europe.

## Origins

Historically, it is well documented that CBPP was carried to South Africa in 1854 with an imported bull from Holland, where it spread rapidly via trek oxen into the Transvaal, killing 100,000 animals within 2 years. It further spread by cattle movements to countries now known as Namibia, Zimbabwe and Botswana, then eventually to Angola, Zambia and possibly the Congo. Windsor (2001) contends that the infection of north-west and East Africa had a separate origin, probably through Abyssinia (now Ethiopia) with an expeditionary force led by the British commander General Napier in 1868 to free some 60 European hostages held by the Abyssinian Emperor Theodore. This seems highly unlikely as accounts by the journalist/explorer Henry Morton Stanley, who accompanied the £9 million mission, details 13,000 troops, 55,000 camp followers, 18,000 mules, 17,000 camels

and 44 elephants but no cattle. Moreover, even if a few cattle were part of this force, what is far from clear, however, is whether India, from where Napier's army originated, was infected at the time. It has long been accepted that India and China became infected in 1910 and 1919, respectively, from Australia, which had itself been infected by a small number of cattle from Britain in 1858 (Hutyra *et al.*, 1938). It is very unlikely that infection could have travelled from Australia to India in such a short time, so some doubt must exist about this second front of infection in Africa. However, there is some molecular epidemiological evidence of two clusters of the causative agent to support Roger Windsor's 'two front' claim, but clearly, amongst many things that General Napier has been held responsible for, introducing CBPP into Africa is not one.

The story of the introduction of CBPP into Australia via a short-horned cow called St Bees after a long sea voyage from Cumbria in England in 1858 and how it took a further 120 years to eradicate has been well told, particularly by Newton (1992). Less well known is that the disease entered New Zealand 5 years later and spread rapidly in cattle in the provinces of Otago and Southland (Fisher, 2006). However, unlike the Australian epidemic, CBPP disappeared 10 years later, probably in part because of the prompt action of stock owners, who were quick to recognize the importance of 'inoculation' of crude vaccines in its control.

Similarly, the establishment of CBPP for over 50 years in the USA in the 19th century is not common knowledge. It appears that the disease entered the USA from Britain (Hutyra *et al.*, 1938) or Holland (Fisher, 2003) as early as 1842 in a diseased cow sold in Brooklyn. It then spread to New Jersey in 1847 and Massachusetts in 1859. Its importance was recognized and attempts were made to eradicate it in the east; however, this was too late to prevent a breakout to Ohio, probably with Jersey cattle bought in Baltimore. CBPP reached the limits of its expansion in 1884, with outbreaks in Missouri, Kentucky and Illinois (Fig. 7.1), by which time such were the concerns by stock owners that Congress was forced to extend powers of appropriation to enable stamping out in most states; the biggest difficulties were, however, seen in New York and New Jersey because of 'the larger territory infected and the density of population'. The disease was finally eliminated from these states in 1891 and 1892, respectively. Salmon writing in 1896 stated that it had been eradicated from the USA and confidently and correctly predicted that 'it is not probable that it will ever be seen in this country again'.

## Economic Impact

A study of the impact of CBPP on the economy of affected countries was carried out in Tanzania, where the estimated loss to the livestock and livestock products trade was US$136 million over a 14-year period since its introduction in 1990. Described as a national disaster by the Minister for Water and Livestock, CBPP has been responsible for the death of 350,000 cattle up to 2004, resulting in the distribution of some 10 million doses of vaccines. A larger study estimated losses in the whole of Africa at 30 million euros as a result of death and disease but the total losses including control, reduced production and trade made it just under 50 million euros (Tambi *et al.*, 2006).

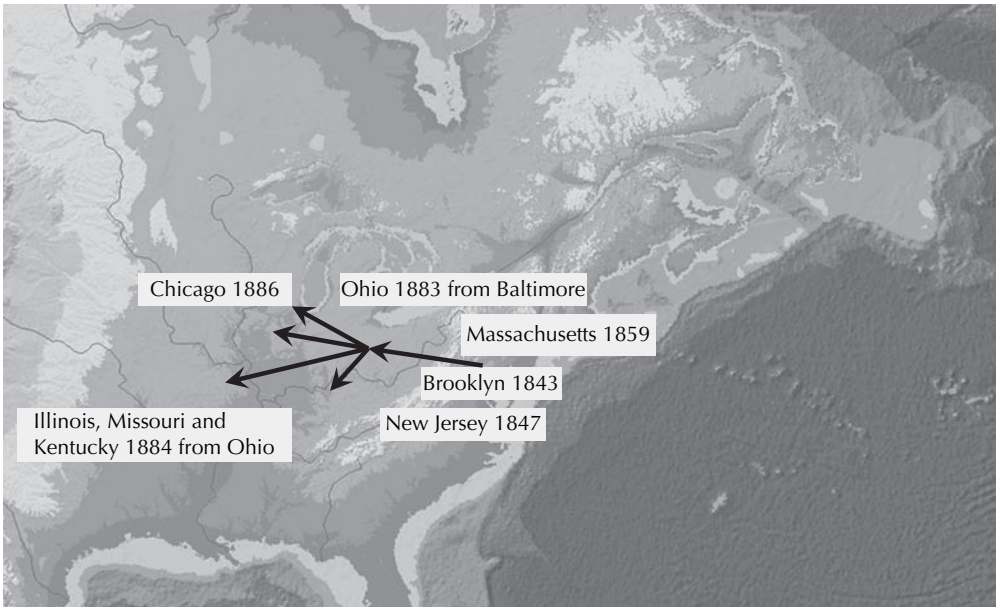


**Fig. 7.1.** Spread of CBPP within the USA.

No official estimates were made of the cost of the Portuguese outbreaks from 1983 to 1999, but nearly 85,000 cattle were slaughtered. Taking into account the costs of veterinary activities in identifying the outbreaks, laboratory diagnosis, slaughter, milk production losses and restocking, Gonçalves (personal communication) estimated a loss of 207 million euros over 17 years. The shorter period of the Italian outbreaks from 1990 to 1993 and the smaller numbers of cattle killed (about 24,000) would make the losses sustained by Italy less than those in Portugal but none the less significant.

## Host Susceptibility

Hutyra *et al.* (1938) stated that under natural conditions CBPP occurs in cattle of the genus *Bos* and allied animals including buffalo, yak, bison and even reindeer. Furthermore, they reported that goats and sheep were susceptible under experimental conditions, but these findings have never been confirmed experimentally in more recent times. Experimental work in Australia showed that buffaloes could be infected by artificial means but did not spread CBPP to in-contact buffaloes (Newton, 1992). However, Santini *et al.* (1992) discovered small sequestrated lesions and isolated *M. mycoides* SC from seropositive buffaloes which had been in contact with CBPP-affected cattle in Italy. They concluded that buffaloes were susceptible, albeit at a low level, and that further work was necessary to clarify the role of buffaloes as a reservoir of infection for cattle.

Small ruminants, in particular goats, have also been shown to harbour the causative mycoplasma (Hudson, 1971). Okoh and Ocholi (1986) isolated *M. mycoides*

from an outbreak of disease in sheep in Nigeria. Brandão (1995) isolated *M. mycoides* SC from the milk of sheep with mastitis as well as from goats with pneumonia in Portugal outside the then endemic region of CBPP. The pathogenicity of these strains for cattle is unknown at present but has been studied in sheep in Portugal. The milk isolate of *M. mycoides* SC induced pleuropneumonia, hydrothorax and pericarditis in experimentally infected sheep and was isolated from trachea, thoracic exudates and lung samples (Machado *et al.*, 1998). The humoral immune response following experimental infection of sheep by the intratracheal–endobronchial route was also monitored over a 3-month period (Gonçalves *et al.*, 2002). A slight cough occurred in some animals and interstitial pneumonia and thickening of pulmonary septa were observed in ten inoculated and six in-contact ewes. One ewe showed hyperplasia of the germinal centres in the lymph nodes and mucosa-associated lymphoid tissue in the lung. *M. mycoides* SC was detected in frozen samples from one ewe by PCR and RFLP. A specific immune response was detected by IBT, starting 2.5 months after inoculation. In all, the clinical and pathological signs appeared too mild for sheep to be considered as models for CBPP infection.

While no evidence has yet been shown of small to large ruminant transmission of CBPP, it is clearly theoretically possible for sheep or goats to harbour the mycoplasma and infect cattle grazing close by.

## Transmission/Clinical Signs

Close and repeated contact by expiration of infectious droplets is the principal mode of transmission, although wind-borne and indirect transmission cannot be completely excluded (Regalla *et al.*, 1996). It was felt very strongly in the USA in the late 19th century that infection was possible from previously infected premises, although by what method these fragile mycoplasmas could survive in the environment was until recently quite difficult to understand. The discovery that mycoplasmas, including *M. mycoides* SC, could produce biofilms enabling increased resistance in the environment may provide an explanation (McAuliffe *et al.*, 2008).

There is considerable variation in the severity of signs observed in cattle affected by CBPP, ranging from hyperacute through acute to chronic and subclinical forms. Respiratory distress and coughing, evident on stimulation of resting animals, are the main signs of CBPP. The average incubation period of the natural disease is between 1 and 2 months but can vary from as early as 3 weeks to as much as 4 months. When healthy CBPP-free cattle were placed in contact with naturally affected cattle from a recent outbreak in Namibia, seroconversion was seen after 6 weeks and rose rapidly for the next 2 weeks, by which time 40% of contacts had died (Hubschle *et al.*, 2006). In other experimental aerosol infections, 8% of cattle developed a hyperacute form, 14% acute, 30% mild and 23% inapparent infection with seroconversion; 25% showed no clinical signs and failed to seroconvert (Provost *et al.*, 1987).

The early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy. Animals show dullness, anorexia, irregular rumination with moderate fever and may show signs of respiratory disease (Fig. 7.2). Coughing is usually

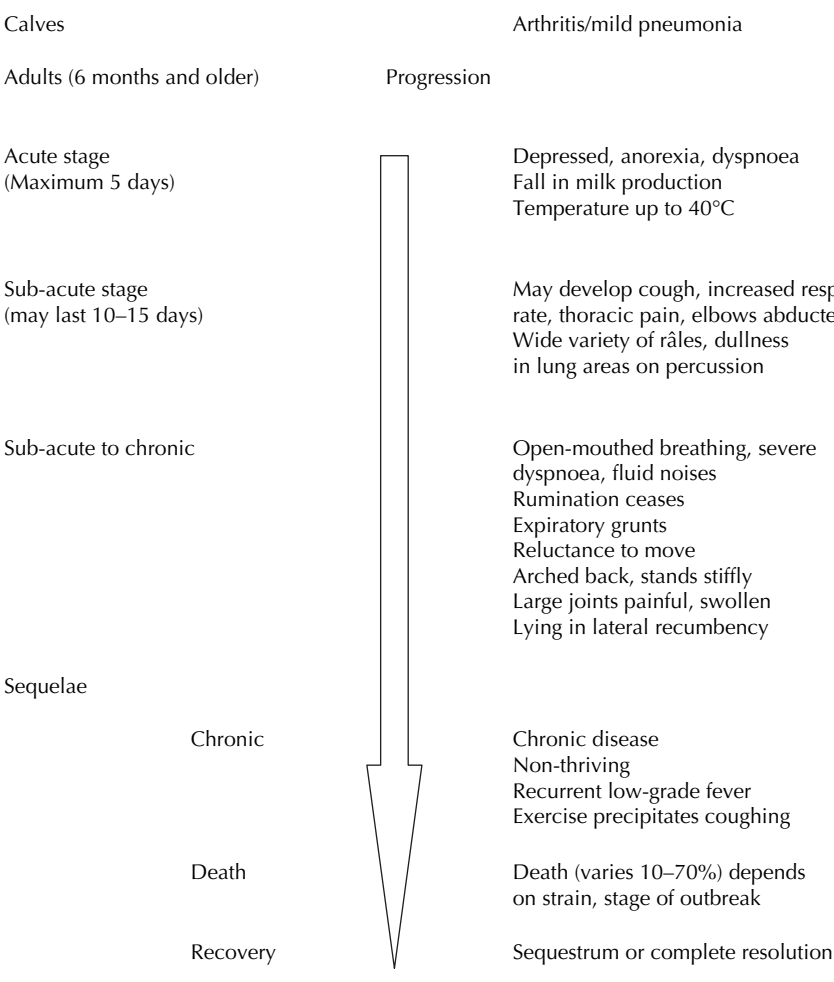


**Fig. 7.2.** Clinical signs of CBPP (from Done *et al*., 1995).

persistent and is slight or dry. Sometimes there is fever with body temperatures of 40–42°C, and the animal prostrates with difficulty of movement (Fig. 7.3). As the lung lesions develop, the signs become more pronounced, with increased frequency of coughing and the animal becomes prostrate or stands with the back arched, head extended and elbows abducted (Fig. 7.4). While classical respiratory signs may be evident in young calves, though pathological lesions are usually slight and restricted to small sequestra, articular localization of the causative agent with attendant arthritis predominates; respiratory disease is the major sign in calves over 6 months.

The clinical signs observed in the acute form are much accelerated. The pathological signs are usually characteristic, with marked pleural adhesion accompanied by exudative pericarditis (Regalla *et al*., 1996). Affected animals may die within a week, exhibiting classical respiratory signs.

**Fig. 7.3.** Prostrate and anorexic cow severely affected by CBPP in the Caprivi region of Namibia.

**Fig. 7.4.** Cow showing classical clinical features of CBPP in Uganda: head low and limbs abducted.

In the subacute form, signs may be limited to a slight cough only noticeable when the animal is exercised. CBPP in Portugal, unlike that caused in Africa, where mortality rates are typically 10–70% in epizootics, was characterized by low morbidity and low or non-existent mortality, with the majority of infected cattle showing chronic lesions. These differences are, in part, due to the fact that European cattle are healthier in general, better fed, subjected to less physical stress and are often permanently housed throughout the year, requiring little movement; they also probably experience strains of lower virulence than in Africa, although this has never been proved convincingly as animal experiments have been limited to one study using a single African and European strain in very few animals (Abdo *et al.*, 1998). However, significant mortality was reported in the Pyrenees in 1980, including deaths in as much as half of one of the three herds affected, many of which showed clinical signs (ter Laak, 1992). In Italy, during the early 1990s, fewer than 5% of cattle in the first outbreak showed clinical signs, possibly suggesting that the disease had been present for several years before it was diagnosed (Guadagnini *et al.*, 1991). The increased and ready use of antibiotics and anti-inflammatory drugs may also help to mask clinical signs in affected cattle in Europe. In Africa, it was estimated that up to a third of cases that recover from acute disease become potential carriers (Nicholas *et al.*, 2000). This figure was probably higher in Europe, where there is a far more widespread use of antimicrobials.

## Pathogenesis/Pathology

In the absence of hard evidence for how the mycoplasma initiates infection, an immunopathological theory has been proposed in which autoimmune complexes are formed as a result of the host response against the pneumogalactan in the lung tissue, stimulated by the reaction to galactan produced by the mycoplasma; this causes thrombi in the lymphatic system, where lesions develop first. As a consequence, the lymph coagulates with distension of the interlobular septa and infiltration of lymphocytes and plasma cells around the blood vessels. The formation of these 'cuffs' or 'organizing centres' around the arterioles is the only histological pathognomic characteristic of CBPP. The secondary lesion involves the alveoli, which become full of exudates from the previous changes. Necrotic foci, surrounded by polymorphic granulocytes, develop and become sequestra if the lesion is too large to resolve.

Lesions are mostly confined to the lungs and thoracic cavity and mostly appear in a single lung. In a study in Portugal, 95% of lesions were unilateral (Egwu *et al.*, 1996), with the diaphragmatic lobes being more commonly affected than cranial lobes. In a small study of recent outbreaks in Namibia, just under 80% of lungs had lesions in a single lung (Aschenborn, personal communication). In Europe the main lesion is the sequestrum but acute lesions are seen, particularly in the early phase of the epidemic; sequestra also predominate in parts of Africa, where the disease has become endemic, suggesting that virulence is cyclic rather than geographic.

Adhesions to the chest causing roughened pleural membranes are common (Fig. 7.5). Many litres of straw-coloured pleural fluid can be found in acute cases,

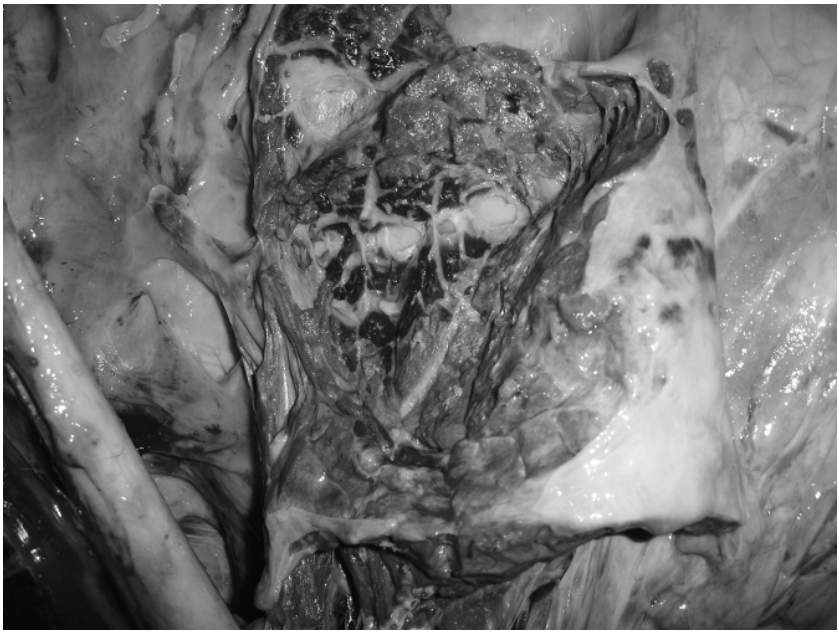

**Fig. 7.5.** Adhesion of the lung to the chest wall of a cow affected by CBPP.

which makes ideal diagnostic material (Fig. 7.6). The interlobular septa are often distended (Fig. 7.7) and lungs show the typical marbled appearance, with lung lobules showing great variations in colour from red, to grey to yellow, depending on the stage of inflammation (Fig. 7.8). Associated lymph nodes undergo hypertrophy (Fig. 7.9). In chronic cases the sequestrum is the main lesion type and consists of necrotic material surrounded by a fibrotic capsule ranging from 10 to 100 mm in diameter (Figs 7.10 and 7.11). Necrotic foci have been reported in the kidneys of affected cattle and pericarditis is occasionally seen in severe cases.

Histopathologically, the CBPP lesion comprises a bronchiolar necrosis and oedema, progressing rapidly to an exudative serofibrinous bronchiolitis with extension to the alveoli and uptake of alveolar fluid into tissue spaces, lymph vessels and ultimately septal lymphatics (Done *et al.*, 1995). These rapidly reach saturation and the process is extended centrifugally to the tracheobronchial lymph nodes and centripetally to the pleural lymphatics. The mediastinal, sternal, aortic and intercostal lymph nodes may then become enlarged, oedematous or even haemorrhagic. With stasis, lymph vessels become thrombosed and ultimately fibrosed (Nicholas *et al.*, 2000). The pulmonary lobules become consolidated with alveolar oedema, fibrin and inflammatory cells. Coagulative necrosis is common. *M. mycoides* SC can be demonstrated in these lobules by immunohistochemistry. Perivascular organization foci or 'organizing centres', found in the interlobular septa, are considered pathognomonic for CBPP (Provost *et al.*, 1987). They consist of a centre occupied by a blood vessel with proliferation of connective and inflammatory cells surrounded by a peripheral zone of necrotic cells.

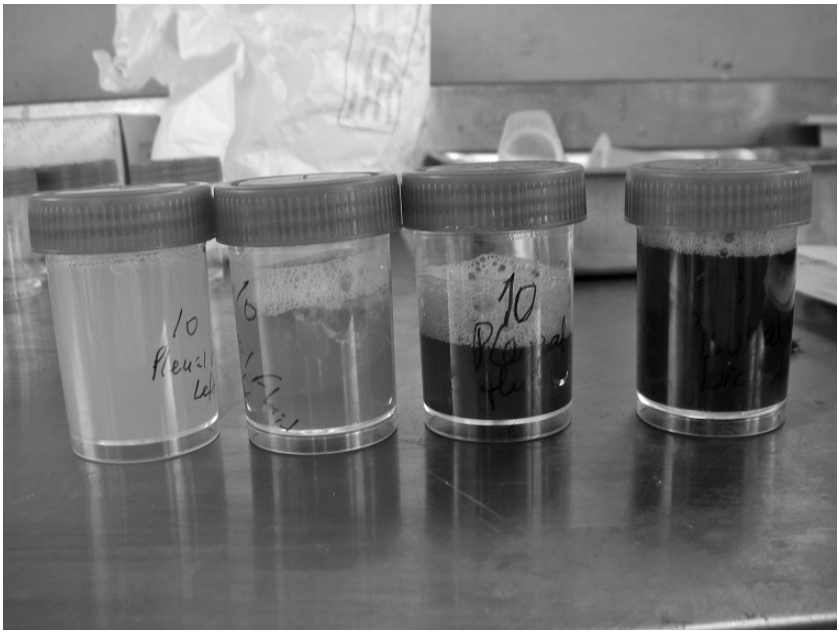

**Fig. 7.6.** Pleural fluid taken from cattle acutely affected with CBPP.

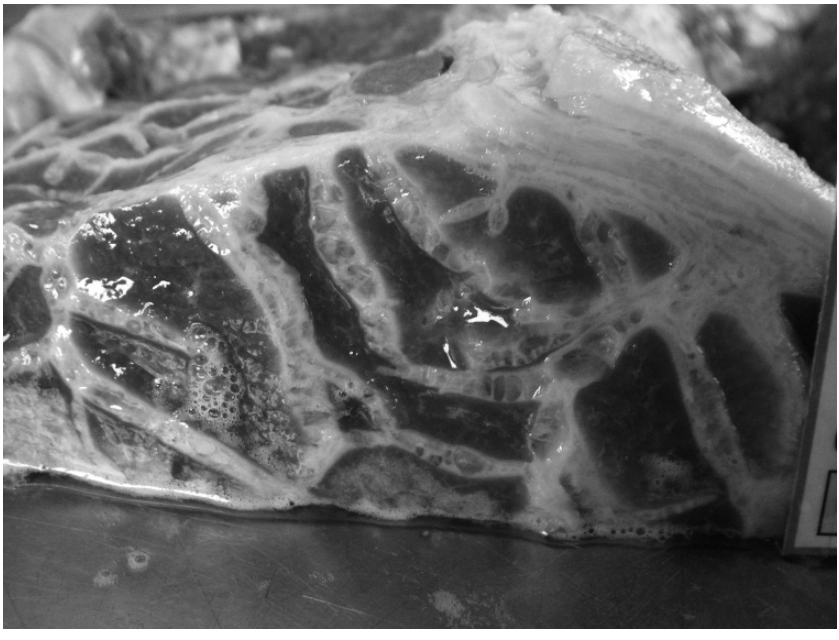

**Fig. 7.7.** Lung of CBPP-affected cow showing oedematous enlargement of interlobular septa.


**Fig. 7.8.** Lung from CBPP-affected cow with classical 'marbled' lesion.


**Fig. 7.9.** Hypertrophic lymph nodes from cow with CBPP.


**Fig. 7.10.** CBPP-affected lung showing a well-encapsulated sequestrum.

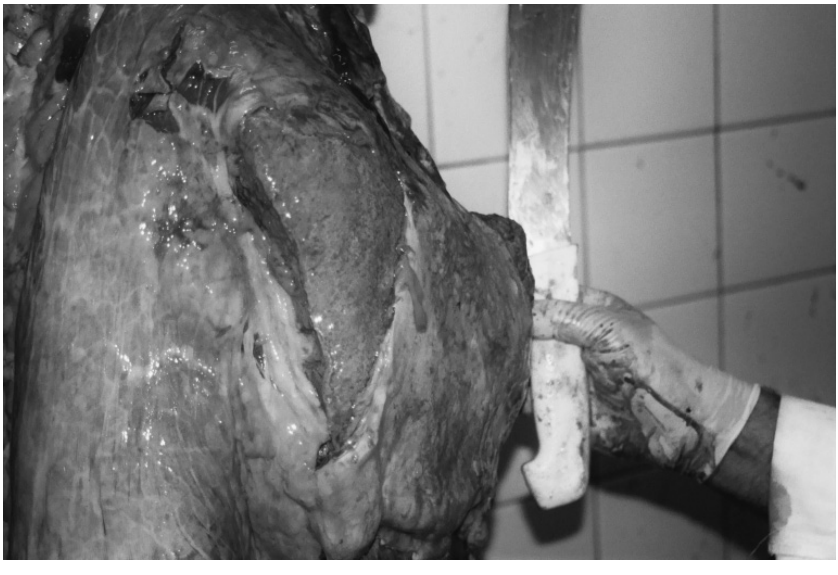

**Fig. 7.11.** Massive sequestrum occupying large part of left lung, caused by CBPP.

## Causative Organism

The sequencing of the entire genome of the type strain PG1 in 2004 made much of the early work on characterizing the *M. mycoides* SC redundant (Westberg *et al.*, 2004). The genome is characterized by a single circular chromosome of 1,211,703 bp with the lowest G + C content (24 mol %) and the highest density of insertion sequences (13% of the genome size) of all sequenced bacterial genomes. The genome contains 985 putative genes with an average length of 982 bp. Only 59% of these have been assigned with a function, while 27% are unique to *M. mycoides* SC and 14% have unknown function but show significant homology to genes of other *Mycoplasma* or bacterial species. A variety of potential virulence factors was identified, including genes encoding putative variable surface proteins and enzymes and transport proteins responsible for the production of hydrogen peroxide and the capsule, which is believed to have toxic effects on the animal.

## Virulence Factors

Little is known of the pathogenicity factors associated with *M. mycoides* SC. The galactan capsule is generally considered to contribute to pathogenicity by promoting binding to host tissue surfaces and enhancing resistance to phagocytosis. In addition, there is some evidence that the capsule of *M. mycoides* SC might have a direct toxic effect on host cells, and its structural similarity to bovine pneumogalactan further suggests that it might induce autoimmune reactions.

The production of hydrogen peroxide and other active oxygen species appears to be an important factor in mycoplasma pathogenicity (Tryon and Baseman, 1992). In mycoplasmas, hydrogen peroxide production may accompany the metabolism of sugars or certain organic acids to acetate plus carbon dioxide. There is a net reduction of $NAD^+$ to NADH during this process and $NAD^+$ is regenerated via NADH oxidase activity; this oxidation requires molecular oxygen, which is reduced to hydrogen peroxide or water. However, in *M. mycoides* SC, only traces of hydrogen peroxide are formed (Miles *et al.*, 1991). An alternative means of hydrogen peroxide production in mycoplasmas is via the metabolism of glycerol. African and Australian but not European *M. mycoides* SC strains oxidized glycerol at high rates which were comparable with those for glucose (Houshaymi *et al.*, 1997). European isolates possessed GP kinase but lacked GP oxidase (Houshaymi *et al.*, 1998). Thus, it appears possible that the apparently reduced virulence of European SC strains, compared with African strains, is specifically associated with the lack of GP oxidase activity. Further evidence of the role of hydrogen peroxide has become evident in studies in which strains from acute cases of disease were shown to be more cytotoxic to cultured bovine epithelial cells than those taken from milder outbreaks in Europe (Bischof *et al.*, 2008). The fact that vaccine strains were also shown to be cytotoxic suggests that they are, as many thought, still largely semivirulent preparations (Mbulu *et al.*, 2004); however, Bischof *et al.* (2008) believe that they may be attenuated in other, though unspecified, ways.

Pilo *et al.* (2007) have proposed that virulence of mycoplasmas is a factor of their ability to evade the host immune response through reversible antigenic

changes, to adhere strongly to the host cell and to persist and disseminate via a blood-borne phase. They believe that damage to the host is caused by the efficient and competitive scavenging of nutrients and the release of toxic metabolites, leading to inflammatory processes that can exacerbate disease. That the host response can often do more damage than the mycoplasma itself can be seen partly in the work of Scacchia *et al.* (2007), where long-term treatment with cyclosporin, which suppresses the cell-mediated immune response, delayed and reduced the size of lesions in some cattle.

In order to evade the host's immune response, many mycoplasmas express surface proteins that undergo reversible changes to alter their antigenic repertoire. Some of these variable surface proteins are also involved in adhesion and immunomodulation. Only two are known in *M. mycoides* SC, one of which, Vmm, is a small lipoprotein of 17 kDa (Persson *et al.*, 2002). Hamsten *et al.* (2008) showed that antibodies in sera from cattle naturally affected with CBPP recognized Vmm and other Vmm-type proteins, suggesting these may be useful as target antigens for the development of diagnostic tests and recombinant vaccines.

## Molecular and Immunological Epidemiology

*M. mycoides* SC is a relatively homogeneous species and few typing methods have been able to detect significant differences between strains. Many studies have found variation in African strains but have been unable to subdivide European strains (Cheng *et al.*, 1995; Gonçalves *et al.*, 1998; Lorenzon *et al.*, 2003). To date, the consensus on the molecular epidemiology of *M. mycoides* SC is consistent with there being two main subtypes: those isolated from recent European outbreaks (post-1980) and those isolated from the African and Australian outbreaks, some of which date back to 1936. Initial studies on the molecular typing of *M. mycoides* SC focused on the restriction analysis of whole DNA (Poumarat and Solsona, 1995) and the chromosomal distribution of insertion sequences (Cheng *et al.*, 1995; Frey *et al.*, 1995). Although these methods could differentiate European from African/Australian strains and distinguish vaccine strains, they were unable to subtype strains further. It must be said that some of the studies performed in the 1990s were often motivated more by a desire to show that the authors' country was not responsible for a neighbour's outbreak rather than a desire for good science.

The source of the unexpected outbreaks of CBPP in Italy (1990–1993) was never successfully traced, although France was heavily implicated because it exports large numbers of cattle to Italy and they had experienced disease in the previous decade. Immunological evidence was presented that most strains from Italy differed from those from outbreaks in Western Europe, leading to the suggestion that 'contamination of Italy did not arise from exportation of CBPP from south western Europe' but from a resurgence in Italy itself or from some unknown foci elsewhere in Europe (Poumarat and Solsona, 1995). However, in the study, two Italian isolates had identical patterns to those from France, Spain and Portugal. A more thorough study by Gonçalves *et al.* (1998), but still lacking sufficient French and Spanish strains, confirmed that two Italian strains were similar to other European strains but others lacked the 98 kDa protein.

Epidemiological evidence compiled during the Italian outbreaks also implicated France, as well as, unexpectedly, Germany and Poland, as outbreaks were linked directly to importation from these three countries (Regalla *et al.*, 1996) but it seemed highly unlikely that Italy, which had been free of the disease for nearly 100 years, should be infected from three different countries within the space of 2–3 years, two of which had not experienced outbreaks for over 50 years. It suggested, perhaps, that tracing the origin and movements of these cattle was far from certain. Of the non-infected countries in Europe, only Switzerland and Hungary have recently conducted targeted surveys to demonstrate freedom from CBPP, although the UK and France actively investigate suspicious cases where other causes have been ruled out.

*M. mycoides* SC strains have also been studied using amplified fragment length polymorphism and pulsed field gel electrophoresis (PFGE); however, these methods only detected limited variation and have not been used on a wide selection of strains (Kusiluka *et al.*, 2001). Based on the polymorphisms of eight housekeeping genes, MLST (see below) differentiated 48 strains into 32 profiles and three main groups and confirmed the distinction between European and African/Australian strains; some separation was also apparent between strains from West and East Africa from those of Southern Africa, suggesting different origins of African strains (Yaya *et al.*, 2007). A recently unearthed European strain from 1967 which lacks the 8.8 kb deletion of other European strains was located at an intermediate position between European and certain African strains and is probably much more closely related to ancestral European strains that are thought to be responsible for spreading CBPP into Southern Africa. This work also postulates an introduction of CBPP into the Horn of Africa based on a single isolate from India, although the date of entry, 1868, as discussed earlier, suggested seems highly unlikely.

Later molecular epidemiological studies have focused on multilocus sequence typing (MLST), which aims to find loci in the mycoplasma genome that show sufficient polymorphisms to enable subtyping. This approach was able to differentiate strains to some extent, with isolates being subdivided into 15 different groups (Lorenzon *et al.*, 2003). Overall, epidemiological studies to date have shown that most European isolates fall into a single homogenous group, while the African and Australian strains form a separate, more heterogeneous, grouping. This heterogeneity is most likely due to the widely separated geographical and temporal origins of the African and Australian strains studied in contrast to those from European outbreaks, which have been collected over a relatively short period of time and from a restricted area (March *et al.*, 2000).

Techniques using variable numbers of tandem repeats (VNTR) have been described for a variety of organisms but have not been used to differentiate mycoplasma strains previously. A recent study has found that despite its minimal size the *M. mycoides* SC genome has 60 VNTRs throughout the *M. mycoides* SC genome and that there was remarkable genetic homogeneity between strains from different geographical origins, with only three VNTRs showing variation between strains (McAuliffe *et al.*, 2007). However, these three VNTRs showed promise as an epidemiological tool, finding variation with African and Australian strains and generating eight different profiles from the 15 strains tested. There was some correlation between the geographic origin of the strains and the VNTR grouping.

Vaccine strains were unusual and most were placed in a single group by VNTR analysis, with the exception of KH3J, which was distinct from all other strains.

Little was known about the origin of CBPP in Asia until relatively recently, although the general view was that the disease was imported from Australia via infected cattle into New Zealand, India, China, Mongolia, Hong Kong and Japan. A possible alternative route was through trade with Russia. Reports from China indicate that outbreaks in the second half of the 20th century were characterized by high morbidity and mortality, suggesting that strains were likely to be similar to the African/Australian cluster. Proof of this was provided by Li *et al.* (2008), who showed that *M. mycoides* SC strains from China isolated between 1953 and 1960 lacked the 8.4 kb deletion of the European strains, possessed the LppB gene and were identical to African/Australian strains by MLST. This confirmed the opinion that China became infected via Australia.

## Participatory Epidemiology

Farmers, including pastoralists, in Africa have a very well-developed knowledge of many aspects of CBPP, covering recognition of the contagiousness, carrier status, immunity and seasonality; they are also very aware of the dangers of trade both legal and illegal but consider these risks worth taking for the socio-economic benefits that arise from these activities. Mariner and Catley (2004), describing their findings from participatory epidemiological surveys of livestock owners in regions endemically affected by CBPP, showed that farmers were aware of the positive effects that antibiotic treatment can have but that these are often ineffective because of under dosage. Mass vaccination programmes on the other hand were seen as having limited or no impact at all. Based on the results of field investigations, mathematical modelling and literature reviews, Mariner and Catley (2004) concluded that mass vaccination alone with the currently available T1/44 vaccine was unlikely to eradicate CBPP, even when applied biannually over a 5-year period, unless linked to a proven treatment regime in which all diseased cattle would be treated while the remainder of the herd was vaccinated; such an option was the only one that approached eradication when modelled.

## Diagnosis

While there are plenty of tools available for CBPP diagnosis, including numerous PCR techniques, there is no single test which can detect all infected animals. The OIE-approved serological tests, complement fixation test (CFT) and the competitive (c)ELISA, lack sensitivity. The cELISA is clearly more robust but is often less sensitive than the CFT (Marobela-Raborokgwe *et al.*, 2003). An indirect ELISA based on a recombinant protein LppQ-N' had similar sensitivity to the CFT and cELISA but was very stable under harsh conditions (Bruderer *et al.*, 2002). Using an ELISA based on this lipoprotein, Xin *et al.* (2007) screened nearly 4000 sera from Tibet and two provinces of China and showed good agreement between the ELISA and CFT, although the former appeared more sensitive. A more sensitive

and specific immunoblotting test has been developed and is presently used to confirm the results of positive and negative tests in the CFT (see Chapter 5 and later in this chapter), but requires expert analysis and does not lend itself to mass screening (OIE, 2004).

The culture and identification of *M. mycoides* SC is still required, particularly in newly affected areas, although PCRs can facilitate diagnosis. The PCRs available for *M. mycoides* SC detection are listed in Chapter 3 and include the PCR/DGGE, which can detect most mycoplasmas and even mixed cultures directly from clinical material (McAuliffe *et al.*, 2005). A PCR based on the insertion sequence IS 1296, which can identify and distinguish European from African/Australian strains without the need for a restriction endonuclease step, was described by Miles *et al.* (2006).

An urgent requirement, particularly in Africa, is the development of rapid pen-side tests. Latex agglutination tests have been developed and are now commercially available (Churchward *et al.*, 2007). The test, which can be performed on fresh, unclotted blood and serum, provides an early warning of outbreaks but confirmation of positive results should be sought from a reference laboratory. A lateral flow device has recently been trialled and appears to be stable and robust (Churchward *et al.*, 2007). Both of these rapid tests are aimed at herd testing and require the sampling of a large number of animals to ensure detection.

## The use of immunoblotting in the eradication of CBPP from Portugal

In Portugal, a national serological survey using the CFT, which began in 1983, provided valuable data on CBPP prevalence. All seropositive and clinically suspect cattle were slaughtered, lungs and lymph nodes examined and the lesions submitted for laboratory investigations. The CFT, with its high specificity, was an essential tool of the eradication campaign but, in its last few years, due to the low prevalence of the disease, its specificity became unacceptable, with false positive results resulting in the slaughter of about 5000 healthy cattle per year. Coupled with the low detection rate of the CFT, there was an urgent need for a more sensitive and more specific test. As a consequence, a strong research effort facilitated by the EU FAIR programme (1995–1998), involving Portugal, Sweden, Spain, Italy and the UK and the EU COST Action on ruminant mycoplasmoses (1995–2000), aimed at improving diagnostic tests and led to the development of the immunoblotting test as a confirmatory test (see Chapter 5). This test greatly aided the eradication of CBPP from the endemically affected region of Entre Douro-e-Minho in northern Portugal in the late 1990s. Over a 10-year period the decrease in the number of cattle with lesions was significant: in 1989 just under half of nearly 2500 had CBPP lesions compared with 19 out of 1747 in 1998 (Fig. 7.12). One year later, only one out of 191 slaughtered animals showed typical CBPP lesions from a total population in the region of over 90,000.

Under the eradication programme, intense serological testing was conducted from 1998 until 2002. During this period, 3.5 million sera were tested by CFT from over 429,000 herds, of which just over 22,000 (0.6%) were CFT-positive. Of these, only 64 (0.3%) from 39 herds were positive by the IBT, all of them located

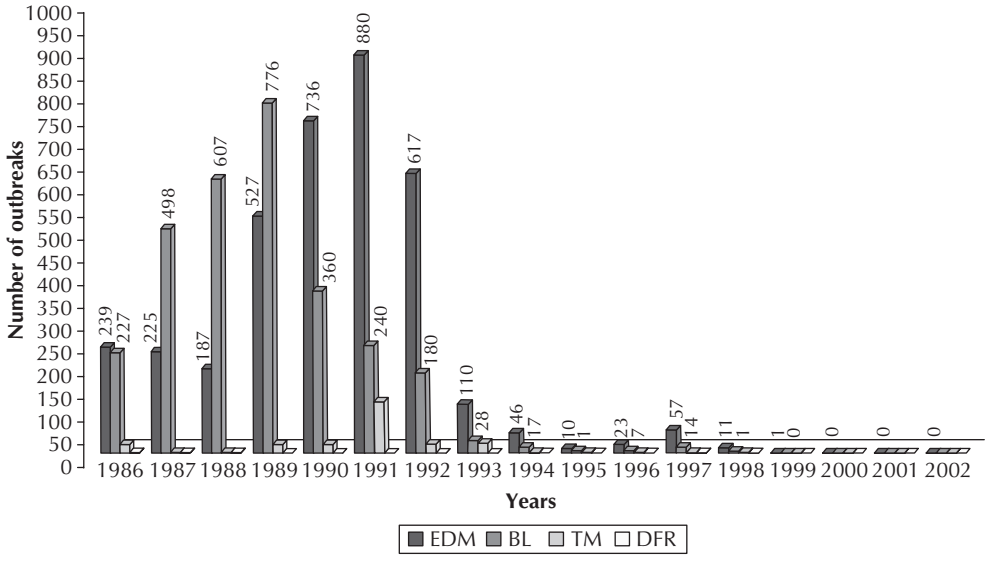


**Fig. 7.12.** Number of CBPP outbreaks seen in the endemic area of Portugal from 1986 to 2002.
EDM – Entre Douro e Minho
BL – Beira Litoral
TM – Trás-os-Montes
DFR – Disease-free regions

in the affected areas. However, with the exception of the last outbreak, which occurred in February 1999, no *M. mycoides* SC isolates were obtained from bacteriological examinations performed on the slaughtered cattle. The last specific profile was detected in 2000 from an animal with no visible CBPP lesions (Regalla, 2004). All CFT-positive sera originating from disease-free areas gave non-specific profiles in the IBT. No positive results were obtained from just under 9000 IBTs performed during 2001 and 2002, most of which came from the previously endemic region. And finally, on May 2003, the International Committee of the OIE declared Portugal free from CBPP.

## Control

CBPP was one of the first infectious diseases of animals for which vaccines were developed and preventive inoculation of 'attenuated' virulent material was practised even before the causative organism was known. Surprisingly, despite being known about for centuries in Europe, it was in Africa where the first attempts at vaccination were made. Virulent material, usually pleural fluid or lung fragments from cattle dying of the disease, was inserted beneath the skin either on the tip of the tail or on the bridge of the nose. The frequent loss of the tail following vaccination was considered a price worth paying. Even today gross lesions at the site of

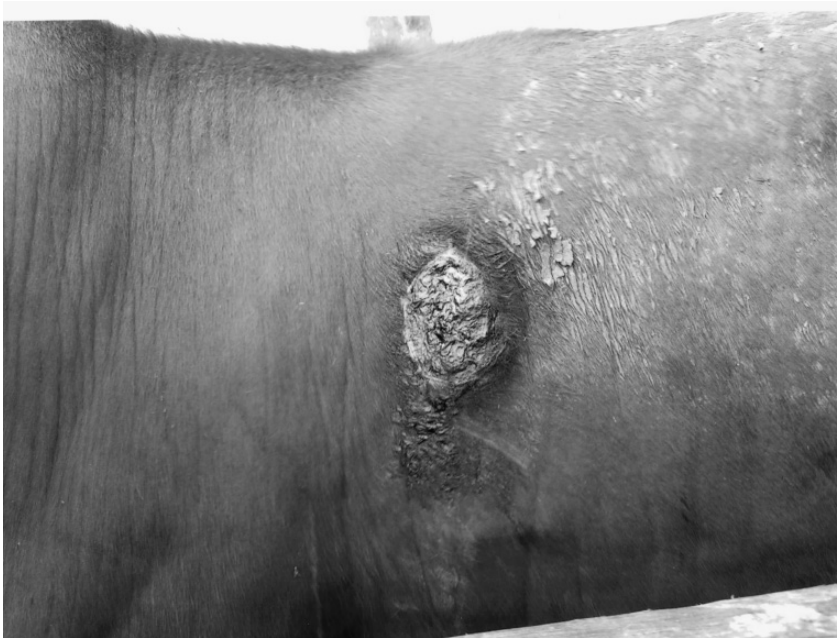

**Fig. 7.13.** Adverse reaction caused by poor administration of T1/44 vaccine against CBPP.

inoculation (Fig. 7.13), often caused by poor vaccine administration, and even occasional death are an accepted side effect of vaccination. Indeed some believe that the production of such lesions is essential for protective immunity (Hudson, 1971).

The OIE-recommended vaccine, which is used throughout Africa, is the T1/44 strain, isolated in Tanzania and attenuated by passaging 44 times in eggs after the Second World War. The vaccine has been in constant use since then and its limitations, including the short duration of immunity, adverse reactions and cold chain dependence, are well documented. It has been proposed that vaccine quality could be improved by simple and inexpensive changes to current vaccines and protocols. Such changes would include the use of HEPES–buffer systems and the inclusion of pH indicators in vaccine media, together with changes to the present vaccine diluent 1 M $MgSO_4$, which is damaging to the mycoplasma (March, 2004). These changes could increase vaccine yields tenfold and stability several 100-fold, increase the ease of production and ultimately produce a vaccine with improved efficacy.

Two new approaches to vaccination against CBPP were reported by Nicholas *et al.* (2005): first, the use of a whole cell mycoplasma vaccine inactivated with saponin, an approach which had previously been found to be protective for calf pneumonia, contagious agalactia and CCPP; and second, the use of a recombinant subunit vaccine prepared from the highly immunogenic lipoprotein LppQ (Abdo *et al.*, 2000). Results showed that, in spite of two vaccinations at 6-weekly

intervals, there was no evidence in the small number of animals immunized of any protection afforded by either preparation; indeed, there appeared to be an exacerbation of pathology in the vaccinated animals compared with unvaccinated contact animals. Lesions and fibrin were most extensive and pleural fluid more abundant in vaccinated animals. In the LppQ group half the cattle died before the end of the experiment while a quarter died in the saponin group. This compared with just under half that died in the control group.

A subunit vaccine based on the capsular polysaccharide (CPS) component of *M. mycoides* SC was tested in mice (Waite and March, 2002). A significant antibody response against CPS was elicited only when CPS was conjugated to ovalbumin. Mice, given a whole cell sonically disrupted vaccine and challenged with live *M. mycoides* SC, exhibited a significantly reduced degree of mycoplasmaemia when compared with non-vaccinated controls; however, mice immunized with the CPS–ovalbumin conjugate did not show a reduction in mycoplasmaemia (Waite and March, 2002). In addition to the fact that the subunit vaccine did not elicit protection, it seems unlikely that these results can be extrapolated to cattle as the mouse model monitored mycoplasmaemia and not clinical or pathological disease.

Recognizing that, in the field, cattle recovering from CBPP often have long-term immunity, Dedieu-Engelman (2008) analysed the immune response of acute and convalescent animals and showed that the latter were characterized by a stronger and persisting local IgA response and a higher *M. mycoides* SC-specific CD4+ T-cell response, with gamma interferon production detected in the blood and persisting in the respiratory lymph nodes. This suggested that both the local humoral and cell-mediated response were playing a role in protection against CBPP. Thus future vaccines will have to be selected for their ability to elicit or prime the mucosal immunity to inhibit the growth of the pathogen or block its virulence factors and to trigger the specific T-cells of the Th1-like type. Ideally these should comprise multicomponent subunit vaccines delivered directly to the mucosal respiratory surface where the mycoplasma initiates infection.

Recently, a novel approach for rapidly testing DNA vaccines using phage library screening was developed for *M. mycoides* SC (March *et al.*, 2006). This approach enabled rapid, high-throughput screening of genomic libraries so that putative vaccine candidates can be selected and tested. Whole bacteriophage $\lambda$ particles offer many advantages as DNA vaccine delivery vehicles and are cheap and stable. They are also thought to be effective as the antigen-presenting cells are targeted and they have the additional advantage that vaccination produces a highly immunogenic signal against phage coat proteins, therefore enabling easy confirmation of the vaccination status of the animals. As has been previously discussed, studies such as these that use a mouse model of mycoplasmaemia are not necessarily relevant to the clinical situation in cattle and therefore it is difficult to extrapolate findings to the host animal species. More studies in cattle are required to evaluate the potential of these vaccine candidates.

A number of strategies have been used to differentiate vaccine from field strains, which is crucial for epidemiological monitoring. Lorenzon *et al.* (2000) developed a PCR which amplified a 700 bp fragment specific to T1. A PCR based on the 16S rRNA gene coupled with electrophoretic separation of PCR products

on a denaturing gradient gel could not only differentiate over 70 *Mycoplasma* species but could clearly differentiate T1 from all other *M. mycoides* SC strains (McAuliffe *et al.*, 2005).

## Treatment

In Europe, apart from a brief period in the second half of the 20th century when vaccines were seriously considered in the Iberian pensinsula following the re-emergence of CBPP, stamping out has been the control method of choice, with the chief detection tools being abattoir surveillance and/or serological monitoring. Control in Africa, where practised, is based on a 60-year-old vaccine which is poorly protective, can cause severe adverse reactions if not performed properly and has residual virulence (Mbulu *et al.*, 2004); movement control is difficult to enforce and antibiotics, officially frowned upon in some countries, are used widely, although their effectiveness is difficult to assess. Evidence from the field has been confusing. Many cattle farmers consider their use essential for keeping affected cattle alive in endemic areas (Mariner and Catley, 2004). Surprisingly then, in a study of CBPP spread within herds in Ethiopia, Lesnoff *et al.* (2004) could find no evidence that antibiotic treatment reduced case fatality risk and average duration of clinical signs; the authors, however, speculated that the most widely used medication, a single intramuscular injection of 10–20 ml of 10% oxytetracycline suspension, was unlikely to achieve recovery. Furthermore, it was suggested that the spread of CBPP throughout Tanzania in the mid-1990s may have been due to inadequate dosing of infected cattle with oxytetracyclines, causing a reduction in clinical signs but failing to prevent mycoplasma shedding (Msami *et al.*, 2001).

Despite this, the relentless spread of CBPP throughout Africa, coupled with shortcomings in the vaccine, has required official bodies, in particular the FAO, to re-evaluate the use of antibiotics in the control of this disease, despite the official view in many African countries that their use be prohibited (Benkirane, 1999). This prohibition is testament to the influence of the late CBPP expert Alain Provost, who stated that 'Chemotherapy, including antibiotic therapy, may give excellent results from the clinical standpoint but it does not sterilise the infection and favours the creation of chronic carriers' (Provost *et al.*, 1987). It is timely to review the use of chemotherapy as data have shown that the newer antibiotics, including the fluoroquinolones, may be mycoplasmacidal, *in vitro* at least (Ayling *et al.*, 2000).

Hubschle *et al.* (2006) examined the effectiveness of danofloxacin (2.5% Advocin, Pfizer Ltd, New York) on naturally affected cattle and on CBPP transmission to uninfected, seronegative, in-contact cattle. Affected adult cattle, taken from a natural outbreak in the Caprivi region of north-east Namibia, were placed into two groups, one of which was treated with danofloxacin for 3 days while the other group was saline treated. CBPP-free, seronegative cattle were placed in contact with each of the two groups. Although apparently ineffective in improving the clinical and pathological condition of the naturally affected cattle, danofloxacin significantly reduced the transmission of the mycoplasma from the treated cattle to the contact cattle and provided support to the view that chemotherapy may have a role in CBPP control.

An opportunity arose to evaluate the effectiveness of antibiotics following new outbreaks of CBPP in the Caprivi region of Namibia originating from neighbouring Zambia. A serological survey of herds in the region identified 15 positive herds comprising nearly 700 animals, all of which were treated with danofloxacin (Nicholas *et al*., 2007). Over the following 18 months, the number of new cases fell significantly, with no deaths reported by the end of the 18-month study. In addition no mycoplasmas could be detected in the lungs of a small selection of culled seropositive cattle after 9 months; these lungs were characterized by extensive fibrosis (Fig. 7.14), very unlike the sequestra of naturally recovered animals. Because of the nature of the emergency, this was not a controlled study as all affected animals were treated, and it is possible that vaccination, performed 3 months before treatment (despite the fact that deaths continued in some herds after vaccination), may have also played some role. However, these results show strategic and targeted chemotherapy using correct dosing can play a part in the control of CBPP alongside vaccination, and further studies are strongly recommended.

A major concern with the use of chemotherapy is the development of antibiotic resistance. Encouragingly, a study of strains isolated mostly between 1996 and 2004 failed to show the appearance of resistant strains even in Europe, where antibiotic usage would be expected to be highest (Table 7.1) (Ayling *et al.*, 2007). This contrasts sharply with *M. bovis*, where *in vitro* resistance to most antimicrobial agents has developed rapidly in European strains (Ayling *et al*., 2000, 2007). The baseline data generated for *M. mycoides* SC strains will allow monitoring of resistance in the next few years.

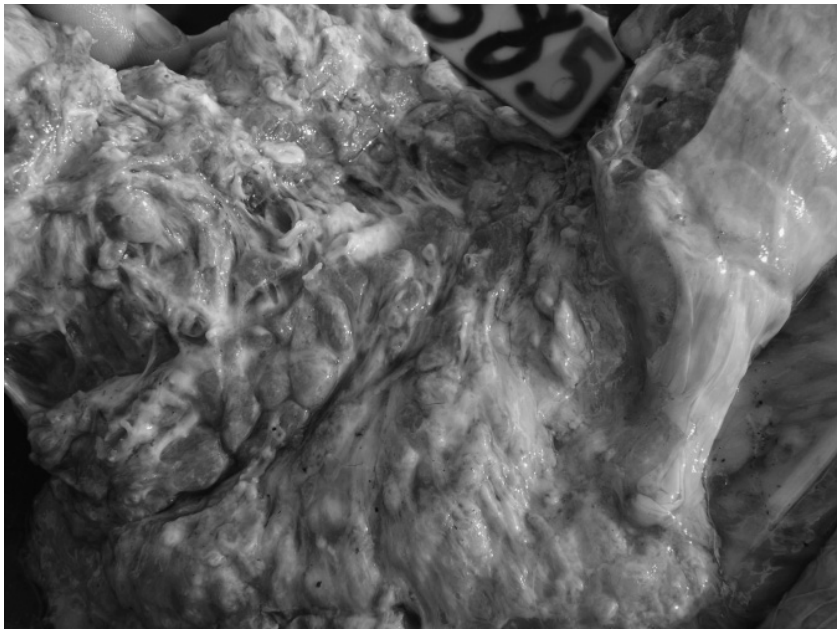

**Fig. 7.14.** Fibrotic lesion in lung from cow treated with antimicrobials and from which no *M. mycoides* SC could be detected.

**Table 7.1.** Summary of MIC range, $MIC_{50}$ and $MIC_{90}$ values, MMC range and $MMC_{50}$ and $MMC_{90}$ values in µg/ml for 50 isolates of *Mycoplasma mycoides* subsp. *mycoides* SC.

| | MIC Range | $MIC^{50}$ | $MIC^{90}$ | MMC Range | $MMC^{50}$ | $MMC^{90}$ |
|---|---|---|---|---|---|---|
| Tilmicosin (TIL) | <0.06–0.12 | <0.06 | <0.06 | 0.12–16.00 | 0.25 | 2.00 |
| Oxytetracycline (OXYTET) | <0.06–0.50 | <0.06 | 0.12 | 4.00–64.00 | 16.00 | 32.00 |
| Erythromycin (ERY) | <0.12–0.25 | <0.12 | 0.25 | 1.00–>32.00 | 8.00 | >32.00 |
| Enrofloxacin (ENRO) | 0.12–0.50 | 0.12 | 0.25 | 2.00–8.00 | 4.00 | 8.00 |
| Clindamycin (CLI) | <0.12–0.50 | 0.25 | 0.25 | 1.00–32.00 | 8.00 | 32.00 |
| Danofloxacin (DAN) | 0.12–0.50 | 0.25 | 0.25 | 4.00–32.00 | 8.00 | 16.00 |
| Ciprofloxacin (CIP) | <0.12–0.50 | 0.25 | 0.50 | 8.00–32.00 | 8.00 | 8.00 |
| Lincomycin (LIN) | <0.12–2.00 | 0.50 | 1.00 | 8.00–>32.00 | 8.00 | >32.00 |
| Chloramphenicol (CHL) | 0.25–2.00 | 1.00 | 2.00 | 32.00–>32.00 | 32.00 | 32.00 |
| Florfenicol (FLO) | 0.25–8.00 | 1.00 | 2.00 | 8.00–>64.00 | 16.00 | 32.00 |
| Norfloxacin (NOR) | 1.00–>32.00 | 2.00 | 8.00 | 32.00–>32.00 | 32.00 | >32.00 |
| Spectinomycin (SPT) | 2.00–16.00 | 8.00 | 16.00 | 64.00–>64.00 | >64.00 | >64.00 |
| Tylosin (TYL) | 0.12–>64.00 | 16.00 | 64.00 | Not tested | Not tested | Not tested |
| Tobramycin (TOB) | 8.00–>32.00 | 32.00 | 32.00 | >32.00–>32.00 | >32.00 | >32.00 |
| Gentamycin (GEN) | 16.00–64.00 | 32.00 | 64.00 | >64.00–>64.00 | >64.00 | >64.00 |
| Naladixic acid (NAL) | 8.00–>32.00 | >32.00 | >32.00 | >32.00–>32.00 | >32.00 | >32.00 |
| Cephalothin (CEF) | 16.00–>64.00 | >64.00 | >64.00 | >64.00–>64.00 | >64.00 | >64.00 |
| Streptomycin (STR) | 32.00–>32.00 | 32.00 | 32.00 | >32.00–>32.00 | >32.00 | >32.00 |
| Rifampin (RIF) | 32.00–>32.00 | >32.00 | >32.00 | >32.00–>32.00 | >32.00 | >32.00 |
| Amikacin (AMK) | >32.00–>32.00 | >32.00 | >32.00 | >32.00–>32.00 | >32.00 | >32.00 |
| Trimethoprim/ sulphamethoxazole (SXT) | >32/608–>32/608 | >32/608 | >32/608 | >32/608–>32/608 | >32/608 | >32/608 |

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# 8 Contagious Agalactia

*Fino a quando non saranno disponibilii metodi diagnostici efficaci ed un vaccino protettivo, l'agalassis contagiosa continuera a rappresentare un incubo per I pastori Siciliani. [Until there are effective diagnostics and a protective vaccine, contagious agalactia will remain a nightmare for Sicilian shepherds.]* Prof Adelmo Mirri (Director of IZS, Sicily (1952)).

## Introduction

Known for nearly 200 years, contagious agalactia (CA) is primarily a disease of dairy sheep and goats, characterized by mastitis, arthritis and keratoconjunctivitis (OIE, 2004). It is mainly caused by *Mycoplasma agalactiae*, but in recent years, *M. capricolum* subsp. *capricolum, M. mycoides* subsp. *mycoides* LC and *M. putrefaciens* have also been isolated in many countries from goats with mastitis, arthritis and, occasionally, respiratory disease. The clinical signs of the infections are sufficiently similar to those of CA for the Office International des Epizooties to include them as causes of this list B disease (OIE, 2004). Furthermore, the consensus of the working group on contagious agalactia of the EC COST Action 826 on ruminant mycoplasmoses (1995–2000), which met in Toulouse in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia. In Italy CA was known as '*mal di sito*', because of its ability to contaminate the environment and infect successive flocks on a farm. How these wall-less organisms could survive in the environment was unknown until it was shown that they were able to produce biofilms that increase their resistance to heat, dessication and, probably, host defences (McAuliffe *et al*., 2006).

## Host Susceptibility

Sheep and goats are equally susceptible to *M. agalactiae* but goats are additionally affected by *M. c. capricolum, M. mycoides* LC and *M. putrefaciens*. Reports from North Africa indicate that *M. c. capricolum* may also be a problem in sheep (Nicholas, 2002). In general, clinical disease is more pronounced in goats. Antibodies to *M. mycoides* LC and *M. c. capricolum* have been detected in South American camelids but no mycoplasmas have yet been isolated (Nicholas, 1998). As alpacas, llamas and vicunas suffer from polyarthritis, pneumonia and pleuritis it is likely that mycoplasmas may eventually be found. Isolations of *M. mycoides* LC have also been made from calves fed with contaminated goat milk, leading to severe arthritis in New Zealand (OIE, 2004). There is no evidence to date of disease transmission to humans.

## Geographic Distribution

CA has been reported in southern Europe, in particular France, Portugal, Spain, Greece and Italy (Bergonier *et al.*, 1997), the Balkans including FYR Macedonia, Bulgaria, Albania (Cokrevski *et al.*, 2001), Turkey (Ozdemir *et al.*, 2005), Israel (Bar-Moshe *et al.*, 1984), Jordan (Al-Momani *et al.*, 2006), India, the USA, South America and North Africa (Nicholas, 2002). Serious problems exist in Iran, where over 1300 cases were reported in 2006 (OIE, 2008). As mentioned above, *M. mycoides* LC has also been reported from arthritic goats and cattle recently in New Zealand (OIE, 2004).

## Epidemiology

CA is predominantly a disease of milking sheep and goats. It often appears in a herd in the spring soon after lactation begins and probably represents the activation of latent infection. The young ruminants become infected directly at suckling, while the adults are contaminated via the milkers' hands, milking machines or by bedding, which often provides a rich source of mycoplasmas. Transmission by aerosol in infective exudates over short distances and ingestion of contaminated water may also lead to infection (Bergonier *et al.*, 1997). The main reservoir of the mycoplasmas causing CA is the infected animal, in which the organisms can persist for over a year after clinical recovery. The introduction of such carriers into a susceptible flock can cause high morbidity and some mortality.

*M. mycoides* LC is likely to be reclassified as *M. mycoides* subsp. *capri* because of its very close similarity to this mycoplasma and will be dealt with in more detail in Chapter 11. Mastitis, arthritis, pleurisy, pneumonia and keratoconjunctivitis may all result from infection with *M. mycoides* LC, which has one of the widest geographical distributions of ruminant mycoplasmas, being found on all continents where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported. However, the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under-reported.

*M. mycoides* LC is mostly confined to goats but has occasionally been isolated from sheep with reproductive disease and cattle with arthritis or respiratory disease. Cases usually occur sporadically, but the disease may persist and spread slowly within a herd. After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, causes high mortality in kids.

*M. c. capricolum* is widely distributed and highly pathogenic, particularly in North Africa, but the frequency of occurrence is low. Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis and severe arthritis may be followed rapidly by death. Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues some distance from the joint.

*M. putrefaciens*, first isolated from mastitic goats in 1955, inflicted severe losses in a goat herd in California in 1987: all but a few died or were destroyed (Bergonier *et al.*, 1997). The outbreak, characterized by mastitis, arthritis and a high rate of abortion, was almost certainly due to unhygienic conditions in the milking parlour and the feeding of infected colostrum to the kids but was compounded by inadequate nutrition and shelter. Similar disease, caused by *M. putrefaciens,* was seen in France in 1982. However, in Spain in 1993, *M. putrefaciens* was isolated from young goats showing only polyarthritis (Rodriguez *et al.*, 1994). This mycoplasma has also been isolated from apparently healthy sheep in Australia (OIE, 2004). *M. putrefaciens* is common in milking goat herds in western France, where it can be isolated from animals with and without clinical signs, although milk production is usually severely affected.

## Molecular Epidemiology

As *M. agalactiae* shows differing prevalence across the world, and as it is currently absent from some countries, notably the UK, there is a need for molecular epidemiological techniques which enable a high degree of strain differentiation, allowing the tracing of the source of disease outbreaks. Recently the genome of *M. agalactiae* was sequenced, making it highly amenable to analysis using sequence-based typing methods such as variable number of tandem repeats (VNTR) analysis and multilocus sequence typing.

Early studies with PFGE analysis showed that *M. agalactiae* was largely homogeneous, revealing no variation in over 80 Italian isolates, although some degree of antigenic variation was evident (Solsona *et al.*, 1996; Tola *et al.*, 1996). Insertion sequence analysis of *M. agalactiae* isolates with wide geographical origin found some degree of variation, with some isolates containing and others lacking the IS element IS*Mag*, although subtyping isolates was not possible (Pilo *et al.*, 2003). McAuliffe *et al.* (2008) found RAPD analysis to be unreliable for typing *M. agalactiae* but found PFGE to be capable of dividing 88 European and Turkish isolates into four groups. VNTR analysis was, however, far more discriminating, enabling the isolates to be divided into 14 groups, which showed some correlation between

geographical origin and the VNTR profiles of the isolates. The *M. agalactiae* genome was found to be rich in VNTRs, which was not surprising as, although mycoplasmas possess a reduced genome with only the genes necessary for replication and survival, they also possess a relatively large proportion of repetitive DNA.

## Clinical Signs and Lesions

The course of disease is more likely to be chronic with *M. agalactiae*, while in goats, *M. mycoides* LC, *M. c. capricolum* and *M. putrefaciens* usually produce acute or hyperacute infection, often with respiratory complications. Animals in the early, acute stage of disease show a general malaise, which probably corresponds to septicaemia. In less than a week the animals can become hyperthermic (over 41°C), prostrated and show inappetence. Pregnant females near full term may abort. While some animals die without showing any other signs, most develop severe mastitis followed by arthritis and keratitis. An unusual feature of outbreaks caused by *M. putrefaciens* is the lack of pyrexia in the affected does and kids (Mercier *et al.*, 2001).

The main target organ of CA is the mammary gland, where a fall in or complete loss of milk production, sometimes within 2–3 days, and morbidity and occasional mortality in young animals can be seen. The milk may become discoloured and granular and take on a thick consistency with milk clots, which may obstruct the teat duct (Fig. 8.1). The causative mycoplasmas can be isolated from milk when mastitis is present. The udder may become hot, swollen and tender (Fig. 8.2). In the later stages of CA, the udder atrophies due to extensive fibrosis of the secretory tissue (Fig. 8.3). The severity of arthritis/polyarthritis may range from

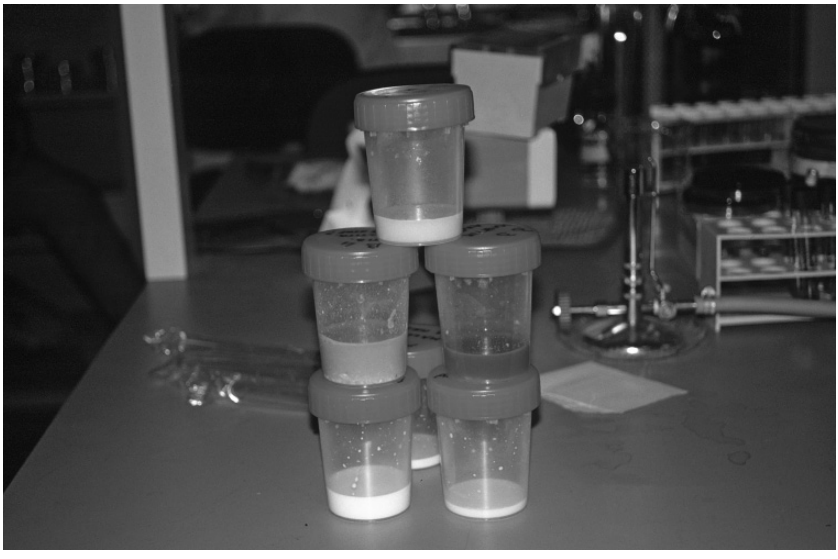

**Fig. 8.1.** Milk from cases of contagious agalactia, showing discoloration and separation into liquid and solid phases.

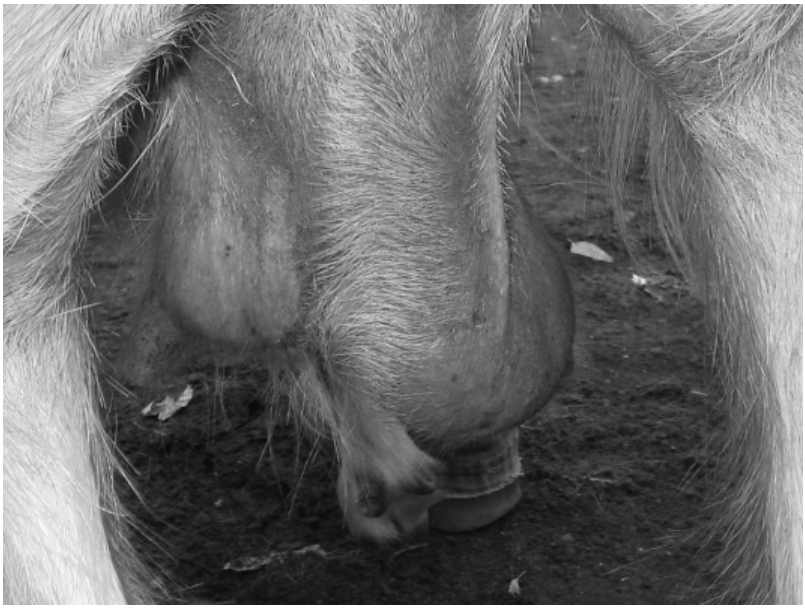

**Fig. 8.2.** Right udder affected by contagious agalactia is swollen, warm and tender.

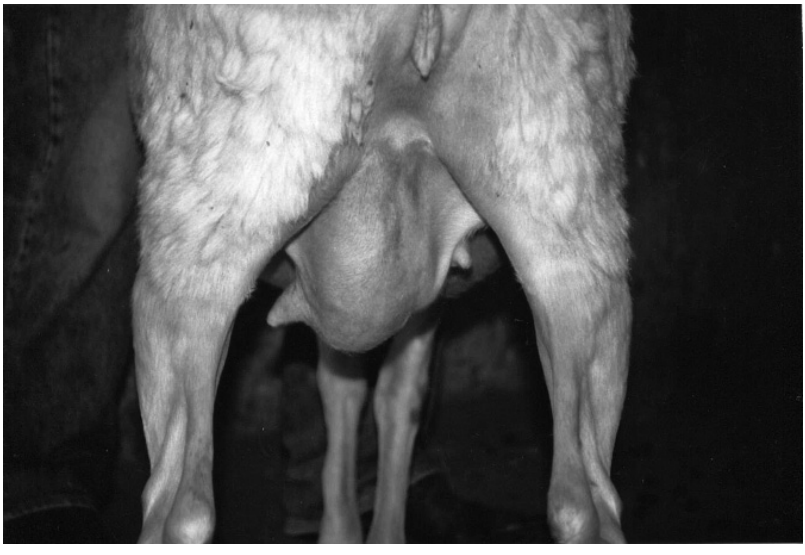

**Fig. 8.3.** Right udder affected by contagious agalactia has atrophied and is no longer secreting milk.

stiffness of the joints to severe lameness in which joints, typically the tarsus, carpus and hock, are swollen with accumulation of synovial fluid (Figs 8.4 and 8.5). This fluid is often a rich source of specific antibody, often at a higher titre than in the serum, and of the causative mycoplasmas themselves (Nicholas, 2002). Ocular

**Fig. 8.4.** Ewe naturally affected with contagious agalactia showing swollen joint in front limb.

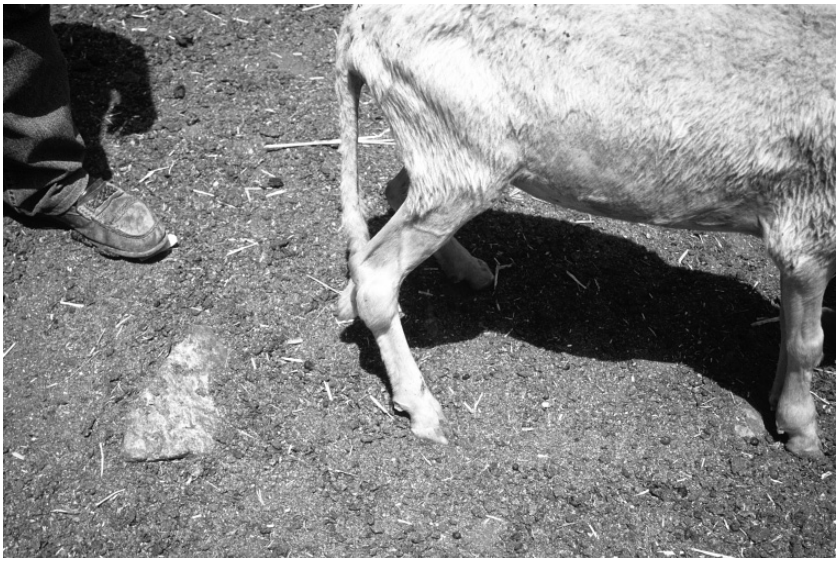

**Fig. 8.5.** Ewe naturally affected with contagious agalactia showing swollen joint in rear limb.

lesions begin with conjunctivitis and congestion, lacrimation and photophobia, followed by vascularization of the cornea, inflammatory foci and parenchymatous keratitis (Fig. 8.6). Severe cases can lead to blindness (Fig. 8.7). Pneumonia has also been reported in cases of CA, especially in young animals, where it may

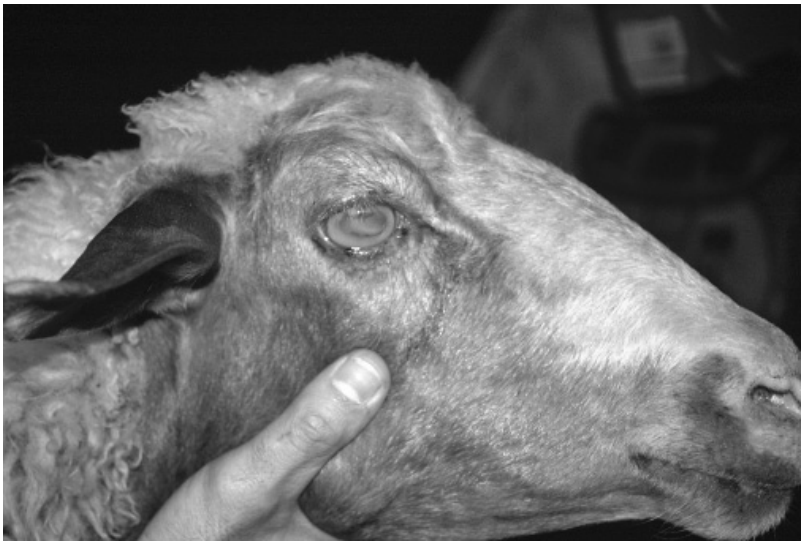

**Fig. 8.6.** Ewe affected by contagious agalactia showing keratoconjunctivitis.

**Fig. 8.7.** Ewe naturally affected with contagious agalactia showing severe keratoconjunctivitis, leading to blindness.

represent the only external sign. Cheese made from milk from subclinically affected ewes can often be spoiled (Fig. 8.8). The young born from affected females have been reported to be ataxic (Fig. 8.9) (Loria *et al.*, 2007), and this may be related to septicaemia and/or the presence of the mycoplasma in the brain causing a non-purulent encephalomyelitis (Fig. 8.10).

**Fig. 8.8.** Cheese made from mastitic milk of ewe subclinically affected by contagious agalactia.

**Fig. 8.9.** Lamb born to ewe affected by contagious agalactia, showing lack of coordination.

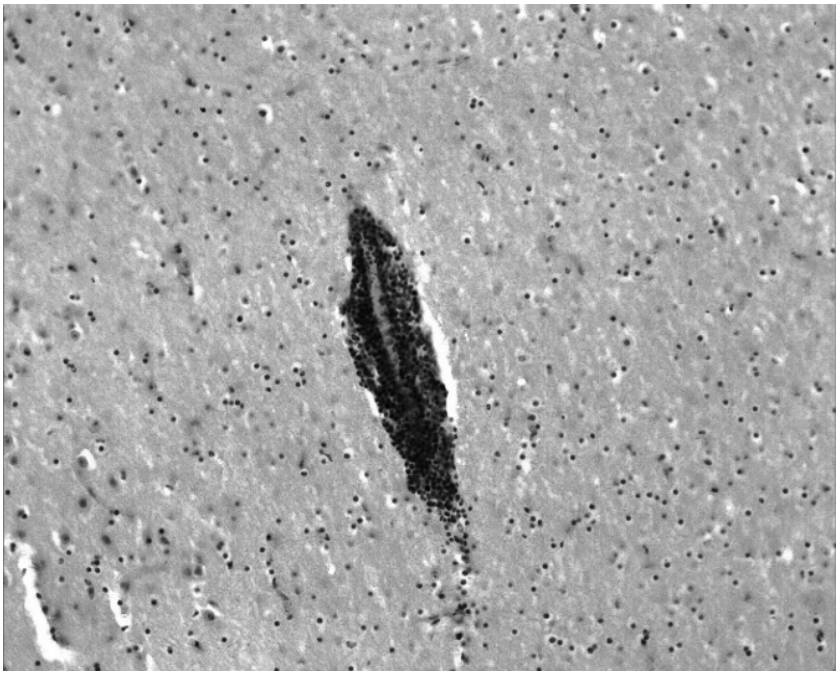

**Fig. 8.10.** Non-purulent encephalitis lesion in the brain of a sheep affected by contagious agalactia.

## Causative Organisms

*M. agalactiae*, like other mycoplasmas, has no cell wall and has a very small genome; it is resistant to penicillin and its analogues but sensitive to osmotic shock and the effect of detergents. Its growth is promoted by aerobic conditions at 37°C (Freundt, 1983; Lambert, 1987) but it does not ferment glucose and hydrolyses neither arginine nor urea, obtaining its energy requirements from organic acids (Khan *et al*., 2004).

The genome, entirely sequenced in 2007, is on the small side for mycoplasma, consisting of 877,438 bp (Sirand-Pugnet *et al*., 2007), but is a typical mollicute with a low GC content (29.7 moles %), which is slightly higher than the average of the other 16 completely sequenced mollicute genomes to date. It has 751 coding DNA sequences, half of which have unknown functions or encode hypothetical proteins, 66 of which are lipoproteins. The genome has 34 tRNA genes, two (nearly identical) sets of rRNA genes with two 16S–23S operons and two 5S operons cluster in two loci separated by 400 bp. It possesses the following predicted virulence factors: (i) phase variable related surface proteins (Vpma); (ii) the P40 protein, which is involved in host adhesion; (iii) P48, which is probably a macrophage stimulatory protein; and (iv) Opp genes, which are known to be important for biofilm formation, antimicrobial production and adaptation to specific environments. The mycoplasma also has the remnants of an integrative conjugative element, which is a conjugative transposon important for gene transfer. An

unusual finding was that 18% of the genome was derived from the *M. mycoides* cluster by horizontal gene transfer, which indicates that it evolved by gene gain from other *Mycoplasma* species.

## Diagnosis

When a flock is severely infected, clinical diagnosis is easy as the three major signs, mastitis, arthritis and keratoconjunctivitis, are present within a flock, though rarely in the same animal. However, an acute form, in which there is septicaemia without specific local signs, can confuse diagnosis.

Laboratory diagnosis provides the only means of confirming the cause of the disease. Preferred samples from living animals include: nasal swabs and secretions, milk from mastitic does or from apparently healthy does where there is a high rate of mortality/morbidity in kids, joint fluid from arthritic cases, eye swabs from cases of ocular disease, and blood for antibody detection from affected and non-affected animals; the ear canal has been shown to be a rich source of pathogenic mycoplasmas (Nicholas and Baker, 1998). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia, although this is rare and transient. From dead animals, samples should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. Culturing the organism presents no real problems to a modern laboratory as all causative mycoplasmas grow relatively well in the majority of reported mycoplasma media, although the addition of pyruvate is to be recommended.

*M. agalactiae* can be distinguished from the other mycoplasmas causing CA by its inability to ferment glucose or to utilize arginine; it also produces phospholipids, which appear as an oily film or spots on the surface of the solid or liquid medium. *M. putrefaciens*, unusually for a mycoplasma, produces a putrid odour in both broth and semi-solid cultures and in milk from affected animals. Final identification is usually achieved by the growth inhibition, immunofluorescent tests using hyperimmune rabbit antiserum, and, increasingly, polymerase chain reaction (PCR) tests, which can be carried out directly on clinical samples including milk.

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However, negative results should not be considered definitive. Several PCRs specific for *M. agalactiae* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (Dedieu *et al.*, 1995; Tola *et al.*, 1997; Subrahamaniam *et al.*, 1998). They can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples, where they have been reported to be more sensitive than culture (Tola *et al.*, 1997), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24-h enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas, 2002). A newly described

PCR-based method called denaturing gradient gel electrophoresis (DGGE), which uses mycoplasma-specific primers, is capable of identifying the majority of small ruminant mycoplasmas, including all the causative agents of CA, by their migration pattern (McAuliffe *et al.*, 2005). A positive PCR result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures. Individual PCRs have been reported for *Mmc* and *M. c. capricolum* (Bashiruddin *et al.*, 1994) and *M. putrefaciens* (Peyraud *et al.*, 2003).

A rapid and highly convenient biochemical test that exploits the C8-esterase activity of *M. agalactiae* has been reported (Khan *et al.*, 2004). The mycoplasma forms red colonies on agar media within 1 h of adding the chromogenic substrate, SLPA-octanoate (a newly synthesized ester formed from a C8 fatty acid and a phenolic chromophore). This activity is shared with *M. bovis,* although this mycoplasma is rarely found in small ruminants. Isolates need not be cloned as *M. agalactiae* can be detected easily in mixed cultures.

Detection of antibodies in serum by complement fixation test or enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis of disease but may not be very sensitive in chronically affected herds and flocks. ELISAs using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *M. agalactiae* (Bergonier *et al.*, 1997). Problems of non-specificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (Lambert *et al.*, 1998). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camelids. A number of commercial ELISA kits are now available and these were used for large-scale surveys in France and the UK (Bergonier *et al.*, 1997; Nicholas, 1998). In a ring trial of serological tests for *M. agalactiae* organized in 1998 under the auspices of the EC COST Action 826 on ruminant mycoplasmoses, commercial ELISAs performed better than 'home-made' kits. In 2006, Kittelberger *et al.* compared four serological tests for detecting antibody to *M. agalactiae* and found the ELISA kit made by Bommeli (Switzerland) to be more sensitive than another commercial kit, Institut Pourquier (France); the CFT was the least sensitive, while the Western blot was unreliable because of the variable response of the immunodominant proteins. It is important that the appropriate antigens are used in the Western blot to mitigate this problem.

ELISAs are not widely available for the other three causative mycoplasmas, although 'home-made' assays are carried out by some laboratories.

Immunoblotting tests have also been described for *M. agalactiae* (Tola *et al.*, 1997; Nicholas, 1998). Strong bands at approximately 80 and 55 kDa were seen in sera with antibodies to *M. agalactiae,* while sera from healthy flocks showed no bands or very faint bands of different sizes.

## Differential Diagnosis

A number of other mycoplasmas such as *M. arginini, M.* ovine/caprine serogroup 11 and *M. bovis* have occasionally been isolated from mastitic milk, eye swabs and joint fluids but their role in disease is not known (Ayling *et al.*, 2004). Other

bacteria causing mastitis include staphlococci, streptococci, *Escherichia coli* and klebsiella; while caprine arthritic encephalomyelitis virus and *Erysipelothrix rhusiopathiae* should also be considered in cases of arthritis.

## Treatment/Control

Regular laboratory monitoring of flocks/herds and replacement animals may help to prevent spread or introduction of disease, and this can be done on serum and/or on individual or bulk tank milk by serology, culturing or PCR. Culling or isolation of infected animals is generally advised because udder damage is considered permanent. Where this is not possible, hygienic measures such as improved milking hygiene practice and pasteurizing milk before feeding to the young should be implemented. In many disease-free countries and regions, a confirmed infected herd would be always be slaughtered.

A study was carried out to examine risk factors associated with CA infection in over 100 small sheep flocks and goat herds in northern Jordan between 2002 and 2003 (Al-Momani *et al.*, 2007). A total of 31 variables including production and health management practices were tested as risk factors for seropositive flocks. Results showed that increasing risk factors were bringing in rams from other farms, improper cleaning of the milking utensils and not separating the young from their clinically affected dams.

Antibiotics such as the penicillins which inhibit cell wall synthesis are not effective against CA. The use of tetracyclines and the macrolides can sometimes bring about clinical improvements but there is always the danger of promoting inapparent carrier animals. Furthermore, the use of erythromycin and tylosin can bring about the destruction of milk-producing tissue in small ruminants. An *in vitro* assessment of antibiotics for *M. agalactiae* strains from endemic disease areas of Sicily showed no strong evidence of resistance, although highest mean MIC values were seen for spiramycin (Loria *et al.*, 2003). However, occasional strains isolated from milk showed rather high values for oxytetracyclines. Overall, enrofloxacin gave the lowest mean values, although there are concerns about the widespread use of these fluoroquinolones in animals because of their potential impact on human health.

## Prevention

Vaccines for the prevention of contagious agalactia due to *M. agalactiae* are used widely in southern Europe and the Middle East but no single vaccine has been universally adopted and no standard methods of preparation and evaluation have been applied.

Live attenuated vaccines for contagious agalactia caused by *M. agalactiae* and *M. mycoides* LC, made at the Pendik Institute, Istanbul, have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (Turkaslan, 1990). However, they can produce a

transient infection with shedding of mycoplasma so they are not recommended in lactating animals (OIE, 2004).

In Europe, where live *M. agalactiae* vaccines are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, must be very high ($10^8$–$10^{10}$ cfus/ml) and are derived from laboratory strains. A trivalent vaccine, Aglovax™ (Intervet, Boxmeer, Netherlands), combines *M. agalactiae*, *M. mycoides* LC and *M. c. capricolum*, all at $10^{10}$ cfus in a 2 ml dose using aluminium hydroxide as the adjuvant. Two subcutaneous treatments 2–4 weeks apart followed by a booster vaccination at 4–6 months are recommended. Autogenous vaccines made from milk, brain and mammary gland homogenates from infected sheep material have been used for many years in parts of Italy, although their efficacy was far from proven. Their use, however, has been discontinued because of their link to severe outbreaks of scrapie in sheep and goats caused by contaminated brains (Agrimi *et al*., 1999).

A formalized vaccine gave some protection against experimental infection of goats with *M. agalactiae* in Spain but, despite three vaccinations per year for 6 years, it could not prevent clinical disease following the introduction of naturally infected animals (Leon Vizcaino *et al*., 1995). Furthermore formalized vaccines did not reduce the excretion of *M. agalactiae* in the milk compared with challenged controls (Pepin *et al*., 2001). It is possible that in some instances the apparent lack of protection given by vaccines could be the result of animals being infected with one of the other four mycoplasmas involved in the contagious agalactia syndrome (Gil *et al*., 1999).

Experimental vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (Tola *et al*., 1999). An oil-emulsified, inactivated vaccine against *M. agalactiae* showed promise in field trials, where sheep developed high levels of antibodies in response to vaccination and, when challenged, were protected from disease signs and colonization by *M. agalactiae* (Greco *et al*., 2002).

There is little recent published information on the availability of vaccines for *M. mycoides* LC, although it is believed that inactivated vaccines are widely used in many Mediterranean countries, suggesting that their production and use is localized (Bergonier *et al*., 1997). In experimental trials in Israel, a preparation of *M. mycoides* LC, inactivated with formalin and emulsified with mineral oil and sorbitol, gave some protection to 1-day-old and 6-week-old kids against a virulent challenge (Bar-Moshe *et al*., 1984). Except for a few field trials, the vaccine was never used widely as the occurrence of the disease caused by *Mmm*LC has declined markedly since the mid-1980s.

Although infections with *M. mycoides* LC and *M. putrefaciens* can be severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections. A multivalent, formalin-inactivated vaccine incorporating all four mycoplasmas appeared to be beneficial in field trials, although in practice a vaccine containing only *M. agalactiae* and *M. mycoides* would be necessary (Ramirez *et al*., 2001).

Much current research is directed at identifying immunogenic proteins that can be utilized in subunit vaccines. In addition, recent developments in epitope mapping are making it much easier to identify immunogenic proteins that may have uses in vaccine development. A strongly immunogenic surface lipoprotein (AvgC) has been identified in *M. agalactiae* and has been shown to be surface exposed, which stimulates specific antibodies in the sera of infected sheep. This protein may show potential for use in future subunit vaccines (Santona *et al.*, 2002).

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# 9 Contagious Caprine Pleuropneumonia

*The rapidity with which the disease had spread among Mr Nierkert's flock, affecting more than 700 goats within a fourteen day period, induced me to believe that its rapid spread could not be due to contagion alone . . . but after carefully watching the progress of the disease for three days, and noting the uniform character of the pathological appearances in the lungs, I began to realise that I had a specific disease to deal with* (Duncan Hutcheon, Colonial Veterinary Surgeon, Cape of Good Hope, South Africa, 1881).

## Introduction

In the autumn of 2002, a severe respiratory disease characterized by high levels of morbidity and mortality was first seen in goat herds in the Thrace region of Turkey, 250 km west of Istanbul (Ozdemir *et al.*, 2005). The disease, which was diagnosed as contagious caprine pleuropneumonia (CCPP), spread throughout the region to an unknown number of herds, some close to the Greek and Bulgarian borders. Goats of all ages were affected and showed a reluctance to walk, a fever of over 41°C and accelerated respiration with frequent coughing. The lungs of dead and euthanized animals showed characteristic lesions with abundant pleural fluid, fibrin and unilateral hepatization; in one herd alone nearly 150 of 400 adults and over 100 of 400 kids died in a single year (Ozdemir *et al.*, 2005). This was the first reported outbreak of CCPP on European soil and today threatens the countries of the EU.

CCPP was first described in 1873 in Algeria and known under the local name of 'bou frida' because, in the majority of diseased goats, only one lung was affected (McMartin *et al.*, 1980). Its contagiousness was not initially recognized because the disease was endemic in most areas under investigation, so climatic conditions were thought to be responsible for disease outbreaks. However, a major outbreak in South Africa in 1881 following the introduction of goats from Turkey led the

colonial veterinary surgeon, Duncan Hutcheon, to conclude that CCPP was highly infectious.

Research into the control of CCPP was initially hampered by the uncertainty over the exact cause of the disease. Two mycoplasmas, *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*, were for some time implicated in the aetiology of the disease because they caused a pleuropneumonia in small ruminants that resembled CCPP. It was not until 1976 that a highly fastidious mycoplasma, designated F38 but later renamed *M. capricolum* subsp. *capripneumoniae*, was isolated for the first time *in vitro* by MacOwan and Minette (1976). Once these workers had developed a suitable medium for the mycoplasma and carried out experimental infections, its role as the primary cause of classical CCPP was confirmed.

However, in spite of this confirmation, respiratory diseases caused by *M. m. capri* and *M. mycoides* LC are still referred to erroneously as CCPP, particularly in the Middle East and India. A condition should only be termed as CCPP when the following criteria have been satisfied:

- *M. c. capripneumoniae* is isolated or there is strong serological evidence of the mycoplasma;
- Lesions are restricted to lung and pleura and consist of a pleuropneumonia;
- The condition is highly contagious with high levels of morbidity/mortality;
- There is no enlargement of the interlobular septa of the lung.

## Geographic Distribution

While the clinical disease has been reported in nearly 40 countries in Africa and Asia, *M. c. capripneumoniae* has only been isolated in 13 countries because few have the facilities for isolating and growing mycoplasmas (Nicholas, 2002) (Table 9.1). Serious problems exist in Oman, where nearly 500 outbreaks were reported in 2006 with a mortality rate of nearly 10% from 15,000 cases, and in Iran, where nearly 300 outbreaks have affected over 13,000 goats (OIE, 2008). The 31 reported outbreaks in Ethiopia almost certainly represents an underestimate, as this disease is having a big socio-economic impact here.

**Table 9.1.** Distribution of CCPP.

| | Confirmed by isolation of mycoplasma | Clinical disease reported or suspected |
|---|---|---|
| Africa | Chad, Eritrea, Ethiopia, Kenya, Niger, Sudan, Tunisia, Uganda | Algeria, Benin, Burkina Faso, Cameroon, Central African Republic, Djibouti, Egypt, Libya, Mali, Nigeria, Somalia, Zaire |
| Asia | Nepal, Oman, Turkey, United Arab Emirates, Yemen | Afghanistan, Bangladesh, India, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Pakistan, Saudi Arabia, Syria |
| Europe | Thrace (Turkey) | |

In 2003, CCPP was diagnosed in Thrace, the region of Turkey on the European mainland bordering Greece and Bulgaria. Prior to this infection, the only previous report of CCPP in Europe dates to the 1920s, when an outbreak occurred in Greece following the seizure of goats from Turkey. Interestingly Greece reported two outbreaks of CCPP in 2006, but it seems likely that this was caused by *M. m. capri*, which is endemic in Greece, rather than by *M. c. capripneumoniae*, as less than 1% of goats died in a herd of over 150. It is probable also that the outbreak of CCPP in the Czech Republic in 1902 was similarly misdiagnosed, although it is clearly impossible to confirm over a century later. There have been no reports of the isolation of *M. c. capripneumoniae* on the American continent, although other members of the *mycoides* cluster have been described there.

## Causative Agent

The taxonomic status of F38 has long been unclear, and it was only in 1993 that it became a subspecies of *M. capricolum* and classified as *M. capricolum* subsp. *capripneumoniae* (Leach *et al.*, 1993). Five distinct groups of mollicutes have been identified by phylogenetic analysis of the 16S rRNA sequences, one of which, the spiroplasma group, contains *M. c. capripneumoniae*, which has been subdivided within the *M. mycoides* cluster. This cluster contains six important ruminant mycoplasmas, including *M. m. mycoides* SC, the cause of contagious bovine pleuropneumonia, *M. m. mycoides* LC and *M. m. capri*, which share immunological and biochemical properties. Their close relationship can lead to problems for diagnosis. Tables 9.2 and 9.3 summarize the properties of *M. c. capripneumoniae* and some members of this cluster as well as other mycoplasmas capable of causing diseases in small ruminants.

## Host Susceptibility

For a long time goats were believed to be the only susceptible host for CCPP (Litamoi *et al.*, 1990), although it was reported that sheep could be infected and seroconvert in the face of exposure (Bolske *et al.*, 1995). Following the introduction of CCPP into Eritrea with the livestock of returning refugees from Sudan, sheep mixing with affected goats were reported to be suffering respiratory disease (Houshaymi *et al.*, 2000). More recently there have been confirmed reports from Qatar of CCPP in captive wild ungulates, including wild goat, Nubian ibex, Larisstan mouflon and gerenuk, kept in animal breeding parks (Arif *et al.*, 2007). Even more surprisingly, however, were the outbreaks of acute respiratory disease in a private collection of captive but free-ranging gazelles and other deer species in the United Arab Emirates, in which over 10% died. The disease was almost certainly introduced via sick goats and spread by close contact with the gazelles at feed stations (Nicholas *et al.*, 2008); it is likely that CCPP is far more widespread in wildlife species in the Middle East as a result of infected escapees from these parks.

**Table 9.2.** Mycoplasmas, including *M. capricolum* subsp. *capripneumoniae*, isolated from small ruminants with respiratory disease.

| Mycoplasma | Host(s) | Primary site of isolation (other) | Disease* | Pathogenicity | *In vitro* growh |
|---|---|---|---|---|---|
| *M. c. capripneumoniae* | Goat (sheep) | Lungs | CCPP | High | Slow (5–7days) |
| *M. m. mycoides LC* | Goat (sheep, cattle) | Resp. tract (udder, joints) | Plp, M, A, C | Moderate | Fast |
| *M. m. capri* | Goat (sheep) | Resp. tract (joints) | Plp, A, C | Moderate | Fast |
| *M. c. capricolum* | Goat, sheep | Joints/resp. tract (udder) | Plp, M, A | High | Fast |
| *M. ovipneumoniae* | Sheep, goat | Resp. tract | P | Low | Moderate |
| *M. conjunctivae* | Sheep, goat | Eyes | KC | Moderate | Moderate |
| *M. agalactiae* | Sheep, goat | Udder (joints, eyes) | M, A, KC, P | High | Fast |
| *M. putrefaciens* | Goat (sheep) | Udder (joints) | M, A | Moderate | Fast |
| *M. arginini* | Ubiquitous | Resp. tract | None | Low/non-pathogenic | Fast |

* CCPP – contagious caprine pleuropneumonia, Plp – pleuropneumonia, P – pneumonia, M – mastitis, A – arthritis, C – conjunctivitis, KC – keratoconjunctivitis

**Table 9.3.** Major biochemical differences between mycoplasmas of small ruminants.

| Mycoplasma | Glucose fermentation | Arginine hydrolysis | Phosphatase activity | Film and spots | Casein digestion | Tetrazolium aerobic | Reduction anaerobic |
|---|---|---|---|---|---|---|---|
| *M. c. capripneumoniae* | +/– | – | – | – | + | varies | weak/+ |
| *M. m. mycoides LC* | + | – | – | – | + | + | + |
| *M. m. capri* | + | – | – | – | + | + | + |
| *M. c. capricolum* | + | + | + | – | + | + | + |
| *M. ovipneumoniae* | + | – | – | – | – | varies | + |
| *M. conjunctivae* | + | – | – | – | – | – | + |
| *M. agalactiae* | – | – | + | + | – | + | + |
| *M. putrefaciens* | + | – | + | + | – | varies | + |
| *M. arginini* | – | + | – | – | – | – | + |

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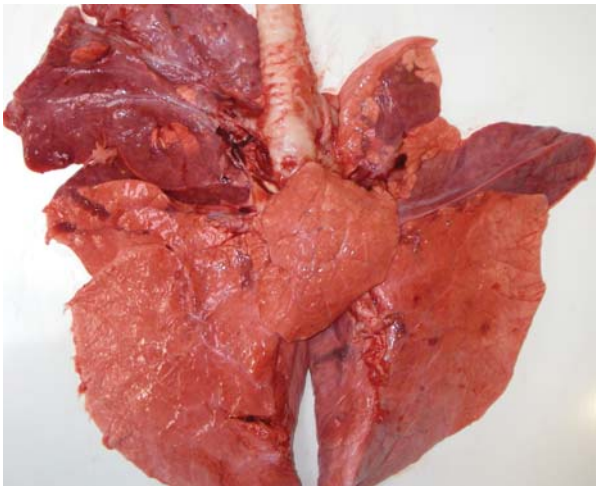

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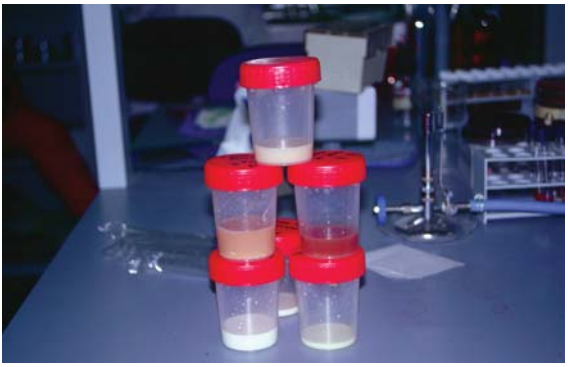

**Plate 1.** Lung of cow infected with *Mycoplasma bovis* showing consolidation (Chapter 10).
**Plate 2.** Ewe with contagious agalactia showing swollen front limb joints (Chapter 8).
**Plate 3.** Ewe affected with contagious agalactia (Chapter 8).
**Plate 4.** Milk from sheep with contagious agalactia (Chapter 8).

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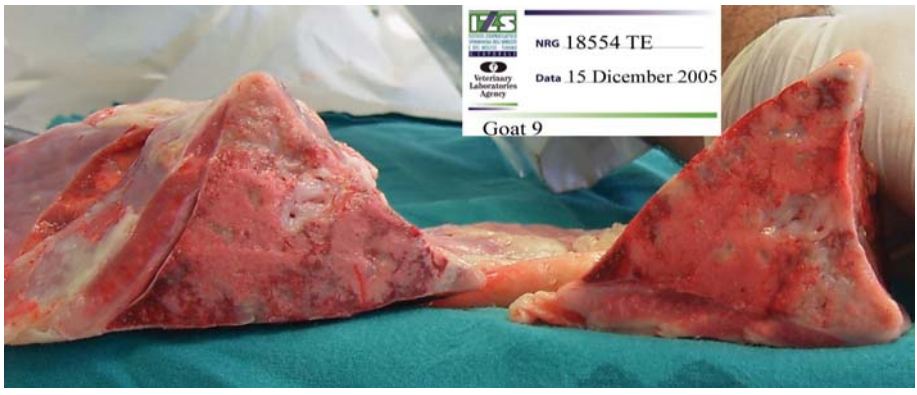


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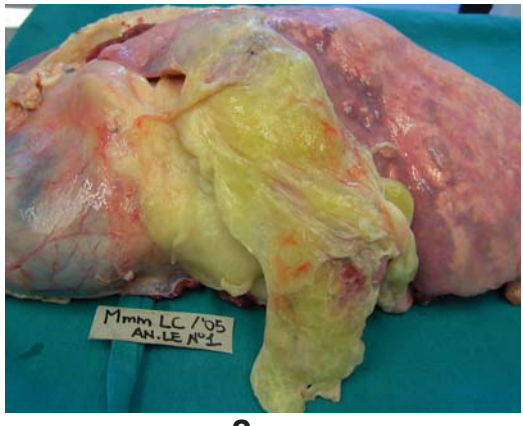


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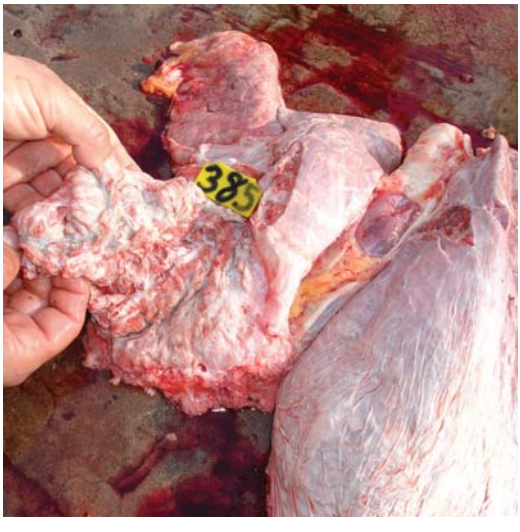


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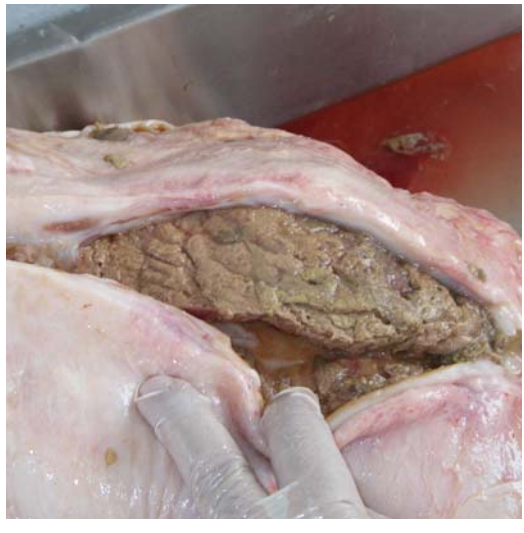

**Plate 5.** Lung of goat infected with *Mycoplasma mycoides* subsp. *capri* showing necrosis and loss of lobular structure (Chapter 11).
**Plate 6.** Fibrin deposition and consolidation of lung of goat with *Mycoplasma mycoides* subsp. *capri* (Chapter 11).
**Plate 7.** Lung of cow infected by CBPP following antibiotic treatment showing fibrotic reaction (Chapter 7).
**Plate 8.** Sequestrum in lung of cow recovering from CBPP (Chapter 7).

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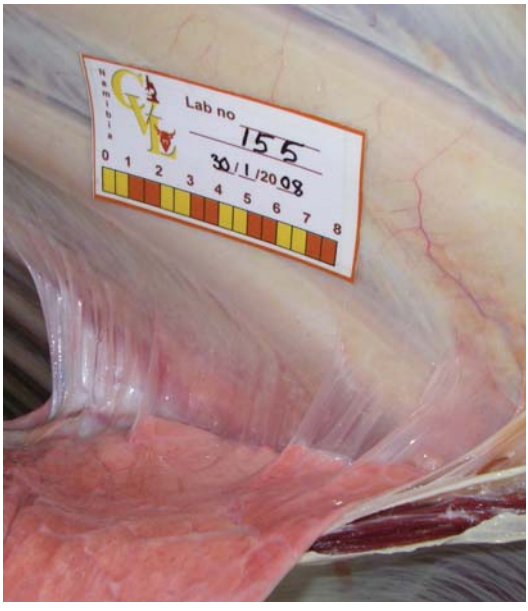


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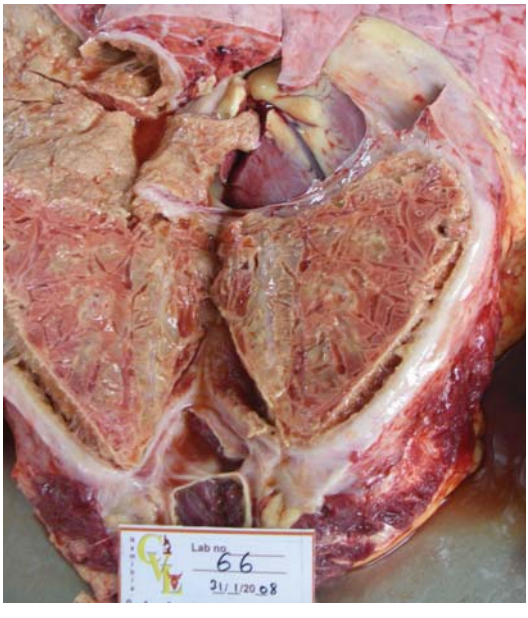


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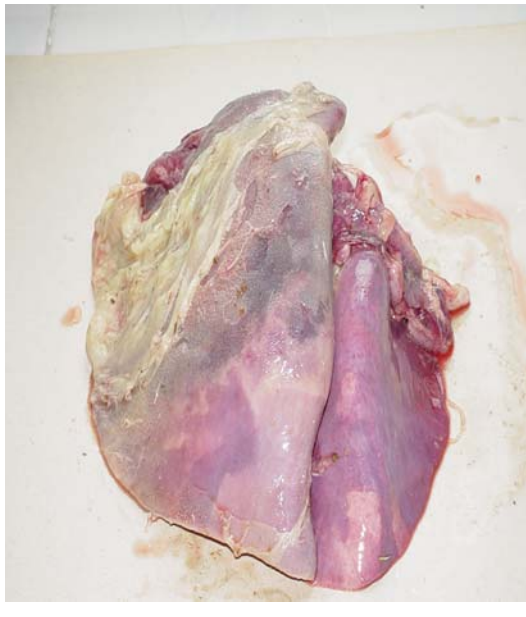

**Plate 9.** Adherence of lung to chest wall in cow affected by CBPP (Chapter 7).
**Plate 10.** Marbling of lung in cow with CBPP (Chapter 7).
**Plate 11.** Sequestrum in lung of cow with CBPP in early stages of recovery (Chapter 7).
**Plate 12.** Lung of goat with CCPP showing 'port-wine' staining and fibrin (Chapter 9).

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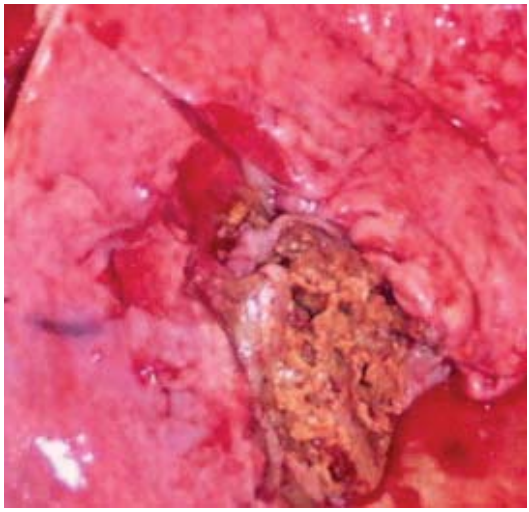

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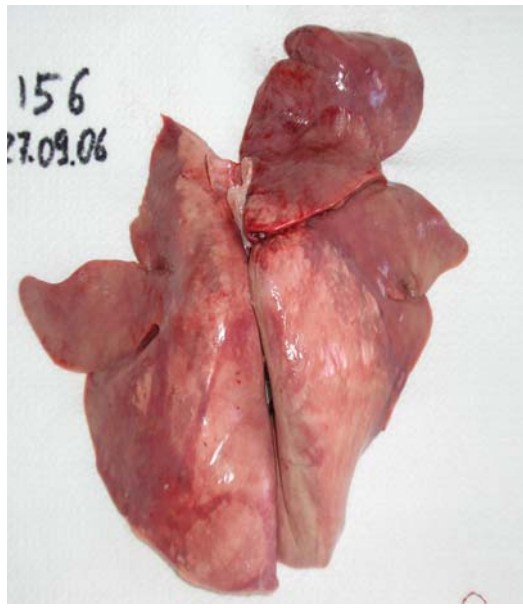


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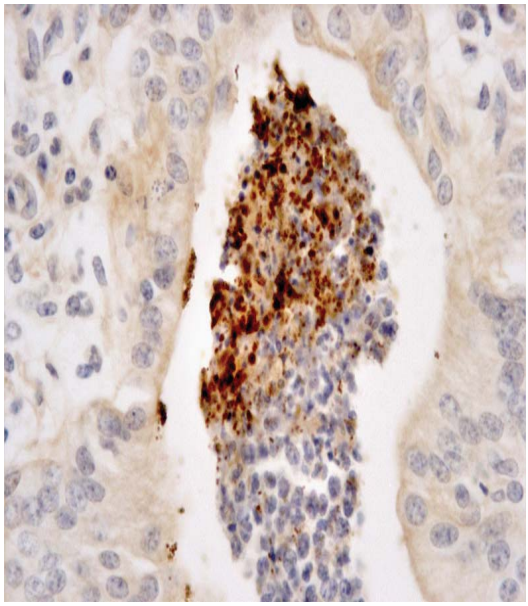

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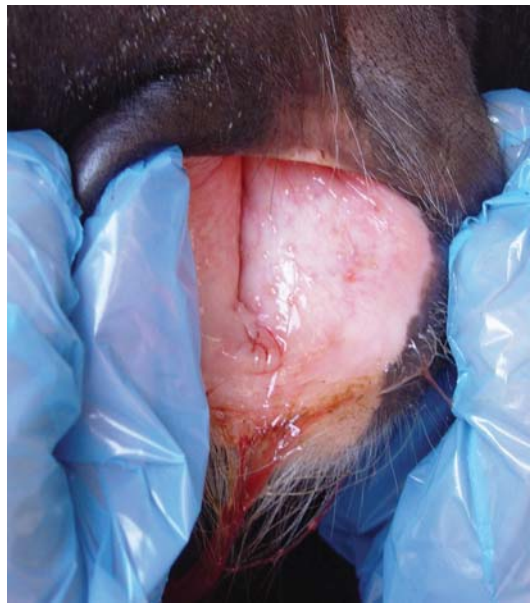

**Plate 13.** Sequestrum in lung of goat with CCPP treated with antibiotics (Chapter 9).
**Plate 14.** Lung of sheep infected with *Mycoplasma ovipneumoniae* showing extensive consolidation (Chapter 11).
**Plate 15.** Lung of sheep with atypical pneumonia showing specific brown immunochemical staining of *Mycoplasma ovipneumoniae* (Chapter 11).
**Plate 16.** Vulvovaginitis in cow infected with *Mycoplasma bovigenitalium* (Chapter 13).

## Clinical Signs and Pathology

Goats of all ages and sex can be affected (Thiaucourt and Bölske, 1996). The acute disease is more noticeable in naive populations in newly affected areas, with high mortality and morbidity rates. The incubation period generally lasts on average 10 days but may vary between 2 and 28 days. The first signs are a reluctance to walk and the onset of fever, typically 41°C and occasionally 42°C, although animals continue to feed and ruminate. Respiration accelerates and becomes painful, with violent coughing episodes. Animals stand with limbs abducted and neck extended (Fig. 9.1). There is continuous salivation and noses become blocked with a mucopurulent discharge. In the terminal stages the goats are unable to move and death follows quickly. In subacute or chronic forms, signs are milder, with coughing usually noticeable only following exercise. High mortality can be seen in kids, where death is usually the result of septicaemia (Fig. 9.2).

The best description of the pathology of the disease was made by the colonial surgeon Hutcheon (1889) writing over a century ago: 'There is no thickening of the interlobular tissue in the diseased lung of the goat, which forms such a striking feature in bovine pleuropneumonia; the section of the diseased lung in the goat has the appearance of a somewhat granular-looking liver'. These features also clearly differentiate disease caused by *M. m. capri* and *M. m. mycoides* LC. In short, the pathological lesions are localized exclusively in the lung and pleura and consist of a pleuropneumonia, unilateral hepatization, adhesions, pleuritis and an accumulation of pleural fluid (Fig. 9.3). The pleural exudate can

**Fig. 9.1.** Goat with acute CCPP showing head lowered and limbs abducted.

**Fig. 9.2.** Kid with severe CCPP probably as a result of septicaemia.

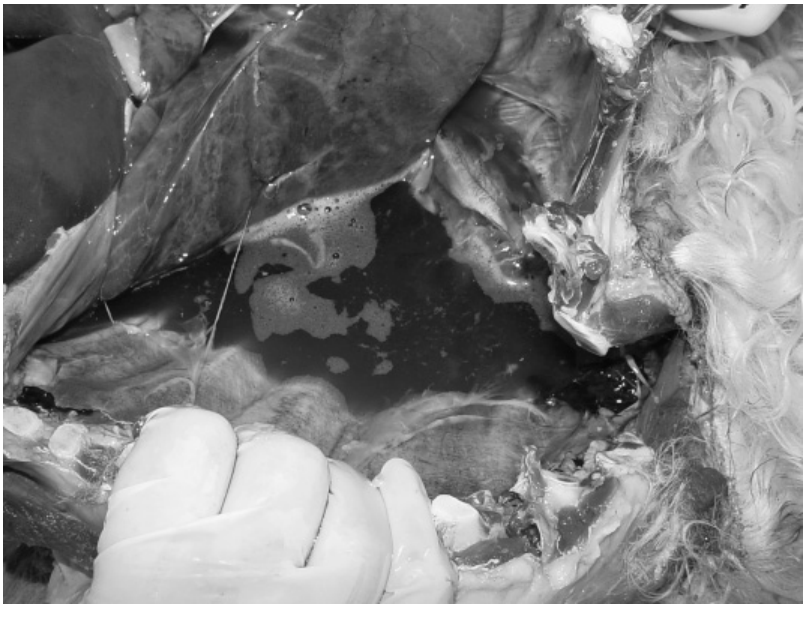

**Fig. 9.3.** Lung of goat with CCPP showing pleural fluid.

solidify to form a gelatinous covering on the lung (Fig. 9.4). Hepatization of the lung as Hutcheon describes is a key feature for differential diagnosis (Fig. 9.5).

A study to correlate clinical signs and early lesions showed that affected goats killed up to a week after contact with affected animals were free of lung lesions or

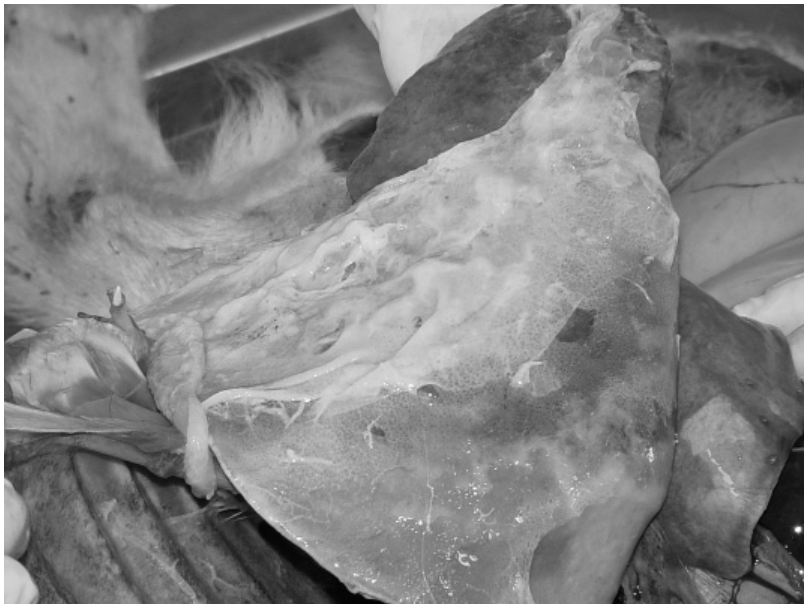

**Fig. 9.4.** Lung of goat with CCPP showing fibrin deposition.

**Fig. 9.5.** Lung of goat with CCPP showing granular hepatization of the cut section.

clinical signs; between 2 and 3 weeks after contact, lung lesions were generally small and superficial, characterized by hyperaemia and oedema with clinical signs being restricted to an infrequent cough; fever was first seen after nearly 4 weeks, which correlated with lung consolidation, the area of which increased as the fever progressed (Wesonga *et al.*, 1993).

In an experiment by Ozdemir *et al.* (2006), it was possible to follow the course of the disease, with and without antibiotic intervention. Healthy Angora goats, confirmed free of CCPP, were exposed to clinically affected animals from a natural outbreak in Thrace, Turkey. After 14 days' exposure, the majority of contacts showed pyrexia (≥41°C). Shortly after, the Angora goats were randomly divided into two groups. Half of these were injected twice over 2 days with danofloxacin; the remaining animals received saline. Goats which survived were euthanazed at day 42. All danofloxacin-treated goats showed resolution of clinical disease by the end of the trial. Two saline-treated goats were euthanized after 4 weeks due to severe disease. Danofloxacin-treated goats showed fewer lung lesions, which were generally sequestrated (Fig. 9.6), and had significantly lower combined clinical scores than saline controls, which showed severe and acute lesions.

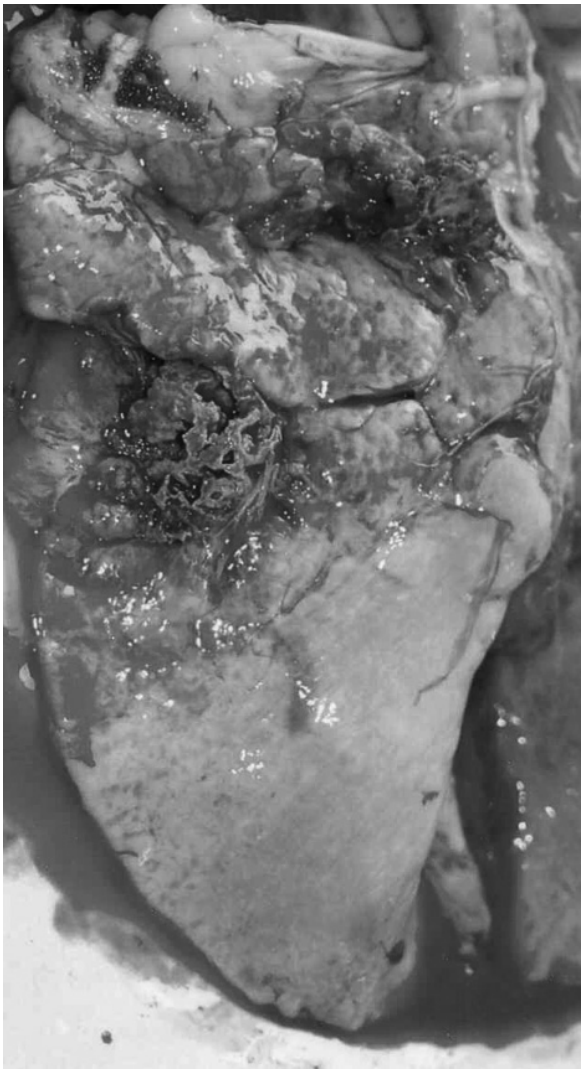

**Fig. 9.6.** Lung of goat with CCPP, treated with antibiotics, showing sequestrum.

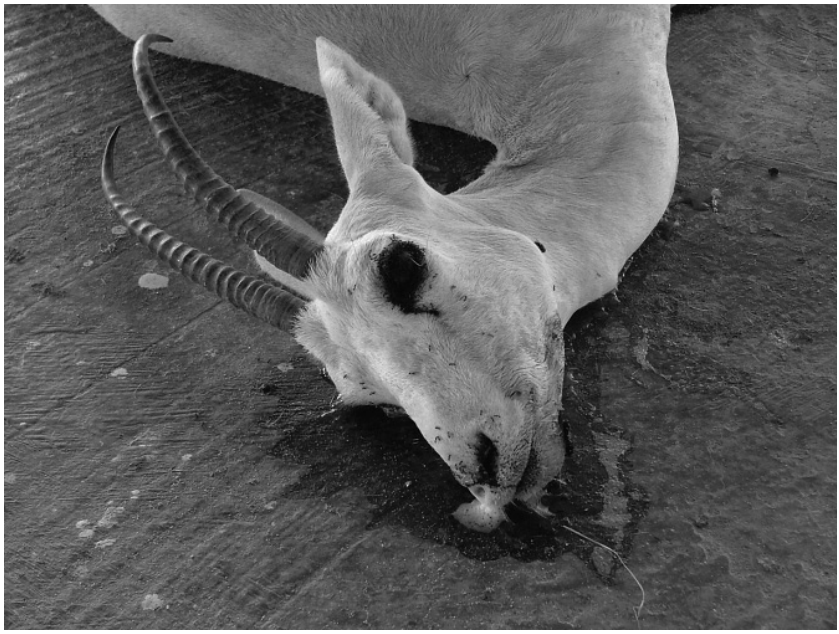

**Fig. 9.7.** Outbreaks of CCPP were seen in semi-captive gazelle species in the Middle East.

The outbreaks of CCPP in gazelles in the Middle East had a very similar clinical (Fig. 9.7) and pathological (Fig. 9.8) appearance to that seen in goats.

Histological examination of the lung tissues often reveals an acute serofibrinous to chronic fibrino-necrotic pleuropneumonia, with infiltrates of serofibrinous fluid and inflammatory cells, mainly neutrophils, in the alveoli, bronchioles, interstitial septae and subpleural connective tissue. Intralobular oedema is more prominent, but interlobular oedema has also occasionally been reported. Peribronchial and peribronchiolar lymphoid hyperplasia with mononuclear cell infiltration is also present (MacOwan and Minette, 1976; Kibor, 1990; Wesonga *et al.*, 1998).

Ultrastructural examination of the lungs of goats naturally affected with CCPP confirms histopathological findings of congested septal capillaries, with inflammatory cells invading thickened septal walls (Johnson *et al.*, 2002). The alveolar lumen contains serous fluid mixed with neutrophils and lymphocytes, some of which are necrotic. The most significant findings are a widespread hyperplasia of type II pneumocytes that have lost most of their characteristic lamellar ultrastructure and large numbers of mycoplasma-like structures lying close to the microvilli and membranes of these cells. It was proposed that the loss of these lamellae may reduce surfactant production, as well as synthesis of key enzymes, leading to increased surface tension within the alveoli and contributing to the atelectasis often seen at post-mortem examination of CCPP cases.

Conditions which may exacerbate CCPP include concurrent viral infections, in particular orf and pestes petits de ruminant (PPR), and possibly other mycoplasma

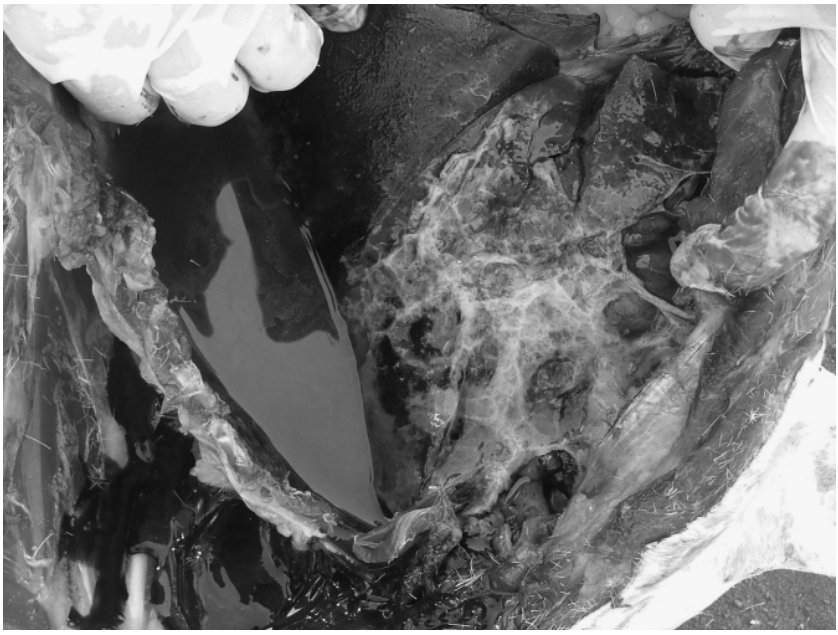

**Fig. 9.8.** Gazelle affected by CCPP showed similar pathological features to those of goats.

infections such as *M. ovipneumoniae*, a cause of disease in its own right; adverse weather conditions and stress caused by transhumance may also compound or accelerate ongoing disease.

## Immunology

Little is known of the immunology of CCPP despite a number of reported experimental infections (Muthomi and Rurangirwa, 1983; Perreau *et al.*, 1984). More recently March *et al.* (2000) monitored the humoral response of goats infected with a multipassaged *M. c. capripneumoniae* strain 19/2 with several serological tests and PCR. While there was little evidence after infection of the infectious agent or clinical or pathological disease, apart from elevated temperatures and a transient cough in one goat, serological responses were detected by latex agglutination test and competitive ELISA. IgG immunodominant bands of 23, 40 and 44 kDa were seen by immunoblotting in all experimentally infected animals, as well as in some sera from a natural outbreak of CCPP in Eritrea, which additionally showed bands of 62, 70 and 108 kDa.

## Transmission

Outbreaks follow the introduction of an infected animal into a susceptible herd. The mycoplasma is transmitted over short distances through the expulsion of

infected droplets during coughing. The disease is very readily contagious and only brief periods of contact are necessary for successful transmission (Thiaucourt and Bölske, 1996). A single surviving seropositive goat from a natural outbreak in Thrace was able to infect all ten disease-free contacts within 2 weeks (Ozdemir *et al.*, 2006). However, the infectious period is quite short as a second contact infection from the same region the following year failed to transmit disease to healthy contacts despite the seropositivity of the naturally infected goats.

No evidence of indirect transmission has been shown as the mycoplasma is highly fragile in the environment. As with many mycoplasma diseases, in particular contagious bovine pleuropneumonia, the disease is introduced into a region by clinically healthy carrier animals.

## Molecular Epidemiology

Unlike other members of the *M. mycoides* cluster, *M. c. capripneumoniae* shows a surprising degree of heterogeneity, particularly in the sequence of its two rRNA operons, which both contain the genes for 5S, 16S and 23S rRNA (Pettersson *et al.*, 1996). These polymorphisms, representing point mutations in either gene, can be used to subtype strains, some of which may have epidemiological and, possibly, evolutionary significance. Sequencing the 16S rRNA genes of African strains identified two distinct lines, I and II, both of which were represented in Central, North and East Africa; isolates from the Middle East were of the type II only, although they could be further divided (Pettersson *et al.*, 1998; Heldtander *et al.*, 2001). Strains isolated from Thrace in Turkey are placed in the groups II and IIb, which suggests that they were introduced illegally across the Dardanelles from Asian Turkey, while those from gazelles are very similar to goat isolates from neighbouring Oman (Nicholas *et al.*, 2008).

Sequencing the amplified products of a different gene(s), the H2 locus, Lorenzon *et al.* (2002) divided strains into four major groups, in which lineage A occurred exclusively in East Africa, B mostly in North Africa and the Middle East, C in Central Africa, and D, represented by only a single strain, from the United Arab Emirates. Subtyping with amplified fragment length polymorphism (AFLP) strongly supported the 16S rRNA sequence analysis by identifying two main lineages (Kokotovic *et al.*, 2000).

On a more local level, ten of 11 strains of *M. c. capripneumoniae* isolated from four different regions of Tanzania had very similar profiles with AFLP. These profiles were also indistinguishable from two Kenyan and one Ugandan strain, indicating the close association between small ruminants in these three neighbouring countries (Kusiluka *et al.*, 2000). Using the PCR/DGGE, it was possible to differentiate strains from Eritrea from all others by their distinctive pattern (McAuliffe *et al.*, 2005).

Isolates from gazelles with CCPP in the UAE (Nicholas *et al.*, 2008) were similar to those from Thrace, Turkey but dissimilar to the type strain F38 and those from Eritrea and mainland Turkey, suggesting disease may have come from contact with affected goats; however, further work is needed to compare with strains from other parts of the UAE.

**Table 9.4.** Substrate utilization by strains of *M. c. capripneumoniae*.

| Substrate | Strain *and origin* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 19/2 Oman | T3 Eritrea | G183 Kenya | F38 Kenya | 7/1a Turkey | G1943 Kenya | G94/83 Kenya | 4/2 LC Oman |
| Lactate | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Pyruvate | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Glucose | +++ | +++ | +++ | – | – | – | – | +++ |
| Glycerol | – | +++ | +++ | – | +++ | – | – | +++ |
| 2-oxybutyrate | – | – | – | +++ | +++ | +++ | +++ | – |

## Biochemistry

Substantial diversity was reported in the metabolism of strains of *M. c. capripneumoniae* (Abu-Groun *et al.*, 1994). In an extension of this work, Houshaymi (1999) divided a range of strains into two major groups, which was also confirmed by DNA–DNA hybridization patterns. Some strains, including the type strain F38, only oxidized organic acids and glycerol but not glucose, while others, including strains from Eritrea, metabolized glucose. The patterns of substrate utilization shown by the non-glucose-oxidizing strains were similar and had a high affinity for 2-oxybutyrate; those for the glucose-metabolizing strains were also similar but failed to oxidize fructose and had a low affinity for 2-oxybutyrate.

Such biochemical diversity within a *Mycoplasma* species is unique and may present diagnostic problems as glucose fermentation is often a key characteristic in their preliminary identification. However, even with glucose-metabolizing strains, the addition of pyruvate to the medium leads to significantly higher yields *in vitro* (Houshaymi *et al.*, 2002). Thus it may be that organic acids are also important energy sources for glucose-oxidizing strains. Table 9.4 illustrates the diversity of strains of *M. c. capripneumoniae* in their substrate requirements.

## Diagnosis

In spite of greatly improved media formulations, the isolation of *M. c. capripneumoniae* remains one of the more difficult tasks for the mycoplasma diagnostic laboratory. The samples of choice are the pleural fluid, which contains high numbers of mycoplasmas, and sections of hepatized lung, preferably at the interface of normal and diseased tissue; however, attempts to isolate mycoplasmas from pleural fluid from goats dying in Thrace were unsuccessful for reasons which remain unclear but may be the result of excessive use of antibiotics. Samples must be sent quickly in a cool condition but will become of little value if journey time is longer than 2 days. Sending samples frozen is recommended but not always practical. During the recent investigation of CCPP in Eritrea, excellent isolation rates of *M. c. capripneumoniae* were achieved from lyophilized lung samples even though isolation was not carried out for several weeks after arrival (Houshaymi *et al.*, 2000). Choice of medium is critical and best results were obtained during the

same investigation with a commercial medium (Mycoplasma Experience, Reigate, UK) (Houshaymi *et al*., 2002). A diagnostic medium for CCPP has also been developed by this company in which *M. c. capripneumoniae* develops coloured colonies in semi-solid medium (Fig. 9.9). Other media which have been shown to support the growth of most strains of *M. c. capripneumoniae* include H25P (Nicholas and Baker, 1998) or FP medium supplemented with 2 g/l of sodium pyruvate (Bölske *et al.*, 1996). Overgrowth of this fastidious mycoplasma by other mycoplasmas is another major problem with isolation. In particular, the 'centreless' colonies of *M. ovipneumoniae* will grow at a much faster rate but can be separated from *M. c. capripneumoniae* by early cloning.

The development of PCR has greatly improved CCPP diagnosis as it is now possible to detect the mycoplasma quickly, even in mixed cultures, directly from clinical material such as pleural fluid and lung, and from this material dried on filter paper (Lorenzon *et al*., 2002). PCRs based on the 16S RNA genes have been reported that enable the detection of all members of the *M. mycoides* cluster followed by specific identification of *M. c. capripneumoniae* by restriction enzyme digestion (Bölske *et al*., 1996). A specific PCR which does not require a restriction enzyme step was described by Woubit *et al*. (2004). The PCR/denaturing gradient gel electrophoresis offers great advantages in detection because it is sensitive, rapid and specific: a single pair of mollicute-specific primers can amplify DNA from all species, which can then be identified by their migration pattern following DGGE (McAuliffe *et al*., 2005). The test also uniquely detects more than one

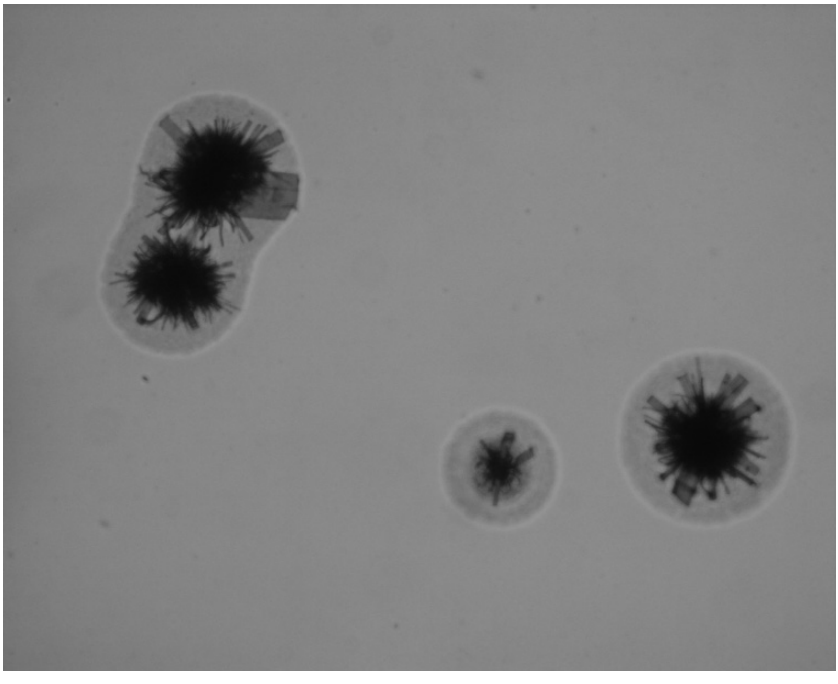

**Fig. 9.9.** Colonies of *Mycoplasma c. capripneumoniae* stained with specific stain (Mycoplasma Experience, Reigate, UK).

*Mycoplasma* species in a sample. However, because of the close similarity of the sequence of the 16S rDNA gene, the target of the PCR/DGGE, in the *M. mycoides* cluster members, a confirmatory PCR such as that described by Woubit *et al.* (2004) should be performed.

Serodiagnosis of CCPP, on the other hand, is a relatively easy task, thanks to a rapid, specific and relatively sensitive test developed initially in Kenya. The latex agglutination test (LAT) uses a carbohydrate extracted from *M. c. capripneumoniae* linked to latex particles which agglutinate in the presence of specific antibodies in the blood of affected goats (Rurangirwa *et al.*, 1987). The test, which takes minutes to complete, is more sensitive than the complement fixation test and easier to perform than the CFT (OIE, 2004) or indirect or competitive ELISA, which should be used for confirmation (Houshaymi *et al.*, 2002). An LAT has also been described for circulating antigen and could provide earlier detection in affected animals before antibodies have appeared (March *et al.*, 2000). The test also provides a convenient means of monitoring growth of the mycoplasma *in vitro*, which is often quite difficult to detect because of the small pH changes produced by some strains; the test requires just a few drops of culture fluid.

## Disease Prevention and Control

Protection against CCPP was shown to be possible more than a century ago when Hutcheon subcutaneously inoculated goats with lung extract from affected animals (McMartin *et al.*, 1980). Later, goats vaccinated with an attenuated broth culture of F38 did not succumb to contact infection (MacOwan and Minette, 1978). This clearly demonstrated control was possible. Since then a number of different preparations have been produced which are reported to produce strong immunity even after 1 year. These include a vaccine composed of sonicated antigens emulsified with incomplete Freund's adjuvant (Rurangirwa *et al.*, 1984) and another in which lyophilized F38 was inactivated with saponin immediately before immunization (Rurangirwa *et al.*, 1987). The latter vaccine has been in use in Kenya for the last few years but this somewhat dangerous practice was modified so that the mycoplasma was inactivated with saponin for at least 12 h at 4°C, enabling successful inactivation to be checked (OIE, 2004). Kids older than 10 weeks of age are vaccinated to avoid maternal antibody, although there is little evidence to show such interference.

In countries where vaccination is not practised, other control measures are used. Antibiotics such as the tetracyclines, fluoroquinolones and the macrolide family are generally effective clinically if used early enough (Onovarian, 1974; Hassan *et al.*, 1984; Ozdemir *et al.*, 2006). However, the complete elimination of the mycoplasma is rarely achieved and treated animals should always be considered as potential carriers.

Movement restrictions and slaughtering of infected and contact animals are recommended for countries or regions that are newly infected.

The risk of introducing CCPP to the USA is probably very small as it does not import small ruminants from Asia or Africa. There is, however, a risk of introducing CCPP to those EU states bordering countries like Turkey, where disease is

endemic, or from Eastern Europe, where surveillance for CCPP rarely occurs. Italy may also be threatened from North Africa, particularly Tunisia and Libya, which are only a short boat ride away. Once introduced into the EU, the disease could theoretically spread throughout the member states, including the UK, via animal trade. The widespread use of antibiotics would suppress the overt clinical signs, reducing the direct economic consequences, with the disease remaining undetectable for several months or years, as was the case in Italy following the introduction of contagious bovine pleuropneumonia in 1990. However, the costs of eradication would be significant. The key to control of this disease, therefore, given an outbreak in Europe, would be the rapid identification of the disease, enabling the destruction of affected and contact animals.

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# 10 Bovine Respiratory Disease

*The outbreak of respiratory disease started during December 1974 . . . to date 27 calves have died out of the 102 calves in the barn* (L.H.Thomas *et al.* describing the first outbreak of respiratory disease caused by *Mycoplasma bovis* in the UK).

## OVERVIEW

Bovine respiratory disease (BRD) is the most common illness affecting housed cattle worldwide and is a major impediment to animal production. It is primarily a problem in calves less than 6 months old but may be seen in calves up to 1 year of age. BRD may also be referred to as 'shipping fever' pneumonia, which has a multifactorial aetiology and is associated with the assembly into feedlots of large groups of calves from diverse geographical, nutritional and genetic backgrounds. Cell-walled bacteria, in particular *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*, are some of the infectious agents involved. Morbidity can approach 35%, while mortality can be as much as 5–10%. In addition to the huge economic losses, there are increasing welfare considerations to be taken into account. BRD is a multifactorial disease which is influenced by:

- Host factors;
- Type and number of infectious agents involved;
- Environmental factors such as crowding and inadequate ventilation;
- Management factors.

The major pathogens involved include the viruses: bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI3), bovine herpes virus 1 (BHV1), infectious bovine rhinotracheitis (IBR) and bovine viral disease virus (BVDV). While virulent strains of BRSV and PI3 have been shown to cause primary lung damage in calves, their immunosuppressive effects on the host's pulmonary defences may also enable the colonization of other pathogens such as pathogenic

bacteria and mycoplasmas. Immunosuppression has been shown more convincingly with BVDV, where a delay in antibody response and greater persistence of BRSV can be seen in co-infections. IBR mostly affects the upper respiratory tract and is present in most herds, causing disease in unexposed or immunosuppressed calves together with bacterial pathogens. BRSV has been increasingly recognized as a respiratory problem in weaner and feedlot animals, in particular young dairy stock.

The major bacterial pathogens most often involved in enzootic calf pneumonia are *M. haemolytica* serotype A, *P. multocida* and *H. somni*, and more often than not two or more of these pathogens can be isolated from the same pneumonic lungs. *Arcanobacterium pyogenes* is frequently isolated from lung abscesses in cases of chronic pneumonia. Generally these bacteria are confined to the upper respiratory tract, and being located in the tonsillar crypts are difficult to isolate from healthy cattle. However, after stress they can invade and cause primary lung damage, with their effects likely to be considerably more extensive if the host has been compromised by previous or current virus or mycoplasma infections. The effects of other pathogens such as lungworm and other parasites should not be overlooked. Compounding the significant animal losses caused by BRD are the inevitable costs of treating animals with antimicrobials, which amounts to the greatest use in cattle husbandry. However, there are growing pressures to reduce the amount being used in food animals because of the fear of drug resistance.

Mycoplasmas cause some of the most serious and economically most costly respiratory diseases of cattle (Table 10.1). Contagious bovine pleuropneumonia, the only bacterial disease classified by the Office International des Epizooties as a list A disease, is caused by *M. mycoides* subsp. *mycoides* small colony (SC) variant. This mycoplasma was isolated just over a century ago but, while Europe has been free since the end of the 1990s, it still presents immense problems in Africa. *M. bovis* is a major, and often overlooked, cause of calf pneumonia, mastitis, arthritis and other conditions. In addition to these respiratory pathogens, *M. dispar*, *Ureaplasma diversum*, *M. bovigenitalium*, *M. bovirhinis* and, more recently, *M. canis* and *M alkalescens* have been isolated from the lungs of pneumonic cattle, though not all are believed to be primary causes of disease. *M. dispar*, a proven pathogen, is an extremely fastidious organism, which accounts for its under-reporting, often involved in pleuropneumonia in older cattle. Distinctive mycoplasma lesions include peribronchial and peribronchiolar lymphoid cuffing and alveolitis. It is being increasingly recognized that, in naturally infected calves, lesions also comprise an exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells. Culture of these organisms requires special media and conditions and may take up to a week for growth of the organisms, although molecular detection tests are speeding up diagnosis and enabling a better assessment of prevalence.

# DISEASES CAUSED BY *MYCOPLASMA BOVIS*

## Introduction

In 1994 such were the concerns about calf pneumonia caused by *M. bovis* in his constituency that Dr Gavin Strong, member of the UK parliament for East

Edinburgh and Musselburgh, asked the then Minister of Agriculture what plans there were to combat the disease (Anon, 1994). The Minister replied that there were no plans to make the disease, which was believed to have been introduced into the UK after the introduction of the EU single market, notifiable. In fact, *M. bovis* was first reported in the UK nearly 20 years earlier, probably introduced from the USA. A second invasion of perhaps more virulent strains entered with imported cattle herds from mainland Europe at the time of the single market, as evidenced by the significant impact it had on Ireland (Byrne *et al.*, 2001).

It is now well accepted that *M. bovis* is a major contributor to calf pneumonia. The evidence for this involvement includes the frequent isolation from the lungs of pneumonic calves, high seroprevalence in affected herds and the significant impact the organism has had since its arrival in Ireland in the 1990s (Nicholas and Ayling, 2003). The effect of *M. bovis* on adult cattle is less well known in the UK and Ireland, although it has been reported as a major and uncomplicated cause of mastitis, arthritis and otitis in North America (Lamm *et al.*, 2004). Recent isolations of *M. bovis* from the aborted fetuses and brains of cattle showing neurological signs and otitis in the UK and Ireland suggest an even wider role in disease than previously thought (Ayling *et al.*, 2005).

In some countries the lesions caused by *M. bovis* are thought to resemble those seen in CBPP, so the comparative properties of *M. bovis* and *M. mycoides* SC, the cause of CBPP, are given in Table 10.1.

**Table 10.1.** Comparative properties of the two most important cattle mycoplasmas.

| Properties | *M. mycoides* subsp. *mycoides* SC | *M. bovis* |
|---|---|---|
| Diseases | Contagious bovine pleuropneumonia in cattle, occasionally arthritis in calves | Calf pneumonia, mastitis, arthritis, abortion, keratoconjunctivitis |
| Distribution | Sub-Saharan Africa, probably in parts of the Middle East, Central Asia | Worldwide |
| Hosts | Cattle, goats (sheep) | Cattle |
| Histopathological lesions | Fibrinous pleuropneumonia with necrosis | Interstitial pneumonia, lympho-histiocytic bronchitis, catarrhal bronchopneumonia |
| Clinical signs | Few signs, respiratory distress evident after exercise | Respiratory distress, mastitis, arthritis |
| Diagnosis | Isolation, serology, PCR, abattoir surveillance | Serology, isolation, PCR |
| Treatment | Recommendation of chemotherapy is contra-indicated because carrier status has been revised (FAO, 2007) | Chemotherapy |
| Control | Vaccination, movement control, slaughter | Management, e.g. improved ventilation, reduced stocking density. Some autogenous vaccines available in USA and UK |

## Distribution

*M. bovis* was first isolated in 1961 in the USA from a case of severe mastitis in cattle (Hale *et al.*, 1962) and, because of its close serological and biochemical similarity with the small ruminant pathogen *M. agalactiae*, was originally designated as *M. agalactiae* var. *bovis*. It then appears to have spread via animal movements worldwide to many countries including Israel (1964), Spain (1967), Australia (1970), France (1974), mainland Britain (1974), Czechoslovakia (1975), Germany (1977), Denmark (1981), Switzerland (1983), Morocco (1988), South Korea (1989), Brazil (1989), Northern Ireland (1993), Republic of Ireland (1994), Chile (2000) (Nicholas, 2002a), South Africa (2005) and the Czech Republic (Zendulkova, 2007).

The prevalence of *M. bovis* is almost certainly underestimated, as *M. haemolytica*, *P. multocida* and *H. somni* are invariably isolated first from pneumonic calves; in arthritis and mastitis, where *M. bovis* is a primary and uncomplicated cause, it is often overlooked as few laboratories routinely monitor for mycoplasmas. However, unlike these conventional bacteria, the occurrence of *M. bovis* in a herd is invariably linked to cases of disease, in particular pneumonia, mastitis and/or arthritis (Pfützner and Sachse, 1996).

## Economic Losses

In the UK, it is estimated that nearly 2 million cattle are affected annually by respiratory disease (Reeve-Johnson, 1999). Furthermore approximately 157,000 calves, which would have a potential market value of £99 million, die annually as a result of pneumonia and related illnesses. Across Europe, with approximately 90 million cattle, this extrapolates to total losses of 576 million euros. It is likely that *M. bovis* is responsible for at least a quarter to a third of these losses, although this is likely to be an underestimate (Nicholas *et al.*, 2000). In the USA, the cost of *M. bovis* infections as a result of loss of weight gain and carcass value has been estimated at $32 million per year (Rosengarten and Citti, 1999). The losses due to bovine mastitis caused by *M. bovis* may be higher than that for respiratory disease, with estimates from the USA of up to $108 million per year, with infection rates of up to 70% of a herd (Rosengarten and Citti, 1999).

## Host Range

*M. bovis* is highly adapted to cattle but occasional isolations have been made in chickens, buffaloes, small ruminants and even humans (Pitcher and Nicholas, 2005). In one case, *M. bovis* was isolated from the throat of a woman who had developed bronchopneumonia after heavy exposure to cow manure (Madoff *et al.*, 1979). The disease subsided following tetracycline therapy with the development of antibodies to *M. pneumoniae*, but not to *M. bovis*, during convalescence, suggesting that *M. bovis* may not have been the primary aetiological agent. In a

second case, *M. bovis* was isolated from a patient with respiratory disease who successfully responded to tetracycline treatment (Pitcher and Nicholas, 2005).

*M. bovis* was isolated from a mastitic goat on a smallholding in Kent, UK in 1999 (Ayling *et al.*, 2004). This was the first case of *M. bovis* from a goat in Britain and caused some concern on its initial isolation because of the close biochemical and immunological similarity to *M. agalactiae,* which causes contagious agalactia and is exotic to Britain. The highly specific PCR of Subramaniam *et al.* (1998) confirmed identification of this isolate as *M. bovis*. In Turkey, multiple isolates of *M. bovis* were detected in the trachea of clinically healthy broiler chickens from a farm where poultry and cattle mixed freely (Ongor *et al.*, 2008).

## Epidemiology

*M. bovis* is not ubiquitous but widely spread within the bovine population in enzootically infected areas. The infection is usually introduced to *M. bovis*-free herds by clinically healthy calves or young cattle shedding the mycoplasma and, once established on multi-age sites, it becomes very difficult to eradicate. Its appearance on some farms suffering low-grade respiratory disease can lead to increased morbidity and mortality (Gourlay *et al.*, 1989). Infected cattle shed the mycoplasma via the respiratory tract for many months and even years, when they act as reservoirs of infection (Pfützner, 1990). Contact animals become infected via the respiratory tract, the teat canal or genital tract; artificial insemination with infected semen is another common route (Pfützner, 1990). The male genital tract can become infected with *M. bovis* through contact with other animals or, possibly, via a heavily contaminated environment. Infection of the prepuce or urethra by *M. bovis* leads to an ascending infection of the testes, causing orchitis, vesiculitis, decrease of semen quality and ultimately shedding in the semen (Kreusel *et al.*, 1989).

In a study of calves in the Netherlands, ter Laak *et al.* (1992a,b) found *M. bovis* in 20% of pneumonic lungs from fattening herds but only in a small number of apparently healthy calves. Following its introduction into the north and south of Ireland in 1994 from mainland Europe, *M. bovis* has been consistently isolated from 13 to 23% of pneumonic lungs (Brice *et al.*, 2000; Byrne *et al.*, 2001; Blackburn *et al.*, 2007). In France, *M. bovis* was isolated from 30% of calf herds with pneumonia (Le Grand *et al.*, 2001) while in Britain, about 20–25% of pneumonic herds contain animals with antibodies to *M. bovis* (Ayling *et al.*, 2004). In the UK, significant antibody titres to *M. bovis* were detected in just under half of 55 pneumonic herds examined, of which only seven had rising titres to viral pathogens: respiratory syncytial virus, infectious bovine rhinotracheitis or bovine viral diarrhoea virus (Nicholas *et al.*, 2001).

Clearly other factors play a role in bovine respiratory disease, such as concurrent viral and bacterial diseases as well as stress and environmental factors, but it is increasingly believed that *M. bovis* is the predisposing factor in the infectious process, leading to invasion by other bacterial pathogens possibly by compromising host defences (Rebhun *et al.*, 1995; Poumarat *et al.*, 2001). In a study of calves which had died of pneumonia, Buchvarova and Vesselinova (1989)

showed that over a third of lungs were infected only with *M. bovis* while the rest contained a combination of *M. bovis* with *P. multocida* and/or *H. somni*. These workers concluded that alterations in the lungs were chiefly due to mycoplasma infection, with the remaining bacteria contributing to complications in the pneumonic process.

A major epidemiology survey of *M. bovis* infection in Britain covering the years 1995–2005 was conducted by Lawes *et al.* (2008). At the herd level this investigation showed that the proportion of pneumonic herds infected with *M. bovis* in England and Wales was very high (42.4%). Other studies have found that the proportion of infected herds is often higher than the percentage of serologically positive animals. Le Grand *et al.* (2001) found that the average number of seropositive herds was 60%, yet the percentage of serologically positive animals ranged from 2 to 13%. This unexpected distribution was also seen in dairy herds in Switzerland (Burnens *et al.*, 1999). Previously published work analysing data between 1994 and 1999 from the UK showed that 22% of herds were seropositive (Nicholas *et al.*, 2000). This showed that the number of infected herds had risen and that this increase could be due to either the pathogen becoming even more widespread or the fact that there was a greater awareness of the infection in the field, reflecting a more accurate picture of the infection status in England and Wales.

Surprisingly, the analysis did not find any seasonality of *M. bovis* infection, which is contrary to other pneumonia-causing pathogens, which are thought to cause higher infection rates in the winter months. On average the seroprevalence was highest in May, June and August (Lawes *et al.*, 2008). Previous studies in the UK have found that outbreaks of *M. bovis* continue in herds where animals are housed all year round and that during the summer months the percentage of herds affected by *M. bovis* increases in relation to the number tested (Nicholas *et al.*, 2000).

The results of spatial analysis in this study showed that *M. bovis* infection is not distributed evenly across England and Wales but that there are areas of very high seroprevalence (hotspots) (Fig. 10.1), which are continually changing on a yearly basis (Lawes *et al.*, 2008). It is possible that the areas of high seropositivity could be related to areas where the numbers of cattle are high. Using census data from 2005, Cornwall was an area with high cattle numbers (greater than 200,000) and in this year the seropositivity for this county was 41–50%. Yorkshire also showed a similar correlation. It is perhaps unsurprising that the 'hotspot' areas are not continually associated with one area, considering the dynamics of the UK cattle industry. Cattle move extensively in their lifetime and cover large distances. The animals may become infected when they move to new premises or they may shed the mycoplasma and consequently infect other herds through the stress of movement. Animals travel further distances now due to the closure of many livestock markets and therefore those remaining may be supplied by animals from many more counties and hence the spread of infection increases. However, despite the organism's persistence on a farm, it is clear that some are able to eliminate the infection, otherwise the prevalence in the UK would continue to increase. Farms operating 'all in, all out systems' are less likely to suffer persistent infection, but some mixed-aged farms also seem to lose infection after a period, although it is not known how. The data set used in this study had some

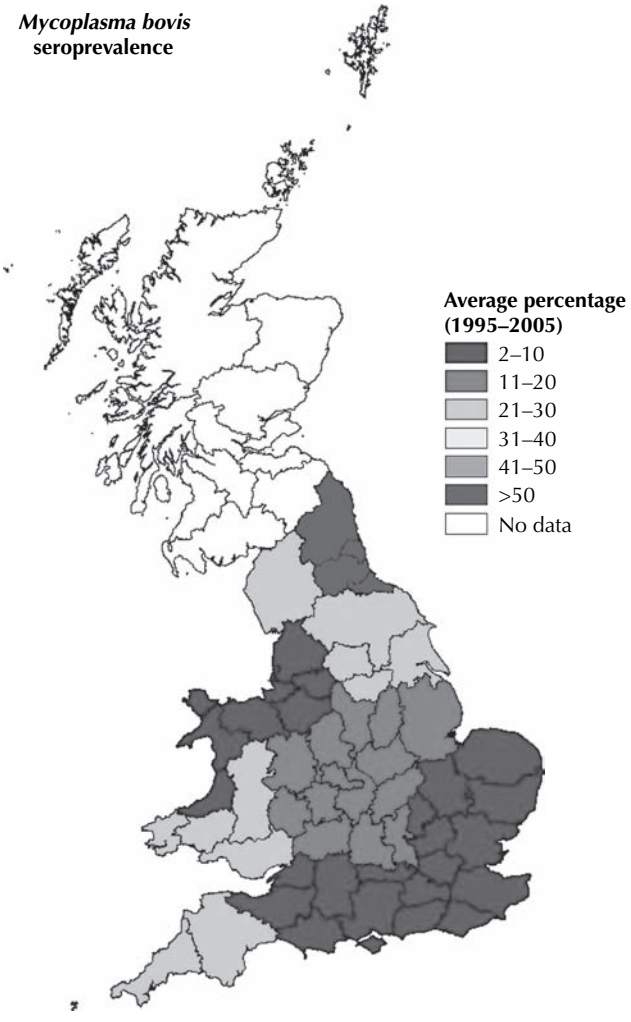


**Fig. 10.1.** Map of England and Wales to show seroprevalence of *M. bovis* by area for 1995 to 2005 inclusive.

limitation in that there appear to be regional differences in the awareness of *M. bovis* as a cause of pneumonia, and this factor therefore affects the number of samples that are taken for diagnosis.

## Molecular Epidemiology

The genetic diversity of 53 isolates collected in the UK between 1996 and 2002 was studied using pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) analysis (McAuliffe *et al.*, 2004). In addition, the influence of variable surface protein (Vsp) profiles on the profiles generated using molecular typing techniques was studied. Both AFLP and RAPD separated the isolates into two distinct groups

comprising over 30 different profiles; however, PFGE showed less congruence with the other techniques. It was possible to speculate that the two distinct groups identified by RAPD and AFLP in this study may represent two clonal lines of descent. It was hypothesized that group A may have entered the UK from mainland Europe around the time of the creation of the European Union single market in 1993, when large numbers of cattle came into the UK from continental Europe (McAuliffe *et al.*, 2004). Group B could have a different and possibly older ancestral origin and have derived from the first strains of *M. bovis* isolated in the UK in 1975, which were thought to have originated in the USA. A later study using RAPD showed a closer similarity between Italian strains and the type strain of *M. agalactiae* than with the type strain of *M. bovis* which originated from the USA (Loria *et al.*, 2007). These two species are very similar and were originally considered subspecies, so molecular typing could provide fresh insights into the evolution of these organisms.

McAuliffe *et al.* (2004) noted a lack of correlation between geographical origin, time of isolation and genomic profiles of the UK strains, and in some instances, strains from farms over 400 km apart had identical profiles. These findings are similar to a study of European *M. bovis* strains using restriction enzyme analysis, which indicated that there was no correlation between the geographical origin, genetic heterogeneity and isolation date (Poumarat *et al.*, 1994) and reflects the extensive cattle movement that occurs within the UK and Europe.

## Causative Organism

*M. bovis* was elevated to species rank and given its present name in 1976 (Askaa and Erno, 1976). However, in spite of the fact that the two mycoplasmas cause different diseases in different animals and have a DNA–DNA reassociation value of only 40%, they show a very close 16S rRNA similarity of 99.8%. *M. bovis* is also biochemically similar to *M. agalactiae,* as neither ferment glucose nor hydrolyse arginine but instead use organic acids, lactate and pyruvate for their energy source for growth (Miles *et al.*, 1988); they also give film and spot reactions *in vitro*, indicating the possession of lipolytic activity. Table 10.2 shows comparative biochemical characteristics of bovine mycoplasmas.

Like all mollicutes, *M. bovis* is small, pleomorphic and lacks a cell wall (Hermann, 1992). It had a genome size of 1080 kbp estimated by PFGE but the complete genome of the type strain *M. bovis* (PG45) was sequenced through a collaboration between the University of Missouri and the J.C. Venter Institute. Traditional shotgun cloning and Sanger sequencing technologies were employed to generate 8X sequence coverage of the genome of a clonal isolate. Analysis of the fully assembled sequence revealed a single circular chromosome of 1,003,404 base pairs, with a G+C content of 29.3%. The current annotation of the genome contained 868 genes, of which 827 are predicted to encode open reading frames (Wise, Calcutt and Methe, personal communication, 2008). Future analysis of the genetic blueprint of this important cattle pathogen is anticipated to reveal features that are critical for virulence and survival, in addition to establishing a database of targets that might be utilized in diagnosis, treatment and control.

**Table 10.2.** Comparative biochemical properties of bovine mycoplasmas, including *M. bovis*.

| | | | | | | | Tetrazolium | |
|---|---|---|---|---|---|---|---|---|
| *Mycoplasma* | Glucose | Arginine | Urease | Film | Casein | Phosphatase | Aerobic | Anaerobic |
| *M. alkalescens* | – | + | – | – | – | + | – | – |
| *M. bovis* | – | – | – | + | – | + | + | + |
| *M. bovirhinis* | + | – | – | – | +/– | +/– | + | + |
| *M. bovigenitalium* | – | – | – | + | – | + | – | + |
| *M. bovoculi* | + | – | – | + | – | +/– | + | + |
| *M. canis* | + | – | – | – | +/– | – | – | + |
| *M. californicum* | – | – | – | – | nk | + | – | Most strains + |
| *M. canadense* | – | + | – | – | nk | weak + | – | + |
| *M. dispar* | + | – | – | – | nk | – | + | + |
| *M. m. mycoides* SC | + | – | – | – | + | – | + | + |
| *U. diversum* | – | – | + | – | – | – | nk | nk |

nk – not known

Despite the fragility of most mycoplasmas in the environment, *M. bovis* can survive at 4°C for nearly 2 months in sponges and milk and for over 2 weeks in water; at higher temperatures survival rates drop considerably (Pfützner and Sachse, 1996). The mechanism of this unlikely survival in a hostile environment, given their lack of cell wall, was largely unknown until McAuliffe *et al.* (2006) showed that mycoplasmas could produce a biofilm which empowered it with increased resistance. It is, however, unlikely that the environment provides a major source of infection compared with that posed by infected animals. On the other hand, frozen sperm contaminated with mycoplasma can remain infectious for years and probably represents an overlooked infection source.

The virulence factors of *M. bovis* and mechanisms of pathogenesis are still largely unknown, but the ability of the agent to vary the expression of a family of membrane surface proteins (*vsp*) with high frequency is shedding light in this area (Pfützner and Sachse, 1996). *M. bovis* has been reported to possess 13 *vsp* genes involved in antigenic variation, which alter the antigenic character of its surface components and may act to enhance colonization and/or adherence or evade the host's immune defence systems (Lysnyansky *et al.*, 2001). These *vsp* genes operate by various mechanisms, including DNA transposition and intrachromosomal recombination within the *vsp* locus (Nussbaum *et al.*, 2002).

Hydrogen peroxide has long been considered a pathogenicity factor for mycoplasmas. Khan *et al.* (2005) showed that UK *M. bovis* strains produced widely variable levels of $H_2O_2$, which may be related to pathogenicity. A large reduction (99%) in $H_2O_2$ was seen in one high-producing strain which was subcultured *in vitro* over 200 times; this reduction appeared to be related to the loss of a 35 kDa protein, possibly the NADH oxidase, after 50 passages. The use of this high-passaged strain or indeed a naturally occurring low-$H_2O_2$ producer as a potential vaccine should be examined.

It has also been suggested that *M. bovis* may cause immunosuppression, leading to invasion by other bacterial and viral pathogens. *In vitro* studies have shown that the mycoplasma can prime the apoptotic cascade in activated bovine lymphocytes and secrete a specific lympho-inhibitor peptide (Vanden Bush and Rosenbusch, 2002, 2004).

## Disease Course

### Respiratory disease

In most experimental infections, even with high doses of *M. bovis* given via the trachea, lesions may be absent or confined to the apical lobes of the lung and usually result in mild clinical lesions with temperature occasionally above 40°C (Fig. 10.2); however, more virulent strains can lead to more extensive lesions (Fig. 10.3) and abscesses (Fig. 10.4) and microabscesses with severe clinical signs (Fig. 10.5), arthritis and death.

A clinical study of endemic pneumonia, in which *M. bovis* and *P. multocida* were frequently isolated from the herd, showed that nearly half of dairy calves were shedding mycoplasmas at 5 days of age and over 90% by 4 weeks (Stipkovits *et al.*, 2001). Clinical disease in the calves, including up to 10% mortality as a result of severe serofibrinous pneumonia, was highest between 10 and 15 days. Surviving calves showed very poor weight gain and remained retarded; other

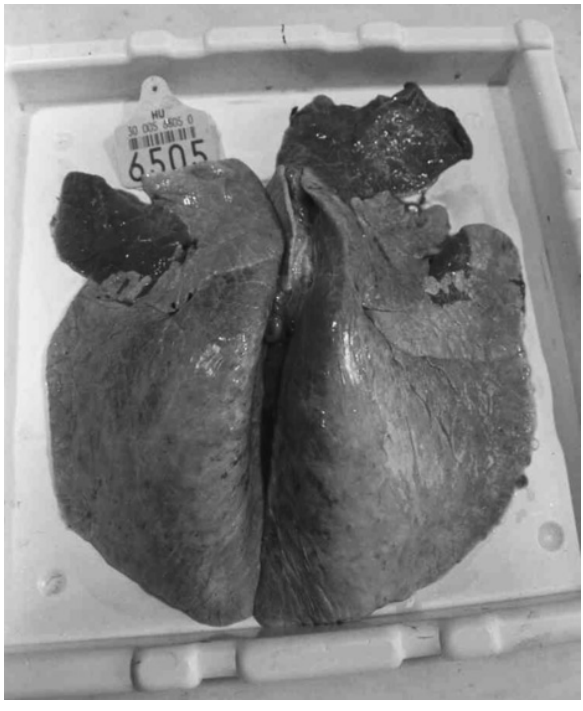


**Fig. 10.2.** Lung from calf experimentally infected with *Mycoplasma bovis* showing lesions in the apical lobes.

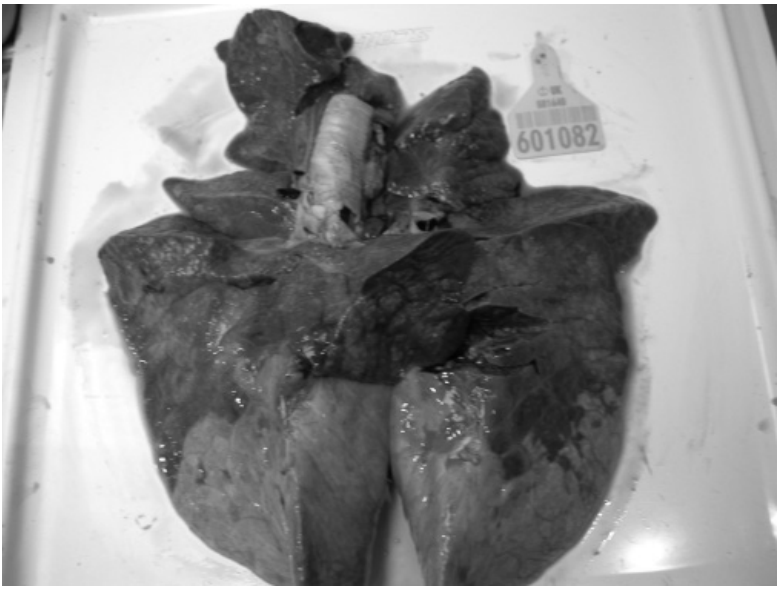


**Fig. 10.3.** Lung from calf experimentally infected with virulent strain of *Mycoplasma bovis* showing more extensive lesions.

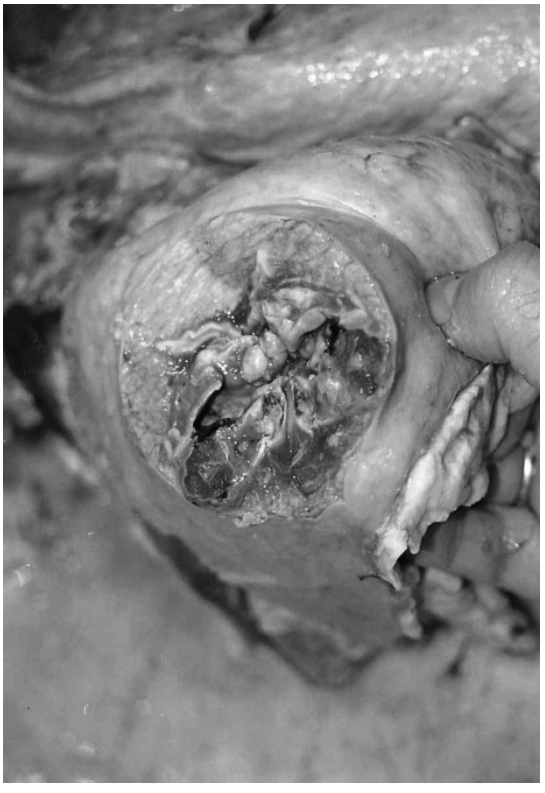

**Fig. 10.4.** Lung from calf experimentally infected with *Mycoplasma bovis* showing abscess and enlarged interlobular septa.

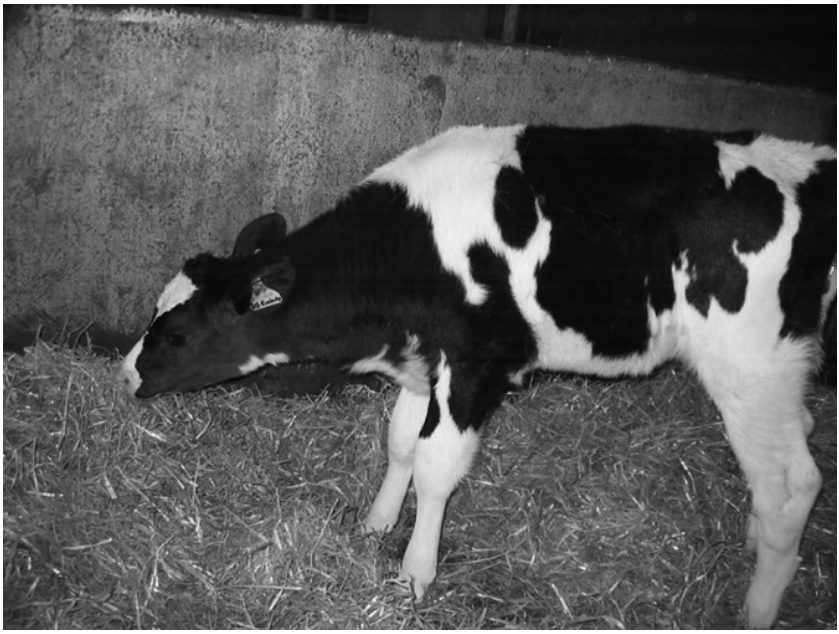

**Fig. 10.5.** Calf experimentally infected with *Mycoplasma bovis* showing clinical signs of respiratory distress.

signs included fever, depression, hyperpnoea, dyspnoea, nasal discharge, mild to continuous coughing and loss of appetite. In the UK, calf pneumonia usually begins in November and peaks around January, but deaths due to *M. bovis* continue to occur in some herds in the spring at pasture, representing relapses because of unresolved lung lesions (Nicholas *et al.*, 2001).

Clinical signs of *M. bovis* infection are not characteristic, but some observers consider the following to be key features of this disease:

- Harsh hacking cough;
- Low-grade fever;
- Mild depression;
- Runny eyes;
- Ear droop;
- Arthritis .

Pulmonary lesions in naturally infected calves comprise an exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells. In studies involving experimental infections of gnotobiotic calves with *M. bovis*, significant pneumonia was induced, involving up to 30% of the lung surface, and was of sufficient severity to cause clinical respiratory disease in some calves (Thomas *et al.*, 1986). Distinctive areas of coagulative necrosis were prominent within the lesions. Chronic infections are often associated with a lymphocytic 'cuffing' pneumonia, where there is marked hyperplasia of peribronchial lymphoid tissue, causing stenosis of airway lumina and compression and collapse of adjacent pulmonary prenchyma. *M. bovis* antigen is mainly detected at

the periphery of the areas of coagulative necrosis, in necrotic exudates and in close association with infiltrating macrophages and neutrophils (Rodriguez *et al.*, 1996).

Chronic pneumonia and polysynovitis caused by *M. bovis* has become an increasing problem in feedlot beef cattle in northern America, characterized by non-responsiveness to antibiotic therapy and frequent relapsing respiratory disease (Gagea *et al.*, 2006). The gross pathology reveals: reddened and consolidated cranioventral lungs with raised, white–yellow, sharply demarcated foci of caseous necrosis typically 2–10 cm in diameter; lung abcesses, which may be dry or purulent (in the later stages) and indistinguishable from that seen in 'shipping fever'; well-differentiated sequestra between 5 and 30 cm; and single or multiple joint lesions. These lesions have caused some concern because of their similarity to CBPP, which has not been seen in America for over 100 years.

In a study in northern Italy, Raedelli *et al.* (2008) examined 140 clinically healthy cattle at slaughter, comprising 70 veal calves and 70 beef cattle. Serology for *M. bovis* was positive in 76% of beef cattle and 100% of veal calves. *M. bovis* was isolated only from veal calves in 16 out of 64 pneumonic cases and was detected by immunohistochemistry in seven culturally positive cases, where it was associated with bronchogenic necrosuppurative or fibrinonecrotizing lesions. Culturally positive and immunohistochemical negative cases were associated with catarrhal–bronchointerstitial pneumonia. Results indicate that *M. bovis* infection, which was the most important disease in beef cattle in northern Italy, may develop into a severe necrosuppurative bronchopneumonia or fibrinonecrotizing pneumonia when associated with a high number of intralesional organisms or, conversely, into a mild catarrhal–bronchointerstitial pneumonia when associated with a low number of organisms. This work also demonstrated the difficulty in isolating mycoplasmas from pneumonic lesions of adult cattle, which may indicate that the lesions represent late and non-specific consequences of respiratory infections which occurred in the first months of life (Raedelli *et al.*, 2008).

Respiratory disease in dairy cattle is relatively rare in the UK. However, a prolonged episode beginning in the early 2000s and characterized by sudden onset and milk drop/pyrexia accompanied by rapid breathing and hard respiratory noises was seen in a 'flying herd' of 1400 animals which had a replacement rate of about 20% from various sources, including Germany and France (Nicholas *et al.*, 2006). At least 10% of the herd was affected per year, of which 20%, predominantly in the 4–5-year-old age group, were culled because of chronic lung disease. A major and consistent finding from the lungs and sera is *M. bovis*, for which chemotherapy provides only transient improvement.

## Mastitis/arthritis

Arthritis associated with *M. bovis* infection may be a sequel to either the respiratory or mastitic form of the disease (Pfützner, 1990). There is lameness, swelling of joints, slight elevation of temperature, failure of antibiotic treatment and, if severe, reduced consumption of feed and debilitation. This condition often arises within 2–3 weeks of housing and may also follow transportation of calves over

long distances. A case of severe keratoconjunctivitis, impairing vision in a number of bullocks, involving *M. bovis* may also have been exacerbated by the stress of transport (Kirby and Nicholas, 1996).

When *M. bovis* entered Britain in 1970 and Ireland in the early 1990s it initially presented as a mastitis, often with severe arthritis, before taking on the more familiar pneumonic form as it became endemic. In the present millennium the number of mastitis cases, often with arthritis, involving *M. bovis* increased dramatically, albeit from a low base. However, reported cases of *M. bovis* mastitis still represent a fraction of the nearly 5000 cases of mastitis diagnoses and the question must be asked: just how many of the 20% of undiagnosed cases of mastitis involve mycoplasma?

Cases of mycoplasma mastitis often have the following characteristics. They are non-responsive to antibiotics and anti-inflammatory drugs and there is decreased milk production in recovered cows. A painful arthritis often develops in bought-in animals within a month of introduction (Fig. 10.6). Other features include high milk somatic cell counts ($>10^6$), atrophy of affected quarters and spread of infection from quarter to quarter. Abortions may also occur, with isolation of *M. bovis* from aborted tissues. Diagnosis is rapid and simple, with *M. bovis* often isolated from joints and milk or detected by PCR; *M. bovis* involvement can be confirmed by the detection of raised antibody levels in sera from cattle with painful joints. Culling is often the only real alternative to non-effective antibiotics,

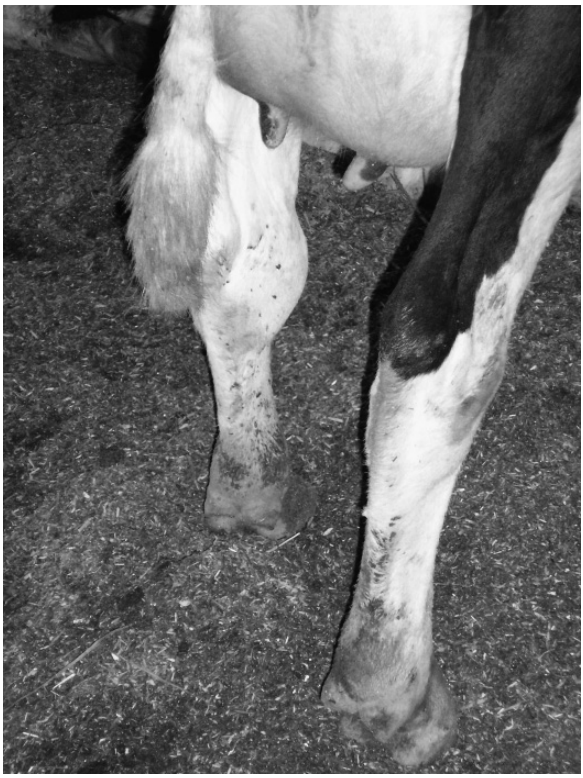

**Fig. 10.6.** Swollen joint of cow naturally infected with *Mycoplasma bovis* which also shows mastitis.

for humane reasons and to prevent the spread of disease from these affected animals to healthy contacts.

## Otitis and related neurological disorders

Otitis in calves may be associated with a variety of bacterial agents, including *H. somni*, *M. haemolytica*, *P. multocida*, streptococcal spp., *Actinomyces* spp., *Arcanobacterium pyogenes* and *M. bovis* (Duarte and Hamdan, 2004; Francoz *et al*., 2005). The pathogenesis of otitis due to *M. bovis* is not fully understood; infection may arise by extension from the pharynx via the Eustachian tube to the middle ear and may extend into the inner ear (Walz *et al*., 1997; Maeda *et al.*, 2003; Lamm *et al*., 2004). Clinical signs associated with infection may include head tilt and neurological signs in dairy and beef calves from 2 to 18 weeks of age (Lamm *et al.*, 2004; Francoz *et al*., 2005). Previous reports of otitis associated with *M. bovis* infection have been from North America and Japan (Maeda *et al.*, 2003; Lamm *et al.*, 2004; Francoz *et* al., 2005).

In 2007, signs of severe otitis media in 20% of dairy calves on one farm were associated with *M. bovis* infection, based on isolation from the external ear canal and nares (Foster *et al*., 2008) (Fig. 10.7). Affected calves also seroconverted to *M. bovis* and no other significant bacteria were isolated. Infection was considered likely to have originated from cows in the milking herd showing low-grade

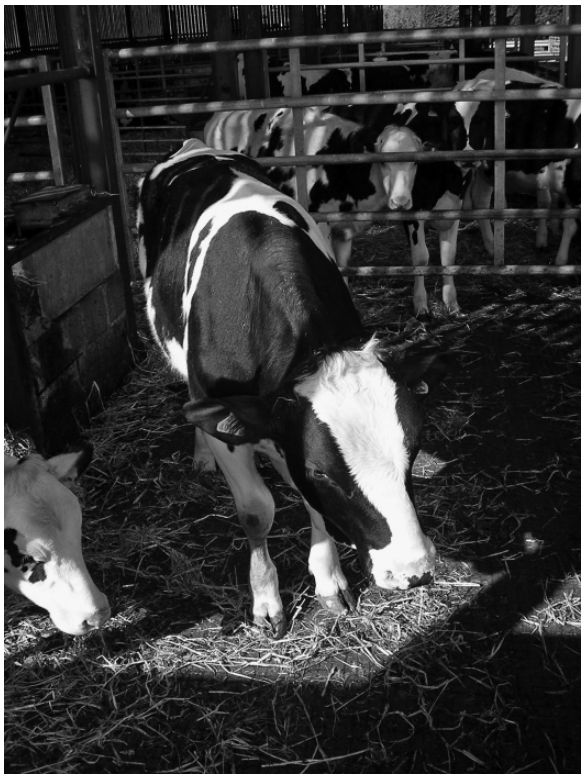

**Fig. 10.7.** Cow with otitis caused by *Mycoplasma bovis*.

mastitis, based on evidence of seroconversion and detection of infection in milk samples.

Two separate cases involving *M. bovis* from calf brains were reported in 2005 in England and Ireland (Ayling *et al*., 2005). In England, calves presented with head tilt and convulsions, suggesting central nervous system (CNS) involvement. One of the affected heifer calves had previously shown signs of head tilt, which appeared to have been treated successfully but after rehousing in the autumn the animal had become ataxic, recumbent and had developed CNS signs including convulsions. Following euthanasia, post-mortem findings included cloudiness and scarring of the left cornea and a purulent meningitis affecting the left side of the cerebellum in the area adjacent to the left middle ear and the ventral part of the brain near the pituitary. *M. bovis*, the only organism isolated, was cultured from the brain close to the left middle ear (Fig. 10.8). Histology indicated a focus of otitis media which had spread to the cranial vault. Serological examination of 13 samples for *M. bovis* showed a range of titres from negative to very high positive samples. Interestingly, the highest titres were from the two affected calves, their dams also being positive.

The Irish case occurred on a farm experiencing outbreaks of respiratory disease. One of the affected and treated calves responded but did not thrive and later developed clinical signs of lethargy, depression, apparent blindness, severe weight loss and was also observed to grind its teeth. Post-mortem examination

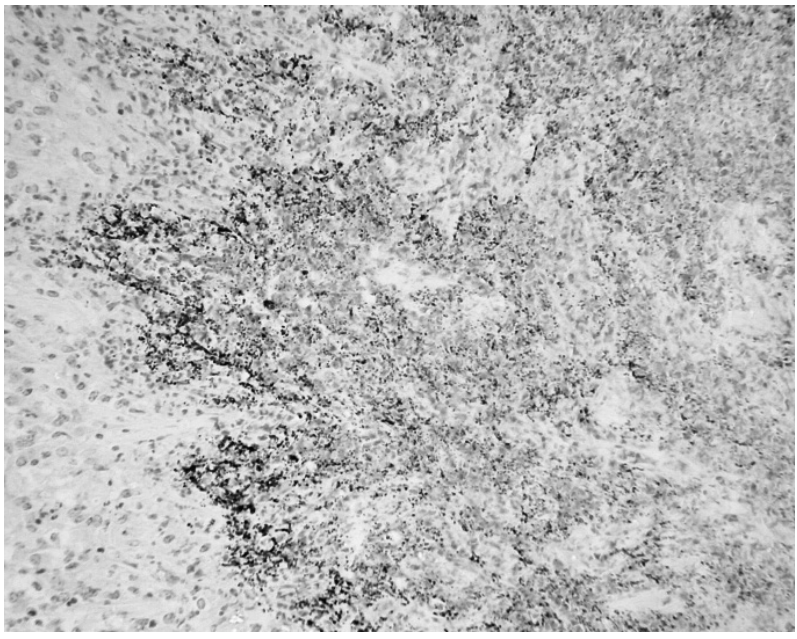

**Fig. 10.8.** Immunoperoxidase technique for *Mycoplasma bovis* antigen showing large numbers of intralesional organisms (dark staining), particularly at the advancing margin of a cerebellopontine abscess in a 7-month-old calf (photo courtesy of Sandra Scholes (VLA) using monoclonal antibody made by Dr Hywell Ball).

revealed a large spheroidal, fibrinous lesion in the heart, which was attached to the wall of the left atrium. There were also multiple areas of necrosis within the cerebral hemispheres of the brain. Only *M. bovis* colonies were isolated from the brain and endocarditis lesions. A second calf showed arthritis in one hock joint but also failed to respond to treatment, resulting in impaired movement and was euthanized. Severe chronic arthritic changes in addition to chronic pneumonic lesions were observed during necropsy examination, from which *M. bovis* was isolated.

These findings suggest an important and possibly under-reported role for *M. bovis* in neurological disease and consideration should be given to its diagnosis when investigating spongiform encephalopathies.

## Diagnosis

Clinical and pathological signs are not always characteristic for *M. bovis,* so laboratory diagnosis is necessary for identification. Samples of choice are listed in Table 10.3. In a study, Thomas *et al.* (2002a) showed that sampling by bronchoalveolar lavage was more predictive of lower respiratory airway pathogens, including *M. bovis*, than nasal swabs, although clearly not as convenient. *M. bovis* grows well in a variety of media, producing 'centred' colonies within 3–5 days (Nicholas and Baker, 1998). In an appropriate medium (such as Eaton's) *M. bovis* produces films and spots and gives an orange colour to the broth. Other biochemical characteristics are shown in Table 10.2 alongside other bovine mycoplasmas. Growth inhibition, film inhibition, fluorescent antibody or metabolic inhibitions tests can be used to identify the mycoplasma using hyperimmune rabbit serum (Poveda and Nicholas, 1998).

Ball and Findlay (1998) described a sandwich ELISA for *M. bovis* in which specific monoclonal antibodies, fixed to the microplate, captured *M. bovis* antigen from the medium; the sensitivity of the test, similar to that of conventional culture diagnosis, can be improved by a short enrichment stage. This test is now available in a commercial format. *M. bovis* can be easily outgrown by

**Table 10.3.** Samples of choice for diagnosis of diseases caused by *M. bovis*.

| Disease | Nasal swabs/ BAL*/affected lung | Milk | Joint fluid/ synovia | Eye swabs | Semen/genital discharge/preputial washings | Serum for antibody detection |
|---|---|---|---|---|---|---|
| Pneumonia | ✓ | | | | | ✓ |
| Mastitis | | ✓ | | | | ✓ |
| Arthritis | | | ✓ | | | ✓ |
| KC** | | | | ✓ | | ✓ |
| Infertility | | | | | ✓ | ✓ |

*bronchial alveolar lavage
**keratoconjunctivitis

opportunistic mycoplasmas like *M. bovirhinis* and acholeplasmas, and occasionally antigenic variability of strains may make serological tests unreliable (Ayling *et al.*, 1997). For these situations, polymerase chain reaction (PCR) tests are very convenient.

The development of new, rapid PCR-based diagnostic tools is also providing a more accurate assessment of the role of mycoplasmas in animal disease. Early PCRs, based on 16S rRNA genes, also amplified *M. agalactiae* DNA (Pfützner and Sachse, 1996; Ayling *et al.*, 1997), but recent PCR developments based on the *uvrC* gene are more specific (Subrahamaniam *et al.*, 1998). PCRs have been used to detect *M. bovis* directly in milk and nasal samples (Hotzel *et al.*, 1996) and even in preservative-treated milk (Pinnow *et al.*, 2001). The denaturing gradient gel electrophoresis (DGGE) test, using PCR of the 16S rRNA gene with mycoplasma-specific primers and separation of the PCR products according to primary sequence on the gel, enables the rapid detection of all 13 bovine species tested, including all mycoplasmas listed in Chapter 2 (McAuliffe *et al.*, 2005). The test can be performed on isolates from culture or directly from clinical samples, often detecting more than one *Mycoplasma* species on a single gel. This detection directly from swabs or tissue precludes the overgrowth of important mycoplasmas by faster-growing opportunists.

Rapid and effective serological diagnosis has been available cheaply for over a decade and has shown that *M. bovis* is involved in 28–35% of pneumonic outbreaks (Ayling *et al.*, 2004). The test has also been used to detect antibodies in *M. bovis* mastitis from either serum or milk, and is capable of identifying infected quarters (Byrne *et al.*, 2000). The ELISA is very useful because the presence of *M. bovis* is nearly always indicative of disease in the herd and is not affected by recent antibiotic treatment, which can hamper isolation of the mycoplasma. The ELISA was used successfully to select *M. bovis*-free cattle following BSE depopulation in Ireland (O'Farrell *et al.*, 2001); its use in maintaining *M. bovis*-free herds by testing newly bought-in animals before introduction to the herd should also be seriously considered by the cattle industry.

Recombinant antigens, consisting of variable surface protein A, expressed in *E. coli* as a fusion protein, were incorporated into an ELISA and were capable of detecting specific *M. bovis* antibodies in naturally infected cattle (Brank *et al.*, 1999), promising more specific diagnostic tests, although to date these are yet to materialize.

Isolation of *M. bovis* from lungs is not always possible, particularly in chronically affected animals; antibiotic treatment, poor lung condition or gross bacterial contamination also inhibit isolation. Here, immunohistochemical techniques, preferably using monoclonal antibodies, may be valuable to visualize the mycoplasma antigens in the affected tissue (Fig. 10.9) (Nicholas *et al.*, 2006; Raedelli *et al.*, 2008).

## Treatment of Disease

Despite Pfützner (1990) warning that diseases due to *M. bovis* were resistant to any chemotherapy, antibiotics are still widely used, sometimes correctly, to reduce

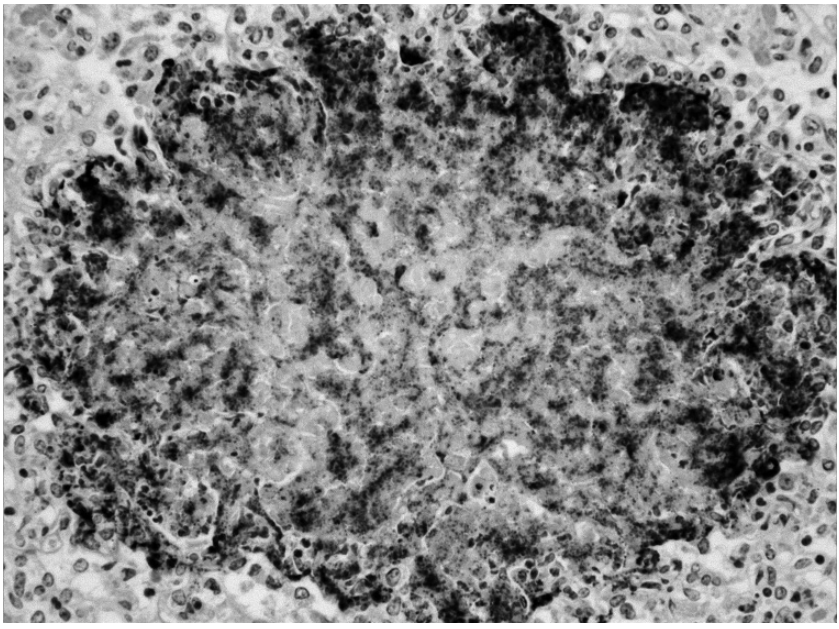

**Fig. 10.9.** Strong staining of *Mycoplasma bovis* antigen in lung of naturally infected calf. (courtesy of F. Twomey and S. Scholes.)

secondary bacterial infections, but often ineffectively to treat the mycoplasma infections. Many *in vitro* studies have compared the susceptibility of *M. bovis* to a range of antibiotics (Table 10.4). While it is clear that antibiotics which are ineffective *in vitro* are unlikely to be effective *in vivo*, those with strong activities *in vitro* will not necessarily perform well in the field, for reasons which are unclear (Ayling *et al.*, 2000). Recent evidence suggests that *M. bovis* strains in Europe and North America are becoming resistant to antibiotics traditionally used for treatment of mycoplasma infections, in particular oxytetracyclines, tilmicosin and spectinomycin (Ayling *et al.*, 2000; Francoz *et al.*, 2005). The fluoroquinolones are still effective but their use in animals is controversial (Nicholas *et al.*, 2000). However, in one study in the UK, monthly treatment by fluoroquinolones for 3 months given at the outset of disease did not prevent respiratory disease in treated calves on a site persistently affected with *M. bovis* (Nicholas *et al.*, 2006). Further field evidence of the ineffectiveness of antibiotics was provided by Haines *et al.* (2001), who found *M. bovis* in the lungs and joints of 80% of cases of feedlot cattle that had failed to respond to antibiotic therapy; bovine viral diarrhoea virus and *M. haemolytica* were found in only 40 and 23%, respectively, of these cases.

Some success against *M. bovis* pneumonia and arthritis in calves was reported by Stipkovits *et al.* (2001) using valnemulin added to the milk from 4 days of age for 3 weeks. Animals in the treated group had fewer clinical signs and reduced clinical scores, although the number of animals with nasal discharge was similar. In spite of this continuous chemotherapy, animals required a considerable number

**Table 10.4.** Effectiveness of antibiotics *in vitro* against field strains of *M. bovis* (minimum inhibitory concentrations, μg/ml).

| Country (source) | No. of strains | Enrofloxacin/ Danofloxacin | Lincomycin | Spectinomycin | Tylosin | Tilmicosin | Oxytetracycline |
|---|---|---|---|---|---|---|---|
| The Netherlands (ter Laak *et al.*, 1993) | 19 | 0.5–2.0/ ND | 0.25–1.00 | 1–4 | 0.06–4.00 | ND | 8–>64 |
| N. Ireland (Ball *et al.*, 1995) | 23 | 1–2/ ND | 0.125–2.000 | 4–6 | ND | 0.06–0.5.00 (9 strains) 4–>32 (14 strains) | ND |
| Italy (Mazzolini *et al.*, 1997) | 23 | 0.06–0.25/ ND | 0.25–1.00 | 0.12–2.00 | 0.12–4.00 | 16–>32 | 0.12–4.00 |
| Britain (Ayling *et al.*, 2000) | 62 | ND/ 0.125–2.000 | ND | 1–8 (50 strains) 128–>128 (12 strains) | ND | 4–<128 | 1–128 |
| (Ayling *et al.*, 2007) | 40 | 0.12–1.00/ 0.12–0.50 | 1–>32 | 2–>64 | ND | <0.06–>64.00 | 0.12–32.00 |

Interpretation (ter Laak *et al.*, 1993):
≤ 1 μg/ml – mycoplasmas susceptible
2–4 μg/ml – mycoplasma show intermediate susceptibility
>8 μg/ml – mycoplasmas resistant
ND – not determined

of individual treatments. The successful treatment of calves with *M. bovis* respiratory disease with the novel microlide tulathromycin (Draxxin) in a challenge model study was described recently despite the MIC values of > 64 μg/ml for isolates, indicating that strains should be resistant (Godinho *et al.*, 2004). Treated calves had lower lung lesion scores and peak rectal temperatures than saline-treated controls, suggesting the treatment reduced secondary invasion of cell-walled bacteria.

The two most important factors in the treatment of mycoplasma pneumonia are early recognition and prolonged therapy. Currin (2007) recommends continuous therapeutic levels of antibiotics for 10–14 days because, without this, 30–70% of the calves will relapse, causing more lung damage, and require further treatment.

The prophylactic use of antibiotics is generally undesirable, but its use may be justified when calves are introduced to a site heavily contaminated with *M. bovis* infection in which high levels of mortality are being sustained. Nagamoto *et al.* (1996) treated one group of introduced calves with leucomycin prior to the development of clinical signs. Untreated groups of calves showed mortality rates of up to 41% while all the calves in the treated group survived, although coughing and nasal discharges were evident. Interestingly, while *M. bovis* antibodies were detected in non-treated groups after 2 months following introduction, antibody development was significantly delayed until 8 months in the antibiotic-treated groups. This suggests that antibody responses are rarely protective in mycoplasma infections.

An alternative but related approach to prophylactic use was proposed by Montgomery (2008), which calls for treatment of calves when clinical disease is seen in 20% of the group sharing a common airspace. This clearly saves time and slows transmission of the mycoplasma as well as reducing lung damage in calves incubating the disease.

Clearly, the more frequent the purchase of replacement animals the more likely is the risk of becoming infected. However, once infected, particularly on multi-age sites, it becomes extremely difficult to eliminate the organism, although the usual husbandry practices such as reducing stocking densities, improving ventilation, separating different age groups and culling any chronically infected animals can help alleviate disease. Wherever possible, consideration should also be given to 'all in, all out' practices to prevent older animals infecting younger ones. If this is not possible, separation of calves from the adults is advisable at the earliest possible opportunity where endemic disease exists and where the particular farm husbandry allows this.

Mastitis caused by *M. bovis* responds poorly to antibiotics, and therefore it is best to segregate or cull carrier cows and to instigate rigid sanitation procedures to prevent transmission from infected to non-infected cows (Pfützner, 1990). Identification of infected cows in the early stages of mastitis may be detected conveniently using the indirect ELISA for specific antibody in the milk; moreover, the test can identify the infected quarter of an individual cow (Byrne *et al.*, 2000). Some success has been seen with treatment for arthritis, although the earlier the treatment begins following detection of *M. bovis*, the more successful the treatment is likely to be (Nicholas, 2002a; Houlihan *et al.*, 2007).

## Disease Prevention and Control

The inability of chemotherapy to control *M. bovis* infections has focused attention on vaccination. Surprisingly there are no vaccines currently available in Europe, although a quadrivalent inactivated vaccine containing respiratory syncytial virus, parainfluenza type 3 and 2 mycoplasmas, *M. dispar* and *M. bovis* was developed and showed some protection against respiratory disease in the field (Howard *et al.*, 1987). A vaccine prepared with formalin-inactivated strains of *M. bovis* and *M. haemolytica* taken from the target herd reduced losses from pneumonia and cost of treatment in newly introduced feedlot calves (Urbaneck *et al.*, 2000). More recently, a saponized-inactivated vaccine was shown to be safe, highly immunogenic and protective against a strong experimental challenge of virulent *M. bovis* (Nicholas *et al.*, 2002). Vaccinated calves showed few respiratory signs while all unvaccinated calves developed signs of pneumonia. There was a statistically significant decrease in body weight gain in unvaccinated calves compared with vaccinates and a significant increase in lung lesions and rectal temperatures in unvaccinated calves. The vaccine also reduced the spread of *M. bovis* to internal organs, including the joints. However, other attempts to vaccinate against *M. bovis* have been less successful. Bryson *et al.* (2002) enhanced the severity of pneumonia in calves using Triton X-114 membrane protein extracts and mixtures of affinity-purified antigens. Vaccination against an experimental infection using an arthritis model was also not successful, although high levels of antibody were detected before challenge (Poumarat *et al.*, 1999). Experimental vaccines against mastitis have not been successful and indeed may make the condition worse (Ross, 1993).

While a variety of commercial vaccines, including autogenous preparations, are available in the USA (although few data exist to assess their effectiveness), they are not licensed for use outside the USA. Experimental work has shown that a vaccine inactivated with saponin can protect in the face of a large mycoplasma challenge (Nicholas *et al.*, 2002). A saponized autogenous vaccine prepared using this technology was recently trialled in the UK on two farms, one presenting pneumonia in bought-in calves (Nicholas *et al.*, 2006) and the other with mastitis and arthritis. Some improvement in the clinical condition was seen in vaccinated calves compared with previous batches but investigations revealed that a third of incoming calves were already infected with *M. bovis*, including some showing clinical signs. Ideally the vaccine should be used in young calves before they are exposed to *M. bovis* and kept isolated until vaccine immunity develops. Vaccination of dams may also be possible and studies need to be addressed at determining whether passive immunity is protective for *M. bovis.* However, on the second farm presenting arthritis and mastitis the use of a vaccine greatly reduced culling rates (Nicholas *et al.*, 2006). Further controlled work is necessary to confirm these findings.

Work carried out in the 1990s at the Institute of Animal Health, Compton, UK and the former Institut fur Bakterielle Tierseuchenforschung, Jena, Germany, clearly demonstrated a major pathogenic role for *M. bovis* in respiratory disease, arthritis and mastitis. Despite this there is still a pervasive view amongst many veterinarians that the role of *M. bovis* in the respiratory disease complex is not

proven (Step and Kirkpatrick, 2001). A major study in the UK on the causes of mastitis in dairy cattle failed to look for *M. bovis* (Bradley *et al*., 2007). Its importance has been shown conclusively following its introduction into both Northern and southern Ireland in the early 1990s, where it rapidly became a major cause of respiratory disease as well as a frequent cause of mastitis and arthritis. Major outbreaks have also been seen in Israel and Greece following importation of affected cattle from Western and Central Europe. The mycoplasma should be recognized by the Office International des Epizooties because of its serious socio-economic effects and the impact it has on international trade. In view of the large amount of evidence showing the ineffectiveness of antibiotics to control respiratory disease caused by *M. bovis*, research efforts should now be directed towards developing effective vaccines which could be used with or incorporated into those already existing for the bacterial and viral respiratory pathogens.

# OTHER BOVINE MYCOPLASMOSES

Most of the important mycoplasmas isolated from cattle are shown in Table 10.5.

## *Mycoplasma dispar*

*M. dispar*, first characterized by Gourlay and Leach (1970), is a frequent isolate from the lungs and nasal cavities of both healthy and pneumonic dairy and

**Table 10.5.** Important mycoplasmas of adult cattle.

| Species | Disease(s) | Distribution |
|---|---|---|
| *M. bovis* | Pneumonia, mastitis, arthritis, IKC, otitis, meningitis, abortion | Worldwide |
| *M. m. mycoides* SC | Contagious bovine pleuropneumonia | Africa |
| *M. dispar* | Pneumonia | Europe, N. America |
| *M. canis* | Pneumonia | Europe, N. America |
| *M. californicum* | Mastitis, pneumonia | Europe, USA |
| *M. bovigenitalium* | Endometritis 'whites', infertility, semen abnormalities | Europe, N. Africa, USA |
| *Ureaplasma* spp. | Granular vulvitis, endometritis, pneumonia | Worldwide |
| *M. alkalescens* | Unknown, possibly pneumonia | Europe, N. America |
| *M. canadense* | Unknown | Europe, N. America |
| *M. bovirhinis* | Probably harmless, may exacerbate pneumonia | Worldwide |
| *M. bovoculi* | Infectious keratoconjunctivitis | Europe, N. America |
| *M. alvi* | Unknown | Europe, N. America |
| *M. verecundum* | Unknown | Europe, N. America |
| *M.* bovine group 7 | Mastitis/arthritis | Australia, Switzerland |

fattening calves. It has been isolated from most parts of the world where cattle are kept, including Europe, Australia, the USA, Canada, Korea and Japan. In Europe there have been reports from the UK, Denmark, Belgium, Holland and France (Nicholas, 2002b). It has been shown to cause pneumonia following experimental infection, although the disease produced is not severe; it has, occasionally, been reported in cases of mastitis (Jasper, 1981). It is certainly under-reported because of its extreme fastidiousness in *in vitro* culture.

It is not possible to determine the significance of *M. dispar* infections as it is rarely the sole cause of disease. Viruses, bacteria and other mycoplasmas are major contributors to calf pneumonia, which has been estimated to cost the UK up to £50 million per year (Rebhun *et al.*, 1995) in terms of set-back costs, calf mortality and treatment; in Europe this figure may exceed 560 million euros (Reeve-Johnson, 1999).

Like all mycoplasmas, *M. dispar* is small with a genome size of 803 kbp (Hermann, 1992), pleomorphic, lacks a cell wall and has a low G+C ratio (28.5–29.3 mol%). However, it is very fastidious in culture, requiring a specialist medium for growth and does not produce typical 'fried egg' colonies, particularly in early passages of fresh isolates. Furthermore these are not always inhibited by hyperimmune serum, making conventional identification difficult in the early stages (ter Laak and Noordergraaf, 1987). *M. dispar* grows slowly in media, requiring 7–14 days to produce colonies. It ferments glucose and reduces tetrazolium chloride under aerobic and anaerobic conditions but does not hydrolyse arginine, digest serum or possess phosphatase activity (Table 10.2).

*M. dispar* has an external capsule composed of a polysaccharide identified as a polymer of galacturonic acid and which can be stained by ruthenium red and polycationic ferritin and observed by electron microscopy (Almeida and Rosenbusch, 1991). It appears to be produced during infection in response to mammalian host cells and may exert an inhibitory effect on the activity of bovine alveolar macrophages by preventing their activation; however, non-encapsulated mycoplasmas may also have an inhibitory effect on neutrophils, so the role of these polysaccharides is unclear (Howard *et al.*, 1980). Antibodies against this capsule are reported to be necessary to enable killing of the mycoplasma by alveolar macrophages (Almeida and Rosenbusch, 1991).

Phylogenetically, *M. dispar* has been assigned to the hominis group on the basis of its 16S rRNA sequence and, within, this close to the *M. neurolyticum* cluster.

*M. dispar* is found widely in the healthy bovine respiratory tract, even in the lower tract, without causing disease. In studies by ter Laak *et al.* (1992a,b) in the Netherlands, *M. dispar* was isolated from 92% of pneumonic lungs from 148 calves and from only 40% of healthy lungs from 270 calves. In Denmark, Tegtmeier *et al.* (1999) isolated *M. dispar* from 13 of 31 lungs showing fibrino-necrotizing bronchopneumonia, 15 of 31 lungs with supperative bronchopneumonia and from 3 of 31 lungs with embolic pneumonia; in all cases other bacteria, including *H. somni*, *P. multicoda*, *M. haemolytica* and *A. pyogenes*, were also present. In Canada, *M. dispar* was isolated from just under half of over 300 pneumonic calf lungs (van Donkersgoed *et al.*, 1993). Results from a study of the development of respiratory disease in dairy calves indicated a possible initiating role for *M. dispar* leading to

invasion of *P. multicoda* (Virtala *et al.*, 1996). In the UK, *M. dispar* is detected frequently from pneumonic calves and was believed to be the cause of a severe pleuropneumonia similar to CBPP in adult cattle which resulted in several deaths, thus requiring differential tests to be readily available (Ayling *et al.*, 2004).

*M. dispar* colonizes the epithelial lining of the respiratory tract, where it may be ciliostatic and even cytopathic to cells of the distal bronchi and bronchioles, leading to severely reduced tracheobronchial clearance of bacteria (Almeida and Rosenbusch, 1991). Immunosuppressive effects have been shown on the response of bovine lymphocytes to mitogens and mixed leucocytes *in vivo* (Nicholas, 2002b).

*M. dispar* causes purple to red consolidation mainly in the cranioventral areas of the lung (Ross, 1993). In experiments with gnotobiotic calves *M. dispar* caused a mild subclinical bronchiolitis with lymphoid cuffing (Howard *et al.*, 1980). Other authors report *M. dispar* causing an alveolitis in which neutrophils, macrophages and oedema fluid accumulated in the alveolar walls and spaces (Gourlay *et al.*,1979; Friis, 1980). Field cases of subclinical pneumonia from which *M. dispar* has been isolated have similar lesions (St George *et al.*, 1973).

Clinical or pathological signs are not characteristic for *M. dispar*, so laboratory diagnosis is necessary for diagnosis. Isolation of *M. dispar* from the dead calf is best achieved by aseptically taking tissue from the interface between affected and non-affected lung and isolating the mycoplasma. Serial dilutions of the lung homogenate should be made to at least $10^{-3}$ in a medium containing a bactericide like thallium acetate and appropriate antibiotics. Aliquots of the homogenate should be inoculated in broth and solid medium. From the living animal, samples should be taken from nasal secretions or ideally from lower respiratory secretions by bronchial–alveolar lavage. A number of medium formulations have been reported which support the growth of this mycoplasma (Gourlay and Leach, 1970; Nicholas and Baker, 1998). A commercial medium is also available from Mycoplasma Experience (Reigate, UK), in which the mycoplasma is reported to grow well.

Identification of *M. dispar* can be achieved by growth or metabolic inhibition or immunofluorescence using hyperimmune serum prepared in rabbits. Ter Laak and Noordergraaf (1987) described an immunoperoxidase test which could be conveniently carried out on fresh isolates on nitrocellulose membranes. Formalin-fixed tissue can also be examined by immunocytochemistry or immunofluorescence using hyperimmune antiserum or preferably monoclonal antibodies.

The introduction of PCR has greatly facilitated detection of this fastidious mycoplasma, although it was not until 2004 that a PCR specific for *M. dispar* was reported (Miles *et al.*, 2005). In this reliable and sensitive test, specific and universal oligonucleotides were used in combination to detect the presence of single polymorphisms within the 16S ribosomal DNA sequence. The presence of mycoplasma 16S rDNA was indicated by the production of a single control fragment while *M. dispar* samples generated an additional smaller, specific product over the same region; a similar test was developed for *M. bovirhinis*, which is generally believed to be avirulent but may confound diagnosis because of overgrowth of more important pathogens like *M. dispar*. Alternatively, McAuliffe *et al.* (2003, 2005) reported a PCR/DGGE technique which detects and identifies over 70 different mycoplasmas, including *M. dispar*.

Serological tests are not widely available for *M. dispar*, although single radial haemolysis, enzyme immunoassays (Howard, 1983) and passive haemagglutination (Martin *et al.*, 1989, 1990) have been reported in the past. It has been reported that antibodies to *M. dispar* are not very prevalent in cattle, possibly because the mycoplasma is restricted to the lung surface (Howard, 1983); however, this may be more a reflection of the poorly sensitive serological test used than the humoral response of the animal. ELISAs have not been reported upon, which may be due partly to the lack of awareness of this mycoplasma as well as to the difficulty in growing sufficiently large quantities of antigen needed in this test.

Calf pneumonias are usually treated with antibiotics, not all of which are effective for those pneumonias caused or exacerbated by mycoplasmas. Two studies carried out on the *in vitro* susceptibility of *M. dispar* to antibiotics showed that oxytetracyclines (often used for mycoplasma infections) were ineffective (ter Laak *et al.*, 1993; Reeve-Johnson, 1999). Tilmicosin, tylosin and enrofloxacin, on the other hand, were effective. Friis and Szancer (1994) also showed the potency of enrofloxacin for *M. dispar*.

Awareness of the importance of this mycoplasma in respiratory disease was sufficient in the 1980s for it to be included as a component of a multivalent vaccine produced at the Institute of Animal Health at Compton, UK, and incorporating respiratory syncytial virus, parainfluenza type 3 virus, *M. bovis* and *M. dispar*, showing some levels of protective immunity (Howard *et al.*, 1987). This vaccine was never widely available.

As there are no widely available serological tests for *M. dispar* it is not easy (although advisable) to prevent the introduction of *M. dispar* with affected calves. Cultural or PCR detection of *M. dispar* is not practical or effective. Control of calf pneumonia (including those caused by mycoplasmas) should include measures to reduce environmental stress and to ensure adequate housing with good circulation of air. Wherever possible consideration should be given to 'all in, all out' practices to prevent older animals infecting younger ones. If this is not possible separation of calves from the adults is advisable at the earliest possible opportunity. Little is known of the persistence of *M. dispar* in the environment but transmission is mostly by close and repeated contact.

## *Mycoplasma bovirhinis*

*M. bovirhinis* was first reported in 1967 from the upper respiratory tract of cattle (Leach, 1967). Since then it has been found in the upper and lower respiratory tracts of both healthy and diseased cattle and buffaloes all over the world. It is not believed to be a primary pathogen but may exacerbate existing disease conditions caused by other pathogens, including *M. bovis* or *M. dispar*. Its main significance may be that of obscuring or overgrowing more fastidious mycoplasma pathogens found in the same tissues, as it grows rapidly in most media. In a study of the mycoplasma populations in the respiratory tract of calves, ter Laak *et al.* (1992a,b) found *M. bovirhinis* to be present in 66% of healthy calves and 88% of pneumonic calves, being isolated equally in the upper and lower respiratory tract of diseased animals. Many other studies have shown it to be

widespread in the respiratory tract of pneumonic cattle (Tegtmeier *et al.*, 1999; Nicholas *et al.*, 2001).

This is very little evidence to indicate that *M. bovirhinis* is capable of initiating disease; it is often associated with infections caused by *M. bovis, M. haemolytica*, *P. multocida*, *H. somni* or viruses such as respiratory syncytial virus, infectious bovine rhinotracheitis or parainfluenza virus 3. Experimental infections of *M. bovirhinis* were not able to successfully reproduce pneumonia in gnotobiotic calves (Gourlay *et al.*, 1979). However, evidence to suggest that *M. bovirhinis* may have some opportunistic role was presented by Komoda *et al.* (1988), who showed that significant increases in its isolation were seen following sampling of pneumonic calves later in the disease process compared with samples taken before clinical signs were apparent. Gourlay *et al.* (1979) reported that *M. bovirhinis* was pathogenic for the mammary tissue, although its role in mastitis has never been considered.

In addition to the respiratory tract, *M. bovirhinis* has been isolated from the preputial washings and sperm samples of bulls in Turkey (Ozdemir and Turkarlsan, 1998), the vaginal mucus of cows in Brazil (Nascimento *et al.*, 1998), the milk of cattle and buffaloes with and without mastitis (El Shabiny, 1994), joints from cattle with arthritis (Reeve-Johnson, 1999), conjunctival swabs from cattle with ocular lesions in Croatia (Naglic *et al.*, 1996) and the kidneys of a bull with urinary obstruction and subacute nephritis (Panagala *et al.*, 1990). Immunohistochemical evidence to enable a link between the mycoplasma and tissue damage is lacking from these studies.

Like all mycoplasmas, *M. bovirhinis* is small, with a genome size of 955 kbp, pleomorphic, lacks a cell wall and has a low G+C ratio (24.5–27.3 mol%) (Hermann, 1992). *M. bovirhinis* grows rapidly in most mycoplasma media and ferments glucose, producing large quantities of acid. It does not hydrolyse arginine, possess phosphatase activity or digest serum but reduces tetrazolium chloride under aerobic and anaerobic conditions (Table 10.2). Phylogenetically, *M. bovirhinis* has been assigned to the hominis group on the basis of its 16S rRNA sequence and, within this, to the *M. synoviae* cluster.

No clinical or pathological signs have been reported for *M. bovirhinis* infections, so laboratory diagnosis is necessary for identification. Isolation of *M. bovirhinis* from the upper and lower respiratory tracts, eyes, joints and reproductive system is easily carried out in most mycoplasma media (Nicholas and Baker, 1998). It grows rapidly within 2–3 days, producing small, 'centred' colonies and can be identified by immunological techniques using hyperimmune rabbit serum (Poveda and Nicholas, 1998). A filter immunobinding assay has been described for detecting and identifying mycoplasmas including *M. bovirhinis* and can detect about $10^4$–$10^5$ colony-forming units (CFU)/ml (Takahata *et al.*, 1997). PCR is not thought necessary to aid identification but a PCR based on the 16S ribosomal RNA gene was described which can detect as few as $10^3$ cfu/ml in pure culture and spiked clinical material (Kobayashi *et al.*, 1998). Miles *et al.* (2005) developed a reliable and more sensitive test where specific and universal oligonucleotides were used in combination to detect the presence of single polymorphisms within the 16S ribosomal DNA sequence. The presence of mycoplasma 16S rDNA was indicated by the production of a single control fragment, while *M. bovirhinis* DNA generated a smaller, specific product over the same region. Alternatively,

McAuliffe *et al.* (2003, 2005) reported a PCR/DGGE technique which detects and identifies over 70 different mycoplasmas, including *M. bovirhinis*. Serological tests are not generally available for antibody detection but could be useful to determine whether *M. bovirhinis* is behaving invasively or merely as a commensal.

Despite the fact that *M. bovirhinis* is not considered pathogenic, a number of antibiotic studies have been carried out *in vitro* and have shown it to be sensitive to most antimicrobials, including enrofloxacin, perfloxacin, erythromycin and lincospectin (Eissa *et al.*, 1999) but resistant to thiamphenicol and oxytetracycline (Katoh *et al.*, 1996). It is not practical, possible or necessary to exclude *M. bovirhinis* from herds as it appears to be present in the respiratory tract of most healthy calves in commercial units.

## *Mycoplasma alkalescens*

*M. alkalescens* was first isolated from the nasal cavity of cattle in Australia in 1963 (Hudson and Etheridge, 1963) and proposed as a new species 10 years later (Leach, 1973). It was originally thought to be a rare cause of arthritis (Whithear, 1983) but has also been associated with cases of otitis (Lamm *et al.*, 2004), mastitis and respiratory disease, often in association with *M. bovis* (Manfrin *et al.*, 1998; Thomas *et al.*, 2002b). Arthritis was induced in calves experimentally infected with an isolate of the mycoplasma (Bennett and Jasper, 1978). In a review of *Mycoplasma* species isolated from ruminants in the UK only two isolates of the mycoplasma were reported between 1990 and 2000 (Ayling *et al.*, 2004). However, since then a major increase in detections has been seen, with 34 positive samples in 2004 and 46 in 2005, from lung, joint fluid, milk, fetal stomach contents, eyes, vaginal swabs and thoracic fluid (Table 10.6) (Lawes *et al.*, 2006). Increases have also been seen in other parts of the world, including Denmark and Israel, where it was isolated from the respiratory tract of pneumonic calves

**Table 10.6.** Isolations of *M. canis* and *M. alkalescens* from cattle in the UK.

| Year | *M. canis* | *M. alkalescens* |
|---|---|---|
| 1994 | 0 | 0 |
| 1995 | 1 | 1 |
| 1996 | 2 | 0 |
| 1997 | 11 | 2 |
| 1998 | 13 | 1 |
| 1999 | 25 | 1 |
| 2000 | 5 | 0 |
| 2001 | 5 | 5 |
| 2002 | 7 | 0 |
| 2003 | 6 | 6 |
| 2004 | 18 | 35 |
| 2005 | 3 | 46 |
| 2006 | 2 | 52 |
| 2007 | 2 | 49 |

in 2007 (Kokotovic *et al.*, 2007) and 2008 (Lysnyansky *et al.*, 2008). Immunohistochemical studies for *M. alkalescens* performed on fixed lung samples from these cases showed good agreement with mycoplasma isolation. In a number of cases, *M. alkalescens* was identified in alveolar exudates and in the cytoplasm of macrophages. The isolation of *M. alkalescens* as the single pathogen, together with localization of *M. alkalescens* antigen in pneumonic lesions of clinical samples, strongly suggests a contributory role in the disease.

An increase in the prevalence of *M. alkalescens* is possibly the result of the use of a more sensitive and discriminatory test in the UK: the PCR/DGGE (Lawes *et al.*, 2006). The culture method of identification used before this favoured rapidly growing mycoplasmas, and therefore fastidious species may have been underreported. This could be the case for *M. alkalescens*, which was possibly outgrown by *M. bovis* and *M. bovirhinis*, resulting in a lower isolation rate. Comparison of the 16S rDNA sequences of *Mycoplasma* species shows that *M. alkalescens* is closely related to other arginine-hydrolysing mycoplasmas such as *M. arginini* and *M. canadense*. An advantage of DGGE is that mixed cultures can be identified because DNA from all *Mycoplasma* species present will be amplified. Alternatively, the prevalence of *M. alkalescens* could be increasing in real terms in the UK cattle population and may be developing into an important cause of disease. The increases in isolation rate seen in the UK could be the result of restocking with imported cattle from mainland Europe following the foot-and-mouth outbreaks in 2001.

## *Mycoplasma canis*

In 1993, ter Laak reported the isolation of *M. canis* from pneumonic calves in the Netherlands. *M. canis* had also been isolated from cattle in Canada. In 1995 the first isolation of *M. canis* was reported from pneumonic cattle in Britain (Nicholas *et al.*, 1995). Two further isolations were made in 1996 from similar conditions. In 1997 and 1998 there were small increases, but in 1999, however, 25 isolations of *M. canis* were seen in cases of calf pneumonia (Table 10.7). Many outbreaks involved animals below the age of 5 months, with some mortality. Most involve other known pathogens such as *M. haemolytica, P. multicoda, H. somni*, RSV or *M. bovis*. However, in a number of cases, no other pathogens were implicated (Table 10.7).

During experimental infections, all calves inoculated by aerosol with *M. canis* showed clinical signs of pneumonia, including two deaths within 2 weeks of infection (Nicholas *et al.*, 2000). Post-mortem examination revealed lung lesions consistent with pneumonia in all animals. *M. canis* was recovered from the lungs with characteristic mycoplasma lesions, including congested apical and median lobes; mycoplasma was also isolated from the liver, blood and kidneys. There was, however, evidence of secondary infection with *M. bovis* and *Pasteurella* but no viral involvement.

The increasing reports of *M. canis*, although relatively small, represent a real increase rather than greater surveillance because the mycoplasma grows readily in most media. So far four countries (Canada 1974, Netherlands 1993, the UK 1995, Belgium 2002) have reported its isolation and there may be others which

**Table 10.7.** Disease data from herds where *M. canis* was isolated.

| Date | Disease | Age (months) | Number affected | Other findings |
|---|---|---|---|---|
| 1/95 | Pneumonia | 3–5 | 3/10* | *H. somni* |
| 4/97 | Pneumonia | 1–5 | 2/10* | None |
| 5/97 | Pneumonia | 2 | 3/20 | None |
| 7/97 | Pneumonia | 1–2 | 1/? | *Clostridium perfringens* |
| 7/97 | Pneumonia | 4–5 | 10/20 | RSV |
| 10/97 | Pneumonia | <6 | 150/150 | RSV/*Pm*/*M. bovis* |
| 11/97 | Pneumonia | 2 | ~20 | None |
| 11/97 | Pneumonia | 3 | 30/100* | None |
| 11/97 | Pneumonia | 3 | not known | *H. somni*/*Mh* |
| 12/97 | Pneumonia | 4 | 5/10 | Mh |
| 12/97 | Pneumonia | 2 | 1 | *Staphylococcus typhimurium*/*Pm* |
| 3/98 | Pneumonia | 3 | 2/10* | None |
| 3/98 | Pneumonia | 5 | 2/10 | *M. bovis*/*H. somni* |

*mortality seen
*Mh – M. haemolytica*
*Pm – P. multicoda*

have not yet considered the organism significant. It is possible that surveillance has not been extended to this mycoplasma or that it has been misidentified as other glucose fermenters such as *M. bovirhinis*. Biochemically the two are similar, apart from the inability of *M. canis* to reduce tetrazolium salts, but immunologically they are quite distinct (Table 10.2).

The occurrence of *M. canis* in cattle may have originally been due to close contact with dogs, in which the organism was first isolated. However, the widespread distribution of *M. canis* in the UK suggests that the organism should now be considered part of the mycoplasma flora of cattle and there is good evidence to suggest involvement in respiratory disease.

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# 11 Respiratory Diseases of Small Ruminants

*The evidence from abbatoir surveys... suggest that we may be underestimating the effect that pneumonic diseases have on our sheep flocks. Pneumonic lesions are seen at necropsy in sheep of all ages and clinical signs are frequently evident when flocks are driven.* (University of Sydney, 2005)

## OVERVIEW

Respiratory disease in small ruminants is responsible for enormous financial losses worldwide, estimated at over one billion dollars in the USA. It may result in sudden death or in a protracted illness causing much suffering to affected animals. In addition to death and sickness, reduced feed efficiency, slaughter condemnations, prevention and treatment measures contribute substantially to losses. It is believed by some that this condition is dominated by pneumonic pasteurellosis caused by *Mannheimia haemolytica*, particularly in newborn and feedlot lambs as well as in mature ewes. Goats, on the other hand, appear susceptible to a wider range of respiratory diseases, many of them caused by infectious agents including mycoplasmas; the role of viruses, however, is largely unknown. It is clear with few exceptions that the aetiology of respiratory disease is multifactorial and complex.

The most common cause of pneumonia, particularly in sheep, is pasteurellosis, which occurs worldwide. Although first described in 1961, pneumonic pasteurellosis became unravelled only with the introduction of serotyping and biotyping in the 1960s, leading, to date, with the recognition of two morphologically identical species, *M. haemolytica* and *Pasteurella trehalosi*, comprising a total of 17 serotypes. *M. haemolytica* (A biotypes) strains are responsible for disease in sheep of all ages, while *P. trehalosi* (T biotypes) strains cause a systemic disease in 6–10-month lambs, often resulting in sudden death. Serotype A2 is most

frequently found in both pneumonic sheep and goats, with A1, A6, A7 and A9 being restricted to sheep. Outbreaks of disease are most common in the summer with a second peak in late autumn. Secondary infections leading to chronic abscess formation are common. The A biotypes are carried in the nasopharynx and many factors such as stress, dipping, castration, transport and other infections, including PI3 and mycoplasmoses, may predispose animals to outbreaks of pneumonia.

Other bacteria which are occasionally isolated from pneumonic small ruminants include *Actinomyces pyogenes*, staphlococcal spp., streptococcal spp., *Haemophilus* spp. and *Klebsiella pneumoniae*. These bacteria are rarely primary causes of disease but may exacerbate an existing condition.

Infection with parainfluenza virus type 3 (PI3) mainly causes a mild respiratory disease, sometimes without clinical signs, but occasionally acute infections can result in high morbidity in lambs under 1 year old. Mainly a threat to livestock in West Africa, Asia and the Middle East, the OIE B-listed peste des petits ruminants (PPR), caused by a paramyxovirus, shows a range of clinical signs, including necrotic stomatitis, foaming at the mouth and ocular discharge; respiratory signs are seen during the acute phase, when a mucopurulent nasal discharge, frequent sneezing and increased respiratory rate accompanied by extended head and mouth breathing are seen. The goats may die several weeks later of a secondary bronchopneumonia or sooner if co-infected with contagious caprine pleuropneumonia. Some goats infected with caprine arthritis encepahalitis (CAE) virus, a lentivirus of the Retroviridae family, develop a progressive interstitial pneumonia characterized by a chronic dry cough and weight loss. This 'slow' disease is very similar to that caused by another lentivirus, Maedi-visna, which is widespread throughout the world except Australia and New Zealand. The two viruses are genetically but not serologically distinct.

Husk, or parastic bronchitis, caused by the adult lungworm *Dictyocaulus filaria,* is seen in young sheep and goats, where it causes coughing, tachypnoea and reduced growth rates. Although not as important as its equivalent in cattle, husk is associated with just under 4% of ovine pneumonias in the UK, appearing late in summer and early autumn.

Goats are highly susceptible to a number of mycoplasmas, including the highly pathogenic *M. capricolum* subsp. *capripneumoniae*, the cause of the OIE B-listed contagious caprine pleuropneumonia (CCPP) (Chapter 9, this volume). Mortality and morbidity rates may exceed 80% in naive herds, with animals dying within days of infection. The disease is present in the Middle East, North and East Africa and Asia but is almost certainly under-reported because of the difficulty in isolating and culturing the mycoplasma. It has been isolated from the respiratory tracts of pneumonic and healthy sheep, suggesting a role for this species as a carrier. *M. ovipneumoniae* is often isolated with *M. c. capripneumoniae*, which can often result in the overgrowth of the latter; early cloning of the two morphologically distinct mycoplasmas may overcome this, as does the use of the PCR directly on lung tissue. *M. ovipneumoniae* is a cause of a non-progressive (atypical) pneumonia in its own right, which may then enable the invasion of *M. haemolytica* with far more serious results. Interestingly, reproduction of atypical pneumonia is far more successful with infected tissue homogenates than with isolated cultures of

*M. ovipneumoniae*. In recent years the term CCPP has been restricted to infections caused by *M. c. capripneumoniae*; however, some workers still refer to infections caused by the very closely related *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC as CCPP. Classical CCPP is highly contagious in goats, and its lesions are severe with fibrinous or fibrinonecrotic pneumonia giving a granular appearance when the lung is cut. The enlargement of the interlobular septa, a feature of *M. mycoides* LC infection, is rarely seen in classical CCPP. *M. mycoides* LC is much more widely distributed, occuring in France and countries bordering the Mediterranean, Asia and America, where it may be seen in goats suffering from contagious agalactia. Pneumonia and/or contagious agalactia are also a feature of infections with *M. capricolum* subsp. *capricolum*, which can be seen sporadically in goats in Europe and in sheep and goats in North Africa. Many other mycoplasmas are occasionally isolated from the lungs of small ruminants, in particular *M. arginini*, but there is little evidence to connect them primarily with respiratory disease. *M. mycoides* subsp. *mycoides* SC, the cause of contagious bovine pleuropneumonia, is occasionally isolated from goats with pneumonia but rarely from sheep.

# ATYPICAL PNEUMONIA OF SHEEP AND GOATS

## Introduction

Respiratory disease in sheep and goats may result in sudden death or in protracted illness, causing suffering to affected animals as well as high financial losses to owners. In addition to death and sickness, reduced feed efficiency, slaughter condemnations, prevention and treatment measures contribute substantially to losses. *M. haemolytica* is the most obvious cause of pneumonic pasteurellosis but the role of mycoplasmas, in particular *M. ovipneumoniae*, is often overlooked. As well as causing disease in its own right, *M. ovipneumoniae* predisposes animals to pasteurellosis and viral infections (Ayling and Nicholas, 2007). Sheep are generally less susceptible to mycoplasmas than goats but a reassessment of the mycoplasma flora of sheep is necessary, particularly in the Middle East, where mixed flocks of small ruminants are kept (Al-Momani *et al*., 2006a).

## Epidemiology and Transmission

*M. ovipneumoniae* is mainly transmitted via the respiratory route by close and repeated contact. Mycoplasmas frequently occur in the upper respiratory tract of healthy sheep, which may act as a major source of infection to lambs. Lambs are thought to become infected within a few days of birth but disease is slowly progressive and often occurs with secondary bacterial infections from 5 to 10 weeks of age. The pneumonia may be severe, but some lambs recover within a few weeks whilst it persists in other lambs for much longer. Outbreaks can occur when groups of lambs from different sources are housed together and may be a result of mixing uninfected lambs with infected lambs or possibly the effect of encountering different strains of *M. ovipneumoniae.* High summer temperatures may be a

precipitating factor in exacerbating a mild condition and principally involves *M. ovipneumoniae* with *Actinobacillus pleuropneumoniae* biovar A (Hervas *et al.*, 1996).

Extensive heterogeneity has been shown amongst *M. ovipneumoniae* strains by DNA and protein analysis, revealing 58 different profiles in 60 isolates (Mew *et al.*, 1985; Ionas *et al.*, 1991). Molecular typing with RAPD and PFGE of 43 different strains in the UK also showed heterogeneity, with 40 different profiles including different profiles within the same flocks (Miles *et al.*, 2006). No link could be made between strains and geographic origin, reflecting extensive animal movements within the UK. The same work also confirmed the highly variable protein expression seen within *M. ovipneumoniae* strains. The observed heterogeneity between this panel of isolates, which were all associated with disease, may represent cell lineages resulting from a much larger population of non-pathogenic precursor clones. Once strains have become established within a host, close contact and animal movement facilitate their spread. This potentially results in a mixture of strains being present on the same farm, or even co-existing within the same host. The presence of such wide variation results in a large gene pool, allowing the population as a whole to respond to changes in its environment, facilitating its survival. Under specific selection pressures it may be expected that the numbers of those isolates best suited to the new conditions will be elevated significantly (Miles *et al.*, 2006).

*M. ovipneumoniae* infections lead to a variable pattern of morbidity and generally cause low mortality. The observed differences might be a result of the existence of variable strain virulence, host response or co-infecting organisms. Bacterial pneumonia in sheep is multifactorial, resulting in conditions that vary in severity and may be chronic or acute. It is believed that primary infection by *M. ovipneumoniae* exacerbates clinical signs resulting from the colonization of tissue by other microorganisms.

Early investigations (Ionas *et al.*, 1991) have indicated that the presence of multiple *M. ovipneumoniae* strains within the same flock leads to more severe disease signs. Although horizontal transfer and recombination of DNA between strains have not been shown, it has been suggested that the unique combination of virulence genes required for successful colonization might only be obtained from a mixture of strains acting together. Such cooperation has been observed for other bacterial species where cells within colonies show spatial organization and cellular differentiation (Shapiro, 1998).

There are few reports of *M. ovipneumoniae* as a cause of severe respiratory disease in goats. In the UK high levels of morbidity, up to 20%, were seen, in which coughing, dyspnoea, pyrexia, inappetance and profound depression were widespread in home-bred goats on a farm where there was sudden mixing with goats from France (Nicholas, 2000). Lesions, which resembled those of CCPP, were confined to the thoracic cavity and included a fibrinopurulent pleurisy and pericarditis with copious amounts of yellow oily fluid in the pericardial sac and thoracic cavity. Lung consolidation (grey hepatization and lung marbling) was particularly noticeable in the anteroventral lung lobes, with up to 70% of the total lung area affected in some goats, often more prominent in a single lung. Well-encapsulated lung abcesses were also evident. The lesions were consistent with an infection

involving *M. haemolytica* and *M. ovipneumoniae*, both of which were isolated profusely. The severity of the infection was linked to overcrowding, sudden mixing of goats from different sources and concurrent infections with caseous lymphadenitis and caprine arthritic encephalitis brought in with the imported animals.

An outbreak of severe respiratory disease, which was restricted to kids and associated with *M. ovipneumoniae*, *M. arginini*, *M. haemolytica* and *P. multocida*, was reported recently in Portugal (Gonçalves *et al.*, 2008). The mortality rates exceeded 20% in kids, with severe pleuropneumonia, lung consolidation, large quantities of pleural fluid and pericarditis seen. Antibiotic treatment with enrofloxacin and florfenicol was administered for 5 days and clinical signs resolved within 10–14 days.

## Clinical Signs

Clinical signs may be mild, with dull lambs showing increased respiratory rates; in contrast, infections may also result in mortality, acute fibrinous pneumonia, consolidated lesions, pulmonary abscesses and pleurisy, depending on exacerbating circumstances. The signs initially present as coughing, temperature rise and depression of appetite and growth rate, with a drop in milk yield. A variable percentage of lambs will be affected but, over a period of weeks, the problem may involve the majority of the lambs. Growth rates may be slowed and carcass weights reduced. The main clinical signs are chronic, persistent and irregular cough, which may lead to rectal prolapses, and a mucopurulent nasal discharge; other bacterial infections may also be involved that cause a more severe inflammatory response and clinical signs (Niang *et al.*, 1998).

Reports from Australia described outbreaks of pneumonia in housed lambs in the first year of life, with high morbidity and low mortality and with poor growth rates and exercise tolerance (Cottew, 1971; St George *et al.*, 1971; St George, 1972). *M. ovipneumoniae* has also been isolated from cases of mastitis and keratoconjunctivitis but its role in these diseases is unknown (Ayling *et al.*, 2004).

## Pathology

Typically, lesions begin with dull red ventral areas of collapse which are accompanied by bronchiolitis in associated airways. They progress to firm red–grey areas of consolidation over 2–3 weeks (Fig. 11.1) but may continue as grey areas of consolidation, often with attached localized pleural adhesions (Alley *et al.*, 1999).

Histologically, a wide range of lung changes are seen, including hyperplasia of the lymphoid nodules and bronchiolar epithelium and a mononuclear cell reaction in the alveolar septa. Also evident are lymphocytic cuffs around bronchioles and vessels, collapsed alveoli and others with exudates containing mononuclear cells. Lymphoid hyperplasia may result in extensive cuffs around airways with consequent compression of the passages. Ultrastructurally *M. ovipneumoniae* has been reported to attach to cilia in airways of lambs and to colonize the upper respiratory tract of sheep (Niang *et al.*, 1998).

**Fig. 11.1.** Lung from sheep naturally affected with atypical pneumonia showing red consolidation of apical, diaphragmatic and cardiac lobes.

In a study of abattoir-derived lungs with characteristic gross lesions of atypical pneumonia, Sheehan *et al.* (2007) detected *M. ovipneumoniae* by culture or immunohistochemistry in 90% of cases. The importance of this mycoplasma was illustrated by the fact that *M. haemolytica* and parainfluenza virus type 3 were only detected in 30% of the cases while *M. arginini* was found in less than 2%. *M. ovipneumoniae* was detected from the majority of cases where lymphoid cuffing was seen, a feature often considered pathogneumonic for mycoplasma infections. However, clear immunohistochemical staining of *M. arginini* in pneumonic lungs was seen and suggests a role for this organism in disease. The absence of other previously reported lesions, including lymphoid hyperplasia and intra-alveolar exudation from just under half of the lungs, may indicate the early stage of some of these infections.

Immunocytohistochemistry, using a specific polyclonal or monoclonal antibody, confirms the presence of the organism in the diseased lungs and provides an indication of the involvement of *M. ovipneumoniae* in lung damage (Fig. 11.2) (Ayling *et al.*, 2005; Sheehan *et al.*, 2007); however, in practice results are often disappointing. It is possible that the poor staining often seen with immunocytohistochemistry may reflect the extensive antigenic variation seen with *M ovipneumoniae*, so a polyclonal sera raised against several strains or a monoclonal antibody to a common protein is to be preferred.

Suitable samples for diagnosis from live animals include sera, nasal swabs, ear swabs or bronchoalveolar lavage. Sheehan *et al.* (2005) described a new

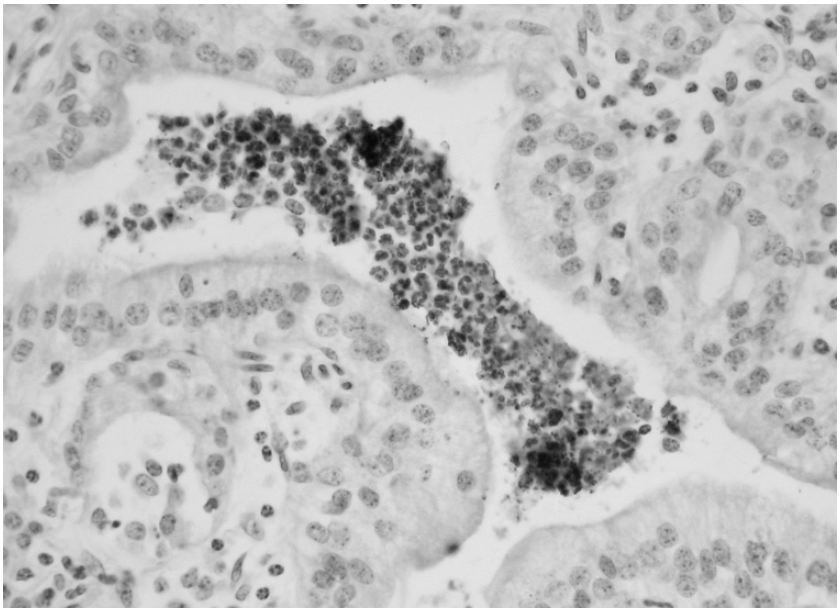

**Fig. 11.2.** Immunoperoxidose staining of *M. ovipneumoniae* in the lung of a sheep with atypical pneumonia.

transtracheal bronchoalveolar lavage technique which successfully recovered *M. ovipneumoniae* and *M. haemolytica.* However, its general use in the field may not be widely accepted. From dead animals, lung taken from the interface between healthy and diseased tissue and pleural fluid is desirable. An alternative approach is to take lung washings using a syringe containing PBS and a sterile tube which can be passed into the lung and withdrawn. The washings can then be examined by culture or molecular methods.

It is important to differentiate atypical pneumonia from the progressive pneumonias maedi and pulmonary adenomatosis. The age, pattern of disease, clinical signs and histological changes are useful distinguishing features. Furthermore, invasion of the lower respiratory tract by *Chlamydia psittaci* can also produce lung damage similar to that seen in atypical pneumonia.

## Causative Organism

*M. ovipneumoniae*, first isolated in New Zealand in 1974, is the cause of atypical or ovine non-progressive pneumonia. Mycoplasma pneumonia is well recognized in both Australia and New Zealand, where it is known as 'summer pneumonia' because of an increase in the prevalence of the disease in the hotter weather. A coughing syndrome identified in the USA in which there was persistent and long-term coughing as well as rectal prolapse was believed to be caused by a combination of *M. ovipneumoniae* and *M. arginini* (Niang *et al.*, 1998). It is believed that a primary infection

with *M. ovipneumoniae* may predispose sheep to invasion of the lower respiratory tract by other organisms such as parainfluenza 3 virus, a frequent pathogen of housed lambs, and *M. haemolytica*, which may enhance the pathological process.

Confirmation of the role of *M. ovipneumoniae* in disease has been shown experimentally following endobronchial inoculation of infected lung lesion homogenates and mixtures of isolates (Jones *et al.*, 1978); inoculation of pure cultures is much less successful and leads to mild disease, suggesting differences in virulence amongst isolates (Gilmour *et al.*, 1979). In the UK there has been an apparent increase in its isolation from pneumonic flocks since 2001, which may be related to overcrowding following restrictions of movement imposed on sheep flocks during the foot-and-mouth disease outbreaks (McAuliffe *et al.*, 2003b); many of these outbreaks were seen in flocks fully vaccinated with pasteurella vaccines.

Low numbers of *M. ovipneumoniae* are often isolated from the lungs of healthy sheep, but during times of stress or bad weather, subclinical infection may predispose sheep to acute fibrinous pneumonia, pulmonary abscessation or pleurisy. In one study over 70% of lambs with lungs affected by chronic non-progressive pneumonia had titres greater than $10^6$ organisms/g of lung, whereas only 25% of non-pneumonic lungs contained mycoplasmas, with only just under 3% having a titre of $10^6$ organisms/g of lung (Alley *et al.*, 1999).

*M. ovipneumoniae* has several mechanisms that may help cause disease or evade the host's immune system. It has a polysaccharide capsule, which is thought to have a role in pathogenicity and may facilitate adherence of the organism to the ciliated epithelium. The capsule may also interfere with macrophage activity and thus contribute to disease (Niang *et al.*, 1997, 1998). The production of autoantibodies to the ciliary antigen of the respiratory tract is thought to be a mechanism involved in the coughing syndrome of lambs. Colonization of the respiratory tract by *M. ovipneumoniae* precedes the production of these antibodies (Niang *et al.*, 1998).

## Biochemistry

Recent studies have indicated that field strains of *M. ovipneumoniae* show wide and varied metabolic activity (Patel, 2008). Substrate utilization patterns were determined using the change in dissolved oxygen tension, employing an oxygen electrode system, as described by Miles and Agbanyim (1998). The substrate utilization studies clearly demonstrate that, regardless of whether these organisms are from abattoir samples, diseased or indeed healthy animals and independent of their geographical location, all strains showed metabolic heterogeneity in terms of substrate utilization patterns. It was noted that, whilst all isolates were able to successfully metabolize glucose and N-acetylglucosamine, metabolism of fructose and maltose was achieved by about 83% of strains. Interestingly, approximately half of the strains were able to metabolize sugars such as mannose. A similar pattern was observed for organic acids: all strains were able to utilize pyruvate with high affinity whilst all but one strain managed to utilize lactate. It was also noted that a high number of strains tested were able to metabolize 2-oxybutyrate with high affinity. The metabolism of alcohol was also examined and demonstrated that all the strains in the test panel were able to metabolize both glycerol and isopropanol with high

affinity. Indeed, the metabolism of isopropanol was so consistently vigorous that this substrate was used as a marker substrate for *M. ovipneumoniae* metabolism. Often mycoplasma metabolic profiles are not generally useful in strain characterization owing to limited metabolic diversity. However, with *M. ovipneumoniae* it may be possible that useful metabolic profiling may be of value because of the variability observed. This apparent heterogeneity is not limited to the metabolic profiles of this organism; Miles *et al.* (2006) showed genetic variability in a similar panel of strains. Taken together these two observations might be important in the identification of the presence of subspecies within the genera. The reasons for this diversity, and the pronounced use of certain substrates such as isopropanol, have yet to be determined; however, it has been speculated that the metabolism of isopropanol to acetone might be important in terms of pathology. These metabolic observations are similar to that seen by Abu-Amero *et al.* (1996); further studies are required to fully ascertain the importance of the metabolic profiles established.

One of the putative virulence factors for mycoplasmas is the production of hydrogen peroxide. Studies by Patel (2008) following on from the metabolic profiles have shown that *M. ovipneumoniae* is no different, producing high levels of hydrogen peroxide following oxidative metabolism of substrates such as NADH and/or α-glycerol phosphate (αGP). *M. ovipneumoniae* metabolism of NADH or αGP showed an interesting departure from what is normally observed. It was clear that the oxidation of either of these two substrates was very vigorous but short lived and produced high levels of hydrogen peroxide, especially when compared with other mycoplasma strains. It is tempting to speculate that this high level of hydrogen peroxide production might be an important contributory component to the respiratory pathology observed. Further study of the biochemistry of organisms such as *M. ovipneumoniae* is of potential importance, as a sound understanding of these characteristics and metabolomic analysis help, in the development of discriminating diagnostic tests, strain differentiation and culture.

## Diagnosis

Gross lung pathology is not pathognomic for mycoplasma pneumonia, so laboratory diagnosis is essential to confirm mycoplasma involvement in disease. Good serological responses have been detected in experimentally infected lambs; however, in the field titres can be much lower and clearly reflect the antigenic heterogeneity of strains. Because of its wide prevalence, examining paired sera for a rising titre gives a better indication of an active *M. ovipneumoniae* infection. The isolation or detection of *M. ovipneumoniae* is only an additional aid to diagnosis of the disease, as many healthy sheep carry low levels of *M. ovipneumoniae*; however, quantification of the mycoplasma concentration in the lungs may be useful. Unlike most mycoplasmas, *M. ovipneumoniae* produces small centreless colonies (Fig. 11.3) but the identity must be confirmed by serological tests like growth inhibition or, increasingly, PCR. Newer molecular methods such as PCR (McAuliffe *et al.*, 2003b) and the 16S rDNA PCR and denaturing gradient gel electrophoresis (McAuliffe *et al.*, 2003a, 2005) offer a rapid method for detecting mycoplasma and can detect multiple mycoplasma infections.

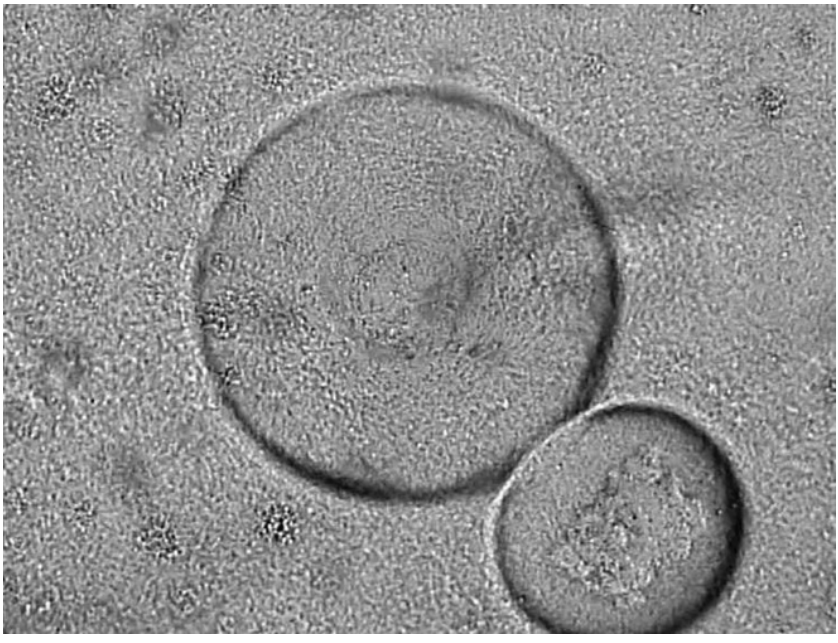

**Fig. 11.3.** Typical centreless colonies of *M. ovipneumoniae*.

A novel medium devoid of ruminant peptone, called TSB-1, was reported recently, which may improve isolation of the mycoplasma from tissues and increase growth yields for antigen and vaccine production (Patel *et al.*, 2008).

## Treatment and Control

Improved husbandry practices, such as lower stocking densities and improved ventilation, are important factors in preventing and reducing the spread of respiratory disease. Contact, even through shared airspace, with older sheep is best avoided and bought-in sheep or lambs should be isolated before mixing with the home flock.

Treatment with antimicrobials effective against *Mycoplasma* species often produces immediate respite, which may be sufficient for the animal to recover and may be the result of elimination of secondary bacteria. However, where *M. ovipneumoniae* is involved, the animals may quickly relapse and require further treatments. Antimicrobials that are likely to be effective include the newer fluoroquinolones, oxytetracycline or a macrolide. However, recent *in vitro* antibiotic trials on UK isolates of *M. ovipneumoniae* indicated variation in antimicrobial susceptibility between strains, particularly to the macrolides, where resistance seems to have developed (Ayling *et al.*, 2005).

The outbreaks of pneumonia seen in pasteurella-vaccinated flocks in the UK recently suggest that consideration should be given to incorporating a mixed

selection of *M. ovipneumoniae* strains into these vaccines to provide greater protection against respiratory disease (McAuliffe *et al.*, 2003b).

## Other Mycoplasmas Associated with Disease

Other *Mycoplasma* species are occasionally associated with respiratory disease in sheep. *M. arginini*, a ubiquitous mycoplasma of many animal species, is frequently isolated from the respiratory tracts of lambs with disease, sometimes mixed with *M. ovipneumoniae*. While it is not thought to be a major pathogen it may increase pathological damage.

*M. agalactiae* is a major cause of contagious agalactia in sheep and goats but has also been isolated from pneumonic lungs (Loria *et al.*, 2003), although its role in respiratory disease is not clear. The cattle pathogen *M. bovis* has also been infrequently isolated from the respiratory tracts of sheep and goats (Ayling *et al.*, 2004). In a recent study in the Middle East the goat pathogens *M. putrefaciens*, *M. mycoides* subsp. *mycoides* large colony variant and *M. capricolum* subsp. *capricolum* were isolated from both nasal swabs and milk samples of pneumonic sheep and goats in mixed herds (Al-Momani *et al.*, 2006a). In North Africa, *M. c. capricolum* appears to be the major cause of pneumonia and contagious agalactia in sheep (Benkirane *et al.*, 1993). Similarly, *M. capricolum* subsp. *capripneumoniae*, the cause of contagious caprine pleuropneumonia, has been isolated from respiratory tracts of sheep in contact with affected goats (Nicholas, 2000).

Whilst only a few *Mycoplasma* species have been reported to cause respiratory disease in sheep, it is apparent that sheep may be equally susceptible to infection by a range of *Mycoplasma* species if the opportunity presents itself. Sheep may also act as carriers of *Mycoplasma* species for other susceptible host species.

# DISEASES CAUSED BY *MYCOPLASMA MYCOIDES* SUBSP. *CAPRI* AND *MYCOPASMA MYCOIDES* SUBSP. *MYCOIDES* LC

## Introduction

While *M. mycoides* subsp. *mycoides* large colony type (LC) produces severe disease in goats, its main significance has been its close similarity to the small colony type, *M. mycoides* subsp. *mycoides* SC, the cause of one of the most serious diseases in cattle, contagious bovine pleuropneumonia (CBPP). It was originally thought that strains of *M. mycoides* subsp. *mycoides* isolated from goats produced large colonies *in vitro* whereas cattle strains gave small colonies; as a result Cottew and Yeats (1978) used this feature to distinguish the two mycoplasmas: *M. mycoides* LC and *M. mycoides* SC. Later, however, Valdiviesio-Garcia and Rosendal (1982) found this to be unreliable as both small and large types could be recovered from goats infected with *M. mycoides* LC. Although serologically related, recent evidence

shows that the two types are clearly distinguishable (Nicholas and Bashiruddin, 1995). However, it is the relationship between *M. mycoides* LC and *M. mycoides* subsp. *capri* which is more contentious and has led many to consider the two to be identical, particularly on the basis of 16S rRNA sequencing, which reveals a 99.9% similarity (Pettersson *et al.*, 1996). Apart from minor serological differences with some strains, these two mycoplasmas are genetically identical, so it is highly likely that these two mycoplasmas will be grouped into the single subspecies *M. m. capri* following ratification by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of the *Mollicutes* (Al-Momani and Nicholas, 2006). Therefore, the name *M. m. capri* will be adopted here.

## Distribution

*M. m. capri* has one of the widest geographic distributions of any ruminant mycoplasma, being found in all continents where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported. New reports of isolations have been made this millennium in New Zealand, Chile and the Netherlands, where it was isolated from calves (Nicholas, 2004).

## Epidemiology

*M. m. capri* is invariably introduced into a disease-free farm/region via clinically healthy or subclinically affected carrier goats. After an incubation period of 2–28 days, it then spreads to the kids, predominantly by the milking process, with high numbers of mycoplasmas being shed in the milk. The adults themselves become infected through contaminated milking machinery or milkers' hands, with the mycoplasmas ascending the teat canal; other routes such as direct contact and aerosol may also be important. Stress caused by changes in husbandry, nutrition and climate is seen as a major factor in outbreaks in adults. Rosendal *et al.* (1979) reported maintenance of infection in a herd for over 10 years with only occasional clinical cases of arthritis in young animals, almost certainly precipitated by an outbreak of coccidiosis and later by de-horning and castration.

In most countries in Europe, *M. agalactiae* is the major cause of contagious agalactia followed by *M. m. capri* (Loria *et al.*, 1999); in the Canary Islands, the seroprevalence of these two mycoplasmas in affected herds was 23 and 16% respectively (Real *et al.*, 1994). In mainland Spain, *M. agalactiae* accounted for 83% of isolates from goats affected by contagious agalactia, while *M. m. capri* accounted for 7% (Gil *et al.*, 1999). While *M. m. capri* is found less frequently, the disease it causes is usually far more severe than that caused by *M. agalactiae*, with mortality reaching 25% in adults and 90% in kids. However, in the goat-milking regions of western France, over 50% of mycoplasma isolates from bulk milk tanks were *M. m. capri*, 27% were *M. putrefaciens* and only 6% were *M. agalactiae* (Mercier *et al.*, 2001). *M. m. capri* is also the most frequently reported caprine mycoplasma infection in the USA, particularly in the eastern and coastal states (Smith and Sherman, 1994).

*M. m. capri*, like other mycoplasmas of small ruminants, has been found in the ear canals, often associated with ear mites (Da Massa and Brooks, 1991) or with fleas (Nayak and Bhowmik, 1990), possibly representing a means of maintaining the carrier state and transmitting new infections to susceptible animals.

## Hosts

*M. m. capri* has been isolated mainly from goats and occasionally from sheep with balanoposthitis and vulvovaginitis (Trichard *et al*., 1993). There have been a few reports from cattle; Perreau (1979) isolated *M. m. capri* from one of four cases of pericarditis, pleurisy and bronchopneumonia in calves in France and Kapoor *et al*. (1989) detected *M. m. capri* in aborted bovine fetuses. In the Netherlands, the mycoplasma was detected in calves showing CBPP-like disease (Nicholas, 2004) and in healthy cattle during the CBPP eradication campaigns in Portugal and Italy, where it was reported to cause serological cross-reactions with *M. mycoides* SC (Machado *et al*., 2000; De Santis, 2005). Perrin *et al*. (1994) isolated *M. m. capri* from captive wild goats in Switzerland.

Antibody responses to *M. m. capri* were detected in 5–15% of Peruvian camelids, including alpacas and llamas, by passive haemagglutination tests (Hung *et al*., 1991) but no mycoplasmas have been isolated to date (Nicholas, 2004). Alpacas, llamas and vicunas suffer from pneumonia, pleuritis and polyarthritis, so it is not unlikely that mycoplasmas are causative agents.

## Economic Impact

Economic losses caused by decreased production, diagnosis and treatment costs can be very high in severe outbreaks, particularly in intensive commercial goat dairy operations. Unfortunately little mycoplasma diagnosis is carried out in countries where these diseases are most prevalent. In one outbreak in the Canary Islands, 150 of 700 goats died of respiratory disease caused by several strains of *M. m. capri* (Rodriguez *et al*., 1996a).

## Causative Organism

*M. m. capri* is a member of the *M. mycoides* cluster, which comprises pathogenic ruminant mycoplasmas that share immunological, biochemical and genetic properties. They include *M. m. capri*/*LC, M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *M. mycoides* SC and the unclassified *M.* bovine group 7 (which have all been isolated from small ruminants). Table 11.1 shows how *M. m. capri* compares clinically with other mycoplasmas of small ruminants.

Like most mollicutes, *M. m. capri* is small, simple, self-replicating and distinguishable from walled bacteria and L-form bacteria by a number of unique properties, including lack of a cell wall and the genetic machinery to synthesize one; its DNA has a low guanine plus cytosine content, typically less than 30 mol %; it has

**Table 11.1.** Comparative characteristics of some mycoplasmas of small ruminants, including *M. mycoides* subsp. *capri*.

| *Mycoplasma* | Host | Primary site of isolation (other) | Disease* | Pathogenicity |
|---|---|---|---|---|
| *M. m. mycoides* LC | Goat (sheep, cattle) | Resp. tract (udder, joints) | Plp, M, A, C | Moderate |
| *M. m. capri* | Goat (sheep) | Resp. tract (joints) | Plp, A, C | Moderate |
| *M. c. capricolum* | Goat, sheep | Joints/resp. tract (udder) | Plp, M, A | High |
| *M. c. capripneumoniae* | Goat (sheep) | Lungs | CCPP | High |
| *M. ovipneumoniae* | Sheep, goat | Resp. tract | Pneumonia | Low |
| *M. conjunctivae* | Sheep and goat | Eyes | KC | Moderate |
| *M. agalactiae* | Sheep and goat | Udder (joints, eyes) | M, A, KC, P | High |
| *M. putrefaciens* | Goat (sheep) | Udder (joints) | M, A | Moderate |
| *M. arginini* | Ubiquitous | Resp. tract | None | Low/non-pathogenic |

*Plp – pleuropneumonia, P – pneumonia, M – mastitis, A – arthritis, C – conjunctivitis, KC – keratoconjunctivitis, CCPP – contagious caprine pleuropneumonia

a small genome and consequently produces small numbers of synthesized proteins. Like all members of the *M. mycoides* cluster, *M. m. capri* has been assigned to the 'spiroplasma' group on the basis of its 16S rRNA gene sequence (Pettersson *et al.*, 1996).

Until the mid-1950s, it was thought that *M. m. capri* was found only in large ruminants, but then Laws (1956) isolated a mycoplasma which was serologically identical to the agent of CBPP from a goat with fibrinous peritonitis. This strain, known as 'Y goat', differed from bovine strains of *M. m. mycoides* in that it grew to greater turbidity and formed larger colonies after the same incubation time. Other strains, similar to Y goat, have subsequently been isolated from goats (Littlejohns and Cottew, 1977) and sheep (von Brack, 1966; Smith, 1983) and were thought not pathogenic in cattle (Hudson *et al.*, 1967). However, strains that more closely resemble the SC type have also been isolated from goats and these have been shown to be pathogenic in cattle (Hudson *et al.*, 1967). *M. mycoides* SC has been isolated from sheep, although its pathogenicity in cattle is as yet unknown (Brandão, 1995). The situation became even more complex with the isolation of a non-pathogenic strain from cattle in Australia (Cottew and Yeats, 1978), and the isolation of strains of uncertain pathogenicity in France (Perreau and Bind, 1981) and Portugal (Machado *et al.*, 2000). Strains from cattle, though occasionally isolated from clinical cases, have not been shown to be pathogenic in sheep or goats (Machado *et al.*, 2000). Thus there is an important need to be able to differentiate SC and LC strains, particularly in regions free of CBPP.

The first attempts at differentiation were by Cottew and Yeats (1978), who confirmed the larger colony sizes (Fig. 11.4) and increased growth rate of the goat

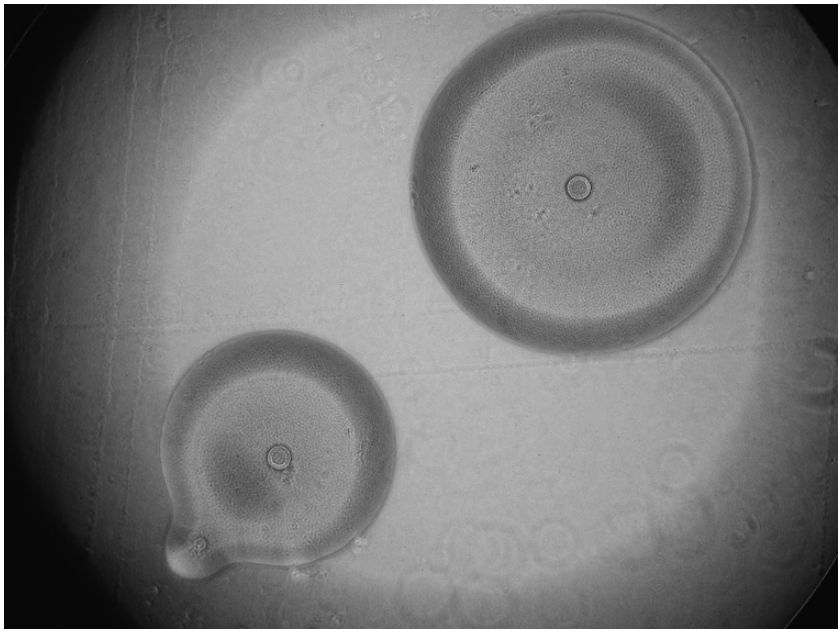

**Fig. 11.4.** Typical colonies of *M. mycoides* subsp. *mycoides* LC/*capri*.

isolates; they also showed that these LC strains digested casein, actively liquefied inspissated serum and survived longer at 45°C than the SC forms. These authors proposed that the latter strains be designated as *M. mycoides* LC and that strains more closely resembling the CBPP agent, which included some goat strains, should be called *M. mycoides* SC. Further confirmation of biochemical, but not serological, differences was demonstrated by Smith and Oliphant (1982): LC strains, but not SC strains, fermented sorbitol to varying degrees. The LC strains were also more resistant to streptomycin than SC strains, while the growth of LC strains was greatly stimulated by the presence of fermentable substrate. The presence of two enzymes, α (alpha) glucosidase and ornithine transcarbamylase, was shown in LC strains but not SC types (Salih *et al.*, 1983). The ability to produce mycoplasmaemia in mice by SC types has also been used to distinguish the two mycoplasmas (Smith, 1983). Later, differentiation between LC and SC types on the basis of colony size was shown to be unreliable by Valdivieso-Garcia and Rosendal (1982), who showed that the two different colony types could be recovered from goats inoculated with a caprine (LC) variant of *M. m. mycoides.* Table 11.2 summarizes the reported differences between *M. m. capri* and *M. mycoides* SC.

*M. m. capri* was thought to be the cause of classical contagious caprine pleuropneumonia (CCPP), but this was later shown by MacOwan and Minette (1977) to be caused by another member of the *M. mycoides* cluster, *Mycoplasma* F38 (renamed *M. capricolum* subsp. *capripneumoniae*). *M. m. capri* can be differentiated from the SC and LC forms by serological techniques, although some cross-reactivity between the two subspecies occurs with some antisera (Cottew, 1979). Biochemically, however, LC reacted identically to *M. m. capri*; the protein patterns of these two mycoplasmas as measured by SDS-PAGE were very similar but distinct from SC forms and other members of the cluster (Costas *et al.*, 1987; Leach *et al.*, 1989).

**Table 11.2.** Some differences between *M. mycoides* subsp. *capri*/LC and *M. mycoides* small colony (SC) types.

| Characteristics | Small colony | *capri*/ LC |
|---|---|---|
| Mean colony size after 137 h at 37°C (mm) | 0.99 | 2.26 |
| Broth turbidity: optical density after 120 h at 37°C | 0.14 | 0.40 |
| Liquefaction of inspissated serum | weak | + |
| Casein digestion | – | + |
| Sorbitol fermentation | – | + |
| Presence of ornithine transcarbamylase | – | + |
| Presence of α-glucosidase | – | + |
| Thermal stability at 45°C | sensitive | resistant |
| Streptomycin resistance | + | +++ |
| Mycoplasmaemia in mice | + | –* |
| Electrophoresis of *Asn*1 digests of PCR products using primers 450/451 | 2 bands | 3 bands |
| Digestion of DNA by *Mbo*I | + | – |
| *Dpn*I | – | + |

* Most strains

Using biochemical approaches, Abu-Groun *et al.* (1994) compared the metabolism of over 50 strains of the *M. mycoides* cluster and showed extensive differences between strains in the range of substrates used, the relative rates of oxidation and the observed saturation constants for substrates. In particular, SC strains were distinguished by their inability to oxidize maltose, trehalose, glucosamine and, at low concentrations, mannose. However, strains of LC and *M. m. capri* could not be distinguished, as their substrate utilization patterns were diverse.

Attempts to clarify the taxonomic position of the *M. mycoides* subspecies have proved contradictory. Results from DNA hybridization studies, using only single strains, revealed an homology of 0.82 between SC and LC types and 0.70 between SC and *M. m. capri* (Askaa *et al.*, 1978). However, large differences in percentage hybridizations were seen between SC and *M. m. capri*. Protein studies gave a slightly different picture. Costas *et al.* (1987) examined cellular proteins of 26 isolates belonging to the *M. mycoides* cluster by SDS-PAGE and showed protein patterns to be generally similar, with many shared bands. However, numerical analysis of the patterns, using a correlation coefficient to compare the similarity of protein distributions, revealed four distinct groups or phenons at a similarity level of 70%: first, *M. m. mycoides* SC strains; second, *M. m. mycoides* LC strains and *M. m. capri*; third, *M. capricolum* subsp. *capricolum* and F38-type strains; and fourth, *M.* bovine group 7. A very close relationship between *M. m. mycoides* LC and *M. m. capri* was revealed, as there were difficulties in distinguishing isolates from each subspecies even at the 75% level. More extensive studies confirmed that *M. m. mycoides* LC and *M. m. capri* were inseparable using protein analysis but showed that in most cases serological tests, in particular immunofluorescence, could distinguish the subspecies designations (Leach *et al.*, 1989). Interestingly, several strains were serologically intermediate between the two subspecies and cross-reacted with both mycoplasmas. Clinically, these two subspecies could be considered as a single species, with some *M. m. capri* strains having a greater tropism for respiratory diseases and some LC strains tending to cause contagious agalactia, with a large group of strains of both mycoplasmas being somewhat intermediate.

## Disease Course

Experimental infections with *M. m. capri* readily reproduce severe pathogenic effects in kids, including fever, anorexia, acute pain, swollen joints, recumbency and dyspnoea, often with death as a result of septicaemia (Nayak and Bhowmik, 1990). Goats and sheep inoculated experimentally by the respiratory or intravenous route frequently developed lung lesions whereas the subcutaneous or intramuscular route resulted in a massive cellulitis at the site of inoculation, accompanied by a marked febrile response. Infection via the teat canal invariably led to mastitis (DaMassa *et al.*, 1983). Whether caprine strains of *M. m. capri* can cause disease in calves is uncertain. In some experiments caprine strains of *M. m. capri* caused significant clinical signs in calves, including arthritis (Rosendal, 1981) and pleuropneumonia (Ojo *et al.*, 1980); in other work, having calves in contact with infected goats or inoculated directly did not lead to clinical disease (Rosendal, 1983).

The most common clinical findings in adult goats with natural infections are fever, mastitis, pleuropneumonia, polyarthritis and occasionally abortion; in kids, arthritis, septicaemia and, occasionally, meningitis and abortion. Keratoconjunctivitis may also be seen in both. Kids are most frequently and more severely affected than adults but mastitis may affect 25–30% of does. The most severe outbreaks occur amongst young kids. Arthritis, more common in kids, always occurs as a result of septicaemia, frequently involving multiple joints and accompanied by generalized malaise and fever. It rarely occurs in isolation and in adults usually follows mastitis, abortion, agalactia, pneumonia and keratoconjunctivitis. In extreme cases septicaemic goats may die without manifestation of localized signs, which may be the result of galactan production by *M. m. capri* (Smith and Sherman, 1994).

## Pathology

Lesions caused by *M. m. capri* are characterized by lungs which are enlarged and firm with hepatization of cardiac and diaphragmatic lobes and marked pleural effusion and fibrinous pleuritis. There is extensive pulmonary oedema and interlobular septa are usually distended and pale. Histopathologically, arterial and arteriolar vasculitis with necrosis of vessel walls and thrombi are common.

Severe respiratory signs and high mortality were the most significant findings in one goat herd with pleuropneumonia caused by *M. m. capri* in Spain (Rodriguez *et al.*, 1995). The adults presented mainly with respiratory disease and/or mastitis, whereas the young animals died showing arthritis and/or keratoconjunctivitis. The most significant lesions were found in the thoracic cavity. Macroscopically, a focal extensive fibrinonecrotic pleuropneumonia was seen and histopathological analysis confirmed a fibrinopurulent and necrotic pleuropneumonia with areas of acute pyogenic bronchopneumonia and fibrinous pericarditis associated with a mucopurulent mastitis and/or a fibrinopurulent arthritis.

An unusual mycoplasma, isolated from a goat presenting contagious agalactia in northern Jordan and identified as *M. mycoides* subsp. *mycoides* LC/*M. mycoides* subsp. *capri* by PCR and DGGE, was used to infect healthy seronegative goats by either aerosol or intracheal routes (Di Provvido *et al.*, 2008). While ELISA and latex agglutination tests detected antibody after about 2 weeks, the complement fixation test remained negative throughout the experiment. After 2 weeks, intubated goats became febrile, showing nasal discharge and slight conjunctivitis, which was followed by respiratory distress and polyarthritis 1 week later. Clinical signs were much milder in the aerosol group, consisting of nasal discharge and slight articular swelling of the carpal joints in one goat. Lesions were seen in intubated goats at post-mortem, including coagulative necrotic pneumonia, fibrinous pleurisy, pleural fluid in the thoracic cavity (Figs 11.5 and 11.6) and inflammatory exudates, necrosis and fibrosis in the joints (Fig. 11.7). Seroconversion was first seen in a group of contacts after 7 weeks by both ELISA and latex agglutination test, which was accompanied by coughing and laboured respiration. Pulmonary lesions in the contacts consisted of fibrinous pleuropneumonia, with focal areas of necrosis and abundant pleural fluid in the thoracic cavity. This work demonstrated

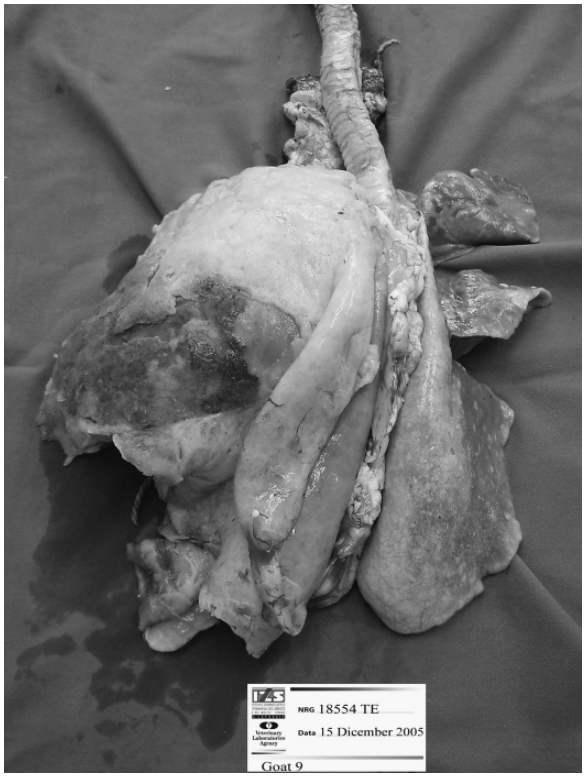


**Fig. 11.5.** Lung of goat experimentally infected with *M. mycoide*s subsp. *capri*, showing fibrin deposition.

the pathogenicity of this unusual serotype in both infected and contact goats. It was interesting to note that the CFT using the type strain as antigen would fail to detect this serotype.

## Diagnosis

Clinical signs and pathological changes caused by *M. m. capri* are not characteristic and it is very important to distinguish between infections caused by *M. m. capri* and those caused by *M. c. capripneumoniae*, the agent of classical contagious pleuropneumonia (CCPP), which is not present in the EU. The key features in CCPP infections are its highly infectious nature, the lack of distention of the interlobular septa in the lungs and its restriction to the lungs. Laboratory diagnosis must always be performed to determine the true cause of the disease (Nicholas and Baker, 1998).

Samples of choice from living animals include nasal swabs and secretions, milk from mastitic does or from apparently healthy does where there is a high rate of mortality/morbidity in kids, joint fluid from arthritic cases, eye swabs from cases of ocular disease, blood for antibody detection from affected and non-affected animals, and ear swabs. From dead animals samples should include lung tissue (at the interface between diseased and healthy tissue) and pleural/

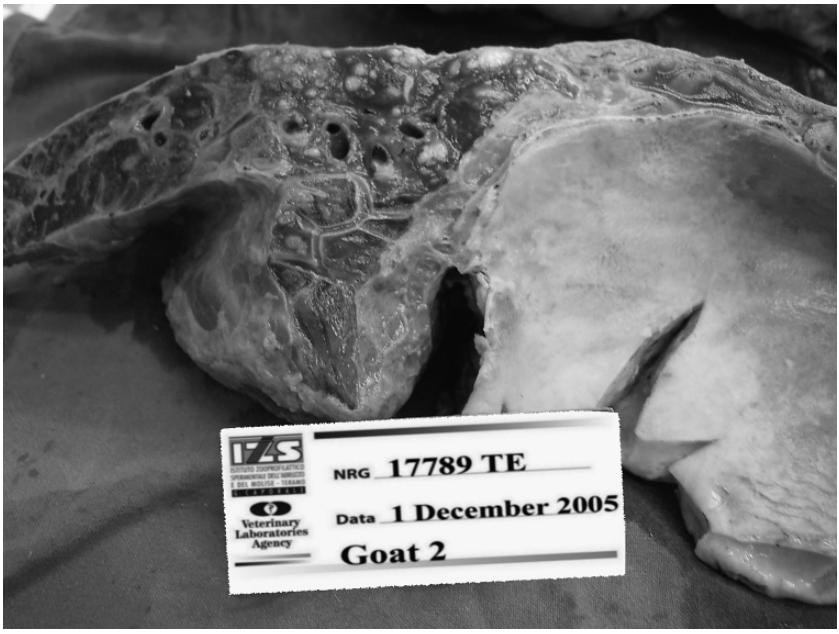


**Fig. 11.6.** Lung of a goat experimentally infected with *M. mycoides* subsp. *capri,* showing necrotic foci and enlargement of the interlobular septa.

pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. *M. m. capri* is readily recoverable from internal organs, joints and milk and grows well in most media, producing large colonies (1–2 mm) in 2–3 days.

Biochemical tests can give some guidance to the identification of the causative mycoplasma (see Table 9.3) but confirmation is definitively achieved by growth inhibition, fluorescent antibody or metabolic inhibition tests using hyperimmune specific rabbit antiserum (Poveda and Nicholas, 1998) and more frequently molecular methods like PCR.

Rodriguez *et al.* (1996a) reported a monoclonal antibody-based sandwich ELISA which could detect $10^5$ cfu/ml of both *M. mycoides* biotypes within 2 h. Sensitivity could be improved significantly by incubating samples for 48 h. This test could not distinguish between the SC and LC types, but coupled with pathological and serological information from affected animals, the test could prove very useful.

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However, negative results should not be considered definitive. PCRs can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples, where they have been reported to be more sensitive than culture (Tola *et al.*, 1997), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a

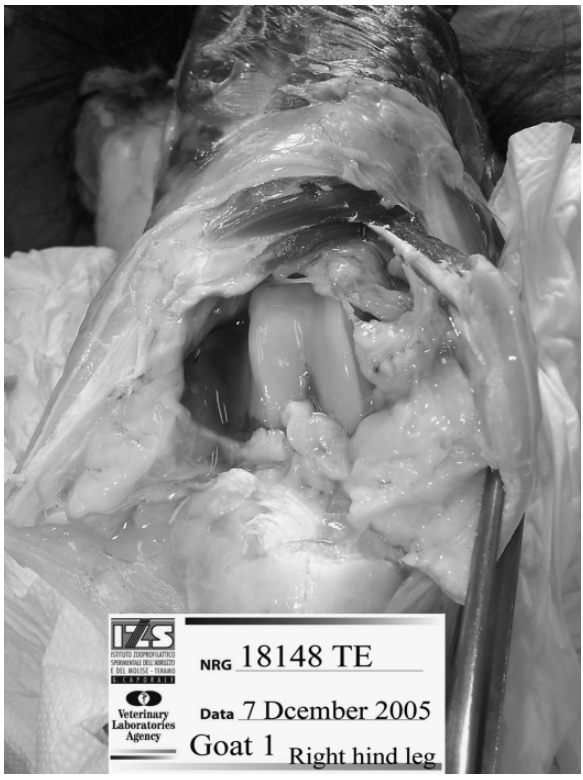


**Fig. 11.7.** Joint of goat experimentally infected with *M. mycoide*s subsp. *capri,* showing fibrin deposition and joint fluid.

24-h enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas, 2002). A newly described PCR-based method called denaturing gradient gel electrophoresis (DGGE), which uses mycoplasma-specific primers, is capable of identifying the majority of small ruminant mycoplasmas, including all the causative agents of CA, by their migration pattern (McAuliffe *et al.*, 2005). A positive PCR result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

Individual PCRs have been reported for *M. m. capri* (Bashiruddin *et al.*, 1994). In addition, a multiplex test has been described which can detect simultaneously *M. mycoides* subsp. *capri*, *M. agalactiae* and *M. c. capricolum* (Greco *et al.*, 2001).

Serological detection of antibodies to *M. m. capri* is not widely performed. Those that have been reported include complement fixation and indirect haemagglutination tests (Lefevre *et al.*, 1987). In-house ELISAs have been produced which can distinguish *M. m. capri* from *M. agalactiae* (Levisohn *et al.*, 1991). In the UK, which is free of contagious agalactia, immunoblotting tests are used on all ELISA positive sera; to date no positive results have been confirmed (Nicholas, 1998).

An immunohistochemical method of detecting *M. m. capri* antigens using monoclonal antibodies on paraffin sections of caprine lungs was described by Rodriguez *et al.* (1996b). This technique detected antigens in the cell debris around

necrotic areas and in macrophages, neutrophils and epithelial cells in lungs from both natural and experimental infections.

## Disease Treatment

Like most mycoplasma diseases, those caused by *M. m. capri* do not respond well to chemotherapy, in particular in cases of mastitis. Although clinical improvements may be seen, the mycoplasma may remain viable in the goats. Infected but apparently healthy does may continue to produce milk containing large numbers of mycoplasmas, causing problems in vulnerable kids. In such circumstances pasteurization of milk may help to control outbreaks; in one case morbidity and mortality was reduced from 90% to less than 5% when only mycoplasma-free milk was fed (Da Massa *et al.*, 1983). Tylosin and oxytetracycline have both been reported to be effective against pneumonia, but all animals in a herd must be treated, including apparently healthy goats, although it should be noted that tylosin is not recommended for treatment of animals under 15 kg. For arthritis, a combination of spectinomycin and lincomycin given intramuscularly for 3 days generated some recovery in sheep and goats (Smith and Sherman, 1994).

## Disease Prevention and Control

No vaccines are currently available for *M. m. capri* as the disease is thought not to be sufficiently widespread in Europe; in Greece, Turkey and the Middle East clinical disease is more prevalent but differential diagnosis is not routinely performed. Vaccines inactivated by saponin have been reported in India, these provoking a strong antibody response and showing some protection. As most disease-free herds become infected following the introduction of a carrier animal, knowledge of the health status of the herd of origin is essential; serious consideration should also be given to maintaining the bought-in animals in quarantine until serological testing has been carried out. Stress such as that triggered by movement or parturition should be minimized. Where mastitis is present, affected animals should be milked last and machines/hands should be disinfected. Where the situation warrants, culling of clinically affected animals should also be encouraged.

The possible involvement of ear mites and fleas in transmission suggests that insecticides such as ivermectin may be worth considering.

# *IN VITRO* SUSCEPTIBILITY OF SMALL RUMINANT MYCOPLASMAS TO ANTIMICROBIALS

Relatively few studies have been carried out on the *in vitro* efficacy of antimicrobials against the mycoplasmas that cause disease in sheep and goats. Bergonier *et al.* (1997) reviewed data on some antimicrobials used for treating CA, but as with all mycoplasma MIC studies they should be examined carefully as the methods used

varied and carried out before the guidelines and recommendations were issued in 2000 (Hannan, 2000). Information on the purity and activity of the antimicrobials used in these studies is also scant. The MIC methods used by Loria *et al.* 2003, Al-Momani *et al.* 2006b and Ayling (unpublished) all used micro-broth dilution methods, and followed Hannan's recommendations. The isolates used for *M. ovipneumoniae* MIC studies were all from sheep in the UK and were isolated between 2003 and 2004.

The results of the various antimicrobial sensitivity studies are given in Tables 11.3–11.7 and have been separated into different classes of antimicrobials tested, providing details of the mycoplasma species tested, the number of isolates (if known), the MIC range and the $MIC_{50}$ value obtained (if known) and the source of the information.

Table 11.3 shows a wide range of MIC values for the macrolides. While later results for *M. agalactiae*, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides* large colony type and *M. putrefaciens* showed relatively low MIC values, earlier data for *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* large colony type showed higher levels. Of more concern are the high MIC values obtained for *M. ovipneumoniae*. The fluoroquinolones, with the exception of flumequine (Table 11.4), gave consistently lower MIC values for most *Mycoplasma* species. It is apparent from the MIC range that some isolates of *M. ovipneumoniae* have

**Table 11.3.** Effect of macrolides on small ruminant mycoplasmas.

| Antimicrobial agent | Organism (number tested)* | MIC range in (μg/ml) | $MIC_{50}$ in (μg/ml) | Reference |
|---|---|---|---|---|
| Erythromycin | *Mcc* (8) | <0.03–<0.03 | <0.03 | Al-Momani *et al.*, 2006b |
| Erythromycin | *Mmm*LC/*Mcc* (15) | 40–200 | NA | Bergonier *et al.*,1997 |
| Erythromycin | *M. put* (18) | 0.25–0.25 | 0.25 | Al-Momani *et al.*, 2006b |
| Erythromycin | *M. ovip* (27) | 8.00–32.00 | 32.00 | Ayling *et al.*, 2006 |
| Spiramycin | *M. agal* (24) | 1.00–4.00 | 1.00 | Loria *et al.*, 2003 |
| Spiramycin | *M. capri* | 0.10–2.00 | NA | Bergonier *et al.*, 1997 |
| Tilmicosin | *M. ovip* (27) | 0.25–>64.00 | 8.00 | Ayling *et al.*, 2006 |
| Tylosin | *M. agal* (17) | 0.10–1.00 | 0.50 | Hannan *et al.*, 1997 |
| Tylosin | *M. agal* (24) | 0.13–2.00 | 0.25 | Loria *et al.*, 2003 |
| Tylosin | *Mcc* (8) | <0.03–<0.03 | <0.03 | Al-Momani *et al.*, 2006b |
| Tylosin | *Mmm*LC/*Mcc* (15) | 0.32–8.00 | NA | Bergonier *et al.*, 1997 |
| Tylosin | *Mmm*LC/*Ma* | 0.02–0.50 | NA | Bergonier *et al.*, 1997 |
| Tylosin | *Mmm*LC | 0.07–8.00 | NA | Bergonier *et al.*, 1997 |
| Tylosin | *M. ovip* (27) | 0.06–>64.00 | 0.12 | Ayling *et al.*, 2006 |
| Tylosin | *M. put* (18) | 0.12–0.12 | 0.12 | Al-Momani *et al.*, 2006b |

*

*M. agal* – *M. agalactiae*
*Mcc* – *M. capricolum* subsp. *capricolum*
*M. capri* – *M. mycoides* subsp. *capri*
*Mmm*LC – *M. mycoides* subsp. *mycoides* large colony type
*M. ovip* – *M. ovipneumoniae*
*M. put* – *M. putrefaciens*
NA – data not available

given high MIC values of 4, 8 and 32 μg/ml for enrofloxacin, danofloxacin and norfloxacin, respectively. Most species and isolates appear to show low MIC results for the tetracyclines (Table 11.5), but again a few isolates have higher MIC values. Generally the chloramphenicols and derivatives (Table 11.6) show MIC values on

**Table 11.4.** Effect of fluoroquinolones on mycoplasmas of small ruminants.

| Antimicrobial agent | Organism (number tested)* | MIC range in (μg/ml) | $MIC_{50}$ in (μg/ml) | Reference |
|---|---|---|---|---|
| Ciprofloxacin | *M. ovip* (27) | <0.12–2.00 | 0.12 | Ayling *et al.*, 2006 |
| Danofloxacin | *M. agal* (17) | 0.05–2.50 | 0.25 | Hannan *et al.*, 1997 |
| Danofloxacin | *M. ovip* (27) | <0.06–8.00 | 0.12 | Ayling *et al.*, 2006 |
| Enrofloxacin | *M. agal* | 0.17–0.22 | NA | Bergonier *et al.*, 1997 |
| Enrofloxacin | *M. agal* (17) | 0.05–1.00 | 0.25 | Hannan *et al.*, 1997 |
| Enrofloxacin | *M. agal* (24) | 0.13–0.50 | 0.13 | Loria *et al.*, 2003 |
| Enrofloxacin | *Mmm*LC (6) | 0.12–0.12 | 0.12 | Al-Momani *et al.*, 2006b |
| Enrofloxacin | *M. ovip* (27) | 0.03–4.00 | 0.06 | Ayling *et al.*, 2006 |
| Enrofloxacin | *M. put* (18) | 0.25–0.25 | 0.25 | Al-Momani *et al.*, 2006b |
| Flumequine | *M. agal* (17) | 5.00–100 | 25.00 | Hannan *et al.*, 1997 |
| Norfloxacin | *M. ovip* (27) | <0.12–32.00 | 0.25 | Ayling *et al.*, 2006 |

**Table 11.5.** Effect of tetracyclines on mycoplasmas of small ruminants.

| Antimicrobial agent | Organism (number tested) | MIC range in (μg/ml) | $MIC_{50}$ in (μg/ml) | Reference |
|---|---|---|---|---|
| Oxytetracycline | *M. agal* (17) | 0.10–0.25 | 0.10 | Hannan *et al.*, 1997 |
| Oxytetracycline | *Mcc* (8) | 0.12–0.25 | 0.25 | Al-Momani *et al.*, 2006b |
| Oxytetracycline | *Mmm*LC/*Mcc* (15) | 0.32–8.00 | NA | Bergonier *et al.*, 1997 |
| Oxytetracycline | *Mmm*LC | 0.50–10.0 | NA | Bergonier *et al.*, 1997 |
| Oxytetracycline | *M. ovip* (27) | 0.06–8.00 | 0.12 | Ayling *et al.*, 2006 |
| Oxytetracycline | *M. put* (18) | 0.25–1.00 | 0.25 | Al-Momani *et al.*, 2006b |
| Tetracycline | *M. agal* (24) | 0.13–4.00 | 0.25 | Loria *et al.*, 2003 |

**Table 11.6.** Effect of chloramphenicol and derivatives on mycoplasmas of small ruminants.

| Antimicrobial agent | Organism (number tested) | MIC range in (μg/ml) | $MIC_{50}$ in (μg/ml) | Reference |
|---|---|---|---|---|
| Chloramphenicol | *Mcc* (8) | 8.00–8.00 | 8.00 | Al-Momani *et al.*, 2006b |
| Chloramphenicol | *Mmm*LC (6) | 8.00–8.00 | 8.00 | Al-Momani *et al.*, 2006b |
| Chloramphenicol | *M. ovip* (27) | 1.00–32.00 | 8.00 | Ayling *et al.*, 2006 |
| Chloramphenicol | *M. put* (18) | 1.00–8.00 | 1.00 | Al-Momani *et al.*, 2006b |
| Florfenicol | *Mcc* (8) | 4.00–8.00 | 4.00 | Al-Momani *et al.*, 2006b |
| Florfenicol | *Mmm*LC (6) | 2.00–4.00 | 2.00 | Al-Momani *et al.*, 2006b |
| Florfenicol | *M. ovip* (27) | 0.50–16.00 | 4.00 | Ayling *et al.*, 2006 |
| Florfenicol | *M. put* (18) | 0.50–4.00 | 1.00 | Al-Momani *et al.*, 2006b |

the high side, with $MIC_{50}$ values ranging from 1 to 8 μg/ml. The remaining antimicrobials in Table 11.7 show a range of results, with some of the *M. mycoides* LC type and *M. ovipneumoniae* MIC values being quite high.

The recent study by Al-Momani *et al.* (2006b) had several important findings, in particular that mycoplasmas thought to be specific for goats could also infect sheep when the opportunity, such as shared grazing, arose. Furthermore, and perhaps surprisingly, different *Mycoplasma* species have different *in vitro* antimicrobial sensitivities. For example, all *M. capricolum* subsp. *capricolum* isolates gave MIC values of just 0.015 μg/ml with erythromycin, whereas all *M. mycoides* LC and *M. putrefaciens* isolates gave higher MIC values of 0.25 μg/ml, possibly indicating that erythromycin is less effective against these two species. This highlights the importance of accurate diagnosis as it is important to know the exact cause or causes of these clinically similar diseases before treatment is decided.

It is also clear from the MIC data that mycoplasmas can develop resistance rapidly, as detected in *in vitro* tests, to a wide range of antimicrobials, which is best seen in the UK isolates of *M ovipneumoniae*. The widespread use of antimicrobials in the UK almost certainly contributes to the development of resistance. In addition, the heterogeneity of *M. ovipneumoniae* strains (Alley *et al.*, 1999; Miles *et al.*, 2006) may enable a greater genetic exchange between the mycoplasmas, providing a mechanism for the development of resistance.

The interpretation of *in vitro* results and how it relates to use *in vivo* is always difficult. While for many bacterial species the relationship between *in vitro* and *in vivo* is given in terms of breakpoints, the MIC values, which are interpreted as susceptible or resistant, have not been determined for animal mycoplasmas. Breakpoints established from other bacterial species can thus only be used as a guide.

In the absence of vaccines, the selection of antimicrobials for treating affected animals should be based on correct diagnosis and accurate information about the effectiveness of the antimicrobials. This will ensure continued antimicrobial efficacy and help avert the development and spread of antimicrobial resistance.

**Table 11.7.** Effect of lincosamides, mutilins and aminocyclitols on mycoplasmas of small ruminants.

| Antimicrobial agent | Organism (number tested) | MIC range in (μg/ml) | $MIC_{50}$ in (μg/ml) | Reference |
|---|---|---|---|---|
| Clindamycin | *M. ovip* (27) | <0.12–2.00 | 0.25 | Ayling *et al.*, 2006 |
| Lincomycin | *Mmm*LC | 0.10–5.00 | NA | Bergonier *et al.*, 1997 |
| Lincomycin | *Mmm*LC | 0.15–>0.50 | NA | Bergonier *et al.*, 1997 |
| Lincomycin | *M. ovip* (27) | 0.25–8.00 | 0.50 | Ayling *et al.*, 2006 |
| Lincomycin/ Spectinomycin | *M. agal* (24) | 0.25–1.00 | 0.50 | Loria *et al.*, 2003 |
| Spectinomycin | *M. ovip* (27) | 0.50–64.00 | 8.00 | Ayling *et al.*, 2006 |
| Tiamulin | *M. agal* (17) | 0.05–0.25 | 0.13 | Hannan *et al.*, 1997 |
| Tiamulin | *Mmm*LC | 0.06–0.14 | NA | Bergonier *et al.*, 1997 |

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# 12 Eye Infections of Ruminants

*"A total of 409 chamois and 33 ibex with clinical signs of IKC were reported. Most of the chamois were shot, primarily because they were blind or in poor general condition. Almost a quarter were observed alive, and 16.9% died as a result of IKC."* Tschopp *et al*. describing the cases of infectious keratoconjunctivitis in wild goats in the Swiss Alps in 2005.

## Introduction

Once the traumatic causes of eye injury such as foreign bodies and feedstuffs have been discounted then infectious agents should be considered. Infectious keratoconjunctivitis (IKC), 'pink-eye' or 'New Forest eye', is the most frequent and costly eye disease of ruminants. A gram-negative bacterium *Moraxella bovis* is considered to be the major cause in cattle but *Mycoplasma bovoculi Pasteurella* spp., *Histophilus somni*, *Neisseria* spp. and infectious bovine rhinotracheitis (IBR) virus have also been associated with conjunctivitis. Ureaplasmas are also frequently isolated from eyes during epidemics of conjunctivitis, a condition which has been reproduced experimentally (Ross, 1993). The role of *M. bovis* in this condition is not entirely clear but it may act as secondary invader following *Moraxella* infection. Other mycoplasmas, *M. bovigenitalium, Acholeplasma* and *M. bovirhinis,* have also been isolated from cases of ocular disease with profuse lacrimation but are thought to be opportunistic. The economic impact of all forms of pink-eye was estimated to be $150 million in 1993 because of the negative effect it has on weight gain and milk production as well as treatment and veterinary costs.

In small ruminants *M. conjunctivae* is recognized as the single major cause of IKC in domestic and wild ovine and caprine species (Giacometti *et al.*, 1999; Hosie, 2007), but *M. agalactiae*, *M. mycoides* LC, *M. arginini* and *Acholeplasma oculi* have also been isolated from sheep with ocular infections (Baker *et al*., 2001). Bacterial

causes of a clinically similar disease include Moraxella *ovis*, *E. coli*, *Chlamydia* spp. and *Rickettsia* spp., which may also be involved in co-infections with *M. conjunctivae* (Lysnyansky *et al*., 2007). It is invariably brought into a flock via sheep with mild or inapparent infection and is spread by close contact and/or possibly by flies, although firm evidence is so far lacking for this insect vector. Generally the disease is milder or inapparent in young animals.

# *MYCOPLASMA BOVOCULI*

## Introduction

While research into *M. bovoculi* has been neglected of late, a substantial body of information was acquired in the 1980s in the USA by Rosenbusch and co-workers, which definitively linked it to eye disease and provided valuable data on its behaviour.

## Distribution and Epidemiology

The mycoplasma has been reported in the UK, Denmark, Switzerland, Croatia, Germany, the Netherlands, Israel, Nigeria, Canada, the USA and India but is probably present wherever cattle are kept.

In a large study in Germany, nearly all mycoplasmas isolated from both natural cases of conjunctivitis and healthy animals were identified as *M. bovoculi* (Schöttker-Wegner *et al.*, 1990) but no attempt was made to correlate the presence of mycoplasma with increased disease. Mycoplasmas could be isolated from the infected eyes for up to 6 months after the appearance of clinical signs. It was noticed that mycoplasmas were more frequently isolated in the autumn, although the reasons for this were unknown; this is puzzling as others have linked IKC with fly infestation, which would be at its worst in the summer months. A serological survey using an ELISA of cattle and buffaloes with and without IKC showed antibody to *M. bovoculi* to be present in 44% of cattle with conjunctivitis and in 15% of healthy cattle; no seroconversion could be detected in buffaloes (Bansal *et al.*, 2002).

In Israel, both *M. bovoculi* and *M. bovis*, but not *Moraxella bovis*, were isolated from conjunctival swabs from young calves with IKC (Levisohn *et al.*, 2004). The authors postulated that the disease was a sequel to pneumonia caused by *M. bovis* and possibly infectious bovine syncitial virus.

## Causative Organism

*M. bovoculi* grows relatively well in most mycoplasma media, fermenting glucose but not hydrolysing arginine or urea, and produces centred colonies. Little work has been done on characterizing strains, although a small study of six isolates showed similar but not identical protein profiles and some antigenic differences detected by immunoblotting with post-exposure calf antiserum, which also revealed a common 94 kDa protein (Salih and Rosenbusch, 1998). Further work with this monoclonal antibody showed some cross-reaction with *M dispar*.

Phylogenetic analysis of the 16S rDNA gene places *M. bovoculi* in the *Mycoplasma neurolyticum* cluster of the hominis group.

## Clinical Signs

Cattle affected with *M. bovoculi* do not appear ill but 10–50% of the herd may have a unilateral or bilateral conjunctivitis with an ocular discharge, initially serous then later mucopurulent, and conjunctival hyperaemia (Fig. 12.1). Photophobia may also accompany these signs. The disease can be produced experimentally by intraconjunctival inoculation of live cultures, appearing within 3–4 days after infection and persisting for over 1 month; the mycoplasma can be recovered from the conjunctival mucosa for at least 2 months (Rosenbusch and Knudtson, 1980). In addition to it being able to cause disease, *M. bovoculi* also has a role in facilitating colonization by other pathogens, including *Moraxella bovis* (Rosenbusch and Ostle, 1986).

## Diagnosis

Disease produced by *M. bovoculi* is not definitive, so laboratory diagnosis is necessary to enable identification. The mycoplasma can be isolated relatively easily, although mixed infections are likely to be encountered, requiring cloning of isolates before the growth inhibition test using hyperimmune serum can be performed. Immunofluorescence tests probably offer a better alternative but interpretation can be subjective (Poveda and Nicholas, 1998). PCR tests are clearly the

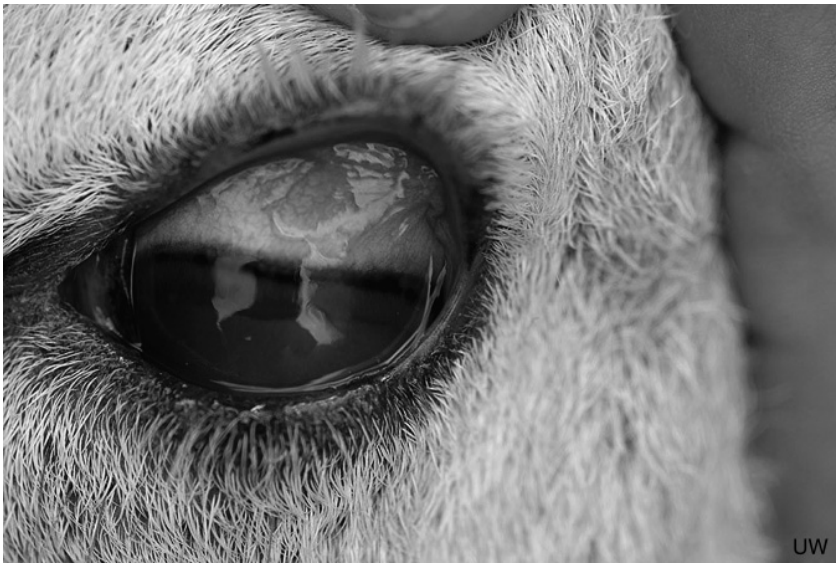


**Fig. 12.1.** Severe infectious keratoconjunctivitis in bovine eye from which *M. bovoculi* and *M. bovis* were isolated.

quickest and most sensitive tests but to date no specific tests have been described. The PCR/DGGE using mollicute-specific primers, on the other hand, can detect *M. bovoculi* and over 70 other mycoplasmas, including mixed mycoplasma cultures (McAuliffe *et al*., 2005).

Specific antibodies can be detected by ELISA in the serum, nasal fluids and lacrimal fluids of infected calves (Salih and Rosenbusch, 1986). In nasal and lacrimal fluids, IgA appeared as early as the first week following exposure to *M. bovoculi* and was present in both of these fluids and lasted for at least 9 weeks. Sera from field cases showed high IgG and IgM activities. However, these tests are not commercially available.

## Treatment and Control

Most cases resolve without chemotherapeutic intervention but not before the mycoplasma has spread, and may affect up to half the herd (Riis, 2008). Topical applications of tetracycline ointments are believed to facilitate recovery.

Until studies are conducted to determine the prevalence of *M. bovoculi* in IKC, no decision can be made as to the need for a vaccine. Theoretically vaccines should be effective because it is known that animals recovered from field infections are protected from reinfection. Furthermore, animals experimentally or naturally infected mount both a significant cell-mediated and humoral response. However, no protective immunity was seen after immunization with three antigen preparations consisting of two non-ionic detergent extracts and a killed whole organism (Salih *et al*., 1987). In view of the possible transmission by flies, methods which reduce infestations should be encouraged.

## Other Mycoplasmas Involved in Eye Infections

An outbreak of severe IKC reported in the UK was probably exacerbated by the stress of transport, as only two of the yearling bullocks presented with signs on arrival at a Welsh farm; however, within 2 days it spread to 13 of the group of 20 and to all but two after several weeks (Kirby and Nicholas, 1996). The disease varied in severity from watering of the eyes to a severe conjunctivitis, with the eyelids so badly swollen that vision was impaired. The worst cases showed a purulent discharge from the eyelids with corneal opacity, some ulceration and transient blindness in three bullocks for 7–10 days. Overt conjunctivitis took about 2 weeks to resolve but some animals still showed corneal scarring 4–6 weeks later. Early cases were treated ineffectively with ampicillin and topical treatment of chlortetracycline powder and benzocaine. While the disease was clearly highly infectious within this newly introduced group, it did not spread to cattle in an adjoining shed or to some animals that had transient contact with some of the infected bullocks. The lack of spread may be due to the fact that the outbreak occurred in winter when there was no fly infestation. *M. bovis* was isolated throughout the outbreak and strong serocoversion was shown a month from onset. The role of *Morexella bovis* in this outbreak was unclear as it was isolated from only one of four swabs taken.

# *MYCOPLASMA CONJUNCTIVAE*

## Introduction

Infectious keratoconjunctivitis is a disease of worldwide distribution in all small ruminant species. It is important to differentiate IKC caused by *M. conjunctivae* and that caused by *M. agalactiae* or *M. mycoides* LC, which are the aetiological agents of the important OIE-listed disease, contagious agalactia.

## Clinical Signs and Epidemiology

In the early stages, the disease may present as a unilateral or bilateral conjuctivitis with hyperaemia of the vessels, but untreated cases may progress to mucopurulent keratitis and corneal ulceration, resulting in opaque or perforated corneas and reversible blindness (Fig. 12.2). Permanent corneal lesions are rare and the occurrence of corneal ulceration is thought to be due to secondary bacterial infection.

Seroprevalence studies in Switzerland have shown it to be present in nearly 90% of 123 sheep flocks in which over half the animals tested were positive (Janovsky *et al*., 2001). Slightly lower overall flock prevalence (70%) was seen in sheep flocks in New Zealand, although the percentage of seropositive animals

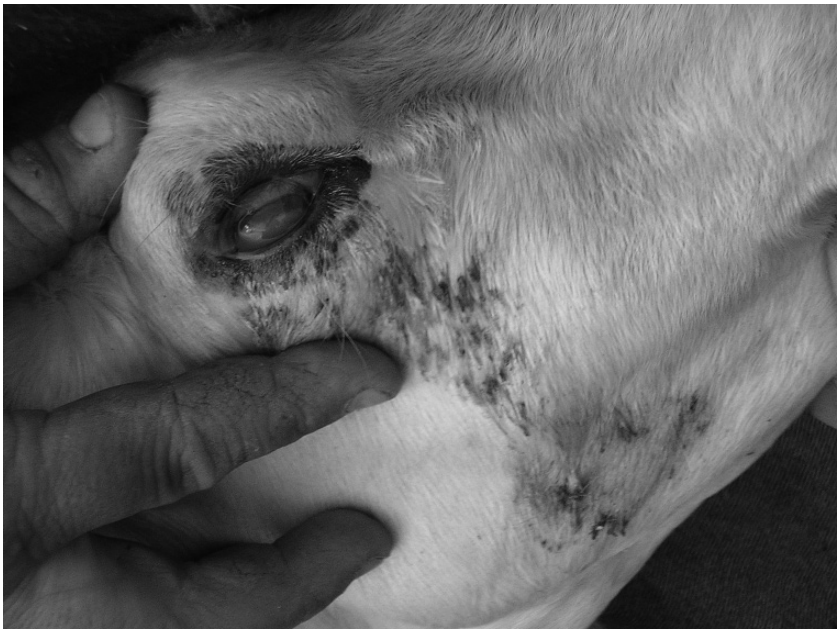

**Fig. 12.2.** Severe infectious keratoconjunctivitis in a sheep in Sicily and from which *M. conjunctivae* was detected.

(65%) in a flock was higher (Motha, 2003). A recent report has implicated *M. conjunctivae* as a cause of conjunctivitis in a human patient following a visit to a zoo (Lysnyansky *et al.*, 2007).

The common features of IKC are persistence of the organism, spontaneous recovery from disease and relapses with incubation times varying between 1 and 21 days, depending on the degree of infection. *M. conjunctivae* may be viable in the conjunctival sac and the nares for several months, providing opportunities for reinfections within a flock (Baker *et al.*, 2001). Severe outbreaks of IKC were seen in wild small ruminants, notably ibex, mouflon and chamois, in Switzerland in the early 1990s, resulting in blind animals falling from cliffs or dying of starvation, with mortality rates estimated at 30% in some areas (Belloy *et al.*, 2003). Outbreaks increased during the summer months as a result of the mixing of wild species with domestic sheep on summer pasture. With individual seroprevalence levels estimated at 53% in sheep throughout the whole country, it seemed highly likely that the wild ruminants were being infected by their domestic relatives. Experimental infections showed that ibexes and chamois were susceptible to sheep strains, and molecular typing based on variations in the *lpps* gene proved convincingly that strains were being transmitted between the wild and domestic species (Belloy *et al.*, 2003). Furthermore, studies showed that the disease was not being maintained in the wild animals but resulted in reinfections from the sheep during shared grazing.

In England a flock of Sufflok sheep was investigated in which animals presented with disorientation as a result of unilateral and transient blindness; some had conjunctivitis or corneal oedema, none of which responded to topical or intramuscular tetracycline treatment (Baker *et al.*, 2001). IKC was diagnosed but the nature of the ocular lesions, which was reminiscent of those seen in sheep infected with *M. agalactiae* and some members of the *M. mycoides* cluster, prompted farm visits for further studies to determine the exact cause. In the first visit 20 sheep were examined and in the second visit, 3 months later, 19 sheep (11 of which were repeat examinations from the first visit) were sampled from this farm. At the onset of the outbreak two rams which had clinical signs of IKC previously were isolated from the flock, but despite this measure, it was evident that the agent had spread throughout the flock and signs appeared in the flock sporadically, which is typical of this epizootic disease. Of the animals which were sampled again, four of 11 had developed pink-eye over the 3-month period. *M. conjunctivae* was detected in 5% of the animals by culture and in 10% of cases by PCR in the samples from the first visit. The detection rate from the second visit rose to 16% by culture and 36% by PCR.

A more severe case of IKC was reported in central Sicily, where about 80% of a milking sheep flock were showing severe IKC affecting about 120 ewes and lambs (Loria *et al.*, 2005). The condition was painful, with milk production and feed consumption greatly reduced. The condition had not been improved by the use of tylosin. Long-acting oxytetracycline reduced the clinical signs but did not prevent relapses and slow spread throughout the herd. In the laboratory, *M. conjunctivae* was detected by specific PCR and a few strains were isolated from recently affected ewes but not from sheep which had been affected for longer than 1 week,

despite clinical signs. Flies were a particular problem on this farm and may have been responsible for the rapid spread of disease.

## Diagnosis

Culture of the mycoplasma is often unsuccessful due to its fastidiousness *in vitro* and may also be due to antimicrobial therapy. The detection of *M. conjunctivae* is better achieved by PCR or PCR/DGGE, which is recommended for diagnosis after gently sampling the eye and fornex behind the third eyelid with saline-dampened swabs (Baker *et al.*, 2001; McAuliffe *et al.*, 2003, 2005). Grattarola *et al.* (1999) also reported improved detection rates with PCR carried out on ocular samples from chamois and ibexes. An immunofluoresent test used to be performed but required local anaesthesia before scraping cells from the conjunctiva for staining and examination.

A strong humoral response is detectable in affected animals, making serological detection convenient, but while ELISAs using antigens consisting of Tween 20-soluble mycoplasma proteins have been reported for serological detection (Belloy *et al.*, 2001), they are not commercially available.

Immunoblot analysis of the humoral and local immune response of alpine chamois, ibex and domestic sheep naturally and experimentally affected with IKC revealed the major immunogenic proteins to be at 83, 68, 60 and 42 kDa, although the 83 and 42 kDa was shared with other small ruminant mycoplasmas (Degiorgis *et al.*, 2000). Naturally infected animals showed much stronger immune reactions than those experimentally infected with specific antibodies.

## Treatment and Control

Treatment is rarely completely effective and the disease spreads slowly through the flock, mainly in adults, despite topical and intramuscular injections of long-acting oxytetracyclines. Isolation of treated animals is recommended because treatment does not prevent spread, and sporadic outbreaks continue for some time. Intramuscular injection of oxytetracycline dehydrate given at the onset of clinical signs prevents further clinical disease but if treatment is delayed until IKC is at its most severe, then clinical cure is delayed for 4 days (Hosie, 2007). For severe cases topical application of auromycin was recommended and often effective for hill sheep, but intensively managed sheep may require repeated doses. Veterinary advice should be sought before using this product.

The reported development of a strong immune response in affected sheep may suggest that a vaccine could be effective, particularly for pedigree sheep, which often acquire infections after visiting shows, or indeed for sheep which may share pasture with rare wild ruminants. Quarantining incoming animals for at least 2 weeks while checking for ocular disease is an effective though often not a practical method of control.

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# 13 Reproductive Diseases of Cattle

## Introduction

Mycoplasmas are commonly isolated from the genital tract of cattle and often from apparently healthy animals, so their role in disease has been unclear. Evidence for their role in infertility, endometritis and the granular vulvitis complex, which is characterized by a chronic cloudy or muco-purulent discharge and raised lesions on the vulvar mucosa, is, however, strong and may be the result of infection with *Ureaplasma diversum*, *M. bovis* or *M. bovigenitalium*. Other possible causes include the gram-negative coccobacillus *H. somni,* campylobacteriosis and trichomoniasis.

## *MYCOPLASMA BOVIGENITALIUM*

*M. bovigenitalium*, first characterized by Freundt (1955), is mainly found in the reproductive tract of cattle and buffaloes but has also been isolated from pneumonic, arthritic and mastitic cattle as well as aborted fetuses (Ruhnke, 1994). Although experimental infections show it is capable of inducing a range of clinical conditions, published evidence from the field that it is a major pathogen is still lacking, with the fastidious ureaplasmas often considered to be the causative agents of reproductive diseases. Reduced fertility and endometritis are conditions often described on farms, but samples are rarely taken for diagnosis of mycoplasma diseases. Even when *M. bovigenitalium* is isolated, other conditions such as mineral deficiency are frequently and erroneously diagnosed. In recent years in the UK, *M. bovigenitalium* has been isolated on numerous occasions from cattle with reduced fertility, endometritis ('whites') (Fig. 13.1) and granular vulvitis (Fig. 13.2), from semen samples and from the respiratory tract.

**Fig. 13.1.** Endometritis ('whites') seen in a cow from which *M. bovigenitalium* was isolated.

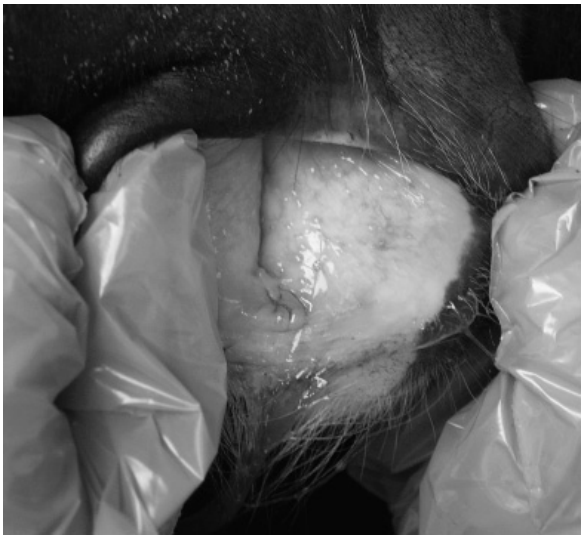

**Fig. 13.2.** Vulvovaginitis in a cow from which *M. bovigenitalium* was isolated.

There have been isolations of an unclassified mycoplasma, *M.* ovine/caprine serogroup 11, which is very similar to *M. bovigenitalium* and causes similar reproductive problems in sheep and goats (Nicholas *et al.*, 1999). Proposals have been made to amalgamate these two mycoplasmas into a single species called *M. bovigenitalium* (Nicholas *et al.*, 2008).

## Geographical Distribution

*M. bovigenitalium* has been found worldwide, being reported from the following countries: UK, USA, Egypt, India, Germany, Austria, Croatia, Brazil, Denmark, Nigeria, Italy Japan, Turkey, The Netherlands, Switzerland, South Africa, France, Canada and Morocco (Nicholas, 2002a).

## Epidemiology

*M. bovigenitalium* is commonly found in semen samples or sheath washings from cattle, often along with *Acholeplasma laidlawii*, *Ureaplasma* species and *M. bovis* (Jasper, 1981; Ball, 1990). It has also been associated with sporadic cases of epididymitis, orchitis, urethritis and seminal vesiculitis, leading to pain on ejaculation or following palpation; the mycoplasma may impair the motility of spermatozoa (Ross, 1993; Ruhnke, 1994). Experimental infections with artificially contaminated semen have shown that it can be isolated easily from washed embryos, where it forms a close association with the surface of the zona pellucida, intact embryos and sperm cells (Bielanski *et al*., 2000); this close attachment of the mycoplasma makes it difficult to eliminate with approved antibiotics.

*M. bovigenitalium* was isolated from dairy herds with vulvovaginitis and infertility, though less frequently than ureaplasmas (Reid *et al*., 1989). In an outbreak of granulomatous vulvitis in feedlot heifers, *M. bovigenitalium* was isolated from affected animals along with bovine herpesvirus type 1, *Moraxella bovis* and *M. bovis*, suggesting a multifactorial aetiology (Gilbert and Oettle, 1990). Serological proof of its involvement in disease has been provided in several reports where antibodies to *M. bovigenitalium* have been detected more frequently in cattle with reproductive disorders than in healthy cattle (Ashwani *et al*., 1996; Garg *et al*., 1999).

*M. bovigenitalium*, like *M. bovis* and *M. californicum*, is commonly isolated from mastitic milk and udder secretions (Jackson and Boughton, 1991; Baumgartner, 1999). It was first isolated in the UK in 1960 then later reported in a more severe form in the USA (Davidson and Stuart, 1960; Boughton, 1979). In one outbreak, *M. bovigenitalium* was isolated from over 50% of a large dairy herd suffering clinical mastitis (Ahmed, 1987). Ter Laak *et al*. (1992a) isolated *M. bovigenitalium* from the respiratory tract of pneumonic calves but not from healthy calves. Similar findings were reported by Naglic *et al*. (1996), who also isolated *M. bovigenitalium* from eye swabs of the same pneumonic cattle, some of which were also showing conjunctivitis.

Chima *et al*. (1995) isolated *M. bovigenitalium* from ewes with vaginal discharge and swollen vulvas. Reports of the biochemically and immunologically similar *M*. ovine/caprine serogroup 11 from identical clinical conditions (Poumarat *et al*., 1992; Nicholas *et al*., 1999) suggest a very close relationship between this mycoplasma and *M. bovigenitalium*. Nicholas *et al*. (1999) consistently isolated *M*. ovine/caprine serogroup 11 from a flock of infertile ewes, some of which were showing vaginal discharge; it was likely that the mycoplasma was introduced by infertile rams showing abnormal sperm morphology and motility.

*M. bovigenitalium* was isolated from the joints of arthritic cattle but was thought not to be a primary cause (Mazzolini *et al*., 1993). *M. bovigenitalium* and *M. canis* were isolated from dogs with and without reproductive disorders, suggesting they played little role in disease (Zoldag *et al*., 1993).

## Hosts

Cattle, buffalo, sheep (Chima *et al*., 1995), goats (Pathak *et al*., 1993) and dogs (Zoldag *et al*., 1993) may harbour *M. bovigenitalium*.

## Causative Organism

Like all mycoplasmas, *M. bovigenitalium* is small, pleomorphic, lacks a cell wall and has a low G+C ratio (28.1–32.6 mol%) (Hermann, 1992). Phylogenetically, *M. bovigenitalium* belongs to the *M. fermentans* group based on 16S ribosomal gene sequence analysis, showing a very close similarity to *M. californicum*. A strong immunological relationship between *M. bovigenitalium* and *M*. ovine/caprine serogroup 11 was first seen by Poumarat *et al.* (1992), who found bovine and ovine isolates to cross-react strongly in immunological tests. This was confirmed by Nicholas *et al.* (1999, 2002), who isolated *M*. ovine/caprine serogroup 11 from infertile sheep. *M. bovigenitalium* does not hydrolyse arginine or ferment glucose but possesses phosphatase activity and reduces tetrazolium chloride under anaerobic conditions. It produces hydrogen peroxide, which may account for its pathogenicity (Cole *et al*., 1968). Two major studies concluded that these two mycoplasmas should be considered a single species called *M. bovigenitalium* because of their identical biochemical, genetic and immunological characteristics (Nicholas *et al*., 2002, 2008). Strains isolated from infertile sheep were compared with the type strain, 2D, and with strains of the cattle pathogen *M. bovigenitalium*, including the type strain, PG11. Examination by growth inhibition and immunofluorescence tests showed strong serological cross-reactivity between *M.* serogroup 11 and *M. bovigenitalium* but not with other ruminant mycoplasmas. Substrate oxidation and growth studies did not show any consistent differences between *M.* serogroup 11 and *M. bovigenitalium* strains; all strains assigned were adapted to the utilization of a small range of organic acids as energy sources. DNA:DNA hybridization, carried out between DIG-labelled reference strains of *M.* serogroup 11 and *M. bovigenitalium* and field isolates of these two mycoplasmas, showed a particularly close relationship, with hybridization rates all greater than 70% and, mostly, closer to 90% (Table 13.1). Sequencing of the 16S ribosomal RNA gene region of the *M.* serogroup 11 and *M. bovigenitalium* strains as well as the respective type strains revealed very high overall homologies of 99.5%. In summary, the results showed a very close phenotypic and genotypic relatedness between these two ruminant mycoplasmas, which justifies their classification into a single species.

Some differences in virulence between *M. bovigenitalium* strains for the bovine mammary gland have been detected following intrauterine infection, although little evidence of disease was seen during the experiment (Ball, 1990).

**Table 13.1.** DNA:DNA hybridization between labelled reference *M. bovigenitalium* /*M.* serogroup 11 DNA and DNA from other strains of *M. bovigenitalium*, *M.* serogroup 11 and other mycoplasmas.

| Species/strains | % hybridization | |
|---|---|---|
| | *M. bovigenitalium* 10122 | *M.* serogroup 11 2-D |
| *M. bovigenitalium* strains | | |
| 10122 Type strain | 100 | 94 |
| 434/81 | 94 | 98 |
| 57B00 | 87 | 96 |
| *M.* serogroup 11 strains | | |
| 2D Type strain | 90 | 100 |
| 47 SR99 | 80 | 91 |
| 3 SR99 | 98 | 97 |
| 126 SR99 | 87 | 98 |
| 95 SR00 | 88 | 92 |
| 52 SR98 | 87 | 76 |
| Salmon sperm DNA | <10 | <10 |
| *M. mycoides* subsp. *mycoides* (LC) Y-goat | <30 | <30 |
| *M.* bovine serogroup 7 PG50 (10133) | <30 | <30 |

Data shown are the mean % hybridization values from duplicate experiments.

## Disease Course

There is plenty of experimental evidence to link *M. bovigenitalium* with a number of clinical conditions. Mastitis was reproduced in all cows experimentally infected via the udder with *M. bovigenitalium*; interestingly, one cow also developed arthritis, although *Bacillus cereus* was also isolated (Roberts, 1968). The mycoplasma persisted in the udder for at least 18 weeks. Only a small number of the bulls experimentally infected with *M. bovigenitalium* via the prepuce showed mild inflammatory lesions of the genital tract, although mycoplasmas were recovered at various times from most animals (Kreusel *et al.*, 1989). Experimental infections of the ovine mammary gland with *M. bovigenitalium* resulted in pathogenic effects, including high milk cell counts and mycoplasma excretion, leading in some cases to subclinical infections (Ball, 1990). Gourlay *et al.* (1979) showed that, when given intratracheally to gnotobiotic calves, *M. bovigenitalium* colonized the lungs and induced pneumonic lesions, producing microscopic cuffing lesions.

## Pathology

Affected seminal vesicles are enlarged and, when sectioned, appear brownish with decreased secretions (Ross, 1993). Respiratory disease associated with *M. bovigenitalium* consisted of cranioventral consolidation of the lungs, although some doubt existed as to whether this was the result of infection with *M. bovigenitalium*

or, more likely, tissue damage caused by other bacteria or mycoplasmas such as *M. bovis*. Experimental infection of gnotobiotic calves with *M. bovigenitalium* produced a mild, subclinical, cuffing-type pneumonia (Gourlay *et al.*, 1979).

Lesions believed to be due to *M. bovigenitalium* were seen in *in vitro* models of buffalo oviduct, hamster tracheal (Kumar *et al.*, 1992) and rabbit Fallopian tube organ cultures (Singh *et al.*, 1997), suggesting a direct pathogenic role for this mycoplasma.

## Diagnosis

Clinical signs are not characteristic for *M. bovigenitalium*, so laboratory investigations are necessary to confirm diagnosis. The mycoplasma grows well in a variety of media, producing 'centred' colonies. In an appropriate medium, such as Eaton's (Nicholas and Baker, 1998), *M. bovigenitalium* produces films and spots and gives an orange colour to the broth very similar to that seen with *M. bovis*. Growth inhibition, film inhibition, fluorescent antibody or metabolic inhibitions tests using hyperimmune rabbit serum can be used to identify the mycoplasma (Poveda and Nicholas, 1998).

Molecular techniques, such as the PCR/denaturing gradient gel electrophoresis (DGGE) method (McAuliffe *et al.*, 2003, 2005), are highly sensitive and have the advantage that they can detect and identify over 70 different *Mycoplasma* species. A specific PCR has been developed for *M. bovigenitalium* based on the 16S ribosomal gene, which detects $10^3$ and $2 \times 10^3$ colony-forming units of the mycoplasma in pure culture and in spiked clinical material, respectively (Kobayashi *et al.*, 1998). As this test lacks some sensitivity, an improved allele-specific PCR was developed which utilizes single nucleotide polymorphisms (SNP), enabling differentiation between the similar sequences found within bovine *Mycoplasma* species (Miles *et al.*, 2005). A comparison was made between traditional culture, PCR/DGGE and the allele-specific PCR on samples from a UK dairy herd presenting prolonged intercalving intervals and endometritis. Three of nine samples taken from the cows with endometritis were positive for *M. bovigenitalium* by DGGE. No other Mycoplasma species were detected by DGGE, although the closely related *Acholeplasma laidlawii* was identified by DGGE in one sample. The *M. bovigenitalium* PCR method was positive for the same three samples, giving the expected 1061 bp amplicon. The standard mycoplasma culture methods yielded just two *M. bovigenitalium* isolates, which highlights the usefulness of the specific PCR and DGGE techniques. Interestingly, no ureaplasmas were detected by either PCR/DGGE or culture using ureaplasma medium.

Serological tests are not widely available for *M. bovigenitalium*, although agar gel precipitin tests, indirect haemagglutination assays and ELISAs have all been described (Ashwani *et al.*, 1996; Garg *et al.*, 1999). ELISAs were considered to be more sensitive and specific but are not commercially available.

## Treatment

For reproductive disorders, only two antibiotics, nourseothricin and lincospectin, of seven tested were effective in eliminating *M. bovigenitalium* and other mycoplasmas

**Table 13.2.** *In vitro* antimicrobial sensitivity data for 11 strains of *M. bovigenitalium* (μg/ml).

| Antibiotic | MIC range | $MIC_{50}$ | $MIC_{90}$ |
|---|---|---|---|
| Tilmicosin | 0.06–0.12 | 0.06 | 0.12 |
| Enrofloxacin | 0.06–0.25 | 0.12 | 0.25 |
| Clindamycin | <0.12–0.25 | 0.12 | 0.25 |
| Ciprofloxacin | <0.12–0.50 | 0.12 | 0.50 |
| Danofloxacin | 0.12–0.50 | 0.25 | 0.50 |
| Oxytetracycline | 0.06–2.00 | 0.12 | 0.25 |
| Florfenicol | 0.50–2.00 | 2.00 | 2.00 |
| Norfloxacin | 0.25–8.00 | 0.50 | 2.00 |
| Lincomycin | 0.12–>32.00 | 0.25 | 0.50 |
| Gentamycin | 2.00–8.00 | 8.00 | 8.00 |
| Spectinomycin | 2.00–32.00 | 4.00 | 8.00 |
| Chloramphenicol | 1.00–32.00 | 2.00 | 32.00 |
| Rifampin | 32.00–>32.00 | 32.00 | >32.00 |
| Erythromycin | <0.12–>32.00 | >32.00 | >32.00 |
| Amikacin | 32.00–>32.00 | >32.00 | >32.00 |
| Naladixic acid | 32.00–>32.00 | >32.00 | >32.00 |
| Streptomycin | 32.00–>32.00 | >32.00 | >32.00 |
| Tobramycin | >32.00–>32.00 | >32.00 | >32.00 |
| Trimethoprim/sulfamethoxazole | >32/608–>32/608 | >32/608 | >32/608 |
| Cephalothin | >64.00–>64.00 | >64.00 | >64.00 |

without adversely affecting the semen (Pfützner, 1989). An assessment of some photosensitive agents, haematopophyrin, a derivative of this and thiopronine, to disinfect bovine semen spiked with various microbial agents including *M. bovigentalium* showed little effectiveness for mycoplasma at concentrations harmless to the sperm (Eaglesome *et al*., 1994).

In a later study, 11 *M. bovigenitalium* isolates from UK herds were tested *in vitro* to assess their sensitivity to 20 different antimicrobials using a broth dilution method (Ayling *et al*., 2005). The results, summarized in Table 13.2, showed that tilmicosin, clindamycin, oxytetracyclines and the fluoroquinalones were effective *in vitro* but all or some strains were resistant or developing resistance to spectinomycin, chloramphenicol and lincomycin, among many others.

## Prevention and Control

With the exception of the reproductive disorders, there is still a weak association between *M. bovigenitalium* and disease, including respiratory, arthritic and mastitic diseases. The only feasible and practical means of control should be directed towards ensuring that mycoplasma-free semen is used for artificial insemination.

Batches of frozen sperm should be tested for contamination by mycoplasmas using cultural techniques and, ideally, PCR. Testing the preputial washings of individual bulls may also be effective, although it may be difficult to find mycoplasma-free samples (Ball, 1990).

To prevent the introduction of disease, purchase of cows should be restricted to herds free of mastitis. Testing of bulk milk tanks should also be undertaken from all herds of origin prior to purchase. Once infection is detected in a herd, infected cows should be segregated from non-infected animals and culling encouraged, as antibiotic treatment is rarely effective for this and other mycoplasma infections.

# UREAPLASMAS

## Introduction

While few laboratories isolate and identify mycoplasmas regularly, even fewer are capable of detecting ureaplasmas because of their unusual and fastidious growth requirements. The existence of ureaplasmas, formerly known as T (for tiny) strains, was first recognized in humans over 50 years ago (Shepard, 1954). Their possession of the urease enzyme made them unique amongst mollicutes and led to their reclassification within the new genus, *Ureaplasma*, which now contains seven named species found mainly in mammals. The entire genome of the human sexually transmitted pathogen, *Ureaplasma urealyticum*, was sequenced in 2000.

Two species of ureaplasma play a major role in disease in animals and man: *U. diversum*, a cause of pneumonia, vulvovaginitis, conjunctivitis and infertility in cattle, and *U. urealyticum*, which can severely affect the newborn. Uncharacterized *Ureaplasma* species are also frequently isolated from the reproductive and respiratory tracts of small ruminants, where their role in disease is unclear. They may also be found in pigs, chickens, cats and dogs.

## Epidemiology

Numerous studies have shown that *U. diversum* plays an important role in bovine reproductive failure (Ruhnke 1994). Clinical signs include granular vulvitis, endometritis, salpingitis, abortion, infertility in the female and seminovesiculitis; all conditions have been reproduced experimentally (Ball *et al.*, 1981, 1987). Ureaplasmas are common contaminants of the prepuce and the distal urethra, having been isolated from 29–100% of samples cultured, where there may be, but not always, inflammation of the prepuce or penis (Pilaszek, 1988). Semen, too, is often contaminated, though not adversely affected, by ureaplasmas, providing an important transmission vector to cows. Embryo transfer fluids have been found to contain ureaplasmas which adhere to the zona pellucida, often resisting removal by washing (Britton *et al.*, 1988). The spread of genital forms of the disease is facilitated by chronic persistence of the mollicute in both the female and male

genital tracts. It is important to differentiate ureaplasma infections from those caused by *M. bovigenitalium*, which causes a very similar clinical disease, although often these two species may be isolated together.

In a study in the Netherlands, ter Laak *et al.* (1992a,b) detected *U. diversum* in the respiratory tract of 80% of pneumonic calves compared with only 9% in healthy calves, indicating a significant role in the aetiology of respiratory disease. This confirms experimental infections of gnotobiotic calves with ureaplasmas in which pneumonic lesions, but not always clinical disease, were seen (Gourlay *et al.*, 1979). The principal reservoir of these infections is the respiratory tract of cattle.

Despite successful experimental reproduction of conjunctivitis in cattle, ureaplasma involvement in natural infections is unclear as known pathogens such as *M. bovoculi*, *M. bovis* and *Moraxella bovis* are often present as well (Nicholas, 2002b).

The role of ureaplasmas in sheep and goat diseases is less clear; they are readily isolated from the urogenital and, occasionally, respiratory tract of both healthy and diseased animals. Curiously, the only reports of isolation from the respiratory tract of small ruminants were during rutting (Ross, 1993). A link, however, has been established between ureaplasmas and urinary calculi in goats on a low-calcium diet, with the infection influencing the total amount and composition of these kidney stones (Ruhnke, 1994).

## Hosts

While cattle are the major species affected, ureaplasmas have been isolated from sheep, goats, camels, pigs and poultry. A number of isolations have also been made from cats and dogs in Japan (Haraswa *et al.*, 1990).

## Economic Importance

Very little data exist for the effects of ureaplasma infections in livestock, but it is clear that infections are under-reported because of the difficulties in isolating the organism. However, Ross (1993) states that up to 27% reduction in fertility may occur in some herds, which would incur significant economic losses.

## Pathogen Characteristics

Within the Mollicute Class, ureaplasmas are grouped within the Order I Mycoplasmatales and Family Mycoplasmataceae but comprise their own genus II, *Ureaplasma*. To date there are seven named species and some as yet unclassified ureaplasmas: *U. urealyticum, U. parvum* (man), *U. diversum* (cattle), *U. gallorale* (poultry), *U. canigenitalium* (dog), *U. cati* (cat), *U. felinum* (cat) and *Ureaplasma* species (sheep, goats). Phylogenetically they are very close to mycoplasmas; *U. urealyticum* has been placed within the pneumoniae group on the basis of its

16S rRNA gene sequence. While similar to mycoplasmas in many respects, ureaplasmas are nutritionally unique, possessing the enzyme urease, which hydrolyses urea to generate ATP, probably through the creation of ionic gradients (Miles, 1992). Like mycoplasmas too, ureaplasmas are small, pleomorphic, lack a cell wall and have a low G+C ratio (Hermann, 1992). They do not hydrolyse arginine or ferment glucose *in vitro* but do require sterols for growth. In 2000 the entire genome of *U. urealyticum* was sequenced, providing unique data on these metabolically unique mollicutes (Glass *et al.*, 2000). The circular genome, the second smallest after *M. genitalium*, consisted of 751,719 base pairs comprising only 652 genes, half of which have unknown functions. The genome size is considerable smaller than the 850–940 kbp estimate based on pulse field gel electrophoresis. Other features of interest include a G+C ratio of 25.5%, the lack of the cell division protein, FtsZ, various chaperonins and ribonucleosidase diphosphate reductase. Finally, the possession of six closely related iron transporters suggests that ureaplasmas have a type of respiratory system not present in other small genome bacteria.

Most *U. diversum* strains can be classified into three serogroups, A, B and C, representing type strains A417, D48 and T44, respectively. Ter Laak *et al.* (1992b) identified 82% of Dutch bovine respiratory isolates as serogroup A, while Howard and Gourlay (1981), in a study of strains from four countries, identified 81% as serogroup B and only 15% as serogroup A. Four serotypes of ureaplasmas have so far been recognized in dogs.

The avian ureaplasma, *U. gallorale*, believed to be pathogenic, was shown to be more closely related to the human *U. urealyticum* than to the other animal ureaplasmas on the basis of sequence homology of the 16S rRNA gene (Stemke and Robertson, 1996).

## Disease Course

Acute granular vulvitis is characterized by an inflamed hyperaemic vulvar mucosa ,with small raised granules usually clustered around the clitoris. A purulent discharge may begin 3 days after infection, which may last up to 2 weeks when the disease becomes chronic, with a reduction in clinical signs; however, the ureaplasma can be isolated from the vulva for up to 7 months (Ruhnke, 1994). There may also be a transient colonization of the uterus. During acute disease, the endometrium and/or Fallopian tubes may become permanently damaged, leading to infertility (Kreplin and Maitland, 1989). In some cases embryo death or abortion, usually in the first or last trimester, stillbirth or extremely weak calves, which die shortly after birth with respiratory failure, may result as a consequence of ureaplasma infection (Ruhnke, 1994).

In studies of heifers experimentally infected with broth cultures of *U. diversum* or vaginal swabs from previously infected heifers, all animals developed granular vulvovaginitis; *U. diversum* was isolated from vagina, urinary bladder and urethra. The pathogenicity of *U. diversum* increased with consecutive passages, making the incubation period shorter and clinical signs more pronounced (Pilaszek and Truszczynski, 1991).

## Pathology

Genital disease in the female is characterized by a purulent vulvar discharge and inflamed hyperaemic vulvar mucosa with varying degrees of granularity (Ross, 1993). Gross lesions in the placenta consist of marked fibrotic thickening of large areas of the amnion and intercotyledonary zones in the chorioallantoic membrane, infiltrated by mononuclear cells. There may be foci of fibrin exudation and haemorrhage. In aborted fetuses, microscopic lesions of alveolitis and placentitis may be seen and antigen may be found in the zona pellucida of the embryo.

Respiratory disease involving ureaplasmas is largely subclinical. However, following endobronchial, but not aerosol, infection of gnotobiotic calves, thick cuffs of lymphoid cells and macrophages are seen surrounding the bronchi, bronchioles and blood vessels, with a lobular catarrhal pneumonia evident (Gourlay *et al.*, 1979; ter Laak *et al.*, 1993a). Macroscopic lesions seen in *U. diversum*-associated respiratory disease consisted of red consolidation in the cranioventral areas of the lung (Ross 1993). In the lung there is a diffuse alveolitis and focal lymphocytic accumulations around the airways. The alveolitis is characterized by degeneration and necrosis of the alveolar epithelium and there is infitration of macrophages and granulocytes (Ruhnke, 1994).

## Diagnosis

Clinical signs of granular vulvitis are considered suggestive of ureaplasma infections, while other signs such as subclinical pneumonia or conjunctivitis are far less definitive. Consequently laboratory diagnosis is necessary to determine the causative agent. Isolation of ureaplasmas in the genital tract is most successful from cultures of vulval swabs. The transient colonization of the uterus makes sampling of this region unreliable. Preputial swabs should be taken with a guarded swab to obtain the sample from as far into the cavity as possible. Semen should be collected following disinfection of the prepuce and should ideally be fresh or kept in liquid nitrogen until ready to culture. Preferred tissues from embryos include the lung, caruncle, cotyledon, stomach contents and amniotic fluid.

From respiratory cases, nasal swabs, lung lavage fluids and lung tissue can be taken. Ter Laak *et al.* (1992a) found considerably higher levels in lavage fluids than in nasal swabs. The appropriate transport medium for samples is crucial, and antibiotics such as streptomycin and gentamycin, commonly used in many media, may prove harmful to ureaplasmas. Recommended media include sucrose phosphate broth (2SP) or standard liquid medium (SLM) (Furr, 1998).

Media recommended for the primary culture of *U. diversum* differ from that for other mycoplasmas as they contain 1% urea as a substrate instead of glucose or arginine; the pH should be adjusted to about 7.0. However, there are many variations in exact composition of ureaplasma media. Whitford *et al.* (1994) list the composition of several media which have given good results, including U4 buffered broth medium. Ter Laak *et al.* (1992a) used Friis media HAU and NHU, and A7B agar medium, which enabled the production of manganese dioxide as a brown deposit when urea was hydrolysed; however, HAU and NHU were superior

to A7B when used in parallel. Commercial media are now available for ureaplasma isolation (Mycoplasma Experience, Reigate, UK).

Once isolated, ureaplasmas can be identified by the production of brown colonies on agar medium containing manganese sulphate caused by the deposition of manganese on the surface of the colony. Isolates can be serotyped by immunofluorescence on colonies grown on agar medium (Howard and Gourlay, 1981).

A PCR based on the 16S rRNA gene was more sensitive and rapid than culture for detecting *U. diversum* in vaginal swabs of cattle (Cardoso *et al.*, 2000). This test offers a lot of advantages over traditional culture techniques. The PCR/DGGE not only offers sensitive detection but enables the differentiation of ureaplasma from *M. bovigenitalium*, which may be found in the same tissue sample (McAuliffe *et al.*, 2003, 2005).

Serological tests are not widely available for ureaplasmas and may be complicated by the existence of different serogroups. However, an indirect haemagglutination test was described for *U. diversum* by Nagamoto *et al.* (1996).

## Disease Treatment

Little information is available on chemotherapeutic strategies for control of ureaplasma infections. An *in vitro* study of antibiotic sensitivity to 17 strains of *U. diversum* in the Netherlands showed the most effective (< 1 μg/ml) to be chlortetracycline, tiamulin and tylosin; antibiotics showing intermediate effectiveness (2–4 μg/ml) included oxytetracycline and enrofloxacin; those to which ureaplasmas were resistant (> 8 μg/ml) included chloramphenicol, spectinomycin and lincomycin (ter Laak *et al.*, 1993b).

In the field, Ball and McCaughey (1984) successfully eliminated ureaplasmas from the urogenital tract of most, but not all, infected sheep with tiamulin or oxytetracycline. In a study by Rae *et al.* (1993), prebreeding treatment with chlortetracycline appeared to improve pregnancy rates in beef heifers with *U. diversum* infections, although there was only a slight decrease in colonization rates of the vagina by ureaplasmas in treated groups.

There was marked and rapid clinical improvement in 2-year-old heifers and bulls with granular vulvitis and ulcerative posthitis following a 5-day intramuscular course of tylosin (Gummow *et al.*, 1992). Ureaplasmas could not be cultured from the external genitalia of either heifers or bulls following clinical recovery, which began 3 days after treatment.

## Disease Prevention and Control

Treatment of semen with combinations of gentamycin, lincospectin and tylosin appears to prevent spread of ureaplasmas and mycoplasmas according to one source (Ruhnke, 1994); however, culling of persistently infected bulls may sometimes be necessary (Reid *et al.*, 1989).

The application of three photosensitive agents followed by irradiation for disinfection of bovine semen had no effect on *U. diversum* at concentrations harmless to sperm (Eaglesome *et al.*, 1994).

No commercial vaccines are presently available for infections caused by ureaplasmas. However, an experimental vaccine provoked some immunity. Mulira *et al.* (1992) measured antibody levels in serum and cervicovaginal mucus (CVM) of heifers vaccinated with killed *U. diversum* strain 2312 in adjuvant; control heifers were given a placebo before both groups were challenged intravaginally. Vaccination stimulated specific IgG1 and IgG2 responses in the serum and CVM but only a slight IgM and no IgA response. It was concluded that vaccination stimulated a specific, albeit non-protective, IgG response in serum and CVM.

While the role of ureaplasmas in respiratory infections is slight or not proven, control measures which reduce environmental stress, including ensuring adequate housing with good circulation of air and adopting 'all in, all out' practices wherever possible, should have beneficial effects. If this is not possible, separation of calves from the adults is advisable at the earliest opportunity where endemic disease exists.

Although survival time of *U. diversum* in the environment, such as floors and slurry, does not exceed 24 h, Pilaszek and Truszczynski (1991) showed that it was sufficient to infect preputial mucosa in bulls but not heifers. These workers also showed that asymptomatic carriers can shed *U. diversum* with urine for prolonged periods. It was concluded that disinfection of cowsheds and treatment of carriers with antibiotics could play an important role in prevention of ureaplasma infection.

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# 14 New, Emerging and Unculturable Mycoplasmas in Ruminants

*"... it is to be expected that as our understanding of mollicute nutrition increases, improved isolation media will enable growth of hitherto unsuspected species."* Roger Miles, Kings College, London (1992).

## Introduction

Increases in the isolations of new or unusual mollicutes from ruminants are seen from time to time; the reason is not always clear but may be due to the introduction of any affected animal from another country or region, leading to a rapid dissemination in the susceptible animal population, such as may have been seen with *M. canis* in cattle. The sudden appearance of *M. canis* in the UK in the mid-1990s represented a real increase in isolations because the species grows quickly and would almost certainly have been detected previously by traditional culture methods. The possibility that the mycoplasmas *M. bovis* or *M. bovigenitalium* may have been introduced through infected semen cannot be discounted. However, with other more fastidious mollicutes, such as *M. dispar*, the use of improved media formulations (Ayling *et al.*, 2004) or molecular detection methods such as PCR would account for an increased prevalence. Genetic-based methods have certainly overcome the problems of serological cross-reactions seen with immunologically related species but are also prone to misidentification because of the close homology of the 16S rDNA sequence of some mycoplasmas, which is the target of many PCR tests.

## Mycoplasma Transmission between Animals and Man

Mycoplasmas are ubiquitous throughout the animal kingdom and virtually every mammal, bird, reptile, amphibian and fish that has been examined for

mycoplasmas has revealed unique species. Although it is often assumed that *Mycoplasma* species are extremely host specific, it is well known that some species, including *M. bovis*, *M. arginini*, *M. agalactiae*, *M. mycoides* SC and LC types and *Acholeplasma laidlawii* have a broader host range and can pass easily between sheep, goats and cattle and that *M. gateae* is found equally in cats and dogs.

Mycoplasmas are most likely to be acquired by their host as a result of intimate contact and transfer of material between mucosal surfaces, for example from dam to calf or through artificial insemination. It is possible that interspecies transfer may also occur on occasion, as between man and farm animals, but whether the foreign mycoplasma will survive for long in its new environment is not known.

Reports of infections in man with animal species of mycoplasma are rare, but infections probably occur more frequently than is generally recognized, the majority of cases remaining undiagnosed because so few laboratories are equipped to detect and identify mycoplasmas.

In some cases there is no certain evidence that the infecting organism was acquired from contact with another animal species. This is the case with *M. fermentans*, a frequent contaminant of laboratory cell lines. The species has been isolated from man on rare occasions but its DNA is very commonly detected by PCR in the synovial fluid of patients with rheumatoid arthritis (Schaeverbeke *et al.*, 1996) and has been associated with infections in patients with AIDS (Pitcher and Nicholas, 2005) and Gulf War Syndrome (Nicolson and Nicolson, 1996). Despite the apparent frequency with which *M. fermentans* is detected by PCR, no evidence of the species' natural habitat has been reported and it is far from certain that humans are its only host. Nicholas *et al.* (1998) reported the first isolation of *M. fermentans* from genital infections in sheep; since then further isolations have been made from both rams and ewes with genital lesions (Fig. 14.1) in three other sheep flocks in England (Nicholas *et al.*, 2000b). No route of transmission from sheep to man or vice versa has yet been identified.

Ruminant species may colonize human body sites as secondary invaders without causing disease. Madoff *et al.* (1979) reported a case where *M. bovis*, a cause of mastitis, arthritis and respiratory disease in cattle, was isolated from the sputum of a woman with bronchopneumonia and central nervous system abnormalities. The only contact the patient had with cattle was exposure to cow manure while gardening 3 weeks before developing respiratory disease. There was serological evidence of *M. pneumoniae* but this species was not isolated. Attempts to identify *M. bovis* antibodies in the patient's blood were unsuccessful and the disease subsided following tetracycline therapy. The patient's symptoms were consistent with *M. pneumoniae* as the primary aetiological agent and the presence of *M. bovis* could not be explained. In another case of *M. bovis* infection in a human that responded to tetracycline treatment, there was no evidence of *M. pneumoniae* (Pitcher and Nicholas, 2005).

*Mycoplasma* infections are particularly common in immunocompromised patients, and cases of human infection with mycoplasmas normally inhabiting domestic animals are probably due to persistent and intimate contact with these susceptible patients. Armstrong *et al.* (1971) demonstrated colonization of the throat by *M. canis* in several family members who were in very close contact with

**Fig. 14.1.** *M. fermentans* was isolated from a severe case of balanitis in a ram in the UK.

their dog. One family member, who was receiving anti-neoplastic chemotherapy, developed a respiratory infection which was treatable by tetracycline.

A fatal infection as a result of pneumonia and septicaemia due to *M. arginini* in an abattoir worker with advanced non-Hodgkin's lymphoma has been reported (Yechouron *et al.*, 1992). The natural habitat of *M. arginini* is unknown and the species has been isolated from a wide range of domestic animals, but most commonly from sheep and goats and is probably the least host-specific mycoplasma known. It is also one of the commonest species to infect laboratory cell lines.

## Mycoplasma Transfer between Animal Species

The canine species, *M. canis*, appears to be accepted as a part of the bovine flora where there is contact between dogs and cattle (ter Laak *et al.*, 1993). Interestingly, while it mainly causes reproductive disease in dogs, in cattle respiratory disease is most frequent. Its widespread occurrence in pneumonic calves in the UK since its first isolation in 1995 indicates its successful colonization of this host (Nicholas *et al.*, 2000a).

The presence of the goat pathogen *M. mycoides* LC in cattle has been described previously but was thought to be avirulent for this species (Nicholas and Bashiruddin, 1995). However, the spread of LC from goats to calves through infected unpasteurized goat milk in New Zealand led to severe outbreaks of polyarthritis, septicaemia and deaths (Jackson and King, 2002). LC and *M. capricolum* subsp. *capricolum* have also been detected serologically in South American camelids (Hung *et al.*, 1991; Nicholas, 1998), but isolates of these mycoplasmas from these animals remain elusive.

*M. bovis*, a major cause of calf pneumonia and mastitis, was originally classified as a subspecies of the small ruminant pathogen *M. agalactiae* because of its close immunological and biochemical relationship; however, distinct genetic differences, particularly in the *uvr*C gene, have been exploited to develop a PCR capable of distinguishing the two species (Subramaniam *et al.*, 1998). This PCR was used to identify *M. bovis* in goats with mastitis in the UK (Ayling *et al.*, 2004); without this test there would have been real concerns that the UK was experiencing its first case of contagious agalactia.

Since its first isolation in 1972 in Australia (Carmichael *et al.*, 1972) the unclassified mycoplasma *M.* ovine/caprine serogroup 11 has been found sporadically in sheep and goats with a variety of clinical conditions, including infertility and vulvovaginitis (Nicholas *et al.*, 2002). The biochemically similar *M. bovigenitalium* causes similar conditions in cattle. In a comparison of strains from these two species, Nicholas *et al.* (2002, 2008) demonstrated very close genetic, biochemical and immunological relationships between the two species and recommended that the *M.* serogroup 11 should be reclassified as *M. bovigenitalium*.

One of the recommended sampling sites for pathogenic mycoplasmas in sheep and goats is the ear canal and it is believed to be due to the presence of ear mites (*Psoroptes cuniculi* or *Railleria caprae*), from which mycoplasmas have been isolated (Da Massa and Brooks, 1991). Goat fleas (order Siphonaptera) too have been implicated in disease transmission; Nayak and Bhowmik (1990) showed that the blood taken from polyarthritic goat kids contained up to $10^5$ organisms/ml of viable *M. mycoides* LC organisms, and when fleas were placed on unaffected kids polyarthritis and septicaemia followed. What is not clear from either study is whether this represents just the passive carriage of mycoplasmas from the blood of acutely affected animals or whether mites and fleas act as reservoirs in which mycoplasmas replicate. Further study is necessary to clarify these findings.

## Unculturable Mycoplasmas

*Mycoplasma wenyonii* is a wall-less haemotrophic prokaryote previously classified as *Eperythrozoon wenyonii* in the order Rickettsiales, but recently assigned to the *Mycoplasma* genus on the basis of 16S rDNA analysis (Neimark *et al.*, 2001). *M. wenyonii* adheres to erythrocytes but may also be found in plasma unattached to erythrocytes (Neimark *et al.*, 2001). Scanning electron microscopic analysis of *M. wenyonii*-infected blood shows deformed erythrocytes with invaginations and

the presence of either rod- or coccoid-shaped organisms embedded in the membrane of erthrocytes (Neimark *et al.,* 2001; Bretana *et al.*, 2002). Although the mode of transmission is unknown, there is evidence that flies, lice and mosquitoes may serve as mechanical vectors and oral transmission also seems likely (Smith *et al*., 1990; Prullage *et al.*, 1993; Hofmann-Lehmann *et al.*, 2004). While the precise role of *M. wenyonii* in disease is still controversial, infections caused by *M. wenyonii* have been reported to result in parasitaemia and anaemia, with infected cattle rarely dying but on occasions showing acute clinical signs (Smith *et al*., 1990). Scrotal and hind limb oedema, tachycardia, pyrexia and infertility were reported in a Charolais bull in the USA that was chronically infected with *M. wenyonii* (Montes *et al*., 1994). In addition, dairy cows with swollen teats, oedema of the distal portion of the hind limbs, transient fever, prefemoral lymphadenopathy, rough coat, dramatically decreased milk production and subsequent infertility and weight loss have been reported (Smith *et al*., 1990). Recently, other as yet unclassified haemotrophic mycoplasmas have been discovered in cattle in Switzerland with severe anaemia that were co-infected with *M. wenyonii* and *Anaplasma marginal* (Hofmann-Lehmann *et al*., 2004); however, these organisms have not yet been found elsewhere.

Previously, diagnosis of *M. wenyonii* has been based on blood smears but these are not specific for *M. wenyonii* and can be difficult to interpret. The PCR and denaturing gradient gel electrophoresis (DGGE), which has previously been described for the detection and differentiation of culturable *Mycoplasma* species (McAuliffe *et al*., 2003, 2005), can be used on blood samples as a rapid and specific test for *M. wenyonii* and can also be used as a screening test for other blood-borne pathogens (McAuliffe *et al*., 2006).

*Mycoplasma ovis*, formerly *Eperythrozoon ovis*, is an reythrocytic agent that causes haemolytic anaemia in sheep and goats and it occurs worldwide. *M. ovis* was previously thought to be a rickettsia, however, phylogenetic analysis revealed that this wall-less bacterium is not a rickettsia but a mycoplasma. This mycoplasma is related closely to several other uncultivated, blood-borne mycoplasmas that comprise a recently identified group, the haemotrophic mycoplasmas (haemoplasmas). It is likely that *M. ovis* is present in many flocks causing asymptomatic infection. In some instances the presence of bacterial infection progesses to disease, characterised by the destruction of red blood cells, leading to anaemia and jaundice. Goats can also be infected. Deaths may occur in severely affected young sheep, especially if they are stressed by handling or movement. Losses of up to 30% of the flock have occurred in these circumstances. As with other haemotrophic mycoplasmas, it is thought that *M. ovis* is transmitted mainly by blood-feeding arthropod vectors.

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