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ISSN 1466-2523

DOI: 10.1017/S1466252307001351

Mycoplasma bovis pneumonia in cattle

Jeff L. Caswell[1]* and Marie Archambault[2]

[1]*Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, N1G 2W1, Canada and* [2]*Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, CP 5000, 3200 rue Sicotte, Saint-Hyacinthe, QC, J2S 7C6, Canada*

Received 15 October 2007; Accepted 25 October 2007

Abstract

Mycoplasma bovis is an important and emerging cause of respiratory disease and arthritis in feedlot cattle and young dairy and veal calves, and has a variety of other disease manifestations in cattle. *M. bovis* is certainly capable of causing acute respiratory disease in cattle, yet the attributable fraction has been difficult to estimate. In contrast, *M. bovis* is more accepted as a cause of chronic bronchopneumonia with caseous and perhaps coagulative necrosis, characterized by persistent infection that seems poorly responsive to many antibiotics. An understanding of the disease has been recently advanced by comparisons of natural and experimentally induced disease, development of molecular diagnostic tools, and understanding some aspects of virulence, yet uncertainties regarding protective immunity, the importance of genotypic diversity, mechanisms of virulence, and the role of co-pathogens have restricted our understanding of pathogenesis and our ability to effectively control the disease. This review critically considers the relationship between *M. bovis* infection and the various manifestations of the bovine respiratory disease complex, and addresses the pathogenesis, clinical and pathologic sequelae, laboratory diagnosis and control of disease resulting from *M. bovis* infection in the bovine respiratory tract.

Keywords: *Mycoplasma bovis*, pneumonia, arthritis, bacteria, cattle, review

Introduction

Mycoplasma bovis (formerly *Mycoplasma agalactiae* subsp. *bovis*) was first identified in 1961 from a case of mastitis (Hale *et al*., 1962), and was described as a cause of respiratory disease in 1976 (Gourlay *et al*., 1976). Since then, *M. bovis* has emerged as an important cause of pneumonia in feedlot cattle and dairy calves. Other disease manifestations include arthritis and tenosynovitis (Singh *et al*., 1971; Gourlay *et al*., 1976; Ryan *et al*., 1983; Adegboye *et al*., 1996; Pfutzner and Sachse (1996); Byrne *et al*., 2001a; Gagea *et al*., 2006a), mastitis (Fox *et al*., 2005), otitis media in dairy calves and unweaned beef calves but uncommonly in feedlot cattle (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004; Gagea *et al*., 2006a), keratoconjunctivitis (Levisohn *et al*., 2004; Alberti *et al*., 2006), decubital abscesses (Kinde *et al*., 1993), metritis, abortion and infertility, seminal vesiculitis (LaFaunce and McEntee, 1982), and perhaps meningitis (Stipkovits *et al*., 1993).

M. bovis has been the subject of considerable investigation in the past 40 years, yet gaps in knowledge limit our ability to control diseases resulting from infection with this pathogen. The challenges of isolating and manipulating mycoplasmas *in vitro*, the high prevalence of infection in some groups of healthy cattle, the discrepancies between experimentally induced and natural disease, and the lack of simple correlation between antibody titers and disease resistance are some factors that complicate the study of the role of *M. bovis* in pneumonia of cattle. Areas of substantial uncertainty include the clinical importance of the genotypic diversity of this pathogen, knowledge of virulence factors and how infection leads to tissue damage, mechanisms of immunity and the potential for development of effective vaccines, the specific role of co-pathogens and environmental factors in development of disease and the overall importance of this agent as a contributor to the bovine respiratory disease complex.

*Corresponding author. E-mail: jcaswell@uoguelph.ca

https://doi.org/10.1017/S1466252307001351 Published online by Cambridge University Press

M. bovis is a member of the family *Mycoplasmataceae* in the class *Mollicutes* ('soft skin') (Waites, 2007). The *Mollicutes* are prokaryotes of gram-positive lineage that evolved from *Clostridium*-like bacteria by gene deletion. The genus *Mycoplasma* comprises the smallest self-replicating life forms and includes over 100 species. Most mycoplasmas are facultative anaerobes, and all have a tri-layered cell membrane but lack the genetic ability to form a cell wall. The lack of a cell wall results in pleomorphic shapes, confers resistance to antimicrobials of the β-lactam group, and interferes with Gram staining. The small genome size of *Mycoplasma* species, ranging from 580 to 1380 kb, may explain their dependency on the host or complex media for essential nutrients, and their limited survival in the environment. The genome of *M. bovis* is 1080 kb with a low G+C ratio of 27.8–32.9 mol% (Hermann, 1992; Nicholas and Ayling, 2003). *M. bovis* and *M. agalactiae* are closely related, with 99% homology in 16S rRNA (Mattsson *et al.*, 1994). *M. bovis* appears to be of no significance as a human pathogen, as there is only one report of its association with human disease: it was cultured from the sputum of a patient with lobar pneumonia and probable myocarditis, nephritis and hemolytic anemia (Madoff *et al.*, 1979).

Laboratory diagnosis

Specimens

Mycoplasmas are extremely sensitive microorganisms, so specimens must be kept refrigerated and delivered to a laboratory within 24 h after collection if immediate transportation to the laboratory is not possible. If shipment or storage times exceed 24 h, the specimen should be placed in transport medium and frozen at −70°C to prevent loss of viability of the mycoplasma. For examination, specimens should be thawed very rapidly in a water bath at 37°C (Murray and Baron, 2003). Samples of choice for the various diseases caused by *M. bovis* have been reviewed (Nicholas and Ayling, 2003) and include bronchoalveolar lavage fluid, pneumonic lung tissue, synovial tissues or joint fluids, mastitic milk, semen, genital discharge, washings of the prepuce and eye swabs. Serum is the appropriate sample for antibody detection. If swabs are used, Dacron, calcium alginate or polyester swabs are preferred over wooden-shaft cotton swabs which are potentially inhibitory (Murray and Baron, 2003). Swabs should be used thoroughly and vigorously to obtain many cells, as mycoplasmas are cell-associated. Swabs should be submitted in transport medium such as Stuart's and Eaton's media. Culture results from nasal swabs do not correlate well with those from the lower respiratory tract (Thomas *et al.*, 2002b), suggesting that bronchoalveolar lavage fluid is preferable as an indicator of lung infection.

Isolation

Samples for *M. bovis* isolation should always be thoroughly mixed before the inoculation of media. Fluids should be centrifuged and the pellet used for inoculation. If contaminants are suspected, samples should be passed through a 0.45 μm filter. To overcome the inhibitory effects of antibodies, antibiotics, bacteria and other inhibitors in the specimen, samples such as bronchoalveolar lavage fluid should be serially diluted in broth, with subculture of each dilution (Murray and Baron, 2003; Arcangioli *et al.*, 2007).

Mycoplasmas require sterols for growth in media, and these are supplied by the addition of serum. *M. bovis* can grow easily in various media such as Eaton's, supplemented pleuropneumonia-like organism (PPLO) medium, N-agar or broth, modified Friis medium, modified Hayflick medium and other commercially available mycoplasma culture media (Nicholas and Baker, 1998). Broth media should contain yeast extract, tryptone, 20% horse serum and 500 μg/ml of ampicillin, and are incubated at 37°C for 3–10 days for recovery of *M. bovis*. The filtration-cloning technique must be used to obtain a pure culture of a field isolate, which involves filtration of the broth culture through a 220–450 nm pore-size membrane filter to remove microcolonies. The filtrate is then serially diluted (10^{-1} to 10^{-4}) in broth medium and plated on agar, where *M. bovis* will usually produce visible colonies within 2–4 days. Single colonies are picked up with a Pasteur pipette or a needle for subcloning back into a series of broth cultures. The classical method recommends that the isolates be cloned three times by filtration to obtain a pure culture (Tully, 1983), but this is sometimes not followed because of the risk of mutations associated with adaptation to *in vitro* growth.

Broths should be incubated at 37°C under atmospheric conditions but agar plates are incubated with 5–10% CO_2 (Murray and Baron, 2003); use of a candle jar may be satisfactory. Each new batch of medium and supplements should be tested with a laboratory control strain such as the *M. bovis* international reference strain PG45, to ensure that the medium sustains growth and does not contain any inhibitory substances. *M. bovis* isolation from chronically affected animals is reportedly more difficult due to inhibitory substances such as antibiotics and contaminants (Ayling *et al.*, 2005). A pure culture of the mycoplasma should be obtained before carrying out identification procedures.

Identification

Colonies of *M. bovis* can be identified at the genus level based on their appearance with magnification, by use of Dienes' stain, and biochemical tests, but species-level identification of the isolates requires testing by ELISA or

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PCR. The agar plates are viewed under a stereomicroscope at 20–60× magnification. It usually requires 2–5 days for *M. bovis* to produce visible colonies, but this is very dependent on the quality of the media and growth in less than 20 h is reported as common to many field strains in appropriate media (Tully, 1983; Nicholas and Ayling, 2003). Colonies of *M. bovis* often have a typical fried-egg appearance, but *M. bovis* produces films and spots in some media such as Eaton's medium (Nicholas and Baker, 1998). Colonies can be small (0.1–0.5 mm), dark and lack halos (Stalheim and Proctor, 1976). Air bubbles, water or lipid droplets may be confused with mycoplasmal colonies. Dienes' stain, a commercially available methylene blue stain, is useful for easier visualization of colonies, and also aids in differentiating mycoplasmas from debris and bacterial L-form colonies. Mycoplasmal colonies stain blue with Dienes' stain and retain their color indefinitely, whereas bacterial L-form colonies are reported to decolorize. The denser center of the colonies, which grows down into the agar, will stain dark blue, whereas the less dense peripheral zone of surface growth stains light blue (Waites *et al*., 1997; Francoz *et al*., 2005).

Mycoplasmas form coccoid cells of about 0.2–0.3 μm in diameter, which cannot be clearly seen by routine light microscopy. Lack of a cell wall prevents staining with the Gram stain, and although they do stain with Giemsa, interpretation is difficult because of their small size (Murray and Baron, 2003). Acridine orange staining has proved to be useful in the recognition and enumeration of mycoplasma cells (Rosendal and Valdivieso-Garcia, 1981; Jasper *et al*., 1984) but this stain is not specific to mycoplasmas.

Biochemical tests such as glucose fermentation, arginine hydrolysis, phosphatase activity, urease activity, film production and reduction of tetrazolium can be used to differentiate *M. bovis* from other mycoplasmas or ureaplasmas (Poveda, 1998). Biochemical characteristics of *M. bovis* and other mycoplasmas have been reviewed (Nicholas and Ayling, 2003). Biochemical tests are of little value to differentiate *M. bovis* and *M. agalactiae*: neither species ferment glucose or hydrolyse arginine, and both use organic acids such as lactate and pyruvate, give an orange color to the broth with an absence of turbidity (Abu-Amero *et al*., 2000; Khan *et al*., 2005a), oxidize ethanol (Abu-Amero *et al*., 2000), and produce film and spot reactions in media as a result of lipolytic activity. Molecular tests are able to distinguish these two bovine pathogens (Subramaniam *et al*., 1998; Thomas *et al*., 2004a; Bashiruddin *et al*., 2005).

Antibody-based tests using hyperimmune rabbit serum have been used to identify *M. bovis*, including growth inhibition, film inhibition, fluorescent antibody and metabolic inhibition tests (Poveda and Nicholas, 1998). These techniques are relatively slow and labor-intensive because the antisera are not commercially available and must be produced in the laboratory. A sandwich ELISA using monoclonal antibodies (Ball *et al*., 1994) is frequently used to confirm *M. bovis* identity and is available commercially (Bio X, Luxembourg). Mycoplasmas can also be identified by dot immunobinding on membrane filtration (MF-dot) using polyclonal antisera prepared against the reference strain PG45 (Poumarat *et al*., 1991; Arcangioli *et al*., 2007). Monoclonal antibody-based ELISA (Boothby *et al*., 1986) and antigen-capture ELISA (Heller *et al*., 1993) have been developed to detect *M. bovis* from milk samples. Other assays to identify *M. bovis* include immunoperoxidase labeling (Nielsen *et al*., 1987; Gourlay *et al*., 1989), immunobinding assays (Infante Martinez *et al*., 1990; Infante *et al*., 2002; Flores-Gutierrez *et al*., 2004) and flow cytometry (Assuncao *et al*., 2006). The sensitivity of many immunological techniques can be improved with an enrichment step (Boothby *et al*., 1986; Infante Martinez *et al*., 1990; Heller *et al*., 1993). Conventional diagnostic methods for the detection of *M. bovis* have been compared previously (Sachse *et al*., 1993b) and molecular tests for the identification of *M. bovis* are described below. Immunohistochemistry is an excellent method for detection of *M. bovis* antigen in formalin-fixed tissues and the distribution of antigen detected by this technique is discussed below (Haines and Chelack, 1991; Adegboye *et al*., 1995a, b; Rodriguez *et al*., 1996b, 2000; Shahriar *et al*., 2002; Haines *et al*., 2004; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006a).

Strain typing

Typing systems are expected to reveal new insights in the epidemiology of *M. bovis* infection, both within herds and across larger populations. Conventional typing systems such as sodium dodecyl sulfate polyacrylamide gel electrophoresis have not revealed sufficient variability to differentiate between strains, although differences in the level of expression of 64–68, 55 and 26 kDa proteins were detected (Sachse *et al*., 1992; Cerda *et al*., 2000). Better discrimination is achieved using molecular methods and are currently recommended.

Random amplified polymorphic DNA analysis (RAPD) identified three groups (A–C) within 54 isolates of *M. bovis* from the United Kingdom (UK), containing 54, 35 and 11% of the isolates. Amplified fragment length polymorphism analysis (AFLP) showed more diversity, with two clusters (A and B) that included 35 banding profiles from 54 UK isolates (McAuliffe *et al*., 2004) and 18 profiles from 42 Danish isolates (Kusiluka *et al*., 2000a). About 80–90% of isolates were assigned to the same group using AFLP and RAPD. For both techniques, the most prevalent group included the type strain PG45, and group A isolates were more diverse than those in group B. Ten isolates from the same herd had identical RAPD results, suggesting transmission between calves or a common source of infection. The banding patterns were considered reproducible, despite variation in the intensity

https://doi.org/10.1017/S1466252307001351 Published online by Cambridge University Press

of bands (McAuliffe *et al.*, 2004). RAPD requires little in the way of specialized equipment, but gel analysis software is needed and careful technique is required to avoid poor reproducibility. In contrast, AFLP has high discriminatory power and better reproducibility, and may be more well suited to analysis of large numbers of samples in different laboratories.

Pulsed-field gel electrophoresis (PFGE) using *Sma*I revealed 23 different profiles from 43 isolates from the UK. The congruency of PFGE with RAPD and AFLP varied by genotype: the concordance between PGFE and RAPD/AFLP was 77–85% for group A isolates but less for group B isolates, although identical PGFE results were obtained for all 10 isolates from a single herd (McAuliffe *et al.*, 2004). Vsp expression was apparently not related to any of the AFLP, RAPD or PFGE typing patterns, implying that recombination in the Vsp locus does not introduce variability to the molecular typing methods. Compared to RAPD and AFLP, PGFE has good discriminatory power but is time-consuming, laborious and cannot type all isolates (McAuliffe *et al.*, 2004).

Arbitrarily primed polymerase chain reaction (AP-PCR) was used to identify seven banding patterns in 50 isolates from three herds. AP-PCR was considered rapid, simple and reproducible, with identical results on isolates obtained from the same animal on different dates (Butler *et al.*, 2001). Analysis of 16S rRNA gene sequences revealed 12 polymorphisms among the eight isolates of *M. bovis*, which may have some value as epidemiologic markers but are not useful for differentiation of *M. bovis* and *M. agalactiae* (Konigsson *et al.*, 2002).

These typing data have been used to suggest that group A isolates from the UK may have been recently introduced from Europe at the time of increased importation of cattle following creation of the European Union, whereas group B isolates derived from those present in the UK prior to this time (McAuliffe *et al.*, 2004). The absence of any relationship between molecular patterns, geographic origin of the isolates, or the year in which the UK isolates were obtained may reflect movement of cattle between herds (McAuliffe *et al.*, 2004). In contrast, AFLP typing data have suggested greater genetic diversity in recent isolates compared to older Danish *M. bovis* isolates (Kusiluka *et al.*, 2000a). Finally, the AP-PCR studies showed high homology and stability over time among isolates obtained from a closed cow-calf beef herd and a 200-head feedlot, but a greater diversity with five banding patterns among isolates from a ranch receiving 20,000 dairy calves, consistent with the diverse sources of calves in this herd (Butler *et al.*, 2001).

Insertion elements are mobile 0.7–2.5 kb fragments of DNA that transfer between bacteria, and their distribution and copy number give clues to the evolution of pathogenic mycoplasmas. A study suggested an older divergence of *M. bovis* and *M. agalactiae*, but more recent transfer of genetic elements from *M. mycoides* ssp. *mycoides*, perhaps from concurrent infection of a single animal by these two species (Thomas *et al.*, 2005b). A similar approach was used to identify variability between 49 UK isolates of *M. bovis* (Miles *et al.*, 2005).

Serology

Serology is effective in indicating exposure to *M. bovis* (Nicholas and Ayling, 2003), and may be more sensitive than culture in chronic cases or those treated with antibiotics, as these factors may interfere with the *in vitro* growth of *M. bovis* (Nicholas and Ayling, 2003). *M. bovis* has both lipid and protein antigens which can elicit antibody responses, and antibody levels remain high for many months (Nicholas and Ayling, 2003). Serology is useful to identify groups of cattle free of infection, but the high seroprevalence in many populations limits its usefulness for routine diagnosis, and antibody titers are not correlated with disease resistance.

Many serological tests have been developed, including indirect hemagglutination, film inhibition and indirect ELISA. Indirect ELISAs using whole cell or treated antigen are the principal methods used for serological testing. Commercial ELISA tests are also available (Biovet, Canada; Bommelli, Switzerland) (Nicholas and Ayling, 2003). The ELISA has been used to assess the seroprevalence of *M. bovis* infection (Grand *et al.*, 2002) and to select *M. bovis*-free cattle for creation of disease-free herds (O'Farrell *et al.*, 2001). Antibodies against *M. bovis* in milk samples have been detected by ELISA, making it possible to identify individual infected quarters (Byrne *et al.*, 2000). A blocking ELISA, which used a monoclonal blocking antibody to an unknown antigen and whole-cell lysate of *M. bovis* as the target antigen, detected serum antibodies to *M. bovis* with high specificity, good sensitivity, low background and lack of cross-reaction (Ghadersohi *et al.*, 2005).

Other ELISAs identify serum antibodies to specific *M. bovis* proteins, for example those using recombinant VspA as a target antigen (Brank *et al.*, 1999) or a membrane lipoprotein homologous to the P48 of *M. agalactiae* (Robino *et al.*, 2005). Antisera raised against the P48 protein identified all *M. bovis* isolates tested, but did not react with six other mycoplasma species of cattle, suggesting that the *M. bovis* P48 ELISA may be useful as a specific marker of infection (Robino *et al.*, 2005).

Molecular tests

Culture and serology have long been the mainstays for diagnosis of *Mycoplasma* infection. Molecular techniques do not eliminate the need for these traditional techniques, but have the advantage of readily available equipment and expertise, rapid sample processing, integration with testing for other pathogens, cost

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efficiency, and the potential for improved sensitivity and specificity. Molecular techniques may be particularly appropriate in situations that interfere with mycoplasma isolation, including samples treated with preservatives, contaminated with bacteria or containing antibodies, from animals treated with antibiotics, or from chronic lesions with tissue debris or autolysis (Pinnow *et al.*, 2001; Cai *et al.*, 2005). The differentiation of *M. bovis* and *M. agalactiae* is an important goal of PCR assays, because distinguishing these two pathogens using conventional techniques is challenging (Bashiruddin *et al.*, 2005).

Amplification of the 16S rRNA gene by PCR does not adequately distinguish *M. bovis* and *M. agalactiae* unless additional methods are used (Subramaniam *et al.*, 1998). One such adaptation is the use of real time PCR for amplification of a 16S rRNA gene fragment, with use of hybridization probes or melting temperature analysis of the product to identify and differentiate *M. bovis* and *M. agalactiae* in milk or lung tissue, in a sensitive, specific, rapid and cost-effective manner (Cai *et al.*, 2005). This RT-PCR is now offered routinely by some laboratories as a same-day diagnostic test for milk samples. Similarly, PCR-based amplification of a 16S rRNA gene fragment can be followed by denaturing gradient gel electrophoresis for rapid differentiation of these two species (McAuliffe *et al.*, 2005).

Other gene targets provide direct identification to the species level. PCR-based amplification of the *uvrC* gene is very useful for identification of *M. bovis* and differentiation from other mycoplasmas including *M. agalactiae* (Subramaniam *et al.*, 1998; Bíró *et al.*, 2004), and was recently modified to allow smaller sample and reaction volumes (Thomas *et al.*, 2004a). Similarly, PCR assays targeting the ATP-binding protein *opp*D/F (Hotzel *et al.*, 1996) or the DNA polymerase III gene (Marenda *et al.*, 2005) were able to identify and differentiate *M. agalactiae* and *M. bovis*. Amplification of membrane protein 81 using multiplex PCR is able to identify *M. agalactiae* and *M. bovis*, and restriction fragment length analysis differentiates these two species (Foddai *et al.*, 2005).

Another *M. bovis*-specific PCR targets an unknown gene sequence (Ghadersohi *et al.*, 1997), and was adapted to a semi-nested PCR with improved sensitivity and reproducibility on milk and mucosal samples (Hayman and Hirst, 2003). A modification of the primers was reported to improve performance of this assay (Tenk *et al.*, 2006).

A study comparing PCR assays routinely used in five laboratories suggested that those targeting the *uvc* and *opp*D/F genes had the highest reliability, and the molecular methods identified four isolates with incorrect species identification by conventional diagnostic methods (Bashiruddin *et al.*, 2005). Antigen capture (Hotzel *et al.*, 1999) or DNA-binding filter membranes (Hotzel *et al.*, 1996) have been used to enhance the sensitivity of PCR for detection of *M. bovis* in milk samples.

Antimicrobial susceptibility testing

Many methods of antimicrobial susceptibility testing used for conventional bacteria have also been used for testing mycoplasmas. Agar dilution has been employed as a reference method (Bebear *et al.*, 1997). However, this technique is not practical to test small numbers of isolates. Agar disk diffusion is not useful for testing mycoplasma species because of the slow growth of these microorganisms and the absence of correlation between zone sizes and minimum inhibitory concentrations (MICs). The microbroth dilution is the most practical and widely used technique since it is economical and permits the testing of several antimicrobial agents in the same microtiter plate. However, this method is labor-intensive because antimicrobial dilutions must be prepared, and the end point tends to shift over time. The 'Sensititer' (Trek Diagnostics) version of the microbroth dilution method removes the labor-intensivity of the technique. The E test (AB BIODISK, Solna, Sweden) has the advantage of simplicity, minimal effect of inoculum size, stable end point and adaptativity for testing single isolates. These methods have all been used to determine the antimicrobial susceptibility of *M. bovis* strains or isolates (Kobayashi *et al.*, 1996; Hannan, 2000).

Irrespective of the method, there are no universally accepted guidelines for performing mycoplasma antimicrobial susceptibility tests. In the absence of accepted breakpoint values, susceptibilities to different antimicrobial agents are usually based on the Clinical Laboratory Standards Institute values for common veterinary pathogenic bacteria (CLSI, formerly National Committee for Clinical Laboratory Standards, 2002) or CLSI values for human pathogens (Francoz *et al.*, 2005). In Japan, the MICs are measured according to a standard method (Japanese Society of Chemotherapy, 1981), in which the breakpoints of MICs for tetracyclines, macrolides, aminoglycosides and fluoroquinolones are 1.0, 0.5–2.0, 2.0–4.0 and 1.0–2.0 $\mu g\ ml^{-1}$, respectively (Japanese Society of Chemotherapy, 1994) based on values for human pathogens (Hirose *et al.*, 2003). In the absence of accepted breakpoint values, published MIC cut-off values for animal mycoplasmas as well as CLSI interpretive criteria can be used as a reference to define *in vitro* activity (Rosenbusch *et al.*, 2005).

Several methodologies have been used to report MIC values for *M. bovis*. In the agar dilution method (Kobayashi *et al.*, 1996; Hannan, 2000), antimicrobial agents were serially diluted in M-broth and mixed with M-agar; the initial MICs were read as soon as the colonies appeared during incubation, while final MICs were read 3 days later (Hirose *et al.*, 2003). A broth microdilution method is described (Rosenbusch *et al.*, 2005), using detection methods including redox indicator (ter Laak *et al.*, 1993), acid production from pyruvate (Thomas *et al.*, 2003a) and growth pellet formation (Ayling *et al.*, 2000). Antimicrobial susceptibility test using alamarBlue

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is described (Rosenbusch *et al.*, 2005), in which diluted cultures were added to custom prepared 'Sensititer' plates, and growth inhibition was detected by changes in the redox status of the resazurin (alamarBlue) indicator dye (Rosenbusch *et al.*, 2005). Finally, in the E test method (Tanner and Wu, 1992; Hannan, 2000; Francoz *et al.*, 2005), commercially available E test strips impregnated with different antimicrobial agents are placed on agar plates that have been inoculated with a diluted culture of *M. bovis*. To date, there are no specified procedures to ensure an adequate inoculum size for performing E tests on *M. bovis*, but inocula of 10^5 CFU ml^{-1} have been recommended (Hannan, 2000; Francoz *et al.*, 2005). Growth inhibition, read with a stereomicroscope, is facilitated by staining with Dienes' stain (Waites *et al.*, 1997).

Antimicrobial resistance (AMR)

The AMR of *M. bovis* isolates has been studied in Europe (Wachowski and Kirchhoff, 1986; Poumarat and Martel, 1989; ter Laak *et al.*, 1993; Ayling *et al.*, 2000; Poumarat *et al.*, 2001; Vogel *et al.*, 2001; Nicholas and Ayling, 2003), Canada (Francoz *et al.*, 2005), the United States (Rosenbusch, 2000; Rosenbusch *et al.*, 2005), Italy (Mazzolini *et al.*, 1997) and Japan (Hirose *et al.*, 2003). Similar AMR profiles were obtained in Europe and the United States, with a high prevalence of resistance (high MIC) to tilmicosin, erythromycin, ampicillin and ceftiofur; intermediate occurrence of resistance to oxy- and chlortetracycline, and infrequent or no resistance (low MICs) to enrofloxacin, florfenicol and spectinomycin (Rosenbusch *et al.*, 2005). The main differences observed between isolates were in the high level of oxytetracycline and chlortetracycline resistance in Dutch and British isolates compared to American ones, and the isolation of florfenicol-resistant *M. bovis* isolates in the British study. In a Canadian study, susceptibility to enrofloxacin was also observed, as was the development of acquired resistance to tetracycline, spectinomycin, azythromycin and clindamycin in *M. bovis*. (Francoz *et al.*, 2005). In Japan, all *M. bovis* isolates tested showed a high susceptibility to tiamulin while erythromycin had no activity on any of the isolates. The *M. bovis* isolates were less susceptible than the type strains to spiramycin, oxytetracycline and tylosin, suggesting that resistance to these antimicrobials may be appearing in field isolates in Japan (Hirose *et al.*, 2003). MICs from this study were higher than those reported in a previous Japanese study (Katoh *et al.*, 1996).

Pulmonary infection with *M. bovis*

Virulence factors

Many mycoplasmas appear to be well adapted to survival on mucosal surfaces, where they are highly dependent on their hosts for provision of nutrients including amino acids, nucleotides, fatty acids and sterols. Although a better understanding of nutrient acquisition and biosynthesis by mycoplasmas is emerging, and the mechanisms by which these bacteria adhere to host cells and evade the immune response are becoming better defined, many other aspects of virulence remain unknown. This situation is particularly true for *M. bovis*, for which evidence of secreted toxins is limited or questionable, and other mechanisms whereby the infection causes injury to host tissues are not clear. This section describes the structural and regulatory aspects of *M. bovis* virulence factors, while their role in pathogenesis is considered below.

The variable surface lipoproteins are the best characterized virulence factors of *M. bovis*. They are a family of immunodominant surface antigens that contribute to phenotypic variability of the bacteria, play important roles in bacterial adherence to host cells and evasion of binding by antibody, and provide a fascinating model for understanding the regulation of protein expression in bacteria. In *M. bovis*, five Vsp proteins have been identified: VspA, VspB, VspC, VspF and VspO (Sachse *et al.*, 2000). High frequency variation in the phase (on/off expression) and size of the expressed proteins are characteristic features (Sachse *et al.*, 2000). Most or probably all strains of *M. bovis* express Vsp proteins; those most commonly expressed are VspB, VspO and VspF, while VspA and VspC expression is less frequent. Many combinations of Vsp protein expression are recorded; for example, VspBO, VspB and VspBF were the most common in one study (McAuliffe *et al.*, 2004). However, it is important to recognize that this marked heterogeneity in Vsp expression, banding patterns and antigenicity is not a stable feature of the bacterial strain, but instead represents recombination and variable expression of the *vsp* genes (Behrens *et al.*, 1994; Rosengarten *et al.*, 1994). Indeed, *in vitro* passage results in altered Vsp expression (Thomas *et al.*, 2005a), and the profile of Vsp protein expression does not correlate with genotype (McAuliffe *et al.*, 2004).

The variable surface lipoproteins are encoded by at least 13 distinct *vsp* genes. The N-terminus is conserved and functions in insertion of the protein into the bacterial membrane. In general, the Vsp proteins contain multiple domains, and many of these domains are formed by repeated sequences of 6–87 amino acid residues (Sachse *et al.*, 2000). The number of domains, the length of each repetitive sequence, and the prevalence of proline, glutamic acid and lysine residues are highly variable among the Vsp proteins. These repetitive sequences and their three-dimensional conformations are presumably critical to their immunogenicity, ligand binding and role in cytoadhesion (Sachse *et al.*, 2000).

The phenotypic variability in Vsp proteins may be explained by the variation in the repertoire of the *vsp* genes between isolates and recombination events in the

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vsp gene locus. Indeed, *M. bovis* isolates differ in their repertoire of expressed *vsp* genes (Poumarat *et al*., 1999). In addition, recombination events within the *vsp* locus involving deletion and addition of repetitive sequences result in size and antigenic variability of the encoded protein, while site-specific DNA inversions mediate the characteristic phase variation and can lead to expression of novel chimeric Vsp proteins (Lysnyansky *et al*., 1996, 2001a, b; Nussbaum *et al*., 2002; Flitman-Tene *et al*., 2003). Further, the presence of antibody to specific Vsp proteins may either influence expression of Vsp proteins or more likely select for bacteria with specific patterns of Vsp expression (Le Grand *et al*., 1996; Lysnyansky *et al*., 2001b). The various roles of the Vsp proteins in the development of disease, described in more detail below, include evasion of host antibody response (Le Grand *et al*., 1996), colonization of mucosal surfaces and adhesion to host cells (Sachse *et al*., 2000; Lysnyansky *et al*., 2001a, b; Thomas *et al*., 2003b), and suppression of lymphocyte blastogenesis and cytokine secretion (Vanden Bush and Rosenbusch, 2004).

Other surface proteins, unrelated to the Vsps, have also been shown to mediate adhesion to host cells. The pMB67 is an immunogenic protein recognized by antibodies that are produced during infection. Like the Vsps, it also undergoes a high rate of phase and size variation *in vitro* (Behrens *et al*., 1996). The P26 antigen is a 32 kDa hydrophilic protein shown to be an important adhesin based on a competitive adherence assay. Neuraminidase treatment revealed the importance of sialyl moieties in adhesive interactions (Sachse *et al*., 1996). A protein of 28 kDa was reported as a factor involved in *M. bovis* adhesion to host cells (Sachse *et al*., 1996). A protein of 40 kDa was reported to be an adhesin in *M. agalactiae* but is a pseudogene in *M. bovis* (Thomas *et al*., 2004b). The role of these proteins in cytoadhesion and pathogenesis is discussed below.

Other virulence attributes, apart from those mediating adhesion, have received much less attention. These include a polysaccharide toxin, other polysaccharides, hydrogen peroxide, heat shock proteins and formation of biofilm. There is a single report of toxin production by *M. bovis*, a heat-stable, 73 kDa, complex polysaccharide which stimulated an inflammatory response following introduction into the mammary gland (Geary *et al*., 1981). A later study was not able to replicate this finding so the presence and importance of toxin production by *M. bovis* remains unconfirmed (Thomas *et al*., 1986). *M. bovis* contains other polysaccharides, and although these are of potential diagnostic use, their role in pathogenesis is unknown (Brooks *et al*., 2004).

Hydrogen peroxide is produced by some mycoplasmas and is thought to play a role in disease by reacting with iron to produce hydroxyl radicals that cause lipid peroxidation and oxidative damage to cell membranes. Hydrogen peroxide production is reported to be variable between strains of *M. bovis* and its production seems to be reduced by *in vitro* passage (Khan *et al*., 2005b).

Heat shock responses occur in mycoplasmas, and the hsp60 genes of *M. bovis*, *M. agalactiae*, *Mycoplasma arthritidis* and *Mycoplasma hyopneumoniae* were recently identified. Sequence analysis revealed high homology between these genes. Investigations with western immunoblotting using recombinant Hsp60 fusion proteins and convalescent sera indicated that mycoplasmal Hsp60 is immunogenic in natural infection (Scherm *et al*., 2002), although there is no evidence that this response is protective or the proteins affect virulence.

Biofilms are usually associated with resistance to heat and desiccation as well as antimicrobial resistance. Biofilm formation has been identified in mycoplasmas, although they lack the genes commonly associated with biofilm formation in other bacterial species. Analysis of mycoplasma biofilms demonstrated that they form a highly differentiated structure with stacks and channels. For *M. bovis*, biofilm formation is reported to be strain-dependent and associated with certain Vsp profiles. For example, strains that expressed VspF showed poor biofilm formation while those expressing VspO or VspB formed abundant biofilms. Oxytetracycline inhibited biofilm formation in planktonic *M. bovis* cells. In an environmental setting, *M. bovis* was shown to survive over 30 h in a biofilm (McAuliffe *et al*., 2006).

Survival, transmission and incubation period

Most mycoplasmas are host-specific commensals on mucosal surfaces, including the respiratory, intestinal and genital tracts and the bovine mammary gland. Survival in the environment is limited and probably of low significance to transmission. However, *M. bovis* survives in the environment for several days if protected from sunlight, remains viable at 4°C in sponges or milk for 2 months and for over 2 weeks in water, but is more sensitive to higher temperatures, with survival of 1–2 weeks at 20°C and 1 week at 37°C (Pfutzner, 1984). Survival on dry paper disks was 126 days at 4°C, 28 days at 30°C and 7 days outdoors (Nagatomo *et al*., 2001). Survival in various materials are reported as follows: manure, 37 days; cotton, 18 days; straw, 13 days; wood and stainless steel, 1–2 days (Ruffo *et al*., 1969). Heat treatment of 65°C for 2 min or 70°C for 1 min resulted in loss of viability (Butler *et al*., 2000). *M. bovis* survived in liquid media for 59–185 days and the survival was not affected by temperatures ranging from 4 to 37°C (Nagatomo *et al*., 2001).

M. bovis infection is acquired by ingestion of infected milk and by close contact with infected calves (Pfützner, 1990). Colonization of the nasal cavity is more common in calves from herds with *M. bovis* mastitis compared to herds free of the disease (Bennett and Jasper, 1977). Aspiration of infected milk is thought to be of particular

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importance in the development of otitis media and pneumonia in young dairy calves. Contact with infected calves is a second major source of infection, and importation of infected calves is a likely method by which farms become infected (Nicholas, 2004). When naïve calves are mingled with infected calves, nasal shedding of *M. bovis* has been detected in the naïve calves within 24 h, and is found in most calves by 7 days after contact (Pfützner, 1990). As discussed below, there is evidence that stress, transport, or handling is associated with increased shedding of *M. bovis* in nasal secretions, suggesting these as possible risk factors for transmission (Boothby *et al.*, 1983). Once established, infection of the respiratory tract is often persistent (Boothby *et al.*, 1983; Allen *et al.*, 1992), which presumably provides a source for infection of in-contact calves.

Defining the incubation period in natural cases is complicated, because it is usually uncertain if the onset of clinical respiratory disease is the result of *M. bovis* or some other unidentified pathogen that was cleared by the time of clinical diagnosis or death, such as viruses or antibiotic-sensitive bacteria. One naturally infected cohort of calves developed respiratory disease 2 weeks after contact with a diseased group of calves (Adegboye *et al.*, 1996). In experimental infections, respiratory disease and arthritis are typically recorded at 8–10 days after infection (Stipkovits *et al.*, 2000a), although respiratory disease in as little as 2–6 days is suggested (Pfützner, 1990).

Immune response: experimental infection and natural disease

The immune response to *M. bovis* infection has been the subject of considerable interest because of the potential for vaccination in disease control. Most studies have focused on experimental challenge, and immune responses in naturally infected calves have not been adequately studied. When seronegative calves were introduced into a group of calves with endemic *M. bovis* pneumonia, the recently introduced calves remained seronegative for at least 29–35 days, then developed serum antibody titers to *M. bovis* by 59–63 days (Nagatomo *et al.*, 1996). This finding indicates a relatively slow spread of infection within the herd, delayed development of a serum antibody response to infection, or both. Naturally infected calves have *M. bovis*-specific IgM and IgG in serum, and low but detectable IgA in serum; whereas nasal secretions and bronchoalveolar lavage fluid contain low levels of *M. bovis*-specific IgM and IgG and more IgA than in serum (Boothby *et al.*, 1983). The half-life of antibodies to *M. bovis* is reported to be 20 days (Tschopp *et al.*, 2001). Naturally infected calves have minimal cell-mediated immune responses, including delayed type hypersensitivity reaction and suppression of *M. bovis*-induced proliferation of blood lymphocytes (Boothby *et al.*, 1983), but cellular immunity has not been studied with more contemporary methods.

Immune responses are more completely characterized in experimental infection. Intratracheal and/or intranasal challenge of seronegative 12-week-old calves resulted in detectable serum IgG1 production by 7 days post-infection (dpi), but this response continued to increase until at least 63 dpi (Vanden Bush and Rosenbusch, 2003). Similarly, another study found no antibody response at 5 dpi, based on ELISA, but antibody was detected at 12 dpi (Nicholas *et al.*, 2002). Following experimental challenge, serum IgM was consistently detected by 7 dpi and maximal at 14 dpi, serum IgG1 was detected in 50% of calves by 1 week and in 100% by 2 weeks, and serum IgG2 was detected in 0% of calves at 1 week and in 80% by 2 weeks after infection (Howard *et al.*, 1986). IgA was the predominant *M. bovis*-specific isotype in tracheobronchial wash fluid and was detected in 100% by 2 weeks after challenge, while IgG1 levels were not increased at 1 week but were increased at 2 weeks (Howard *et al.*, 1986).

The antigen specificity of the antibody response has been described. The variable surface lipoproteins are immunodominant antigens, and contain immunodominant epitopes of 3–7 amino acids (Behrens *et al.*, 1994; Sachse *et al.*, 2000). Other surface proteins that are targets of the antibody response include pMB67, a phase- and size-variable protein distinct from the Vsp family (Behrens *et al.*, 1996), and the surface lipoprotein P48 (Robino *et al.*, 2005).

Experimentally infected cattle had activation of CD8+ T cells with a lower response by CD4+ and $\gamma\delta$-T-cells, based on lymphocyte blastogenesis and enhanced expression of the IL-2 receptor-α (CD25) (Vanden Bush and Rosenbusch, 2003). IFN-γ-producing cells were present in similar numbers as IL-4-producing cells, and antigen-specific serum antibody responses showed increased amounts of IgG1 with minimal IgG2. These results suggest skewing of the immune response with a Th2 bias (Vanden Bush and Rosenbusch, 2003).

The effector mechanisms necessary for immunity to *M. bovis* infection are poorly understood. Based on the extracellular location of the bacteria in the airways and alveoli and their association with airway epithelial cells, it is assumed that an effective antibody response might block attachment, opsonize the bacteria, or activate complement-mediated killing. *M. bovis* activated complement in bovine serum by the classical pathway in the presence of either IgG1 or IgG2 from an immunized donor, or in the presence of serum from conventionally reared cattle, and led to dose-dependent killing of *M. bovis*. IgG1 was more effective at complement-mediated killing than was IgG2. In contrast, *M. bovis* did not activate complement by the alternate pathway, and immunoglobulin alone did not kill the bacteria (Howard, 1981).

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Morphology and development of disease

Many studies have reported gross and histopathologic findings in experimentally induced and in natural infections. The literature on this topic suffers from a lack of critical analysis, in particular regarding the causal association of *M. bovis* infection and disease, and from inconsistent use of the terms coagulation and caseous necrosis. Experimental challenge studies carry the limitation that the lesions are often more mild or of different appearance than those seen in natural cases, perhaps because experimentally infected calves are usually examined within 2 weeks of challenge whereas natural disease is often more chronic, or because other factors are necessary for development of lesions. In contrast, in studies of natural cases there is often uncertainty in the causal relationship between *M. bovis* infection and the associated lesions because of the complicating factors described below. A critical analysis of the causal role of *M. bovis* in development of lung lesions will require consideration of both experimental and natural cases.

Cattle may be infected with *M. bovis* in the absence of detectable clinical signs or lesions. *M. bovis* can be isolated from the nasal cavity and bronchoalveolar lavage fluid from about half of feedlot cattle at the time of entry into the feedlot, and from nearly all calves at 12 days after arrival (Allen *et al*., 1992). Necropsy studies have identified *M. bovis* in the lung of 3% of healthy calves (ter Laak *et al*., 1992), 25% of those lungs with no macroscopic lesions (Tenk *et al*., 2004), and from 46% of grossly and histologically normal lungs (Gagea *et al*., 2006b). Bronchoalveolar lavage fluid samples infected with mycoplasmas did not differ in their leukocyte populations from fluid samples without detectable mycoplasmal infection (Boothby *et al*., 1983). Finally, seroepidemiology studies found no association (Martin *et al*., 1989, 1999) or an uncertain association (Martin *et al*., 1990) between seroconversion to *M. bovis* and the prevalence of respiratory disease. Thus, calves may remain healthy despite being infected with *M. bovis*, implying that *M. bovis* may not necessarily be the cause of pneumonia in infected calves, so it is essential to correlate the findings in natural and experimental infections to properly understand the spectrum of lesions caused by this pathogen.

Clinical and pathologic findings in natural infection and disease

The clinical signs of *M. bovis* pneumonia are usually indistinguishable from other causes of pneumonia in feedlot cattle and dairy calves (Pfützner, 1990; Pollock *et al*., 2000). Non-specific clinical signs of bronchopneumonia include anorexia, depression, lethargy, fever, hyperpnea, respiratory distress and weight loss (Adegboye *et al*., 1996; Stipkovits *et al*., 2000a, b). Features that specifically suggest *M. bovis* pneumonia are lameness, chronicity and failure to respond to antibiotic therapy, but even these signs are not specific for *M. bovis*: lameness can also be caused by *Histophilus somni* infection, and chronic non-responsive pneumonia can result from pulmonary abscessation, bronchiectasis and sequestration. Arthritis manifests clinically as lameness and pain in one or more joints, and some but not all cases have externally visible swelling of the affected joints. Stifle, hock, shoulder and elbow joints are most commonly affected in the disease.

Chronicity and failure to respond to antibiotic treatment is a common feature in cases that have postmortem lesions of caseonecrotic bronchopneumonia (Pollock *et al*., 2000; Shahriar *et al*., 2002). However, it is unknown whether other cases of *M. bovis* pneumonia recover, because *M. bovis* would not be recognized as the cause of such cases of pneumonia unless arthritis was present. Feedlot calves that have postmortem lesions of caseonecrotic bronchopneumonia attributed to *M. bovis* infection were first treated at a mean of 14–15 days after arrival, and died at a mean of 44 days after arrival (Pollock *et al*., 2000; Gagea *et al*., 2006b). This duration of illness was longer than for other forms of bronchopneumonia, reflecting the chronicity of disease. However, the interval between arrival in the feedlot and first treatment was similar to that for other forms of bronchopneumonia, reinforcing the idea that the early stages of *M. bovis* pneumonia and pneumonic pasteurellosis are usually indistinguishable. Most death losses from chronic pneumonia were the result of euthanasia, and timely euthanasia is an important welfare consideration to prevent unnecessary suffering (Pollock *et al*., 2000).

M. bovis infection is associated with four main patterns of lesions: caseonecrotic bronchopneumonia, bronchopneumonia with foci of coagulation necrosis, suppurative bronchopneumonia without necrosis and chronic bronchopneumonia with abscessation. Caseonecrotic bronchopneumonia is a distinctive lesion and there is a strong evidence that *M. bovis* plays a causal role, as discussed below. The lesions most consistently affect the cranial and middle lung lobes, but severe cases may have involvement of over 80% of the lung tissue, with only a thin band of unaffected lung in the dorsocaudal aspect of the caudal lobes. The affected areas of lung contain nodules of caseous necrosis separated by areas of reddened and collapsed or consolidated lung. The nodules vary from pinpoint to several centimeters in diameter, and are generally circular, white, dry, crumbly and bulging from the pleural or cut surfaces of the lung (Fig. 1 and 2) (Rodriguez *et al*., 1996a; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006a). These caseous foci may develop into sequestra of necrotic tissue that occupy the entire lobe (Shahriar *et al*., 2002; Gagea *et al*., 2006a), and bronchiectasis may be present (Khodakaram-Tafti and Lopez, 2004). In other

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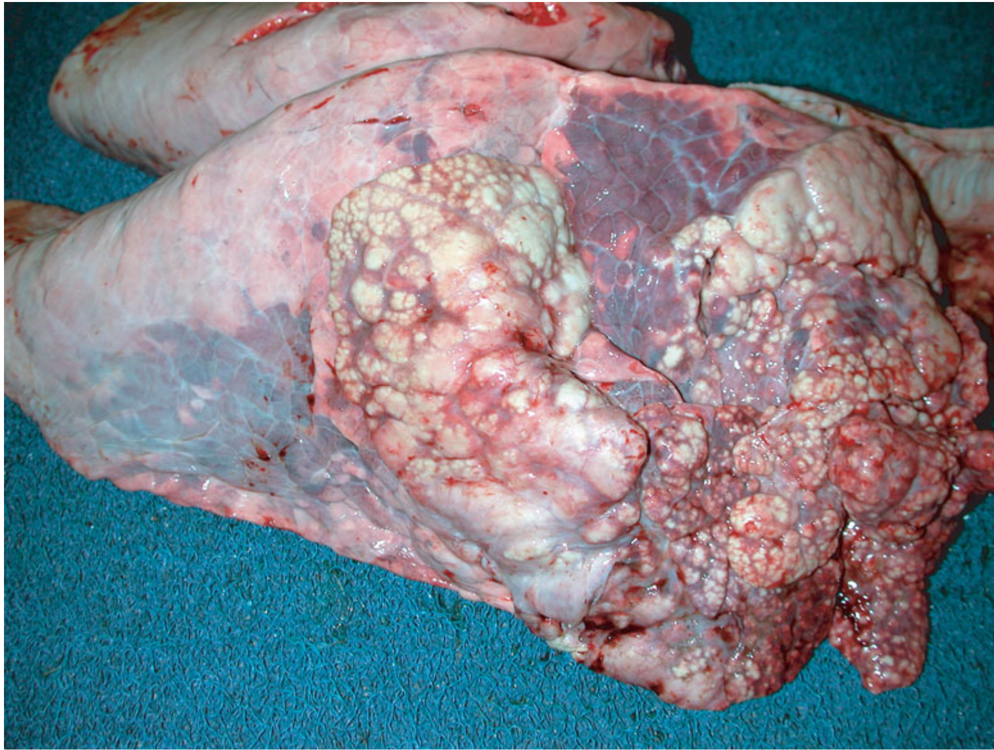

Fig. 1. Gross lesions of *M. bovis* pneumonia. Multifocal white nodules are present in the cranioventral areas of lung, and the intervening tissue in these areas is atelectatic and reddened. Reprinted from (Gagea *et al*., 2006a) with permission of the American Association of Veterinary Laboratory Diagnosticians.

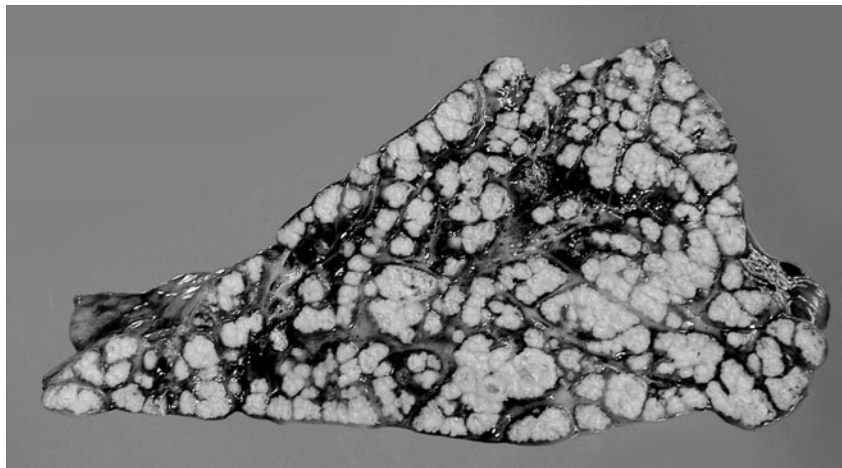

Fig. 2. Gross lesions of *M. bovis* pneumonia. On section, the lung contains multiple circular raised nodules containing white friable or crumbly caseous material. Reprinted from (Gagea *et al*., 2006a) with permission of the AAVLD.

calves, presumably when the lesions are co-infected with *Arcanobacterium pyogenes* or other bacteria, the foci of caseous necrosis develop into abscesses with liquid pus instead of dry friable caseous material. Rare cases have unilateral lesions affecting the middle and caudal lobes, and contagious bovine pleuropneumonia is an important differential diagnosis in such cases with unilateral lesions (Bashiruddin *et al*., 2001).

Histologically, the foci of caseous necrosis originate in small bronchioles, alveoli and interlobar septa. In the most mild and probably earliest lesion, leukocytes fill the lumen of bronchioles or alveoli, but undergo a distinctive form of necrosis in which they retain their ghost-like cellular outlines and have hypereosinophilic cytoplasm and inapparent or fragmented nuclei (Fig. 3). In contrast to lesions caused by *Mannheimia haemolytica*, the

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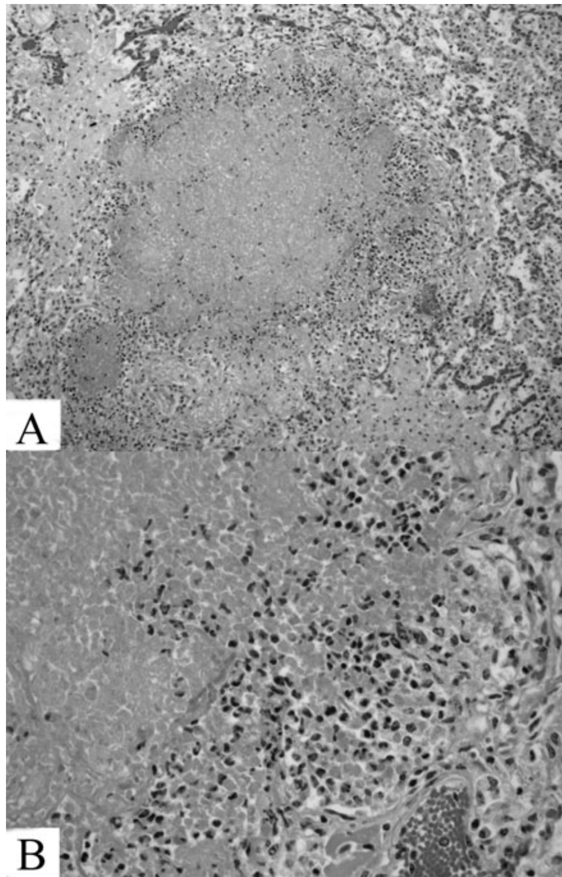

Fig. 3. Histologic lesions of caseonecrotic bronchopneumonia caused by *M. bovis*. (A) A focal area of caseous necrosis in the lung tissue, with loss of the alveolar architecture. 40×. (B) The focus of necrosis contains faintly visible outlines of necrotic eosinophilic cells, and is delineated by necrotic neutrophils containing pyknotic nuclei. 200×.

streaming chromatin of necrotic leukocytes ('oat cells') is not expected. More advanced lesions contain a coagulum of eosinophilic material with ghost-like remnants of necrotic leukocytes at the periphery, erosion of bronchiolar epithelium, and peripheral layers of necrotic cells with pyknotic nuclei, macrophages and lymphocytes and fibrosis (Rodriguez *et al*., 1996a; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006a). Lymphocytes infiltrating these lesions include CD4+ and CD8+ positive T cells and IgG-producing plasma cells (Howard *et al*., 1987). Descriptions of this lesion in the literature are confusing because different reports describe the same lesion as coagulation or as caseous necrosis. Although the histologic appearance of ghost-like remnants of necrotic leukocytes within the eosinophilic cellular debris gives credence to the term 'coagulative', 'caseous' is considered more appropriate because of the gross appearance of dry friable material and the histologically evident loss of alveolar architecture. The situation is further complicated because both lesions may co-exist, in which a central mass of lung tissue that has undergone coagulation necrosis (in which the bronchiolar and alveolar architecture remains visible) is surrounded by caseous eosinophilic cellular debris (Gagea *et al*., 2006a).

Other pulmonary lesions associated with *M. bovis* infection include bronchopneumonia containing foci of coagulation necrosis. The caseonecrotic foci described above differ in their gross appearance from these foci of coagulation necrosis, which are irregular in shape, red-tan, non-friable, and not raised, and on histologic examination the architecture of the coagulated alveolar and bronchiolar tissue remains visible. Such lesions are often infected with *M. bovis*, but are indistinguishable from those caused by *M. haemolytica*. The possible role of *M. bovis* in their development is discussed below. Lung abscesses are frequently infected with *M. bovis* and with other bacteria; the abscesses are filled with liquid pus that differs from the dry friable content of the caseonecrotic foci.

Clinical and pathologic findings resulting from experimental infection

Several reports describe the lesions that result from inoculation of *M. bovis* into the trachea or bronchi of calves that had undetectable serum antibody to *M. bovis* and no history of *M. bovis* infection in the source herds (Gourlay *et al*., 1976; Martin *et al*., 1983; Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996a). In some, experimentally challenged animals showed no clinical signs (Rodriguez *et al*., 1996a), while others had fever, lethargy and lameness with increased amount of joint fluid. Inconsistently reported signs included nasal discharge, cough, tachypnea and inappetance (Gourlay *et al*., 1976; Rodriguez *et al*., 1996a). In one study, the clinical signs were transient and lasted only 6–9 days (Gourlay *et al*., 1976).

In calves euthanized at 7 or 14 days after challenge, gross lesions were minimal (Lopez *et al*., 1986), or affected up to 25% of the lung with consolidation and reddening in the cranial and middle lobes, increased prominence of interlobular septa and enlarged lymph nodes (Gourlay *et al*., 1976; Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996a). Histologic lesions were usually restricted to peribronchiolar aggregates of mononuclear cells, neutrophils filling the lumen of bronchioles, infrequent attenuation of bronchiolar epithelium, and increased numbers of macrophages and few neutrophils in the alveoli (Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996a). Necrotic debris in alveoli was occasionally reported (Gourlay *et al*., 1976). Although atelectasis was prominent, accumulation of leukocytes in alveoli was not (Thomas *et al*., 1986), similar to the situation in natural cases.

It is notable that lesions of caseous necrosis were not described in these studies of less than 2 weeks duration, whereas lesions of multifocal necrosis are described in two more chronic studies. Two gnotobiotic calves

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euthanized at 35 and 42 days after intratracheal challenge with *M. bovis* had grossly apparent raised white nodules in the cranial lung. Histologic lesions in these calves included focal coagulative necrosis in which the necrotic alveolar tissue remained visible, as well as foci in which pale amorphous eosinophilic material was surrounded by an inner band of pyknotic cells, a layer of macrophages and plasma cells, and an outer rim of fibroblast proliferation. In contrast to lesions caused by *M. haemolytica*, the foci of coagulation necrosis resulting from *M. bovis* challenge were less severe and less frequent, did not cross the interlobular septa, and had few neutrophils, little fibrin, no thrombosis of lymphatics, and lacked the leukocyte necrosis with fusiform streaming of nuclear chromatin (oat cells). *M. bovis* was isolated from the lung of these two calves, but other pathogens were not (Thomas *et al.*, 1986). In a second study, naïve calves were challenged by aerosol with *M. bovis*, and lesions at 18 and 19 days after infection included foci of necrosis consisting of amorphous eosinophilic debris surrounded by a layer of pyknotic cells and an outer layer of macrophages (Stipkovits *et al.*, 2000a). Although these foci were described as coagulative, the description is identical to the caseonecrotic lesions described in natural cases (Shahriar *et al.*, 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al.*, 2006a), and probably reflects inconsistent use of the terms coagulation and caseous necrosis in the literature. Although these descriptions are based on only four calves, these lesions in chronically infected calves are noteworthy for their association with *M. bovis* and their similarity to lesions in natural cases described below.

Pathogenesis and localization of antigen

The distribution of *M. bovis* antigen has been investigated using immunohistochemistry. In the lesions of caseonecrotic bronchopneumonia, *M. bovis* antigen is identified throughout the foci of caseous necrosis but most prominently at their periphery, and is both within the cytoplasm of macrophages and free in the necrotic material. Antigen was similarly present in the bronchiolar lumen, within the eosinophilic cellular debris and between and within leukocytes (Thomas *et al.*, 1986; Rodriguez *et al.*, 1996b; Khodakaram-Tafti and Lopez, 2004; Gagea *et al.*, 2006a).

In lesions of fibrinosuppurative bronchopneumonia with foci of coagulation necrosis, *M. bovis* antigen was mainly localized at the periphery of the foci of coagulation necrosis, and also in the cytoplasm of necrotic neutrophils and macrophages, occasionally within non-necrotic neutrophils, and free in edematous alveoli (Khodakaram-Tafti and Lopez, 2004; Gagea *et al.*, 2006a).

M. bovis antigen has been clearly identified in the cytoplasm of macrophages in natural lesions (Rodriguez *et al.*, 1996b; Khodakaram-Tafti and Lopez, 2004; Gagea *et al.*, 2006a) and within mononuclear cells in lymphoid follicles (Khodakaram-Tafti and Lopez, 2004). However, it remains unknown whether this intracellular immunolabeling represents *M. bovis* bacteria that have been phagocytosed and killed by macrophages, or survival of these bacteria in macrophages as a strategy for evasion of the immune response.

M. bovis is able to adhere to and invade between tracheal and bronchial epithelial cells. Antigen was identified, albeit infrequently, among ciliated epithelial cells of the trachea and bronchi in natural and experimental infections (Rodriguez *et al.*, 1996b; Gagea *et al.*, 2006a). When tracheal explants were infected with *M. bovis*, bacteria and bacterial antigen were detected on the epithelial cell surface and between epithelial cells, which may be a portal for dissemination via the blood (Thomas *et al.*, 1987). The association appears to represent specific adhesion of *M. bovis* to bovine bronchial epithelial cells, and is partially mediated by the variable surface proteins (Sachse *et al.*, 2000; Thomas *et al.*, 2003b, 2005a) and protein P26 (Sachse *et al.*, 1993a, 1996). Adhesion to epithelial cells is temperature-dependent, abrogated by trypsin and by *in vitro* passage, and may be associated with more highly pathogenic strains of the bacterium (Sachse *et al.*, 1996; Thomas *et al.*, 2003b, 2005a). Attachment of *M. bovis* to ciliated epithelial cells has no effect on ciliary activity (Thomas *et al.*, 1987), nor does experimental challenge with *M. bovis* alter pulmonary clearance of *M. haemolytica* (Lopez *et al.*, 1982).

The issue of whether *M. bovis* infects airway epithelial cells is controversial. Some studies have identified *M. bovis* antigen within the cytoplasm of bronchiolar epithelial cells (Adegboye *et al.*, 1995b; Rodriguez *et al.*, 1996b), whereas this was specifically addressed but found to be absent (Gagea *et al.*, 2006a) or sparse (Khodakaram-Tafti and Lopez, 2004) in other investigations. It has been suggested that this invasion across the epithelium occurs within 7 days of infection and may not be detected in more chronic stages (Thomas *et al.*, 1986). Infection of tracheal explants with *M. bovis* did not reveal cytoplasmic localization of the bacteria (Thomas *et al.*, 1987).

Endothelial cells are susceptible to infection with *M. bovis in vitro* (Lu and Rosenbusch, 2004), although localization of antigen in vascular walls is not reported in natural or experimental cases so the significance of this infection may be limited. Infected endothelial cells became activated, with induced surface expression of the adhesion molecule VCAM-1 and transcription of several cytokines, but there was minimal or no necrosis of infected endothelial cells (Lu and Rosenbusch, 2004).

The mechanisms by which *M. bovis* infection incites inflammation and the characteristic lesion of coagulation necrosis remain unknown. *M. bovis* induces activation of pulmonary alveolar macrophages and production of tumor necrosis factor-α (Jungi *et al.*, 1996); it is not known whether membrane lipoproteins are responsible for this effect as has been shown for other mycoplasmas

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(Herbelin *et al*., 1994). A polysaccharide toxin is described and is reported to stimulate an inflammatory response characterized by increased vascular permeability, complement activation and infiltration of eosinophils and neutrophils (Geary *et al*., 1981). However, others have been unable to replicate these findings or induce a similar response using culture filtrate (Thomas *et al*., 1986). Some strains of *M. bovis* produce hydrogen peroxide, and it is suggested that peroxide may react with iron to produce hydroxyl radicals that cause lipid peroxidation and tissue injury (Khan *et al*., 2005b), as a possible explanation for the characteristic appearance of necrotic leukocytes within the lesions of caseous necrosis.

Hematogenous dissemination from the lung to other tissues

Bacteremia results from infection of the respiratory tract with *M. bovis*. Following intranasal and intratracheal challenge, *M. bovis* can be recovered by culture from blood, up to 9 days after infection but not at later times (Thomas *et al*., 1986). However, infection of other tissues persists, and *M. bovis* can be recovered at 21 days after infection from the spleen, liver and kidney of experimentally challenged calves (Stipkovits *et al*., 2005). *M. bovis* antigen has been localized by immunohistochemistry to macrophages in many tissues, hepatocyte cytoplasm, bile ductules, renal tubules and rarely axons in facial nerve (Maeda *et al*., 2003). Thus, bacteremia is a rapid sequel to challenge of the respiratory tract with *M. bovis*. *In vitro* findings and immunohistochemical examination of lesions suggests that bacteremia may result from invasion across the airway or alveolar epithelium, or by lymphatic drainage from alveoli through the interlobular septa (Thomas *et al*., 1987; Adegboye *et al*., 1995b; Rodriguez *et al*., 1996b; Gagea *et al*., 2006a).

Arthritis is mainly the result of pulmonary infection. *M. bovis* arthritis in the absence of pneumonia is rare in feedlot cattle (Gagea *et al*., 2006a), and clinical signs of pneumonia and arthritis often occur concurrently (Byrne *et al*., 2001a). Clinical signs and lesions of arthritis developed in 14–66% of calves following intranasal or intratracheal challenge with *M. bovis*, and *M. bovis* was identified by culture or immunohistochemistry in the affected joints (Gourlay *et al*., 1976; Thomas *et al*., 1986; Stipkovits *et al*., 2005). These findings imply that infection of the lung with *M. bovis* leads to hematogenous dissemination to the joints, and subsequent development of arthritis.

Mechanisms of evasion of the immune response

The observation that *M. bovis* infection of the lung is often chronic in natural cases (Allen *et al*., 1992; Shahriar *et al*., 2002; Gagea *et al*., 2006a) and experimental infection (Thomas *et al*., 1986; Stipkovits *et al*., 2000a, 2005) implies that the immune response is ineffective in eliminating infection. The effects on innate immunity in the lung have not been adequately investigated. Although *M. bovis* antigen is often identified within alveolar macrophages, this seems to induce macrophage activation and cytokine secretion, and not suppression of the macrophage functions studied to date (Jungi *et al*., 1996). *M. bovis* adheres to neutrophils and inhibits oxidative burst, chemiluminescence and degranulation in these cells (Finch and Howard, 1990; Thomas *et al*., 1991). Although impairment of neutrophil function might predispose to other bacterial infections, this probably has little effect on perpetuating the mycoplasmal infection. The association of *M. bovis* with ciliated epithelial cells has not been shown to affect ciliary beating or clearance of bacteria from the lung (Lopez *et al*., 1982; Thomas *et al*., 1987). Although other infectious agents are capable of impairing antimicrobial peptide expression in airways (Al-Haddawi *et al*., 2007), it is unknown whether *M. bovis* has a similar effect.

Variable expression of immunodominant proteins on the bacterial surface is probably a major mechanism by which *M. bovis* evades the developing humoral immune response. Incubation of *M. bovis* bacteria with serum that contained antibody to *M. bovis* resulted in altered expression of Vsp and reduced expression of pMB67 surface antigen. Further, incubation with Vsp-specific monoclonal antibodies reduced expression of the corresponding Vsp, which returned following incubation in media containing no antibody. These findings suggest that Vsp are a major target of the humoral immune response, but high frequency mutation of Vsp as well as antibody-driven alteration in Vsp expression allows the bacterium to evade this antibody response (Le Grand *et al*., 1996).

M. bovis is also able to suppress the *in vitro* proliferative response of lymphocytes to mitogens (Finch and Howard, 1990; Thomas *et al*., 1990) and induce apoptosis of lymphocytes *in vitro* (Vanden Bush and Rosenbusch, 2002). The inhibition of mitogen-induced lymphocyte function is mediated by a C-terminal fragment of Vsp-L, termed *M. bovis* lympho-inhibitory peptide (Mb-LIP) (Vanden Bush and Rosenbusch, 2004). The impact of lymphocyte dysfunction and apoptosis on the magnitude, timing, or character of the immune response has not been investigated.

Role of co-infection with other pathogens

Most calves with *M. bovis*-associated pneumonia are also infected with other pathogens, which complicates the interpretation of the role that *M. bovis* plays in development of these lesions. *M. bovis* was the sole pathogen isolated from the lung in 34% of cases of fatal pneumonia

https://doi.org/10.1017/S1466252307001351 Published online by Cambridge University Press

(Byrne *et al*., 2001b). In contrast, many cases of pneumonia are infected not only with *M. bovis*, but also with *M. haemolytica*, *Pasteurella multocida*, *A. pyogenes*, or *Mycoplasma arginini*, and less frequently with *H. somni*, bovine herpesvirus-1, bovine respiratory syncytial virus, or parainfluenza-3 virus (Byrne *et al*., 2001b; Shahriar *et al*., 2002; Thomas *et al*., 2002a; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006b; Arcangioli *et al*., 2007). Co-infection with bovine diarrhea virus is of particular interest, as it is identified in 30–40% of cases of caseonecrotic bronchopneumonia (Haines *et al*., 2001; Gagea *et al*., 2006a) and 62% of cases of chronic pneumonia (Shahriar *et al*., 2002). However, since bovine viral diarrhea virus (BVDV) infection was also identified at a similar prevalence in calves with fibrinosuppurative bronchopneumonia typical of *M. haemolytica* infection, it was suggested that BVDV may impair innate immune responses in the lung and thereby predispose to bacterial pneumonia or enhance its severity, but not preferentially predispose to *M. bovis* pneumonia (Gagea *et al*., 2006a; Al-Haddawi *et al*., 2007).

Several studies have investigated whether co-infection of *M. bovis* and other agents results in exacerbation of disease. Infection with both *M. bovis* and *M. haemolytica* appears to result in more severe clinical disease and lesions than either infection alone (Houghton and Gourlay, 1983), particularly when *M. bovis* infection preceded *M. haemolytica* infection by 1 or 2 days (Gourlay and Houghton, 1985). In contrast, in calves with natural *M. bovis* infection, challenge with *P. multocida* induced only mild lesions (Martinez *et al*., 1982). Although one study found higher mortality in calves that were naturally infected with both *M. bovis* and bovine respiratory syncytical virus (BRSV) compared to BRSV infection alone (Howard *et al*., 1990), experimental studies found no exacerbation of illness in co-infected calves (Thomas *et al*., 1986).

Caseonecrotic bronchopneumonia is the result of chronic *M. bovis* infection, and it has been suggested that this lesion may arise from lesions initiated by *M. haemolytica* (Gagea *et al*., 2006a). This hypothesis is based on several observations: the early stages of these two infections are clinically indistinguishable and are first detected at similar times after calves arrive in the feedlot, suggesting that they might begin as the same disease; *M. bovis* and *M. haemolytica* often co-infect the areas of coagulation necrosis (Fig. 4); some lesions have a central area of coagulation necrosis encircled by a peripheral area of caseous necrosis, perhaps representing caseation at the edge of an earlier lesion of coagulation necrosis; and the caseonecrotic lesions are more chronic than those of coagulation necrosis, consistent with the former being a complication of the latter (Gagea *et al*., 2006a). Arguing against this hypothesis is the observation that more severe lesions develop when experimental *M. bovis* infection is followed by *M. haemolytica* challenge than the converse, and fact that caseonecrotic lesions were not described in the only study in which calves were sequentially infected with *M. bovis* and later *M. haemolytica* (Gourlay and Houghton, 1985). Thus, it remains unresolved whether these severe caseonecrotic lesions are a direct consequence of *M. bovis* infection, or whether co-infection or prior infection with *M. haemolytica*, *M. arginini*, or other factors contribute to the development of severe disease. Nevertheless, these are important considerations, because they dictate whether disease prevention might best be accomplished by control of virulent strains of *M. bovis*, or whether a better strategy is to control co-infections, underlying diseases, or other factors that enhance the disease resulting from *M. bovis* infection.

M. bovis *and pneumonia: is the association causal?*

As described above, naturally occurring cases of *M. bovis* infection are associated with four main patterns of lesions: caseonecrotic bronchopneumonia, bronchopneumonia with foci of coagulation necrosis, suppurative bronchopneumonia without necrosis and chronic bronchopneumonia with abscessation. Caseonecrotic bronchopneumonia is a characteristic lesion that is almost always infected with *M. bovis* (Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006a). Experimental studies confirm the causality of this association: the same lesion develops following experimental challenge, and *M. bovis* but not other respiratory pathogens are isolated from the ensuing lesions (Thomas *et al*., 1986; Stipkovits *et al*., 2000a).

Less certain is the role of *M. bovis* in other forms of bronchopneumonia. A critical interpretation of the causal role of *M. bovis* in development of pneumonia in feedlot cattle is complicated by several factors outlined in Table 1. Calves with *M. bovis* infection detected by nasal culture were more likely to have a fever than uninfected calves (Wiggins *et al*., 2007), and *M. bovis* was isolated more commonly from the lung of calves with suppurative bronchopneumonia (even in the absence of caseous necrosis) than from healthy lungs or those with viral pneumonia (Gagea *et al*., 2006a). Further, experimental challenge with *M. bovis* results in neutrophil infiltration of alveoli and bronchioles, with caseonecrotic lesions developing in only a minority of infected calves (Thomas *et al*., 1986; Rodriguez *et al*., 1996b; Stipkovits *et al*., 2000a). Thus, it is clear that *M. bovis* is capable of inducing suppurative or catarrhal bronchopneumonia, and that the lesions may not progress to caseous necrosis.

The question of whether *M. bovis* is an important cause of pneumonia at the herd level is distinct from whether this pathogen is capable of causing bronchopneumonia. The question is complicated by the lack of measures to specifically prevent the disease, which would otherwise allow an estimate of the attributable fraction – the proportion of pneumonia that would not occur if *M. bovis* were absent from the herd. However, the

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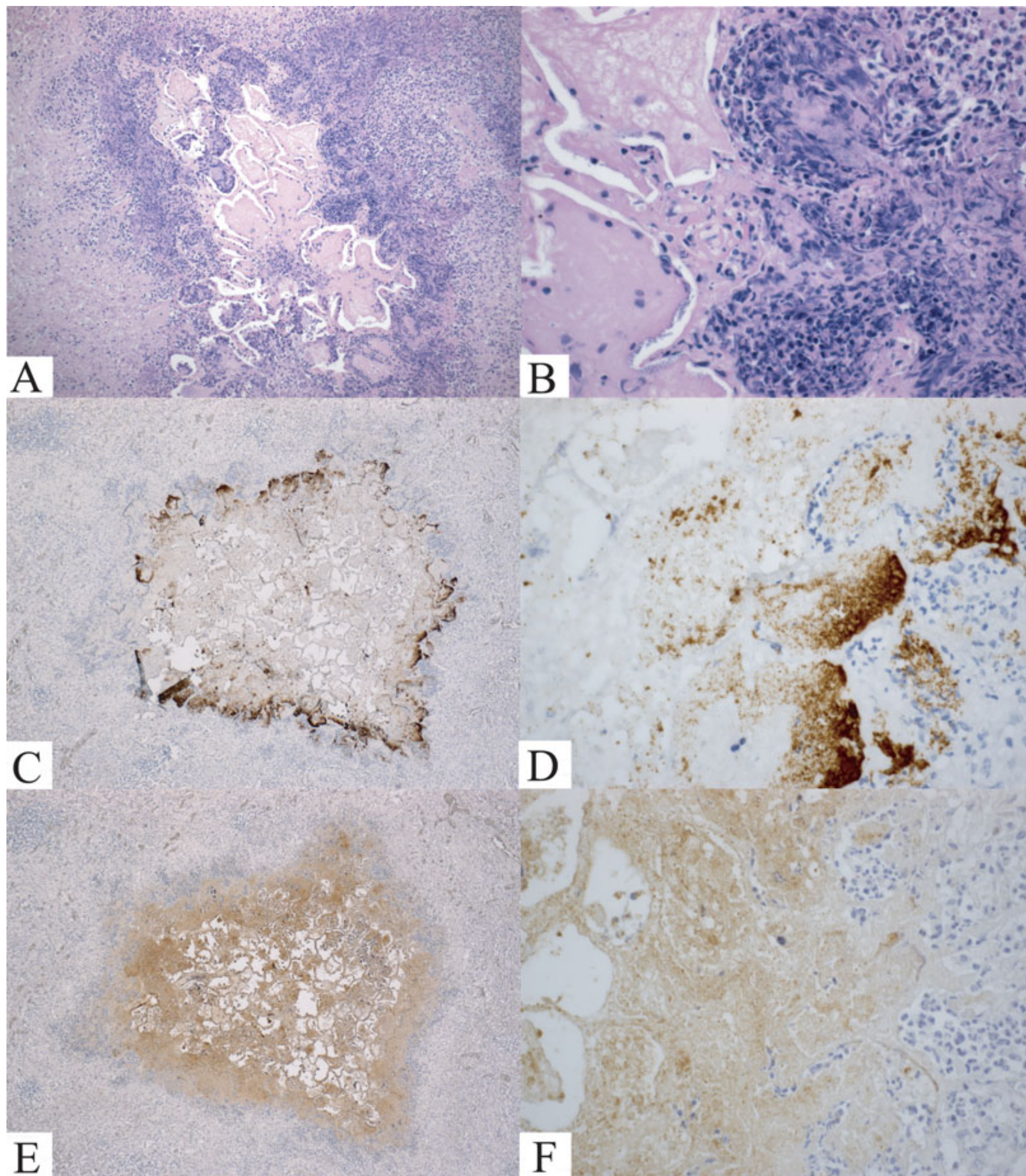

Fig. 4. Bronchopneumonia and coagulation necrosis associated with *M. bovis* and *Mannheimia haemolytica* infection. (A, B) A lesion of bronchopneumonia contains focal coagulation necrosis. The neutrophils at the periphery undergo necrosis with streaming of chromatin (oat cells), typical of that caused by *M. haemolytica* infection. Haematoxylin and eosin stain. (C, D) *M. bovis* antigen is present at the periphery of the focus of necrosis, and to a lesser extent within this lesion. Immunohistochemistry for *M. bovis*, DAB chromogen, haematoxylin counterstain. (E, F) *M. haemolytica* antigen is present throughout the focus of necrosis. Immunohistochemistry for *M. haemolytica*, DAB chromogen, haematoxylin counterstain. A, C, E: 100×; B, D, F: 400×.

following suggest that *M. bovis* infection does indeed contribute significantly to the prevalence of pneumonia in cattle herds. Calves that seroconverted to *M. bovis* were more likely to be treated for pneumonia than those that did not seroconvert (Rosendal and Martin, 1986; Martin *et al.*, 1990), although this association was not repeated in other studies (Martin *et al.*, 1989, 1999). The mortality rate from pneumonia increased from 10 to 36 deaths per year following introduction of *M. bovis* infection to a single farm, for which laboratory data were also available prior to introduction of this pathogen (Gourlay *et al.*, 1989). Finally, the timing of infection is appropriate, as initial *M. bovis* infection followed by challenge with *M. haemolytica* results in more severe disease than the reverse order (Gourlay and Houghton, 1985). Thus, the available evidence indicates that *M. bovis* is a cause of caseonecrotic bronchopneumonia, but that *M. bovis* infection is also capable of causing and is probably an important contributor to other forms of bronchopneumonia in cattle.

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Table 1. Challenges in interpreting the role of *Mycoplasma bovis* in individual cases of bronchopneumonia

(1) The lungs of feedlot cattle may be infected with *M. bovis* in the absence of detectable clinical signs or pulmonary lesions. As a result, the mere presence of *M. bovis* in pneumonic lungs does not imply that the lesions were caused by this agent.

(2) Lesions resulting from experimental infection with *M. bovis* are generally more mild and often differ from those associated with *M. bovis* infection in field cases, suggesting that other factors are important in development of severe disease.

(3) Most calves with chronic pneumonia are treated with antibiotics prior to death, so it is often impossible to exclude the possibility that lesions were caused by earlier or undetected infection with other bacteria.

(4) More than one pathogen can be isolated from most cases of pneumonia in cattle, so attributing causation to one agent is perhaps simplistic.

(5) The lesions associated with infection with *M. bovis* may appear similar to those caused by other bacterial pathogens, making it challenging to declare *M. bovis* as the cause in a particular case of suppurative bronchopneumonia. Even if *M. bovis* is the only pathogen isolated from such cases, it would be reasonable to suggest that the mycoplasmal infection was incidental or secondary and the early lesions were caused by *M. haemolytica*, but antibiotic treatment precluded detection of this pathogen at the time of death.

Prevalence and economic impact

Prevalence of M. bovis *infection*

M. bovis infection has been reported in the United States, Canada and most European countries. Recent reports suggesting an absence of this pathogen from any country are rare, apart from a single study in Finland (Härtel *et al.*, 2004). Northern Ireland and the Republic of Ireland were thought to be free of *M. bovis* until it was detected in 1993 and 1994, probably from importation of infected cattle associated with relaxation of trade barriers (Reilly *et al.*, 1993; Doherty *et al.*, 1994).

Estimates of the prevalence of infection vary widely between reported studies, and may depend on the amount of mixing of calves from different sources, the prevalence of pneumonia of any cause, the animal management system and presence of recent stresses, the samples analyzed, and the sensitivity and specificity of methods used for identification of the pathogen. Prevalences reported in older studies are generally lower than from more recent studies (Springer *et al.*, 1982; Schulz and Umlauft, 1987; Schulz *et al.*, 1990; Kusiluka *et al.*, 2000b), but there are examples of high prevalence in older studies (Muenster *et al.*, 1979; Allen *et al.*, 1992). One report mentioned that *M. bovis* was more frequently isolated from bronchoalveolar lavage fluid than from nasopharyngeal swabs (Allen *et al.*, 1992).

Many herds have serologic evidence of *M. bovis* exposure, but the prevalence of seropositive herds varies substantially (from 28 to 90%) between studies and between geographic regions in the same study (Grand *et al.*, 2002; Tenk *et al.*, 2004; Ghadersohi *et al.*, 2005). The prevalence of seropositivity within herds is also not uniform: many groups of pre-weaned beef calves have <20% prevalence while others have >40% seropositivity (Grand *et al.*, 2002), and the seroprevalence in dairy cows at slaughter was also variable between herds (Tenk *et al.*, 2004). Although calves with high serum antibody titers to *M. bovis* are at increased risk of having pneumonia (relative risk=1.7), many calves with high titers do not have chronic pneumonia (Pollock *et al.*, 2000).

Most studies have found only 0–7% prevalence of *M. bovis* infection in the lung of healthy calves that were not recently transported or introduced to feedlots (ter Laak *et al.*, 1992; Grand *et al.*, 2002; Thomas *et al.*, 2002a; Hirose *et al.*, 2003; Rifatbegovic *et al.*, 2007; Wiggins *et al.*, 2007). When healthy calves of different ages were compared in the same study, differences in prevalence were not found (Vogel *et al.*, 2001).

In contrast, several studies indicate that the prevalence of *M. bovis* infection is higher in feedlot calves and in calves that have been recently transported or co-mingled, even when these calves have no evidence of pneumonia. The prevalence of infection in healthy beef cattle was 40–60% on arrival in feedlots and increased to nearly 100% by day 12 after arrival (Allen *et al.*, 1991, 1992); and a different study reported that 55% of calves seroconverted to *M. bovis* during the first 7 weeks in the feedlot (Tschopp *et al.*, 2001). Similarly, *M. bovis* was isolated from bronchoalveolar lavage fluid of 79% of healthy veal calves in feedlots, at the onset of respiratory disease outbreaks (Arcangioli *et al.*, 2007). In contrast, the prevalence of *M. bovis* in nasal swabs was only 2% in calves sampled within 10 days of arrival in stocker and backgrounding operations (Wiggins *et al.*, 2007). Of calves dying within 2 months of introduction to a feedlot, *M. bovis* was isolated from 46% of those lungs that had no gross or histologic evidence of inflammation (Gagea *et al.*, 2006b). Infection of feedlot cattle is persistent: studies involving sequential sampling show that when *M. bovis* is isolated from bronchoalveolar lavage fluid, subsequent samples from the same calf are likely to also be positive (Allen *et al.*, 1992). Thus, pulmonary infection with *M. bovis* is uncommon in most groups of healthy non-transported calves, but becomes much more prevalent and persists after transport, co-mingling, or introduction to a feedlot.

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Possible explanations for the higher prevalence of *M. bovis* infection in feedlot cattle include increased exposure both to *M. bovis* and to other pathogens as a result of co-mingling of animals from different farms or different age groups, or stress or altered respiratory defenses as a result of transportation and re-ordering of social groups. Epidemiologic assessment identified the following risk factors that were significantly associated with seroconversion to *M. bovis* in feedlot cattle: mixing calves of different ages and groups, contact with a seropositive animal and prophylactic antibiotic treatment (Tschopp *et al*., 2001). In a group of 30 seronegative calves from a single facility in which *M. bovis* could not be isolated from nasal swabs prior to transportation, this bacterium was isolated from nasal swabs and bronchoalveolar lavage fluid in 20 and 47% of the calves respectively after transportation to a research facility (Boothby *et al*., 1983). This finding suggests that transportation stress or exposure to a new environment may enhance proliferation or spread of *M. bovis*, independent of assembly of groups from different sources. A recent study found *M. bovis* infection in a recently purchased group of heifers as well as in a fattening herd, but not in 20 other groups of cattle (Rifatbegovic *et al*., 2007), indicating an effect of transportation or mixing of groups of calves independent of the feedlot environment itself. Thus, mixing and transporting animals increases the risk of *M. bovis* infection, although the precise predisposing factors and mechanisms are unknown. It is notable that similar factors increase the risk for pneumonic pasteurellosis in feedlot cattle.

M. bovis infection is more prevalent in the lungs of calves with pneumonia than in healthy lungs, with estimated prevalences ranging from 20 to 90% (Allen *et al*., 1991, 1992; Kusiluka *et al*., 2000b; Byrne *et al*., 2001b; Vogel *et al*., 2001; Thomas *et al*., 2002a; Tenk *et al*., 2004; Gagea *et al*., 2006b; Godinho *et al*., 2007). The infection is particularly common in the lungs of cattle with chronic pneumonia (71%) (Haines *et al*., 2001) and particularly those with chronic caseonecrotic bronchopneumonia (98%), a lesion that was identified in 54% of calves dying in beef feedlots in the first 2 months after arrival and was considered an important contributor to death in 36% (Gagea *et al*., 2006b). The prevalence of chronic pneumonia varies substantially among feedlots and pens, but an overall prevalence of 1.3% is typical (Pollock *et al*., 2000).

It has been hypothesized that the recent emergence of *M. bovis* pneumonia in feedlot cattle might be a consequence of the routine use of metaphylactic antibiotic treatment, and perhaps a tendency for producers to monitor at-risk cattle less intensively if they are treated with antibiotics. Although there is currently little published evidence to support this idea (Tschopp *et al*., 2001), it certainly merits further investigation. Alternatively, since *M. bovis* pneumonia seems to have been under-recognized in the past (Gagea *et al*., 2006a), increased awareness of the disease by clinical veterinarians and diagnosticians may also have contributed to its apparent emergence.

Estimated economic impact

The economic costs of *M. bovis* infection are likely to include reduced weight gain or feed efficiency, pharmaceutical and labor costs for treatment of ill animals, death losses, and a portion of the cost of preventative measures such as conditioning. However, it is not possible to estimate the economic impact of *M. bovis* with any accuracy, because the proportion of acute pneumonia attributed to this pathogen is not known. In particular, the clinical signs in the early stages of *M. bovis* pneumonia cannot be distinguished from those in other forms of bacterial pneumonia, unless arthritis is also present, and many cases of pneumonia involve interactions between more than one pathogen. These factors make it impossible to reliably attribute economic losses to a single pathogen, and it is not yet possible to estimate the economic advantage of eliminating *M. bovis* from a herd. Thus, caution is warranted in interpreting estimates of the economic impact of this disease. In one study, calves that seroconverted to *M. bovis* experienced a 7.6% reduction in average weight gain during the first 7 weeks in the feedlot, and had about 2 times more antibiotics prescribed than calves that remained seronegative (Tschopp *et al*., 2001). Whether *M. bovis* infection was the cause of this reduced weight gain is unknown, as it is plausible that stress or other predisposing factors may have enhanced infection with and seroconversion to *M. bovis*. Within these limitations, *M. bovis* infection has been estimated to cost €144 million per year for the European cattle industry and $32 million per year in the United States (Nicholas and Ayling, 2003), and €25 for calves of milking cattle and €58 per veal calf (Gevaert, 2006). Finally, the non-economic costs are highly significant: chronic pneumonia is an important welfare issue, and prolonged treatment of affected animals with a variety of antibiotics contributes to development of antimicrobial resistance in other pathogens.

Control of the disease

Treatment of affected cattle

Studies that investigate the treatment of *M. bovis* pneumonia address three disease conditions: 'enzootic' pneumonia in young dairy and veal calves, in which viruses, *M. bovis*, and other bacteria contribute to the disease complex; acute shipping fever pneumonia of feedlot cattle, in which the role of *M. bovis* is poorly defined; and chronic pneumonia and arthritis syndrome of feedlot cattle, in which *M. bovis* plays an important and

https://doi.org/10.1017/S1466252307001351 Published online by Cambridge University Press

causal role. In a western Canadian feedlot, 40% of calves with chronic pneumonia and arthritis syndrome were euthanized or died in the chronic hospital pen. The 60% that responded adequately enough to treatment to return to their home pen required on average 4 weeks of treatment and weighed 58 kg less than their cohorts (Pollock *et al*., 2000). No reports have described the efficacy of antibiotics compared to placebo for treatment of chronic *M. bovis* pneumonia of feedlot cattle, although most such calves are treated with a succession of different antibiotics.

This poor response to treatment in cases of chronic pneumonia and arthritis syndrome should not be taken as proof that other forms of *M. bovis* pneumonia have a similarly grim prognosis. The difficulty in clinical diagnosis is a major problem in designing and evaluating trials that address the treatment efficacy in acute *M. bovis* pneumonia: the clinical signs of acute *M. bovis* pneumonia are indistinguishable from those of pneumonic pasteurellosis except for cases in which arthritis is also present, isolation of *M. bovis* is of limited significance because of its prevalence in clinically normal calves, and the lesions of caseonecrotic bronchopneumonia can only be detected at necropsy and presumably resolve in calves that respond to therapy. Thus, it may be that some cases of acute *M. bovis* pneumonia respond well to antibiotic treatment, but the etiologic role of *M. bovis* in these cases cannot be easily determined. Experimental studies have the advantages of a more standardized exposure to *M. bovis* and improved control of other pathogens compared to field cases, but the experimental disease may not resemble that seen in naturally occurring cases. In the reported efficacy studies, the experimental challenge models induce acute disease that would be indistinguishable from other causes of acute bacterial pneumonia, but do not reliably induce the chronic disease, arthritis, or caseonecrotic bronchopneumonia that typify those cases recognized clinically and pathologically as chronic *M. bovis* pneumonia.

In an experimental challenge model, spectinomycin had no effect on clinical signs and a small effect on isolation of *M. bovis* from lung tissue (Poumarat *et al*., 2001). Treatment with enrofloxacin had no effect on development of arthritis that was experimentally induced by intra-articular inoculation of *M. bovis* (Belli *et al*., 1993). Treatment of arthritis with tylosin is reported to be effective if administered early in the course of disease (Henderson and Ball, 1999), but there is no indication of its effectiveness in field cases.

The use of valnemulin and tulathromycin have been more extensively investigated. One study involved intratracheal *M. bovis* challenge of 3–9 weeks old dairy calves, followed 4 days later by treatment with tulathromycin or saline. Tulathromycin-treated calves had a significantly lower prevalence of severe disease, lower lung lesion scores, lower rectal temperatures, and greater body weight gains compared to saline-treated calves (Godinho *et al*., 2005a). In this study, the experimental challenge resulted in consolidation of the lung, but there was no mention of the caseonecrotic lesions typical of that seen in field cases, nor was arthritis described. These experimental studies are complemented by others showing that both tulathromycin and tilmicosin are effective treatments for naturally occurring acute bovine respiratory disease. Although the disease in these studies was associated with infection by *M. bovis*, *M. haemolytica* and other bacteria, it is unknown whether elimination of *M. bovis* was the reason for treatment efficacy in these cases because these antibiotics are also expected to be effective against bacteria of the family *Pasteurellaceae* (Godinho *et al*., 2005b; Skogerboe *et al*., 2005).

The efficacy of valnemulin has been evaluated in an experimental challenge model. Seronegative 10–35 days old dairy calves were challenged by aerosol, then valnemulin or enrofloxacin therapy was initiated 10 days later. Treatment with either drug resulted in improvement in clinical signs, appetite, weight gain and reduced number of *M. bovis* bacteria isolated from the lung (Stipkovits *et al*., 2005). Although lameness and arthritis were detected and lesions of bronchopneumonia were described, the lung lesions lacked the caseonecrotic foci seen in natural disease. The effectiveness of valnemulin therapy was also reported in a group of dairy calves in which *M. bovis* and *P. multocida* were the major bacterial pathogens isolated from calves with enzootic pneumonia. Treatment was associated with reduced prevalence of depression, respiratory signs and arthritis. *M. bovis* was isolated less frequently in nasal swabs but was identified with similar frequency by PCR, in treated and control calves (Stipkovits *et al*., 2001).

Early intervention with antibiotic therapy is critical for successful treatment of pneumonic pasteurellosis. There are no published studies to address the importance of early treatment for *M. bovis* pneumonia, but it is highly likely that delayed treatment reduces the response to therapy, in part because the caseonecrotic lung lesions are probably poorly penetrated by many antibiotics and by the host leukocyte response.

Prevention

Metaphylactic antibiotic treatment

Metaphylactic treatment with antibiotics reduces morbidity and mortality from respiratory disease in beef feedlots (Vogel *et al*., 1998; Duff and Galyean, 2007), although the impact on the prevalence of chronic pneumonia and arthritis is unknown. Similarly, in 3 weeks to 12 months old calves experiencing outbreaks of febrile respiratory disease, treatment of clinically normal in-contact calves with tulathromycin compared to saline was associated with reduced prevalence of disease and reduced number of relapses following treatment. However, the contribution of *M. bovis* to disease in these calves is unknown, and

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would be difficult to reliably assess (Godinho *et al.*, 2005b).

Vaccination

Because of the poor therapeutic response in cases of severe chronic *M. bovis* pneumonia, and concerns surrounding the preventative use of antibiotics in beef production, vaccination is an attractive method for control of *M. bovis* pneumonia. This approach has proven problematic because of a lack of knowledge of whether and how calves develop immunity to pulmonary infection with this pathogen. As described above, antibody responses to the immunodominant antigens, the variable surface proteins, are thought to be non-protective because they stimulate a shift in antigen expression by the bacteria. It remains unknown whether this evasion of the immune response can be overcome in naturally infected cattle or in vaccinates, as a result of limitations in the variability of this Vsp system, humoral immune responses to other antigens, or other mechanisms of innate or acquired immunity. Two vaccines for *M. bovis*, both bacterins, are currently licensed by the United States Department of Agriculture for an aid in prevention of pneumonia in cattle: Pulmo-Guard™ MpB (Boehringer Ingelheim Vetmedica, Inc.) and Myco-Bac™ B (Texas Vet Lab, Inc.). A third vaccine, Mycomune™ (Biomune Co.), is licensed for control of mastitis (USDA-APHIS, 2007), and autologous vaccines are also in use (Thomson and White, 2006).

Many studies have shown that vaccination is capable of stimulating a measurable antibody response, including development of *M. bovis*-specific IgM, IgG1, IgG2, and IgA in serum, and IgA in the lung lining fluid (Boothby *et al.*, 1983; Urbaneck *et al.*, 2000; Boehringer Ingelheim Vetmedica, Inc., 2003). In one study, titers were detectable in vaccinates within 16 days and remained for at least 6 months (Nicholas *et al.*, 2002). However, it is clear that serum and local antibody titers do not necessarily correlate with protection based on the prevalence of titers in cattle soon after arrival in feedlots (Allen *et al.*, 1991, 1992; Tschopp *et al.*, 2001) and in calves dying of *M. bovis* pneumonia (Gagea *et al.*, 2006b).

Vaccines have shown promise in some experimental challenge studies, whereas other reports have suggested an increased level of disease in vaccinates (Nicholas *et al.*, 2002). Use of a vaccine based on saponin-inactivated *M. bovis* bacteria was reported to be safe in 3–4 week old dairy calves. Following aerosol challenge with *M. bovis*, vaccinates had a lower prevalence of *M. bovis* detected in lung and joints, reduced severity of fever and clinical signs of respiratory disease, and less extensive and severe pulmonary lesions at necropsy (Nicholas *et al.*, 2002). Lesions were described mainly as interstitial pneumonia and lymphohistiocytic bronchitis with less frequent catarrhal bronchopneumonia, so the relevance of this model to severe field cases may be questionable. Similarly, when calves were vaccinated with formalin-inactivated *M. bovis* and later challenged by intratracheal inoculation of *M. bovis*, vaccinates had reduced colonization of the lung at 3 weeks after infection. Although these findings suggest a role for local immune responses in protection against experimental challenge, the relevance of these findings to control of natural disease is unknown (Howard *et al.*, 1977). One commercially available vaccine is claimed to have resulted in 29–89% reduction in lung lesions following experimental challenge, but detailed data are not available and the results are not published in the peer-reviewed literature (Boehringer Ingelheim Vetmedica, Inc., 2003).

Field studies of vaccines share design challenges with the antibiotic efficacy studies described above, because of the difficulty in establishing the precise role of *M. bovis* in field cases of pneumonia. An autologous formalin-inactivated vaccine is reported to have reduced both infection and mortality in a herd with ongoing respiratory disease caused by *M. bovis* and *M. haemolytica* (Urbaneck *et al.*, 2000). A quadrivalent inactivated vaccine containing respiratory syncytial virus, parainfluenza type 3, *Mycoplasma dispar* and *M. bovis* conferred an approximately 45% reduction in 4 of 7 groups of calves, and this effect was greater than that of the BRSV vaccine alone (Howard *et al.*, 1990). In contrast, in a field trial in a herd that suffered up to 25% mortality attributed in part to *M. bovis*, calves vaccinated on arrival but receiving no antibiotics had higher prevalence of severe disease than non-vaccinated calves that were treated with in-feed antibiotics (Nicholas *et al.*, 2006). There are no published reports of field studies describing the effectiveness of *M. bovis* vaccines in preventing pneumonia of beef feedlot cattle.

Other disease control strategies

Conditioning of beef calves prior to their transport to feedlots is an effective method to prevent pneumonic pasteurellosis, and requires that calves be castrated and dehorned at an early age, and weaned and introduced to roughage and concentrated feed at least 30 days before transport to the feedlot. Some conditioning programs also require prior vaccination against respiratory viruses, BVDV, *M. haemolytica* and/or *H. somni* (Macartney *et al.*, 2003; Duff and Galyean, 2007). Anecdotal evidence suggests that a similar strategy may minimize the prevalence or severity of *M. bovis* pneumonia, but objective published data to support this approach are lacking. Similarly, prevention of *M. bovis* pneumonia in dairy calves is often feasible by ensuring adequate ventilation and stocking densities, and segregating calves of different ages or different sources (Nicholas and Ayling, 2003).

Eradication is a theoretically possible control strategy because of limited survival of *M. bovis* in the environment, but is not currently practical in many herds because of the high prevalence of infection. Several reports describe

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eradication of *M. bovis* from dairy herds. Strategies include depopulation followed by repopulation with pregnant or weanling heifers that were from seronegative herd status, isolation on arrival, and on-arrival antibiotic treatment (O'Farrell *et al*., 2001); or by identification of infected cows using serology and culture of mammary gland or nasal cavity, followed by segregation then culling of cows that tested positive (Bicknell *et al*., 1983; Byrne *et al*., 1998). Herds were monitored by serologic testing and culture of imported cattle, or by a program of bulk tank milk culture (Bicknell *et al*., 1983; O'Farrell *et al*., 2001).

Conclusion

M. bovis has emerged as an important cause of pneumonia, both in young dairy and veal calves and in feedlot cattle. Recent investigations have yielded important insights into the biology of this pathogen, the host response to infection and the role of *M. bovis* in disease. However, important questions still remain regarding genotypic diversity, the role of co-pathogens and other risk factors, disease mechanisms and immunity. Addressing these questions will be important in the development of improved strategies to control this disease.

Acknowledgments

We thank Drs Ken Bateman, Fernanda Castillo, Ted Clark, Mihai Gagea, Gord Mitchell and Tony van Dreumel for insightful discussions of this disease. Mycoplasma research by the authors is supported by the Ontario Cattlemen's Association, Canadian Cattlemen's Association and the Natural Sciences and Engineering Research Council of Canada.

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