Mycoplasma bovis INFECTION OF DAIRY CALVES

By

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To my parents, Pauline and John; and my husband, Fred.

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Mycoplasma bovis is an important cause of pneumonia, otitis media and arthritis in young dairy calves, and there is a critical need to develop improved preventative strategies for this disease. Because there is a lack of efficacy data for *M. bovis* vaccines, especially in young calves, we evaluated a commercial *M. bovis* vaccine in this age group. However, our major research focus was to define local immune responses to *M. bovis* in young calves. Specific objectives were to develop a reproducible model of *M. bovis* infection of the upper respiratory tract (URT) that mimicked natural infection and to define lymphocyte responses generated along the respiratory tract during *M. bovis* infection in young calves.

A field trial to determine the efficacy of a commercial *M. bovis* vaccine for the prevention of *M. bovis*-associated disease in calves was conducted on three Florida dairies. Vaccination had no effect on rates of nasal colonization with *M. bovis*, age at first treatment, incidence of respiratory disease, or mortality to 90 days of age. In one herd, vaccination was associated with an increased risk of otitis media.

We defined a model of *M. bovis* infection that mimics natural disease by feeding milk containing *M. bovis* to young calves. *Mycoplasma bovis* consistently colonized the eustachian tubes as well as the tonsils of inoculated calves, and otitis media and pneumonia developed in a

subset of calves. Evaluation of immune responses along the respiratory tract showed that the infection site corresponded to the distribution of immune responses. The URT lymphoid tissues were major sites for B and T cell responses after oral infection, and *M. bovis*-specific mucosal IgA responses were observed. Overall, we found that local immune responses are important in the pathogenesis of *M. bovis*. The oral inoculation model will facilitate further study of host-pathogen interactions during colonization, expansion of infection and dissemination to the lungs and middle ear, as well as providing a tool for evaluating new control strategies for *M. bovis* infection of young calves.

CHAPTER 1
Mycoplasma bovis AND BOVINE IMMUNOLOGY: REVIEW OF LITERATURE

Overview

Mycoplasmas belong to the class *Mollicutes* (from the Latin *mollis*, soft; *cutis*, skin), a group of bacteria so named because they lack cell walls, instead being enveloped by a complex plasma membrane. Mollicutes are also characterized by their tiny size, small genomes (0.58 to 2.2 Mb), and low G + C content (24 to 33 mol %) (Razin *et al*., 1998). Perhaps as a direct consequence of the limited biosynthetic capacity of their small genome, mycoplasmas usually form an intimate association with host cells to obtain growth and nutritional factors necessary for survival (Rosengarten *et al*., 2001). Mollicutes are found in a wide range of hosts including mammals, birds, reptiles, fish, arthropods and plants (Razin, 1992). Their individual relationship with the host varies from primary or opportunistic pathogens to commensals or epiphytes.

In mammalian hosts, mollicutes typically inhabit mucosal surfaces, including those of the respiratory, urogenital and gastrointestinal tracts, eyes, and the mammary gland (Rosengarten *et al*., 2000). As is typical of many mucosal pathogens, pathogenic species of mollicutes may inhabit some mucosal sites without causing disease (Rottem and Naot, 1998; Hickman-Davis, 2002). Disease occurs when host and/or pathogen factors result in dissemination to other sites (e.g. from the nasal mucosa to the lower respiratory tract [LRT]), invasion and destruction of host tissues, or a detrimental inflammatory response. Hematologic dissemination from mucosal surfaces can occur, with the joints being a frequent site of secondary colonization (Simecka *et al*., 1992). Mollicutes are very effective at evading and modulating the host immune response and the immune response contributes significantly to the pathogenesis of many mollicute-associated diseases (Simecka *et al*., 1992; Rosengarten *et al*., 2000).

Mycoplasma bovis was first isolated from a case of severe mastitis in a U. S. dairy cow in 1961 (Hale *et al*., 1962), and is now recognized as a world-wide pathogen of intensively-farmed cattle. At least nine pathogenic and numerous non-pathogenic mycoplasma species have been isolated from cattle (Simecka *et al*., 1992). The most severe ruminant disease is caused by *Mycoplasma mycoides* subsp. *mycoides* biotype small colony (SC), the etiologic agent of contagious bovine pleuropneumonia, an Office International des Epizootes List-A disease. In North America and most of Europe, where contagious bovine pleuropneumonia has been eradicated, *M. bovis* is considered the most pathogenic of the bovine mycoplasmas (Nicholas and Ayling, 2003). It causes mastitis in dairy cows (Gonzalez *et al*., 1992; Pfutzner and Sachse, 1996; Fox *et al*., 2003; Gonzalez and Wilson, 2003) and pneumonia and arthritis in feeder and stocker cattle (Kusiluka *et al*., 2000b; Haines *et al*., 2001; Tschopp *et al*., 2001; Shahriar *et al*., 2002; Thomas *et al*., 2002a; Gagea *et al*., 2006). In addition, in the past decade *M. bovis* has emerged as an important cause of pneumonia and otitis media in dairy calves (Stipkovits *et al*., 2001; Nicholas and Ayling, 2003; Francoz *et al*., 2004; Lamm *et al*., 2004).

Calf Specific Disease

Evidence for *M. bovis* as an Etiologic Agent of Calf Disease

It is now well established that *M. bovis* is a primary cause of respiratory disease, otitis media and arthritis in calves. There are many reports of respiratory disease outbreaks where *M. bovis* was the predominant bacteria isolated from lungs of affected calves (Buchvarova and Vesselinova, 1989; Gourlay *et al*., 1989a; Brown *et al*., 1998a; Stipkovits *et al*., 2000; Bashiruddin *et al*., 2001; Rosenbusch, 2001; Stipkovits *et al*., 2001). In addition, although bovine pneumonia rarely involves a single infectious agent, experimental infection studies have shown that inoculation with *M. bovis* alone can cause pneumonia in calves (Gourlay *et al*., 1976; Thomas *et al*., 1986; Poumarat *et al*., 2001). Seroconversion to *M. bovis* is associated with

increased respiratory disease rates (Martin *et al*., 1990) as well as decreased weight gain and increased number of antibiotic treatments in feedlot calves (Van Donkersgoed *et al*., 1993; Tschopp *et al*., 2001). However, as with most bovine respiratory pathogens, colonization is not always sufficient cause for disease. *Mycoplasma bovis* can be isolated from the upper respiratory tract (URT), trachea, and LRT of calves without clinical disease or gross lesions (Bennett and Jasper, 1977c; Springer *et al*., 1982; Allen *et al*., 1992a; Virtala *et al*., 1996b; Tenk *et al*., 2004), although its presence in the LRT may cause subclinical inflammation (Allen *et al*., 1992b). Despite these findings, isolation of *M. bovis* as the predominant pathogen in numerous outbreaks of respiratory disease and experimental confirmation of its ability to cause pneumonia in calves verify its role as an important respiratory pathogen.

Field cases of respiratory disease caused by *M. bovis* are sometimes accompanied by arthritis, and *M. bovis* has been isolated in pure culture from affected joints, as well as from the lungs of calves with concurrent respiratory disease (Stalheim and Page, 1975; Stipkovits *et al*., 1993; Rosenbusch, 1995; Adegboye *et al*., 1996; Butler *et al*., 2000; Byrne *et al*., 2001). Consistent with the observations of natural disease, arthritis has been induced by inoculation of *M. bovis* into joints or lungs, or intravenously (Stalheim and Page, 1975; Gourlay *et al*., 1976; Chima *et al*., 1980; Ryan *et al*., 1983; Thomas *et al*., 1986; Linker *et al*., 1998). Variation among clinical isolates of *M. bovis* in their ability to cause arthritis in an experimental infection model has been reported (Rosenbusch, 1995).

In addition to causing disease of the LRT and arthritis, *M. bovis* is the predominant pathogen isolated from the middle ear of young calves with otitis media (Dechant and Donovan, 1995; Walz *et al*., 1997; Maeda *et al*., 2003; Francoz *et al*., 2004; Lamm *et al*., 2004). However, other bacteria, including *Mycoplasma bovirhinis*, *Mycoplasma alkalescens*, *Mycoplasma*

arginini, *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Arcanobacterium pyogenes* are isolated sporadically, and some have been associated with outbreaks of otitis media, especially in feedlot cattle (Jensen *et al*., 1983; Nation *et al*., 1983; McEwen and Hulland, 1985; Henderson and McCullough, 1993; Dechant and Donovan, 1995; Lamm *et al*., 2004; Gagea *et al*., 2006). In tropical regions of the world, parasitic otitis followed by secondary mixed bacterial infections of the external and middle ear occurs (Duarte and Hamdan, 2004).

Susceptibility to *M. bovis*-induced otitis media is age-related, with the peak incidence of clinical disease at 2-6 weeks of age (Dechant and Donovan, 1995; Walz *et al*., 1997). In one recent study of feedlot cattle (Gagea *et al*., 2006), *M. bovis* was frequently isolated from the tympanic bullae of animals with no clinical or gross lesions of otitis media, suggesting it is the expression of clinical disease rather than dissemination to the middle ear which is affected by age-related factors. Nonetheless, in the past 15 years, outbreaks of otitis media in groups of North American dairy calves have been largely attributable to *M. bovis* infection (Dechant and Donovan, 1995; Walz *et al*., 1997; Lamm *et al*., 2004). Experimental infection studies using *M. bovis* to induce otitis media have not been published, however, nor has otitis media been reported as a sequelae following experimental inoculation of *M. bovis* in studies of respiratory disease. Therefore, although current information strongly supports the role of *M. bovis* as a cause of otitis media, more studies are required to fulfill Koch's postulates and to determine the host pathogen interactions contributing to disease expression.

Clinical Disease in Dairy Calves

Clinical disease associated with *M. bovis* infection of dairy calves typically presents as pneumonia, otitis media or arthritis, or any combination of these (Stalheim and Page, 1975; Rosenbusch, 1995; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000; Stipkovits *et al*.,

2001; Francoz *et al*., 2004; Lamm *et al*., 2004). *Mycoplasma bovis* has also been associated with a variety of other less common clinical manifestations in calves, including tenosynovitis, decubital abscesses and meningitis (Kinde *et al*., 1993; Stipkovits *et al*., 1993; Adegboye *et al*., 1996). The age of onset of clinical disease in affected calves is typically between 2 and 6 weeks (Walz *et al*., 1997; Brown *et al*., 1998a; Stipkovits *et al*., 2000; Stipkovits *et al*., 2001) but has been reported as early as 4 days of age (Stipkovits *et al*., 1993). Clinical disease caused by *M. bovis* tends to be chronic, debilitating and unresponsive to therapy (Gourlay *et al*., 1989a; Allen *et al*., 1992a; Adegboye *et al*., 1995a; Apley and Fajt, 1998; Shahriar *et al*., 2000; Stipkovits *et al*., 2000; Gagea *et al*., 2006). Chronic endemic disease as well as epizootics can occur (Rodriguez *et al*., 1996).

Mycoplasma bovis-associated respiratory disease has a similar clinical presentation to other types of calf pneumonia (Figure 1-1). Fever, loss of appetite, nasal discharge, coughing, and both increased respiratory rate and effort are typically reported, and concurrent cases of otitis media and arthritis may occur (Adegboye *et al*., 1996; Walz *et al*., 1997; Brown *et al*., 1998a; Stipkovits *et al*., 2001; Francoz *et al*., 2004; Lamm *et al*., 2004). As for undifferentiated calf pneumonia, auscultation reveals abnormal breath sounds including increased bronchial sounds, crackles, wheezes, and areas of cranioventral consolidation in severe cases (Ames, 1997). Both acute and chronic disease can occur, and mixed infections are common (Howard *et al*., 1987b; Gourlay *et al*., 1989a; Virtala *et al*., 1996b; Mosier, 1997; Stipkovits *et al*., 2000; Poumarat *et al*., 2001; Vogel *et al*., 2001; Thomas *et al*., 2002a). Calves with chronic pneumonia often develop extreme dyspnea and emaciation (Ames, 1997).

Otitis media has been an increasingly recognized form of *M. bovis*-associated disease in North American dairy calves over the past 15 years (Figure 1-2) (Dechant and Donovan, 1995;

Walz *et al*., 1997; Lamm *et al*., 2004). The clinical signs of otitis media observed include loss of appetite, fever, listlessness, ear pain evidenced by head shaking and scratching at or rubbing ears, epiphora, ear droop and signs of facial nerve paralysis (Walz *et al*., 1997; Brown *et al*., 1998a; Maeda *et al*., 2003; Francoz *et al*., 2004; Van Biervliet *et al*., 2004). One or both tympanic bullae can be affected. In some cases, purulent discharge from the ear canal is observed following rupture of the tympanic membrane (Walz *et al*., 1997; Francoz *et al*., 2004). In addition, calves with *M. bovis*-induced otitis media often have concurrent pneumonia (Dechant and Donovan, 1995; Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). Bacteria gain access to the middle ear by several possible routes that include extension of external ear infections via the tympanic membrane, colonization of the oropharynx and extension into the tympanic bulla via the eustachian tubes, or by hematogenous spread (Duarte and Hamdan, 2004; Morin, 2004). In pigs, otitis media due to *Mycoplasma hyorhinis* has been shown to occur by extension of URT infections to the middle ear via the eustachian (auditory) tube (Morita *et al*., 1995; Morita *et al*., 1999). Although the route of infection has not been studied in calves, it is likely that a similar mechanism occurs given the frequent colonization of the nasopharynx with *M. bovis* in young calves (Bennett and Jasper, 1977c).

Otitis interna is a common sequelae to otitis media in calves, and affected animals exhibit varying degrees of vestibulocochlear dysfunction including head tilt, horizontal nystagmus, staggering, circling, falling, and/or lateral recumbency (Dechant and Donovan, 1995; Maeda *et al*., 2003; Lamm *et al*., 2004; Van Biervliet *et al*., 2004). Meningitis can occur as a complication of otitis interna (Stipkovits *et al*., 1993; Francoz *et al*., 2004; Lamm *et al*., 2004; Van Biervliet *et al*., 2004). Meningitis secondary to otitis media-interna may be localized, so cerebrospinal fluid samples collected for diagnostic purposes should be from the atlanto-occipital, rather than the

lumbo-sacral space (Van Biervliet *et al*., 2004). Spontaneous regurgitation, loss of pharyngeal tone and dysphagia have also been reported in calves with *M. bovis*-associated otitis media-interna, indicative of glossopharyngeal nerve dysfunction with or without vagal nerve dysfunction (Van Biervliet *et al*., 2004). Whether these nerves are affected by inflammation associated with meningitis or with inflammation at the site where the nerves pass over the tympanic bullae is unknown (Van Biervliet *et al*., 2004). As is observed with *M. bovis*-associated respiratory disease, calves with chronic otitis media-interna may become emaciated (Walz *et al*., 1997; Van Biervliet *et al*., 2004).

In contrast to *M. bovis* infections of the upper and lower respiratory tracts, *M. bovis*-induced arthritis is presumed to be a consequence of mycoplasmemia (Chima *et al*., 1981; Thomas *et al*., 1986). Arthritis was preceded by mycoplasmemia in one calf that was inoculated intratracheally with *M. bovis* (Thomas *et al*., 1986). Infections of other body systems that occasionally accompany polyarthritis are also likely to be a consequence of mycoplasmemia (Stipkovits *et al*., 1993). Clinical cases of *M. bovis*-induced arthritis in dairy calves tend to be sporadic and are typically accompanied by respiratory disease within the herd and often within the same animal (Gonzalez *et al*., 1993; Stipkovits *et al*., 2005). Although uncommon, outbreaks of disease where arthritis was the predominant clinical presentation have been reported (Stipkovits *et al*., 1993; Butler *et al*., 2000). Clinical signs are typical of septic arthritis; affected joints are painful and swollen, and calves exhibit varying degrees of lameness and may be febrile in the acute phase of disease (Figure 1-3) (Stipkovits *et al*., 1993; Byrne *et al*., 2001; Step and Kirkpatrick, 2001a). Large rotator joints such as the shoulder, elbow, carpal, hip, stifle and hock joints are most frequently involved (Thomas *et al*., 1975; Stipkovits *et al*., 1993; Adegboye *et al*., 1996; Step and Kirkpatrick, 2001a; Clark, 2002; Gagea *et al*., 2006). One or multiple joints

can be affected, and calves with *M. bovis* arthritis are frequently culled due to poor response to therapy (Adegboye *et al*., 1996; Byrne *et al*., 2001; Stokka *et al*., 2001).

Mycoplasma bovis also may cause a variety of less common clinical syndromes in calves, with or without concurrent respiratory disease. In addition to its occurrence as a sequelae of otitis media (Maeda *et al*., 2003; Francoz *et al*., 2004), meningitis has occurred as a consequence of mycoplasmemia in very young calves (Stipkovits *et al*., 1993). For example, in one case report, 3 to 21 day-old calves developed polyarthritis and meningitis with an associated high mortality rate. *Mycoplasma bovis* was the only pathogen isolated from joints and meninges of affected calves (Stipkovits *et al*., 1993).

Mycoplasma bovis infections in or around tendons and synovial structures have been reported, and tenosynovitis and bursitis are commonly reported in feedlot calves with concurrent chronic *M. bovis* arthritis (Adegboye *et al*., 1996; Step and Kirkpatrick, 2001a; Clark, 2002). In addition, intra-articular inoculation of *M. bovis* in calves resulted in arthritis plus tenosynovitis (Stalheim and Page, 1975; Ryan *et al*., 1983). In an unusual presentation of *M. bovis* infections, an outbreak of subcutaneous decubital abscesses over carpal and stifle joints and in the brisket was reported in fifty calves fed unpasteurized waste milk on a Californian calf ranch. *Mycoplasma bovis* was the only pathogen isolated from abscesses, which occurred at the sites of pressure sores. Whether the bacteria gained entry through skin abrasions or via hematogenous spread is unknown, but the authors hypothesized that *M. bovis* in nasal secretions may have contaminated pressure sores when calves licked these areas. There was no evidence of joint involvement in affected calves, but at least one calf had concurrent *M. bovis*-associated respiratory disease (Kinde *et al*., 1993).

Mycoplasma bovis can be isolated from the conjunctiva of cattle in infected herds (Boothby *et al*., 1983b), although *M. bovis*-associated ocular disease is considered uncommon (Brown *et al*., 1998b). However, there are several reports of outbreaks of keratoconjunctivitis involving *M. bovis* alone, or in mixed infections with *Mycoplasma bovoculi* (Jack *et al*., 1977; Kirby and Nicholas, 1996; Levisohn *et al*., 2004; Alberti *et al*., 2006). An outbreak of severe keratoconjunctivitis, from which *M. bovis* was the only consistently isolated pathogen, was reported in a group of 20 calves. Clinical signs included mucopurulent ocular discharge, severe eyelid and conjunctival swelling, and corneal edema and ulceration. Most clinical signs resolved within 2 weeks but some animals had residual corneal scarring (Kirby and Nicholas, 1996). In a recent report (Alberti *et al*., 2006), an outbreak of *M. bovis*-associated keratoconjunctivitis in beef calves in Italy was followed by cases of pneumonia and arthritis.

In summary, *M. bovis* infections primarily result in pneumonia, otitis media and arthritis in young calves, but other more unusual clinical presentations affecting a wide variety of body systems can occur. *Mycoplasma bovis* is an important cause of mastitis in adult cows, but discussion of this clinical syndrome is beyond the scope of this dissertation.

Prevalence

Mycoplasma bovis appears to be widespread within the North American dairy cattle population (Uhaa *et al*., 1990; Gonzalez *et al*., 1992; Van Donkersgoed *et al*., 1993; Kirk *et al*., 1997; Fox *et al*., 2003; USDA:APHIS, 2003). In the National Animal Health Monitoring System (NAHMS) Dairy 2002 study, 7.9% of 871 dairies tested positive for mycoplasmas upon culture of a single bulk tank milk sample; *M. bovis* was identified in 86% of the positive herds. States in the Western region had a greater percentage of operations with positive mycoplasma culture (9.4%) than states in the Midwest (2.2%), Northeast (2.8%) and Southeast (6.6%) regions. These

values are likely an underestimate of true prevalence, as subclinically infected cows shed mycoplasmas intermittently in milk (Jasper, 1981; Biddle *et al*., 2003) and milk from cows with clinical mastitis is usually withheld from the bulk tank. Reported prevalence in individual cows varies widely among herds and among studies; Gonzalez *et al*., (1992) reported that 11.7% of cows sampled between 1970 to 1990 in 165 New York herds were infected, whereas Wilson *et al*., (1997) reported that only 85/105,083 (0.1%) of individual cow cultures collected in Northeast U.S. dairy herds between 1991 and 1995 were positive for mycoplasmas. In a study of 463 dairy operations in the Northwest U.S., 93 (20%) of herds had at least one mycoplasma positive bulk tank milk sample between 1998 and 2000.

Studies of the prevalence of *M. bovis*-associated disease in dairy calves in North America have not been published, but in Europe it has been estimated that *M. bovis* is responsible for 25% to 35% of calfhood respiratory disease (Nicholas and Ayling, 2003). Although the prevalence of *M. bovis*-associated disease is unknown, there are data on the prevalence of undifferentiated respiratory disease in North American calves. In fact, after diarrheal diseases, respiratory disease is the second most important cause of morbidity and mortality in U.S. dairy heifers. In the NAHMS Dairy 2002 study (USDA:APHIS, 2003), which represented 83% of U.S. dairy operations, respiratory disease accounted for 21.3% of all heifer deaths; pre-weaning and post-weaning mortality were 8.7% and 1.9%, respectively. Morbidity rates were not reported, but in the 1991-1992 National Dairy Heifer Evaluation Project (NDHEP), which included 906 dairies from 28 states, the cumulative incidence risk of respiratory disease to 8 weeks of age was 10%; 24.5% of mortality was due to respiratory disease (Wells *et al*., 1997). To the best of the author's knowledge, more recent national estimates of respiratory disease rates in dairy heifers have not been published. The incidence and severity of respiratory disease vary markedly among

individual farms and among regional studies. In 18 commercial New York dairy operations, the incidence of pneumonia in the first 3 months of life was 25.6% and the case fatality rate was 2.2% (Virtala *et al*., 1996b). In a 1991 study of 30 Minnesota dairies, the incidence rate for pneumonia was 0.10 per 100 calf days at risk, the case fatality rate was 9.4%, and pneumonia accounted for 30% of mortality during the first 4 months of life (Sivula *et al*., 1996). In a study of calf health on two large Florida dairies, mortality to 6 months of age was 11.7%, 22% of which was attributed to respiratory disease (Donovan *et al*., 1998a). Although these prevalence studies are not pathogen specific, it is clear from the reports of outbreaks of *M. bovis*-associated respiratory disease in North American dairy calves that *M. bovis* can be a significant contributor to overall rates of disease and mortality in affected herds (Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). For example, in a 1996 prospective study of five New York dairies, 40 cases of pneumonia occurred in 78 calves that were prospectively followed for the first 3 months of life; 22 (55%) of these cases were attributed to *M. bovis* infection (Musser *et al*., 1996).

Economic Losses

There are limited data available on the economic impact of *M. bovis*-associated disease. Financial losses were estimated at approximately $350 per case per lactation for mycoplasmal mastitis, based on records from 105,083 cows in the Northeast U.S. and a milk price of $13.00/hundred pounds of milk (Wilson *et al*., 1997). Losses to the U.S. beef industry as a result of reduced weight gain and carcass value due to *M. bovis*-associated disease have been estimated at $32 million per year, and in the U.K., it is estimated that *M. bovis* contributes to at least a quarter of economic loss due to bovine respiratory disease (Rosengarten and Citti, 1999). However, the cost of *M. bovis*-associated disease in dairy heifers has not been reported. In addition, there is scant recent information available on the cost of undifferentiated respiratory disease in dairy heifers in North America. In a 1990 study of Michigan dairy herds, the cost of

respiratory disease in calves was estimated at $14.71 per calf year (Kaneene and Hurd, 1990). Esslemont and Kossaibati (1999) estimated that the average cost of respiratory disease in dairy heifers in the U.K. was $61 per calf in the herd, based on 30% morbidity and 5% mortality rates. Economic costs associated with calf respiratory disease include treatment costs, labor costs, veterinary services, increased mortality, increased premature culling, reduced weight gain, reduced fertility, increased age at first calving, and possibly reduced milk production (Waltner-Toews *et al*., 1986; Warnick *et al*., 1995; Virtala *et al*., 1996a; Ames, 1997; Warnick *et al*., 1997; Donovan *et al*., 1998b). Without pathogen-specific data being available, it is reasonable to assume that *M. bovis*-associated disease incurs many of the same costs.

Mycoplasma bovis-associated disease tends to be debilitating and unresponsive to therapy (Gourlay *et al*., 1989a; Adegboye *et al*., 1995a; Apley and Fajt, 1998; Pollock *et al*., 2000; Stipkovits *et al*., 2000; Rosenbusch, 2001). Tschopp *et al*., (2001) gives an example of an outbreak of *M. bovis*-associated disease in which 54% of 415 calves introduced into an *M. bovis*-endemic facility seroconverted to *M. bovis.* Calves that seroconverted within 7 weeks of arrival experienced an 8% reduction in weight gain and required twice as many antibiotics as did seronegative calves. The proportion of clinical episodes of respiratory disease attributable to *M. bovis* in these calves was 50.3%. In another report of an *M. bovis*-associated disease outbreak, 70% of the calves in one dairy herd required treatment for respiratory disease or otitis media prior to 3 months of age (Brown *et al*., 1998a). On the individual farm affected with *M. bovis*-associated calf disease, losses resulting from treatment costs, death, and culling can be substantial, and economically devastating outbreaks with very high morbidity rates and loss of up to 30% of calves have been observed (Figure 1-4) (Gourlay *et al*., 1989a; Kinde *et al*., 1993;

Stipkovits *et al*., 1993; Dechant and Donovan, 1995; Walz *et al*., 1997; Butler *et al*., 2000; Stipkovits *et al*., 2000; Stipkovits *et al*., 2001; Tschopp *et al*., 2001).

Animal Welfare

In addition to any economic consequences, *M. bovis* must be considered important from a calf welfare perspective. *M. bovis*-associated disease is often chronic, responds poorly to antibiotic therapy, often affects a substantial proportion of calves in a herd, may cause permanent health issues for affected calves, and available vaccines are, at best, poorly efficacious (Gourlay *et al*., 1989a; Allen *et al*., 1992a; Adegboye *et al*., 1995a; Apley and Fajt, 1998; Stipkovits *et al*., 2000; Nicholas and Ayling, 2003; Gagea *et al*., 2006). Taken together, these characteristics result in affected calves that may be subject to long periods of illness for which the producer or veterinarian can provide only limited relief. There is therefore a critical need to develop improved preventative and treatment strategies for *M. bovis*-associated disease in young calves.

Epidemiology

Colonization and Shedding

Mycoplasma bovis is a frequent colonizer of the URT of healthy or diseased calves, with nasal prevalence ranging from < 5% to 100% of calves in a herd (Bennett and Jasper, 1977c; Springer *et al*., 1982; Allen *et al*., 1992a; ter Laak *et al*., 1992b; Brown *et al*., 1998a; Mettifogo *et al*., 1998). Within-herd prevalence is generally higher in herds with a history of *M. bovis*-associated disease than in herds without such a history. For example, Bennett and Jasper (1977c) reported a nasal prevalence of 34% in dairy calves < 8 months of age in herds with *M. bovis*-associated disease, compared with 6% in non-diseased herds. Cattle can remain infected for long periods of time and may shed *M. bovis* intermittently for many months and even years, acting as reservoirs of infection in the herd (Bennett and Jasper, 1977c; Pfutzner and Sachse, 1996). Chronic colonization of tonsils, with or without nasal shedding, has been described for

mycoplasmal respiratory pathogens in other hosts (Goltz *et al*., 1986; Friis *et al*., 1991), but whether the bovine tonsils are the primary site of URT colonization for *M. bovis* has not been established.

The significance of *M. bovis* colonization as a risk factor for the development of clinical disease in the individual animal is unknown. On a herd level, high prevalence of nasal colonization is associated with increased rates of clinical disease and with isolation of *M. bovis* from the LRT (Bennett and Jasper, 1977c; Springer *et al*., 1982; Allen *et al*., 1991; Brown *et al*., 1998a). However, isolation of *M. bovis* from nasal swabs in individual calves is generally poorly correlated with both clinical disease and the presence of *M. bovis* in the LRT (Bennett and Jasper, 1977c; Allen *et al*., 1991; Thomas *et al*., 2002b), although a positive correlation between *M. bovis* isolation from nasal swabs and clinical disease was reported in one study (Wiggins *et al*., 2007). Based on the current level of understanding, colonization of the URT precedes the development of clinical disease in calves, but is not always sufficient cause for disease.

Little is known about the typical age of onset and duration of nasal shedding of *M. bovis* in endemically-infected herds. Bennett and Jasper (1977c) reported that in calves less than 1 week of age, nasal prevalence was 38% in herds with *M. bovis*-associated disease and 7.5% in non-diseased herds. Prevalence peaked at 48% between 1 and 4 months of age. *Mycoplasma bovis* was still detected in nasal swabs from some calves at 8 months of age and from pre-partum heifers, although whether these represented new or chronic infections was not determined. Other investigators reported that almost 50% of calves in a herd with severe *M. bovis* and *P. multocida* pneumonia were shedding *M. bovis* at 5 days of age and over 90% were shedding *M. bovis* by 4 weeks; the onset of clinical disease peaked between 10 and 15 days of age. Approximately 10% of the calves died as a result of severe pneumonia, and surviving calves had poor weight gain

(Stipkovits *et al*., 2001). On a Florida dairy experiencing an outbreak of *M. bovis*-associated disease, *M. bovis* was isolated prior to 14 days of age from nasal swabs of all of 50 calves sampled, and 70% of these calves required treatment for respiratory disease or otitis media (Brown *et al*., 1998a). It is apparent from these studies that calves in infected herds are often colonized when they are very young, even at less than 1 week of age, and that the highest rates of nasal shedding occur in the first 2 months of life. In addition, Bennett and Jasper (1977c) found that *M. bovis* may be shed in nasal secretions of calves in herds with no history of *M. bovis*-associated disease.

Although the URT is the most common site of infection, *M. bovis* may similarly colonize and be shed from other body systems without causing clinical disease. Subclinical *M. bovis* mastitis is common, and infected cows may intermittently shed the bacteria in milk for months to years (Ruhnke *et al*., 1976; Jasper, 1981; Pfutzner and Sachse, 1996). *M. bovis* has also been isolated from the conjunctiva (Boothby *et al*., 1983b), semen and vaginal secretions (Feenstra *et al*., 1991; Pfutzner and Sachse, 1996) of cattle without clinical disease. Although both respiratory tract and mammary gland shedding have been implicated as reservoirs of infection within a herd (Pfutzner and Sachse, 1996), colonization at other sites does not seem to play a major role in the epidemiology of *M. bovis*.

Transmission and Risk Factors

Mycoplasma bovis is thought to be introduced to *M. bovis*-free herds by clinically healthy cattle that are carrying this microorganism (Jasper, 1981; Burnens *et al*., 1999; Tschopp *et al*., 2001; Gonzalez and Wilson, 2003). Spread to uninfected animals may occur at the time of introduction into the herd or may be delayed until, and if, shedding occurs (Fox *et al*., 2005). Little is published on the epidemiology of *M. bovis* within young calf populations, but there are several potential routes of initial exposure. Calves could become infected from their dams or

from other adult cows in the maternity area that are shedding *M. bovis* in colostrum, vaginal or respiratory secretions (Pfutzner and Sachse, 1996). The isolation of *M. bovis* from vaginal secretions of cows at calving (Feenstra *et al*., 1991; Brown *et al*., 1998a) and congenital infection of calves (Bocklisch *et al*., 1986; Stipkovits *et al*., 1993) have been reported, although both events appear to occur infrequently and probably do not play a major role in transmission.

One of the major means of transmission to young calves is thought to be ingestion of milk from cows shedding *M. bovis* from the mammary gland (Figure 1-5) (Pfutzner and Schimmel, 1985; Bocklisch *et al*., 1986; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). Colonization of the URT by *M. bovis* occurs more frequently in calves fed infected milk than in those fed uninfected milk (Bennett and Jasper, 1977c), and clinical disease has been documented following feeding of *M. bovis*-contaminated waste milk to calves or nursing of cows with *M. bovis* mastitis (Stalheim and Page, 1975; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). Because milk in modern husbandry systems is typically batched for feeding to calves, a single cow shedding *M. bovis* can potentially expose a large number of calves to infection, and calves may be repeatedly exposed over the milk-feeding period. In a field study to determine the method of transmission of *M. bovis* in one Florida dairy herd, 100% of 50 calves exposed to *M. bovis* contaminated waste milk became colonized in the URT by 14 days of age (Brown *et al*., 1998a). Culture of nasal and vaginal swabs of cows at calving was only positive for *M. bovis* in one instance each. This led the authors to conclude that the main method of spread of *M. bovis* from dam to calf was through contaminated waste milk. This hypothesis was supported by other investigators (Walz *et al*., 1997; Butler *et al*., 2000), although experimental infection by this route has not been published. Feeding of unpasteurized waste milk is clearly not the only important factor in the epidemiology of *M. bovis* in calves, as clinical disease can occur

in herds that only feed milk replacer or in herds that effectively pasteurize bulk tank or hospital milk prior to feeding (Lamm *et al*., 2004). The importance of colostrum as a source of *M. bovis* infection in calves is unknown, although in one study (Brown *et al*., 1998a), investigators did not isolate *M. bovis* from 50 colostrum samples collected during an outbreak of *M. bovis*-associated disease.

Whatever the mechanism (infected milk, colostrum, respiratory or vaginal secretions, or congenital infection) by which calves become infected, they may then shed *M. bovis* in respiratory secretions and potentially transmit it to other calves. Once established on multi-age sites, *M. bovis* becomes extremely difficult to eradicate, suggesting that continual transmission from older animals to incoming calves occurs (Bennett and Jasper, 1977c). Transmission is likely to be a result of direct or indirect contact of uninfected calves with calves that are shedding *M. bovis* in respiratory secretions (Bennett and Jasper, 1977c; Tschopp *et al*., 2001; Nicholas and Ayling, 2003).

In general, for bacterial pathogens involved in multifactorial diseases, the risk of infection and of developing clinical disease depends on a large number of pathogen, host and environmental factors. With the exception of exposure to *M. bovis*-contaminated milk (discussed above), few specific risk factors for the transmission of *M. bovis* or for outbreaks of clinical disease have been identified. Mixing of calves from different sources and the presence of at least one seropositive animal in new purchases increased the risk of *M. bovis*-associated disease on a ranch that raised dairy bull calves (Tschopp *et al*., 2001). This result is in agreement with epidemiological studies of *M. bovis* mastitis, where one of the few consistently identified herd-level risk factors has been a history of purchasing cattle (Gonzalez *et al*., 1992; Burnens *et al*., 1999). Herd size is the only other commonly identified risk factor for mycoplasmal mastitis.

Herd size was identified as a risk factor for an *M. bovis*-positive bulk tank in the NAHMS Dairy 2002 study, with 21.7% of herds of 500 head or more having positive samples, compared with 3.9% and 2.1% of medium (100 to 400 head) and small (< 100 head) herds, respectively. In several smaller-scale regional studies, large herds have been identified as being at increased risk of *M. bovis* mastitis (Thomas *et al*., 1981; Uhaa *et al*., 1990; Fox *et al*., 2003). However, some investigators have not identified herd size as a risk factor after analyses were adjusted for purchase of animals (Gonzalez *et al*., 1992). Larger herd size was associated with increased rates of undifferentiated respiratory disease in calves in the NDHEP study (Wells *et al*., 1997), but the effect of herd size on *M. bovis*-associated disease in young calves has not been reported.

Despite the lack of published studies, other potential risk factors for *M. bovis* infection in young calves can be identified from the limited studies of *M. bovis* epidemiology in calves, from studies of *M. bovis* mastitis and by extrapolating from what is known about risk factors for other respiratory pathogens in calves. For example, calves with clinical *M. bovis*-associated disease shed huge numbers of bacteria (Bennett and Jasper, 1977c) and are therefore likely to be the greatest contributors to the load of bacteria within a calf-rearing facility and the most important factor in calf-to-calf spread of disease. For undifferentiated respiratory disease, high bacterial counts in the air of calf pens are associated with increased disease prevalence (Lago *et al*., 2006). Large numbers of *M. bovis* can be isolated from the air in barns housing calves with *M. bovis*-associated disease (Jasper *et al*., 1974), and, therefore, factors that influence airborne bacteria counts in calf pens, such as pen design, barn ventilation and stocking density (Lago *et al*., 2006) may affect transmission rates. Independent of effects on bacterial load, poor air quality compromises respiratory defenses, which may increase the risk of respiratory disease (Ames, 1997), although this has not been specifically evaluated with respect to *M. bovis* infections.

Mechanical transmission via fomites has been implicated in udder-to-udder spread of *M. bovis* mastitis. Milking of uninfected and infected cows at the same time increases the risk for new cases, and milking equipment, teat dip, hands, sponges, washcloths, and poor hygiene during intramammary infusion of antibiotics have been implicated in the spread of *M. bovis* (Jasper *et al*., 1974; Bushnell, 1984; Step and Kirkpatrick, 2001b; Gonzalez and Wilson, 2003). It is plausible that similar mechanical means of transfer could occur in calf facilities. Despite being enveloped by only a thin plasma membrane, some mycoplasmas survive well in the environment. *Mycoplasma bovis* survives at 4°C for nearly 2 months in sponges and milk, over 2 weeks on wood and in water, and 20 days in straw, although at higher temperatures survival drops considerably (Pfutzner and Sachse, 1996). In general, survival is best under cool, humid conditions (Pfutzner and Sachse, 1996). In surveys of Florida dairy farms, *M. bovis* was commonly isolated from cooling ponds and from dirt lots with recently calved cows on farms that had a history of *M. bovis*-positive bulk tank milk culture (Bray *et al*., 1997; Bray *et al*., 2001). These studies demonstrate that *M. bovis* can survive well in the dairy environment, and that mechanical transmission via fomites could theoretically occur among calves. However, further studies are required to examine the role of fomites in the epidemiology of *M. bovis* infection in calf-rearing facilities.

In a study of the effect of temperature and humidity on nasal shedding of mycoplasmas in calves, an abrupt change from warm (17°C) to cold (5°C) conditions was associated with increased rates of nasal shedding of *M. bovis*. In addition, calves that were permanently housed at 5°C had higher rates of nasal shedding of *M. bovis* than calves housed at 16°C (Woldehiwet *et al*., 1990). Other investigators subjected healthy calves to extreme environmental temperatures (5°C or 35°C) for 4 hours; calves were housed at 18 to 20°C before and after the exposure.

Calves exposed to environmental extremes experienced significantly higher rates of respiratory disease over the following 3 weeks than did unexposed control calves. *Mycoplasma* spp. were identified as the cause of respiratory disease in calves that were exposed to 5°C, whereas no mycoplasmas were isolated from the lungs of calves exposed to 35°C or in control calves (Reinhold and Elmer, 2002). Together, these findings suggest that mycoplasmal nasal shedding and, perhaps, clinical disease are favored by low environmental temperatures. However, epidemiological studies to evaluate the association between temperature and clinical *M. bovis*-associated disease have not been published.

Season may have some effect on *M. bovis* infections in calves. Lamm *et al*., (2004) reported that there was a seasonal distribution in the number of cases of mycoplasmal otitis media in calves submitted for necropsy to a Californian diagnostic laboratory, with the highest proportion of cases submitted in the spring and the lowest in the summer months. Seasonal effects have been observed in some studies of mycoplasmal mastitis (Bayoumi *et al*., 1988; Gonzalez *et al*., 1992), but not in others (Kirk *et al*., 1997; Fox *et al*., 2003). For example, Gonzalez *et al*., (1992) reported a significantly higher incidence of mycoplasmal clinical mastitis in New York dairy herds in winter than in other seasons. Kirk *et al*., (1997) reported that there was no seasonal pattern to *M. bovis* isolation from bulk tank milk in 267 Californian herds, contrary to previous findings where a higher incidence of *M. bovis* mastitis was observed from January to April in California dairies than at other times of the year (Bayoumi *et al*., 1988). The reasons for these discrepancies are unknown. There are several possible explanations for increased rates of *M. bovis*-associated disease in winter or early spring compared with other times of year. Survival of mycoplasmas in the environment is best in cool, humid conditions (Pfutzner and Sachse, 1996) and the risk of indirect transmission between animals may be

greatest when these conditions predominate. Secondly, a seasonal distribution could reflect an association of *M. bovis* infection with exposure to cold environmental temperatures, as discussed above (Woldehiwet *et al*., 1990; Reinhold and Elmer, 2002). Lastly, air quality in enclosed cattle facilities may be worse in winter than at other times of the year, predisposing animals to increased rates of respiratory disease (Ames, 1997; Lago *et al*., 2006). Further epidemiological studies are required to definitively determine if there is a seasonal distribution of *M. bovis*-associated disease in calves.

The immune status of the calf is important in determining susceptibility to respiratory infections. The calf is born with little or no humoral immunity and is dependent upon absorption of maternal immunoglobulins from colostrum for disease protection during early life (Davis and Drackley, 1998). Numerous investigators have found a strong association between failure of passive transfer of maternal immunoglobulins and increased risk and severity of respiratory disease in young calves (Thomas and Swann, 1973; Williams *et al*., 1975; Davidson *et al*., 1981; Blom, 1982; Corbeil *et al*., 1984; Van Donkersgoed *et al*., 1993; Donovan *et al*., 1998a). However, whether maternal antibodies have any protective effects against *M. bovis* infection is not clear. In one study (Van Donkersgoed *et al*., 1993), there was no significant association between *M. bovis*-specific serum antibody titers in the first 2 weeks of life and occurrence of pneumonia in 325 colostrum-fed dairy calves. Likewise, Brown *et al*., (1998a) did not find an association between *M. bovis*-specific serum antibody concentrations at 7 days of age and occurrence of *M. bovis*-associated disease in 50 Holstein calves. Specific immunity to *M. bovis* will be further discussed later in this chapter.

Non-specific respiratory defenses are important in protection from mycoplasmal respiratory infections in other hosts (Cartner *et al*., 1998; Hickman-Davis, 2002), and it is logical

that they would also be important in *M. bovis* infections. The non-specific respiratory defenses of calves can be compromised by a variety of factors including infection with viral pathogens, sudden changes in environmental temperature, heat- or cold-stress, overcrowding, transportation, poor air quality and inadequate nutrition (Bryson, 1985; Ames, 1997). However, further studies are required to define the role of factors affecting the non-specific respiratory defenses of calves as well as the role of passive immunity in *M. bovis*-associated calf disease.

Genetic background is thought to play an important role in the susceptibility of cattle to infectious disease (Uribe *et al*., 1995; Kelm *et al*., 1997; Kelm *et al*., 2001; Abdel-Azim *et al*., 2005). Genetic background is also important in determining susceptibility or resistance to mycoplasmal respiratory infections of non-bovine species. In many cases, genetic susceptibility to mycoplasmal respiratory disease appears to be a result of increased immunoreactivity of the host when compared with resistant animals. For example, resistance to *M. pulmonis* lung disease in inbred strains of rats is a result of a more controlled host immune response after mycoplasmal inoculation into the lung, compared with susceptible strains of rats (Davis *et al*., 1982). Inbred strains of mice that are susceptible to *M. pulmonis* lung disease have reduced alveolar macrophage clearance of mycoplasmas from the lung early in the infection process compared with resistant strains of mice (Hickman-Davis *et al*., 1997). In addition, mice that are genetically susceptible to asthma-associated symptoms, a phenotype mediated by impaired interferon (IFN)-γ secretion and strong Th2 responses, have a much greater susceptibility to *M. pulmonis* infection than do immunocompetent mice (Bakshi *et al*., 2006). The resistance to *M. pulmonis* is multifactorial, and has been mapped to several chromosomal locations (Cartner *et al*., 1996). Interestingly, males are more susceptible than females, suggesting that hormonal regulation may also be important in disease susceptibility (Yancey *et al*., 2001). Genetic susceptibility to

mycoplasmal infections is not limited to rodents. In pigs that were bred for high or low cellular and humoral immune responses, high responders that were experimentally infected with *M. hyorhinis* had more severe arthritis than did pigs bred for low immune response (Wilkie and Mallard, 1999). These findings coupled with the fact that immune responsiveness in cattle has a strong genetic influence implies that genetic background is likely to be associated with susceptibility to *M. bovis*-associated disease in cattle. However, to date no studies have addressed the role of genetics in susceptibility of cattle to mycoplasmal infections.

Bovine respiratory disease frequently involves a number of viral and bacterial pathogens (Bryson, 1985; Ames, 1997), and *M. bovis*-associated respiratory disease is no exception (Howard *et al*., 1987a; Rodriguez *et al*., 1996; Virtala *et al*., 1996b; Mosier, 1997; Stipkovits *et al*., 2000; Poumarat *et al*., 2001; Vogel *et al*., 2001; Thomas *et al*., 2002a; Gagea *et al*., 2006). In fact, *M. bovis* infection may predispose the respiratory tract to invasion by other bacterial pathogens (Houghton and Gourlay, 1983; Virtala *et al*., 1996b; Poumarat *et al*., 2001; Gagea *et al*., 2006); similarly, other pathogens may enhance *M. bovis* infection. Viral infections can damage the respiratory mucosa, reduce ciliary activity, and impair secretory and cellular immune defenses in the respiratory tract (Ames, 1997; Kapil and Basaraba, 1997). Any or all of these changes could increase susceptibility to mycoplasmal infection. Studies in feedlot calves with chronic, antibiotic-resistant pneumonia suggest that there may be synergism between Bovine Viral Diarrhea virus (BVDV) and *M. bovis* (Shahriar *et al*., 2000). Experimental infection studies have confirmed that *M. bovis* plays a synergistic role with other pathogens (Gourlay and Houghton, 1985; Lopez *et al*., 1986; Thomas *et al*., 1986), especially *P. multocida* and *M. haemolytica*.

Mixed infections can also occur in otitis media, although their significance is unknown (Dechant and Donovan, 1995; Lamm *et al*., 2004). In several other host species, viral infections of the URT are important risk factors for increased incidence, severity and chronicity of bacterial otitis media; one mechanism by which viral infections can potentiate bacterial otitis media is by perturbing the ciliary clearance mechanisms of the eustachian tubes (Bakaletz *et al*., 1993; Eskola and Hovi, 1999; Tong *et al*., 2000). Specific viral etiologies have not been identified in the lungs of pre-weaned calves with *M. bovis*-associated otitis media (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004; Van Biervliet *et al*., 2004). However, attempts to isolate viruses from lesions in the tympanic bullae have been reported only once (Maeda *et al*., 2003), and no attempts to isolate viruses from the nasopharynx or eustachian tubes of affected calves have been reported. In cases of *M. bovis*-associated arthritis, mixed infections in affected joints are uncommon, although calves with arthritis often have concurrent respiratory disease from which multiple pathogens may be isolated (Butler *et al*., 2000; Haines *et al*., 2001; Lamm *et al*., 2004).

Risk factors that have been identified for otitis media in other species include viral or bacterial infection of the nasopharynx, eustachian tube dysfunction, age (with neonates being at greatest risk), host factors such as impaired immunological status, URT allergies, genetic predisposition, feeding method (breast vs. bottle in human infants) and environmental factors such as mixing of different age groups and exposure to respiratory irritants (Bluestone, 1996; Duffy *et al*., 1997). With the exception of an apparent age-related distribution, these factors have not been evaluated with respect to *M. bovis*-induced otitis media in calves.

In summary, young calves can be infected at a very early age by ingestion of milk from cows infected with *M. bovis* or, probably, by direct or indirect transmission from other calves shedding *M. bovis* in nasal secretions. However, other than the feeding of infected milk, few

specific risk factors have been identified, and factors associated with dissemination from the upper to the LRT and clinical disease expression are poorly understood. Clearly, well designed epidemiological studies would be helpful to establish risk factors and to provide guidance for dairy producers to reduce *M. bovis*-associated disease.

Molecular Epidemiology

Mycoplasma bovis is well equipped to generate genetically diverse populations, and has been observed to undergo DNA recombination and rearrangement events at high frequency (Lysnyansky *et al*., 1996; Poumarat *et al*., 1999; Nussbaum *et al*., 2002). The *M. bovis* genome contains a large number of insertion sequences which are also likely to lead to heterogeneous populations (Miles *et al*., 2005; Thomas *et al*., 2005b). There have been several molecular epidemiological studies of *M. bovis* utilizing a variety of DNA fingerprinting techniques including randomly-amplified polymorphic DNA analysis, amplified fragment length polymorphism analysis, restriction fragment length polymorphism analysis, pulsed-field gel electrophoresis (PFGE) analysis, and insertion-sequence profile analysis (Poumarat *et al*., 1994; Kusiluka *et al*., 2000a; Butler *et al*., 2001; McAuliffe *et al*., 2004; Biddle *et al*., 2005; Miles *et al*., 2005). Considerable genomic heterogeneity among field isolates of *M. bovis* has been reported, especially when isolates were collected from diverse geographical regions and over a period of several years (Poumarat *et al*., 1994; McAuliffe *et al*., 2004; Miles *et al*., 2005). Correlations between particular DNA fingerprint types and geographic location, year of isolation, and type or severity of pathology have not been identified (Poumarat *et al*., 1994; Kusiluka *et al*., 2000a; McAuliffe *et al*., 2004; Miles *et al*., 2005). This may reflect the frequent movement of cattle among herds in modern management systems, as well as the ability of *M. bovis* to create genetically diverse populations.

Comparison of PFGE patterns for isolates of *M. bovis* or *Mycoplasma californicum* obtained at necropsy from multiple body sites in seven cows with mycoplasmal mastitis was reported (Biddle *et al*., 2005). Within each cow, the same PFGE pattern was found in 100% of isolates from sites in the mammary system (milk, mammary parenchyma and supra-mammary lymph nodes). Forty-one percent of isolates obtained from the respiratory system and 90% of isolates obtained from other body systems had PFGE patterns identical to that of the mammary isolates. These findings indicate that the same strain of *M. bovis* often colonizes multiple body sites, but also that multiple strains may be present within an animal. Isolates of *M. bovis* from multiple sites of pathology within the same animal or from multiple animals in the same disease outbreak typically are closely related or identical by DNA typing methods, especially when the herd is closed (Gonzalez *et al*., 1993; Kusiluka *et al*., 2000a; Butler *et al*., 2001; McAuliffe *et al*., 2005). In contrast, endemically-infected open herds, including dairy calf ranches, harbor numerous genetically diverse strains of *M. bovis*; this has been attributed to introduction of animals from multiple sources over time (Butler *et al*., 2001).

Pathology

The macroscopic and microscopic lesions of the respiratory tract in experimental *M. bovis* infection vary considerably among studies, probably reflecting differences in the route of inoculation, the dose and strain of *M. bovis*, the age and health status of the host and the duration of infection. Macroscopic lesions have consisted of cranioventral lung consolidation, sometimes accompanied by multiple necrotic foci (Gourlay *et al*., 1976; Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996). Histologically, experimental lung infections with *M. bovis* are characterized by peribronchiolar lymphoid hyperplasia or cuffing, often accompanied by acute or subacute suppurative bronchiolitis, thickening of alveolar septa due to cellular infiltration, atelectasis, and, in some cases, foci of coagulative necrosis (Gourlay *et al*.,

1976; Martin *et al*., 1983; Bryson, 1985; Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996). In one study, immunohistochemical staining of *M. bovis* antigen was present at 14 days post-infection in bronchioles, peribronchiolar tissue and within inflammatory exudates in alveoli; necrosis was not observed (Rodriguez *et al*., 1996). Other investigators identified large amounts of *M. bovis* antigen at the edges of lesions of coagulative necrosis and in bronchiolar exudates (Thomas *et al*., 1986).

Lesions described for the lungs of calves with natural *M. bovis* infections are similar to those described for experimental disease, although often of much greater severity. Macroscopically, affected lung lobes are a deep red color and have varying degrees of consolidation, often accompanied in subacute to chronic cases by multifocal necrotizing lesions (Gourlay *et al*., 1989a; Adegboye *et al*., 1995a; Adegboye *et al*., 1996; Clark, 2002; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Lesions usually have a cranioventral distribution, but can involve whole lung lobes and the cranial portions of the caudal lobes. Necrotic lesions can vary from 1-2 mm to several centimeters in diameter and contain yellow caseous material. They are distinct from typical lung abscesses in that they are not usually surrounded by a well-defined fibrous capsule (Clark, 2002; Khodakaram-Tafti and Lopez, 2004). Diffuse fibrinous or chronic fibrosing pleuritis are sometimes observed, and interlobular septae may contain edema fluid or linear yellow necrotic lesions (Rodriguez *et al*., 1996; Bashiruddin *et al*., 2001; Step and Kirkpatrick, 2001a; Clark, 2002; Gagea *et al*., 2006). Occasionally, chronic cases of *M. bovis* pneumonia contain areas of lung sequestration, consisting of a central area of necrotic tissue surrounded by red-brown exudate, and enclosed in a fibrous capsule that separates the sequestra from surrounding lung (Gagea *et al*., 2006). Fibrinosuppurative tracheitis has been

reported in calves with mycoplasmal lung infections (Dungworth, 1993; Hewicker-Trautwein *et al*., 2002).

Histologically, lung lesions in naturally-occurring *M. bovis* infections are characterized by a subacute to chronic suppurative bronchopneumonia that is usually necrotizing (Adegboye *et al*., 1995a; Rodriguez *et al*., 1996; Clark, 2002; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Mixed infections are common and often complicate characterization of lesions (Adegboye *et al*., 1995a; Adegboye *et al*., 1996; Clark, 2002; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Bronchioles are filled with purulent exudate that contains abundant *M. bovis* antigen, accompanied by varying degrees of peribronchiolar lympho-histiocytic cuffing, thickening of alveolar septa due to cellular infiltration, and atelectasis.

Two distinct types of necrotic lesions have been reported in *M. bovis* pneumonia, the most common being multifocal pyogranulomatous inflammation with centers of caseous necrosis (Adegboye *et al*., 1995a; Rodriguez *et al*., 1996; Clark, 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). These well-delineated necrotic foci have centers of amorphous eosinophilic material in which degenerative neutrophils are sometimes visible, especially at the periphery, and are surrounded by a band of lymphocytes, plasma cells, macrophages and fibroblasts. In many cases, it appears that foci of caseous necrosis are centered on obliterated bronchioles. Edema fluid, fibrin and variable numbers of neutrophils and macrophages are often present in adjacent pulmonary parenchyma. The second, and less common, type of necrotic lesion described is fibrinopurulent bronchopneumonia accompanied by multifocal irregular areas of coagulative necrosis, surrounded by a dense zone of necrotic cells, especially neutrophils (Bashiruddin *et al*., 2001; Shahriar *et al*., 2002; Maeda *et al*., 2003; Khodakaram-Tafti and

Lopez, 2004). Edema, fibrin deposition, and vascular and lymphatic thromboses in the interlobular septa may accompany these types of lesion (Rodriguez *et al*., 1996; Khodakaram-Tafti and Lopez, 2004). Large amounts of *M. bovis* antigen have been demonstrated in both caseous and coagulative necrosis by immunohistochemical staining, especially at the periphery of lesions (Rodriguez *et al*., 1996; Clark, 2002; Maeda *et al*., 2003; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Whether the two distinct types of necrosis are a result of temporal events, co-infection with other pathogens, variation among strains of *M. bovis*, or variation in the host response is unknown. In one study, (Khodakaram-Tafti and Lopez, 2004), investigators hypothesized that foci of coagulative necrosis evolve over time into foci of caseous necrosis, but this has not been demonstrated experimentally nor are both types of lesions usually observed in the same lung. Further studies are required to better characterize *M. bovis*-associated lung lesions.

Experimental and natural *M. bovis*-associated respiratory disease is typically accompanied by hyperplasia of the lymphoid tissues in both the URT and LRT (Thomas *et al*., 1986; Gagea *et al*., 2006). Foci of caseous necrosis in bronchial and mediastinal lymph nodes of affected calves have been observed (Gagea *et al*., 2006).

Lesions in the joints and tendon sheaths of calves after experimental inoculation of *M. bovis* are characterized as necrotizing fibrinosuppurative arthritis or tenosynovitis (Chima *et al*., 1981; Ryan *et al*., 1983; Thomas *et al*., 1986). Similar lesions have been reported in naturally-occurring *M. bovis* arthritis (Adegboye *et al*., 1996; Clark, 2002; Hewicker-Trautwein *et al*., 2002; Gagea *et al*., 2006). Macroscopic lesions vary from minimal to severe, but chronically affected joints usually contain non-odorous, turbid, yellow, and fibrinous to caseous exudate accompanied by thickening of the joint capsule. Histologically, affected joints usually

have severe erosion of articular cartilage, hyperplasia and caseous necrosis of synoviae, and thrombosis of subsynovial vessels (Ryan *et al*., 1983; Gagea *et al*., 2006). Adjacent soft tissues, including ligaments and tendons are frequently involved (Adegboye *et al*., 1996; Clark, 2002; Gagea *et al*., 2006). Large amounts of *M. bovis* antigen in the periphery of necrotic lesions and within joint exudates have been demonstrated by immunohistochemical staining of the joints of cattle with natural and experimental *M. bovis* arthritis (Thomas *et al*., 1986; Adegboye *et al*., 1996; Clark, 2002; Gagea *et al*., 2006).

In calves with *M. bovis*-associated otitis media, affected tympanic bullae are filled with fibrinosuppurative to caseous exudate (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). Histologically, extensive fibrinosuppurative exudates fill the tympanic bullae and normal architecture may be obliterated (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). The tympanic mucosa may have areas of ulceration and/or squamous metaplasia and is markedly thickened due to infiltrates of macrophages, neutrophils, and plasma cells, and proliferation of fibrous tissue. There is usually extensive osteolysis and/or remodeling of adjacent bone (Walz *et al*., 1997; Lamm *et al*., 2004; Van Biervliet *et al*., 2004). Lesions are accompanied by fibrinosuppurative eustachitis (Lamm *et al*., 2004). Large quantities of *M. bovis* antigen have been observed within necrotic exudates and, particularly, at the margins of necrotic lesions within the tympanic bullae, similar to findings in *M. bovis* pneumonia (Maeda *et al*., 2003). In chronic cases, lesions frequently extend into the inner ear and include petrous temporal bone osteomyelitis (Maeda *et al*., 2003; Lamm *et al*., 2004). Meningitis as a consequence of otitis interna is usually localized to the regions adjacent to the affected petrous temporal bone and characterized as fibrinous to fibrinosuppurative and sometimes necrotizing (Lamm *et al*., 2004; Ayling *et al*., 2005). In addition, diffuse fibrinous meningitis has been described in neonatal

calves with *M. bovis* meningitis which likely originated from mycoplasmemia (Stipkovits *et al*., 1993).

Mycoplasma bovis-associated lesions have occasionally been identified in other body systems in both experimentally- and naturally-infected calves (Thomas *et al*., 1986; Adegboye *et al*., 1995a; Maeda *et al*., 2003; Ayling *et al*., 2005). Ayling *et al*., (2005) described a 10-month-old calf with a history of respiratory disease that had lesions of endocarditis and encephalitis from which *M. bovis* was the only pathogen isolated. In another report (Thomas *et al*., 1986), intratracheal inoculation of *M. bovis* resulted in arthritis in one calf, and mycoplasmas were isolated from the blood during the first week post-inoculation. At necropsy, investigators observed perivascular mononuclear cell infiltration in portal areas of the liver, and immunohistochemical staining revealed *M. bovis* in association with these lesions. Other investigators identified *M. bovis* antigen within foci of mononuclear cell infiltrates in the liver and kidneys of 2 calves with chronic *M. bovis* pneumonia (Adegboye *et al*., 1995a).

Diagnosis

The occurrence of *M. bovis* is generally underestimated for several reasons. Mycoplasma culture requires special equipment and expertise (Gourlay and Howard, 1983; Waites and Taylor-Robinson, 1999), and few laboratories routinely monitor for this organism. Cows with *M. bovis* mastitis can shed the bacteria intermittently, so repeated milk cultures may be required to determine true infection status (Jasper, 1981; Biddle *et al*., 2003). In respiratory disease, multiple pathogens are often present, and as other bacteria such as *M. haemolytica* and *P. multocida* are easier to culture, the presence of *M. bovis* may be missed (Ames, 1997; Gagea *et al*., 2006). Recent studies suggest that *M. bovis*-associated disease is under-diagnosed, perhaps because veterinarians and pathologists fail to recognize the infection during routine physical, gross and microscopic examination (Nicholas and Ayling, 2003; Gagea *et al*., 2006). In otitis

media and arthritis, where *M. bovis* is often the only pathogen present, it may also be missed unless culture for mycoplasmas is specifically requested. In addition, the physical location of the tympanic bullae makes sample collection difficult in otitis media cases. *Mycoplasma bovis* is sometimes associated with a variety of unusual clinical presentations in which its involvement is not widely recognized, and so appropriate diagnostic tests to detect this pathogen may not be requested.

A history of respiratory disease that is poorly responsive to antibiotic therapy is suggestive of *M. bovis* involvement, especially when accompanied by cases of arthritis and/or otitis media. Although the associated lung pathology can be variable, multiple nodular lesions of caseous necrosis are strongly suggestive of *M. bovis* infections (Adegboye *et al*., 1995a; Gagea *et al*., 2006). However, as there are no pathognomonic clinical or pathological signs for *M. bovis*-associated disease, a definitive diagnosis is based on isolation of *M. bovis* from the affected site, and/or by demonstrating its presence in affected tissues by polymerase chain reaction (PCR), capture enzyme-linked immunosorbent assay (ELISA) or by immunohistochemistry (IHC).

The culture of bovine mycoplasmas requires the use of nutritionally complex media as well as a moist carbon-dioxide enriched atmosphere (Jasper, 1981; Gourlay and Howard, 1983; Nicholas and Baker, 1998; Waites and Taylor-Robinson, 1999). Growth of *M. bovis* in appropriate media is often apparent after 48 hr, but may take up to 10 days (Jasper, 1981; Gourlay and Howard, 1983; Nicholas and Ayling, 2003). Mycoplasmal colonies on solid media are identified by their characteristic morphology; growth in broth is indicated by turbidity, film formation, and by subculture onto solid media (Gourlay and Howard, 1983). A number of pathogenic and non-pathogenic bovine mycoplasmal species may be isolated from the URT or from sites of pathology, either alone or in mixed infections (Nicholas and Ayling, 2003; Lamm

et al., 2004; Gagea *et al*., 2006). Many of these cannot be differentiated morphologically, so speciation by immunological methods (direct or indirect immunofluorescence or immunoperoxidase testing) or by PCR is necessary (Jasper, 1981; Kotani and McGarrity, 1986; Poumarat *et al*., 1991; Rosenbusch, 2001).

In live calves with clinical signs of respiratory disease, mycoplasmal culture of transtracheal wash or broncho-alveolar lavage (BAL) fluids are suitable for the diagnosis of *M. bovis* infections (Allen *et al*., 1991; Virtala *et al*., 2000; Thomas *et al*., 2002b). Comparisons of paired culture results for nasopharyngeal swabs and BAL samples in cattle with respiratory disease indicate that, in individual animals, isolation of *M. bovis* from the URT is not well correlated with its presence in the LRT or with clinical disease (Allen *et al*., 1991; Thomas *et al*., 2002b). For example, in one study nasal swabs had a sensitivity of only 21% for predicting *M. bovis*-associated lung disease (Thomas *et al*., 2002b). Nasopharyngeal swabs can be used at the group level to indicate the presence of *M. bovis* within a calf facility (Bennett and Jasper, 1977c), although the sensitivity of this test has not been determined. In calves with arthritis or tenosynovitis, affected joints and tendon sheaths can be aspirated for culture (Byrne *et al*., 2001). Due to difficulties with access to the site of infection, samples are not usually collected from the tympanic bulla in live calves with otitis media.

Mycoplasma culture of necropsy specimens can be performed directly from homogenates of fresh tissues, aspirates, swabs collected from lesion sites and lavage samples (Gourlay and Howard, 1983; Rosenbusch, 2001; Thomas *et al*., 2002b). As for other infectious diseases, calves that are selected for necropsy for the diagnosis of a herd problem should be representative of the cases seen in that herd. Culture of BAL samples collected at necropsy may be preferable to culture of lung tissue when tissues cannot be processed immediately; mycoplasmas remain viable

in BAL fluids for months at -20°C or -70°C, for a few days at 4°C and for several hours at room temperature, whereas isolation rates from lung tissue decrease markedly over a few hours after collection due to release of mycoplasmal inhibitors from disrupted tissue (Gourlay, 1983; Taylor-Robinson and Chen, 1983; Nicholas and Baker, 1998). Complete agreement between mycoplasmal cultures of paired BAL fluids collected at necropsy and corresponding lung tissue cultured immediately after collection from cattle euthanized for respiratory disease has been reported (Thomas *et al*., 2002b).

Sample handling and transport are particularly important to ensure the survival of *M. bovis.* Swabs should be collected into transport media such as Ames (without charcoal) or Stuart's (Clyde and McCormack, 1983). Swabs, lavage fluids, aspirates, milk and colostrum samples should be refrigerated and tissue samples should be collected as soon as possible after death and placed in sealed plastic bags on ice (Clyde and McCormack, 1983). Samples should be transported to the laboratory within 24 hr (Clyde and McCormack, 1983; Biddle *et al*., 2004). If samples such as milk are stored frozen, they should still be submitted within 7 to 10 days of collection, as longer storage significantly decreases the isolation of *M. bovis* (Biddle *et al*., 2004). Detection of mycoplasmas in clinical samples can potentially be improved by using enrichment techniques and large inoculum sizes (Biddle *et al*., 2003). Limitations of mycoplasma culture include the requirement for specialized equipment and expertise, the need to speciate any mycoplasmas that are isolated, the length of time before results are obtained, the overgrowth of slower growing species by other more rapidly growing mycoplasmas or other bacteria and fungi, the need to process samples rapidly after collection to maximize sensitivity, and the occurrence of false negative cultures due to the presence of antibiotics or other inhibitors in clinical samples (Tully, 1983).

In part to address frustrations with conventional culture techniques, a variety of PCR systems have been developed for the diagnosis of *M. bovis* infections. A few are designed to permit differentiation of multiple bovine mycoplasmal species within a single assay (Ayling *et al*., 1997; Bashiruddin *et al*., 2005) whilst most are designed to be specific for *M. bovis* (Hotzel *et al*., 1996; Ghadersohi *et al*., 1997; Subramaniam *et al*., 1998; Pinnow *et al*., 2001; Bashiruddin *et al*., 2005). Three PCR systems have been most widely adopted for clinical diagnostics, including (1) amplification of the 16S*rRNA* gene with species or class-specific primers followed by digestion with various restriction enzymes to permit differentiation of several species of mollicutes within a single assay (Ayling *et al*., 1997; Bashiruddin *et al*., 2005), (2) amplification of the 16S*rRNA* gene with species-specific primers (Chavez Gonzalez *et al*., 1995; Bashiruddin *et al*., 2005), and (3) amplification of the housekeeping gene *uvr*C with species-specific primers (Subramaniam *et al*., 1998; Thomas *et al*., 2004; Bashiruddin *et al*., 2005). PCR can be used for the speciation of mycoplasmas that have already been isolated by routine culture methods (Ayling *et al*., 1997; Subramaniam *et al*., 1998; Thomas *et al*., 2004), as well as for the direct detection of *M. bovis* in clinical samples (Chavez Gonzalez *et al*., 1995; Hotzel *et al*., 1996; Hotzel *et al*., 1999; Pinnow *et al*., 2001). However, PCR performed directly from clinical samples can have variable sensitivity, and some authors report that samples containing $< 10^2$ colony forming units/ml were often detected as negative by PCR (Hotzel *et al*., 1999), a detection level that is no better than standard culture procedures. Sensitivity has been improved by antigen capture prior to PCR using an *M. bovis*-specific monoclonal antibody (Hotzel *et al*., 1999). A nested PCR was slightly more sensitive than assays based on culture of fresh milk samples, but was much more sensitive than culture (100% compared with 27%) for detection of *M. bovis* in milk after 2 years of frozen storage (Pinnow *et al*., 2001).

Because of the very close genotypic relationship between *M. bovis* and *Mycoplasma agalactiae* there has been considerable work invested in developing assays that accurately differentiate these two species (Mattsson *et al*., 1991; Chavez Gonzalez *et al*., 1995; Tola *et al*., 1996; Subramaniam *et al*., 1998; Thomas *et al*., 2004; Bashiruddin *et al*., 2005; Foddai *et al*., 2005). In a recent study (Bashiruddin *et al*., 2005) five laboratories evaluated the specificity of four PCR detection systems for differentiating *M. bovis* and *M. agalactiae.* PCR based on detection of the housekeeping genes *opp*D/F or *uvr*C had better specificities (both at 100%) than did detection of the 16S*rRNA* gene combined with restriction enzyme analysis (96%) or detection of species-specific sequences in the 16S*rRNA* gene (95.8%). However, because *M. agalactiae* is a pathogen of small ruminants that is presumed to be absent from North America and is rarely isolated outside of its typical hosts, differentiation from *M. bovis* is less of a concern on this continent than in regions where both pathogens exist.

A sandwich ELISA has been developed to capture *M. bovis* antigen from culture medium or clinical samples, and is commercially available in Europe (Bio-X Diagnostics, Belgium) (Ball and Finlay, 1998). The ELISA has a similar sensitivity to conventional culture when performed directly from clinical samples, but sensitivity is improved when samples are incubated in broth culture medium for a brief period prior to antigen capture.

Immunohistochemical demonstration of *M. bovis* antigen within tissues is a sensitive and specific means of determining the involvement of *M. bovis* in observed pathology (Haines and Chelack, 1991; Adegboye *et al*., 1995b; Rodriguez *et al*., 1996; Haines *et al*., 2001; Clark, 2002; Shahriar *et al*., 2002; Maeda *et al*., 2003; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Advantages of IHC are that it performs well using formalin fixed, paraffin embedded tissues, and can be performed retrospectively, especially when other findings suggest a *M. bovis*

infection but culture is negative. An additional advantage of IHC is that it reveals the location of *M. bovis* within lesions. In one recent retrospective study (Gagea *et al*., 2006), 98% and 100% of cases of caseonecrotic bronchopneumonia from feedlot calves submitted to a diagnostic laboratory were positive for *M. bovis* by culture and IHC, respectively. In cases of fibrinosuppurative pneumonia where *M. haemolytica* was isolated, *M. bovis* was also isolated in 82% of cases, and was demonstrated by IHC within lesions in mixed infections with *M. haemolytica* by IHC in 85% of cases (Gagea *et al*., 2006). The involvement of *M. bovis* in lesions at a variety of other body sites has also been verified by IHC (Thomas *et al*., 1986; Kinde *et al*., 1993; Stipkovits *et al*., 1993; Adegboye *et al*., 1996; Clark, 2002; Maeda *et al*., 2003; Gagea *et al*., 2006). An indirect fluorescent antibody test using polyclonal antisera has been described for the detection of *M. bovis* in fresh, frozen lung tissue (Knudtson *et al*., 1986).

A variety of methods for the detection of *M. bovis*-specific antibodies in serum and other body fluids have been described (Boothby *et al*., 1983a; Rosendal and Martin, 1986; Brank *et al*., 1999; Ghadersohi *et al*., 2005). An indirect hemagglutination test (IHA) has been successfully used to demonstrate the presence of *M. bovis*-specific antibody in serum, colostral whey and joint fluid (Cho *et al*., 1976; Boothby *et al*., 1983a; Rosendal and Martin, 1986; Gagea *et al*., 2006). However, the most widely applied method to detect *M. bovis*-specific antibodies is an indirect ELISA (Le Grand *et al*., 2002; Nicholas and Ayling, 2003). Most studies have used whole cell or membrane protein antigens derived from various reference or field strains of *M. bovis*. Laboratory-grown strains of *M. bovis* vary over time in their variable surface protein (Vsp) expression profiles, and it has been proposed that this may effect the reliability of immunological assays (Rosengarten and Yogev, 1996), although studies addressing whether this issue is of practical concern in diagnostic ELISA have not been published. Le Grand *et al*.,

(2002) developed an indirect ELISA using membrane proteins derived from a phenotypic clonal variant of the *M. bovis* type strain PG45 with a high-level of expression of Vsp A; the assay performed well in experimentally- and naturally-infected cattle populations, although whether the antigen was superior to traditional antigens was not determined. A variety of ELISA tests for serological detection of *M. bovis* antibodies are now commercially available in North America and Europe; for example, Biovet in Canada, Bio-X Diagnostics in Belgium, and Bommelli in Switzerland all manufacture ELISA kits that detect *M. bovis* antibodies.

Mycoplasma bovis-specific serum immunoglobulin (Ig) is detectable as early as 6 days (IgM) to 10 days (IgG) after experimental inoculation of *M. bovis* into the respiratory tract (Brank *et al*., 1999; Le Grand *et al*., 2002). Specific serum immunoglobulin concentrations remain elevated for months to years after *M. bovis* infection, so a high titer does not necessarily indicate very recent exposure (Le Grand *et al*., 2001; Nicholas and Ayling, 2003). Maternal antibody can also result in high antibody levels in young calves, although with a half-life of 12 to 16 days this typically wanes by a few months of age (Tschopp *et al*., 2001). Virtala *et al*., (2000) reported that of 75 pneumonic dairy calves less than 3 months of age in which *M. bovis* was isolated from tracheal wash samples, only 57% had a 4-fold or greater increase in *M. bovis* serum antibody titers by IHA. The authors concluded that paired serum samples were not a good predictor of *M. bovis*-associated respiratory disease, possibly due to the presence of maternal antibody titers. Other investigators also failed to find a correlation between serum antibody titers to *M. bovis* and *M. bovis*-associated respiratory disease in naturally infected individual animals (Rosendal and Martin, 1986; Martin *et al*., 1989). However, on a group level, seroconversion has been predictive of *M. bovis*-associated respiratory disease (Martin *et al*., 1990; Tschopp *et al*., 2001). Therefore, serology is of limited diagnostic value in individual animals and is really most

useful in epidemiological surveillance (Rosendal and Martin, 1986; Le Grand *et al*., 2002). Serology has also been effective as a biosecurity tool to screen new purchases prior to introduction into a herd, but this would only be applicable to animals more than a few months of age, after maternal antibodies have waned (Byrne *et al*., 2000; Nicholas and Ayling, 2003).

Treatment

The fact that *Mycoplasma* species lack a cell wall has important implications for treatment, as it means the beta-lactam antibiotics are ineffective (Taylor-Robinson and Bebear, 1997). *Mycoplasma* species are also naturally resistant to sulfonamides. Currently, only one product, containing the triamilide antibiotic tulathromycin (Draxxin®; Pfizer, Inc.) is approved for treatment of *M. bovis*-associated disease in dairy calves in the U.S. Other antimicrobials that have a theoretical basis for efficacy against *M. bovis*, and that are approved in the U.S. for treatment of respiratory disease in dairy heifers less than 20 months of age, include florfenicol, oxytetracycline, spectinomycin, tilmicosin, and tylosin. Recent evidence suggests that antimicrobial resistance to antibiotics traditionally used for treatment of mycoplasma infections is increasing in field isolates of *M. bovis* in North America (Francoz *et al*., 2005; Rosenbusch *et al*., 2005) and Europe (Ayling *et al*., 2000; Thomas *et al*., 2003a); isolates from both continents show widespread resistance to tetracyclines and tilmicosin, and European isolates show increasing resistance to spectinomycin. Although *in vitro* antibiotic susceptibility profiles of *M. bovis* may be useful in making broad generalizations about antibiotic resistance, data have not been published on the relevance of these profiles to clinical efficacy on an individual or a herd level. The antibiotic susceptibility profiles of paired *M. bovis* isolates obtained from nasal swabs and BAL samples in calves with respiratory disease were found to differ considerably within

animals, suggesting that if susceptibility profiles are used, they need to be based on isolates obtained from the site of infection (Thomas *et al*., 2002b).

In spite of the limited choice of potentially effective antibiotics available, antibiotics are widely used to treat *M. bovis*-associated disease. However, treatment is frequently unrewarding, with affected calves requiring a long duration of treatment or failing to respond (Stalheim and Stone, 1975; Romvary *et al*., 1979; Allen *et al*., 1992a; Stipkovits *et al*., 1993; Adegboye *et al*., 1996; Pfutzner and Sachse, 1996; Walz *et al*., 1997; Apley and Fajt, 1998; Poumarat *et al*., 2001; Stokka *et al*., 2001; Francoz *et al*., 2004; Van Biervliet *et al*., 2004). Calves with chronic and/or multisystemic disease are reported to have an especially poor response to treatment (Stalheim and Stone, 1975; Stipkovits *et al*., 1993; Adegboye *et al*., 1996; Apley and Fajt, 1998; Stokka *et al*., 2001). There are few controlled clinical trials evaluating the efficacy of various antibiotics available for treatment of *M. bovis*-associated disease, and the few efficacy studies published must be interpreted with caution, as most use experimentally infected calves and treatment is often started early in the disease course (Gourlay *et al*., 1989b; Poumarat *et al*., 2001; Godinho *et al*., 2005). In an industry-sponsored study, tulathromycin was an effective treatment for respiratory disease in dairy calves that had been experimentally infected with *M. bovis*, when treatment was initiated at 3 or 7 days after inoculation (Godinho *et al*., 2005). Likewise, tilmicosin administered 6 hrs prior to inoculation or at the onset of clinical disease was effective in reducing lung colonization by *M. bovis* in calves that had been experimentally infected with *M. haemolytica* plus *M. bovis* (Gourlay *et al*., 1989b). However, treatment with spectinomycin did not alter the clinical course of disease in calves with *M. bovis* plus *P. multocida* pneumonia when treatment was started 6 days after inoculation, although the numbers of *M. bovis* in the lung were reduced in treated calves (Poumarat *et al*., 2001).

Scant information is available regarding treatment of *M. bovis*-associated disease in field situations, and most studies have come from Europe. Marbofloxacin, a fluoroquinolone antibiotic, was an effective treatment for naturally-occurring *M. bovis*-associated respiratory disease (Thomas *et al*., 1998), but this antibiotic cannot be used in cattle in the U.S. Available therapies that have resulted in clinical improvement in calves with *M. bovis*-associated respiratory disease in field trials include oxytetracycline, tilmicosin, or a combination of lincomycin and spectinomycin (Picavet *et al*., 1991; Musser *et al*., 1996). However, given the recent evidence that resistance against these drugs is increasing, these antibiotics may no longer be appropriate choices. Without other data to guide choice of an antibiotic, selection of a specific treatment regimen from the list of potentially effective antibiotics based on past performance in the affected herd is frequently recommended (Apley and Fajt, 1998; Step and Kirkpatrick, 2001a).

In addition to antibiotics, short term use of anti-inflammatory drugs can be beneficial in the treatment of bovine respiratory disease (Bednarek *et al*., 2003). Although these therapeutic agents have not been specifically evaluated for the treatment of *M. bovis*-associated disease, there is a logical basis for their use, as the inflammatory response may contribute significantly to the pathology of *M. bovis* infections (Howard *et al*., 1987c; Rosenbusch, 2001). Non-specific supportive therapy including oral or intravenous fluids and nutritional support may be indicated in specific animals (Van Biervliet *et al*., 2004).

Irrigation of the middle ear after the tympanic membrane has ruptured has been recommended for treatment of otitis media in calves (Morin, 2004), although studies of the efficacy of this procedure were not identified in a literature search. Puncture of the tympanic membrane (myringotomy) followed by insertion of tympanostomy tubes is commonly used in

the treatment of children with chronic or recurrent otitis media (Lous *et al*., 2005; Poetker *et al*., 2006), and some veterinarians have promoted blind myringotomy using a sharp object such as a knitting needle in the treatment of otitis media in calves (Schnepper, 2002). To the best of the author's knowledge, studies on the risks and efficacy of this procedure in clinical cases have not been published. The potential benefit of myringotomy is the relief of pain and pressure caused by the build-up of exudate in the middle ear, as well as access to the middle ear for irrigation (Rosenfeld *et al*., 2004). Whether the procedure might provide relief for calves that have the thick, caseous exudate characteristic of chronic *M. bovis* otitis media is not clear. In a recent study using calf cadavers, investigators reported that blind insertion of a 3.5 mm diameter straight knitting needle approximately 3 cm into the ear canal to perforate the ear drum was anatomically feasible (Villarroel *et al*., 2006). Studies are clearly needed to evaluate the effect of myringotomy on drainage from the middle ear and the health and recovery of the calf.

Another more aggressive surgical treatment of otitis media/interna was described in one case report (Van Biervliet *et al*., 2004). A bilateral tympanic bulla osteotomy was performed on a 4-week-old calf with severe, chronic *M. bovis*-associated otitis media-interna that had failed to respond to antibiotic treatment. Post-surgically, the tympanic bullae were lavaged daily with warm saline for 3 days, and antibiotics were continued for 16 days. Surgery coincided with a dramatic improvement in clinical signs and the calf was reported to be clinically normal at 1 year of age. Because of the cost and complexity of this procedure, as well as the requirement for general anesthesia, its application is probably limited to refractory cases of otitis media in high-value calves without concurrent respiratory disease.

To summarize, antibiotic treatment of *M. bovis*-associated disease is often unrewarding, especially in calves with chronic or multisystemic infections. Improved efficacies are reported in

experimental infection studies when treatment is initiated early in the disease course, suggesting that early intervention or, perhaps, metaphylactic therapy in high risk calves (discussed below) may be more rewarding. Extended duration of antimicrobial therapy is frequently recommended. Further studies are needed to determine field efficacy of particular antibiotic regiments for treatment of clinical disease in U.S. dairy calves and to evaluate the safety and efficacy of myringotomy and irrigation of the middle ear in calves with otitis media.

Control and Prevention

Results of epidemiological studies of mycoplasmal mastitis suggest that the best way to prevent *M. bovis* infections is to maintain a closed herd or to screen and quarantine purchased animals (Gonzalez *et al*., 1992; Burnens *et al*., 1999; Step and Kirkpatrick, 2001b; AABP, 2005). Results of such studies also suggest that *M. bovis*-associated mastitis can be effectively eliminated from dairy herds through aggressive surveillance and culling of cows with *M. bovis* mastitis (Brown *et al*., 1990; Fox *et al*., 2003). In feedlot cattle, where these types of biosecurity measures are not practical, recommendations for the control and prevention of *M. bovis*-associated respiratory disease and arthritis focus on limiting stress, vaccinating to reduce the incidence of other respiratory pathogens and segregating affected groups of calves from new arrivals to reduce exposure of high-risk animals to *M. bovis* (Step and Kirkpatrick, 2001a; Stokka *et al*., 2001). Dairies that are expanding and calf ranches that rear animals from multiple sources obviously cannot maintain closed herds, and calf ranches are not usually able to screen new calves prior to introduction into the facility. However, calf ranches do have the ability to be selective in purchasing calves, and animals could be screened on arrival to determine if a particular supplier is consistently providing *M. bovis*-infected calves. Prevention of *M. bovis*-associated disease is hampered in dairy calf operations by the extremely limited understanding of its epidemiology and risk factors.

Current recommendations for prevention of *M. bovis*-associated calf disease are based on reducing exposure to *M. bovis.* Potential sources of exposure that could be controlled include unpasteurized bulk tank or waste milk, colostrum, and indirect or direct contact with respiratory aerosols from infected calves. Exposure to *M. bovis* in milk could be limited by culling infected cows or avoiding feeding milk from cows that are infected, by on-farm pasteurization of milk prior to feeding, or by feeding milk replacer (Pfutzner and Meeser, 1986; Walz *et al*., 1997; Butler *et al*., 2000; Stabel *et al*., 2004). On-farm batch pasteurization of discard milk to 65°C for 1 hr or 70°C for 3 min (Butler *et al*., 2000) or the use of a high-temperature short-time pasteurizer (Stabel *et al*., 2004) will inactivate *Mycoplasma* species. Frequent monitoring by culture of pasteurized milk samples to ensure that pasteurization has been effective is important in any on-farm pasteurization program (Godden *et al*., 2005). Pasteurization of colostrum is also possible; authors of some recent studies reported that on-farm batch pasteurization at 60°C for 30 min eliminated viable *M. bovis* while immunoglobulin concentration and colostral consistency were not adversely affected (Godden *et al*., 2006; McMartin *et al*., 2006). Pasteurization methods that use higher temperatures have resulted in reduced colostral quality and unacceptable feeding characteristics (Godden *et al*., 2003; Stabel *et al*., 2004; Godden *et al*., 2006). If colostrum is not pasteurized, it has been recommended that it should not be pooled to minimize potential exposure of calves to *M. bovis* (Rosenbusch, 2001).

Large numbers of *M. bovis* can be shed in respiratory secretions of calves with clinical *M. bovis*-associated disease (Bennett and Jasper, 1977c; Pfutzner and Sachse, 1996). It has therefore been recommended to segregate affected and healthy calves, although this is frequently impractical (Step and Kirkpatrick, 2001a). Other recommendations that have been made include taking appropriate precautions to prevent potential transfer of *M. bovis* between calves by

personnel or equipment (Nicholas and Ayling, 2003). Nipples, bottles, tube feeders and buckets should be adequately sanitized, and pens disinfected between calves. As discussed earlier, *M. bovis* survives surprisingly well in the environment, but it is highly susceptible to heat and to most commonly used chlorine-, chlorhexidine- acid- or iodine-based disinfectants (Pfutzner *et al*., 1983b; Boddie *et al*., 2002). 'All in, all out practices' have been recommended to prevent older animals from infecting younger ones, but are often impractical in dairy calf facilities (Nicholas and Ayling, 2003).

Management practices that help control other respiratory diseases by maximizing the ability of the calf's respiratory system to resist and control infection have been recommended for *M. bovis*, although none of these has been specifically evaluated with respect to this pathogen (Ames, 1997; Rosenbusch, 2001; Step and Kirkpatrick, 2001a). Such measures include providing proper nutrition, adequate ventilation at the pen level and reducing environmental stressors such as overcrowding and heat- and cold-stress. Because viral respiratory pathogens, especially BVDV, may predispose to *M. bovis* infection (Ames, 1997; Shahriar *et al*., 2000), herd vaccination protocols for infectious bovine rhinotracheitis virus (IBR), parainfluenza type 3 virus (PI_3), BVDV and bovine respiratory syncytial virus (BRSV), as well as the herd BVDV monitoring program, should be evaluated to ensure that they are appropriate. Although the role of passive transfer of *M. bovis*-specific antibodies in protection of calves from *M. bovis*-associated disease is unclear, a sound colostrum feeding program can reduce the risk of infection with other respiratory pathogens (Ames, 1997), and may therefore decrease the risk of secondary *M. bovis* infections.

There are no *M. bovis* vaccines approved for use in the U.S. in young dairy calves, although at least two are approved for prevention of respiratory disease in older cattle, and one

for prevention of mastitis. Autogenous vaccines are also used by some producers in an attempt to prevent *M. bovis*-associated disease in calves (Thomson and White, 2006). Although some vaccines have appeared promising (Chima *et al*., 1980; Chima *et al*., 1981; Howard *et al*., 1987a; Nicholas *et al*., 2002), others have failed to protect from or have worsened clinical disease (Rosenbusch, 1998; Bryson *et al*., 1999). Vaccination will be further discussed in the sections on relevant experiences with mycoplasmal vaccines for diseases other than *M. bovis*, and vaccination against *M. bovis*, below.

The prophylactic or metaphylactic use of antibiotics is generally undesirable but its use may be justified when high levels of morbidity and mortality are being sustained. Strategic antibiotic treatment of calves that are deemed to be at high risk for respiratory disease upon arrival at feedlots has clearly been demonstrated to reduce the incidence and severity of respiratory disease (Galyean *et al*., 1995; Schunicht *et al*., 2002; Thomson and White, 2006). In addition, feeding metaphylactic levels of antibiotics in milk replacer to dairy calves on calf ranches reduces disease incidence and delays the onset of clinical disease during the pre-weaning period (Berge *et al*., 2005). For *M. bovis*-associated disease, the response to treatment when antibiotics are given prior to, or early in the course of experimentally induced disease, is often better than the response rates reported in field cases, suggesting that metaphylactic treatment might be more successful than treatment after disease is clinically apparent. In one European study, investigators found that valnemulin added to the milk from 4 days of age for 3 weeks was effective in limiting *M. bovis*-associated disease in calves (Stipkovits *et al*., 2001). Animals in the treated group had fewer clinical signs and reduced clinical scores, although disease was not eliminated and calves still required a considerable number of individual treatments. Nagatomo *et al*., (1996) treated calves that were at high risk of *M. bovis*-associated disease with

chloramphenicol. Untreated calves had high mortality rates (up to 41%), while the onset of clinical disease was delayed in treated calves and all treated calves survived. Prophylaxis or metaphylaxis with antibiotics that are approved for use in U.S. cattle have not been evaluated with respect to *M. bovis*-associated disease in young dairy calves.

Microbial Pathogenesis

Antigenic Variation

Mycoplasmal lipoproteins are involved in many diverse functions including modulation of essential cellular pathways, acquisition of nutrients, immune modulation and cytadhesion (Citti and Rosengarten, 1997; Chambaud *et al*., 1999). Surface lipoprotein variation in mycoplasmas is thought to be a means of adapting to varying environmental conditions, including the host immune response, and may be important in determining the chronic nature of many mycoplasmal infections (Citti and Rosengarten, 1997; Chambaud *et al*., 1999). Many of these immunodominant mycoplasmal antigens undergo phase and/or size variation (Jan *et al*., 1995; Razin *et al*., 1998). There is some evidence that lipoprotein variation in mycoplasmas is involved in protection from the immune response. Antigenic variation is observed *in vivo* (Levisohn *et al*., 1995; Rasberry and Rosenbusch, 1995), and particular variants of *M. bovis* can be selected *in vit*ro by the addition of antibodies to culture medium (Jensen *et al*., 1995; Le Grand *et al*., 1996). The accessibility of antibodies to a mycoplasma colony depends on the size of Vsps (Levisohn *et al*., 1995) and elongated surface lipoproteins protect mycoplasma cells from growth inhibiting antibodies (Citti *et al*., 1997), possibly by limiting epitope accessibility. The variable surface antigens (Vsa) of *M. pulmonis* are hypothesized to form a "molecular shield" that prevents lysis by complement (Simmons and Dybvig, 2003; Simmons *et al*., 2004).

Consistent with other mycoplasmal infections, cell-surface lipoproteins are the preferential target of the humoral immune response in *M. bovis* infections (Behrens *et al*., 1996;

Brank *et al*., 1999). A large family of immunodominant Vsp lipoproteins has been characterized in *M. bovis* (Behrens *et al*., 1994; Lysnyansky *et al*., 1996; Beier *et al*., 1998; Brank *et al*., 1999; Sachse *et al*., 2000; Nussbaum *et al*., 2002). Structurally, Vsp molecules contain extensive regions of tandemly reiterated sequences that can comprise over 80% of the entire protein (Behrens *et al*., 1994). The members of the Vsp family undergo independent high frequency phase and size variation to generate diversity in the Vsp repertoire (Behrens *et al*., 1994; Lysnyansky *et al*., 1996; Lysnyansky *et al*., 1999). Phenotypic switching in Vsp antigens is associated with high frequency chromosomal rearrangement in the *vsp* genomic locus, which consists of a large cluster of related but divergent single-copy *vsp* genes (Lysnyansky *et al*., 1999). Because of the processes producing antigenic variants, a given population of *M. bovis* cells always comprises variants differing in their lipoprotein repertoire (Rosengarten and Yogev, 1996). The expression of particular Vsp antigens has not been associated with geographical location, year of isolation, clinical manifestation, mode of infection, or pathology (Rosengarten *et al*., 1994; Brank *et al*., 1999; McAuliffe *et al*., 2004). However, compared to the type strain of *M. bovis*, field strains have been shown to possess modified versions of the *vsp* gene complex in which there is extensive variation in the reiterated coding sequences of the *vsp* structural genes, indicating a vast capacity for antigenic variation within *M. bovis* populations (Nussbaum *et al*., 2002).

In addition to the Vsp family, *M. bovis* may possess other methods to increase its capacity for antigenic variation. For example, *M. bovis* has recently been found to contain genes with homology to the abundantly expressed MALP-404 surface lipoprotein of *Mycoplasma fermentans* (Lysnyansky *et al*., 2006). Posttranslational processing of MALP-404, involving specific cleavage of part of the molecule into the extracellular environment, results in dramatic

changes in the surface phenotype of *M. fermentans* (Davis and Wise, 2002). Whether these types of events also occur in *M. bovis* is currently unknown, but may be feasible given the genetic data that has been reported (Lysnyansky *et al*., 2006).

Adhesion

Adherence is an important feature of mycoplasma pathogenicity. Adherence is thought to be the initial step in the disease-causing process of pathogenic mycoplasmas (Baseman and Tully, 1997; Rottem and Naot, 1998; Rosengarten *et al*., 2000). Once attached, the mycoplasma effectively colonizes the host respiratory surface, can induce physiological changes such as ciliostasis, and establishes a persistent and chronic infection (Rottem and Naot, 1998; Rosengarten *et al*., 2000). The microbe then elicits a host immune response, and it is the character and intensity of the host response that is critical in lesion severity (Jones and Simecka, 2003).

Unlike some pathogenic mycoplasmas (Krause, 1998; Rosengarten *et al*., 2000), *M. bovis* lacks a defined attachment tip. Although little is known about the ligands involved in *M. bovis* cytadherence, neuraminidase-sensitive sialyl moieties are important for adherence of many mycoplasmas, including *M. bovis* (Sachse *et al*., 1996). *Mycoplasma bovis* adheres *in vitro* to neutrophils, embryonic bovine lung cells, and primary cultures of bovine bronchial epithelial cells in a specific manner (Thomas *et al*., 2003b; Thomas *et al*., 2003c). Immunohistochemical and electron microscopic studies have demonstrated *in vivo* adherence of *M. bovis* to respiratory and other mucosal surfaces, including the joint and mammary gland (Stanarius *et al*., 1981; Thomas *et al*., 1987; Adegboye *et al*., 1995b; Adegboye *et al*., 1996). Surface molecules of *M. bovis* that have been identified as important in adhesion include the protein P26, as well as members of the Vsp family, particularly Vsp C, Vsp F, and an as-yet uncharacterized Vsp (Sachse *et al*., 2000; Thomas *et al*., 2005a). Recently, *M. bovis* has been shown to form a biofilm

in vitro (see discussion below), and surprisingly, the inability to form biofilms was linked to expression of Vsp F (McAuliffe *et al*., 2006). Completion of *M. bovis* genome sequencing projects are anticipated in the near future and likely will provide additional information on putative adhesins for this pathogen.

Biofilms

Although flocculent growth of mycoplasmas in liquid medium as well as development of microcolonies have been observed (Pollock and Bonner, 1969; Miyata *et al*., 2000), little attention has been given to the development of biofilms by these microorganisms. However, the ability of mycoplasmas to colonize mucosal surfaces, the development of persistent, chronic infections even in the face of a robust host immune response, and the refractory nature of many mycoplasmal infections to antibiotic therapy are characteristics that have been associated with biofilm formation (Donlan, 2000; Donlan, 2002; Donlan and Costerton, 2002). Biofilm formation is well established as a mechanism by which bacteria, alone or in concert with other microbes, form sessile microbial communities that facilitate persistence within the host and development of chronic infections (Costerton *et al*., 1999; Donlan, 2000; Donlan, 2002; Donlan and Costerton, 2002; Morris and Hagr, 2005). A key feature of biofilms that contributes to persistence is the increased resistance to antibiotic therapy, often rendering microbes within the biofilm refractory to standard treatment regimens (Donlan, 2000). Additionally, microbes in biofilms are protected from components of the host immune response (Costerton *et al*., 1999; Donlan and Costerton, 2002).

Biofilm formation has now been documented for several ruminant mycoplasmas (McAuliffe *et al*., 2006) as well as the rodent pathogen *M. pulmonis* (Simmons *et al*., 2007; Simmons and Dybvig, 2007). Strains of *M. agalactiae, M. bovis, Mycoplasma cotewii, Mycoplasma putrefaciens,* and *Mycoplasma yeatsii* all produced substantive biofilms, with other

mycoplasmal species isolated from ruminants producing limited biofilms. *In vitro* analysis demonstrated that *M. bovis* in the biofilm were resistant to desiccation and heat stress, but did not alter the minimum inhibitory concentrations for standard antibiotics (McAuliffe *et al*., 2006). Somewhat surprisingly, the most virulent bovine mycoplasma *M. mycoides* subsp. *mycoides* biotype SC, did not produce a biofilm; in fact none of the 24 SC strains tested could produce a biofilm (McAuliffe *et al*., 2006).

Biofilm formation was influenced by the strain of *M. bovis*, and the inability to form biofilms was linked to expression of Vsp F (McAuliffe *et al*., 2006). In fact, Vsp F was not expressed by any strain that was capable of forming prolific biofilms. Conversely, expression of Vsp B and O was more common in strains that produced strong biofilms. A similar association of Vsa expression and biofilm formation has been reported for *M. pulmonis* (Simmons *et al*., 2007; Simmons and Dybvig, 2007). In *M. pulmonis*, the size of the Vsa as determined by the tandem repeat length rather than the specific Vsa type was the critical determinant. Expression of short Vsa proteins was associated with strong surface attachment and production of a substantial biofilm, whereas expression of longer Vsa proteins resulted in free-floating microcolonies that failed to attach to surfaces (Simmons *et al*., 2007; Simmons and Dybvig 2007). The attached *M. pulmonis* were more resistant to complement-mediated lysis but were sensitive to gramicidin. The relationship between Vsa size and susceptibility to complement killing was shown previously (Simmons and Dybvig, 2003; Simmons *et al*., 2004), but more recent studies suggest that resistance to complement killing is localized to the tower structures of the biofilm (Simmons and Dybvig 2007), which contained the most complex and dense association of *M. pulmonis.*

Although biofilms are regulated by environmental factors and quorum sensing in both Gram negative and Gram-positive bacteria (Sauer, 2003; Stanley and Lazazzera, 2004),

mycoplasmas appear to lack the classic two-component regulatory systems. It has been suggested that in *M. pulmonis,* slipped strand mispairings that generate variation in tandem Vsa repeats provide a stochastic mechanism for control of biofilm formation (Simmons *et al*., 2007) and thus represent a simplistic model to study biofilm development in the absence of known regulatory elements. It is important to note that to date these studies have been done with *in vitro* systems, and definitive proof of the role of biofilms in the pathogenesis of mycoplasmal infections will require studies in animal models.

Recently, biofilm formation has been given greater consideration as a potential mechanism by which microbial pathogens establish chronic, nonresponsive infections of the ear in humans (Post, 2001; Roland, 2002; Fergie *et al*., 2004; Post *et al*., 2004; Morris and Hagr, 2005; Vlastarakos *et al*., 2007). Bacterial biofilms have been detected in biopsy material from the ears of children with a history of chronic otitis (Hall-Stoodley *et al*., 2006). Biofilms were confirmed in 90% of these patients. The formation of biofilms by *Haemophilus influenzae* during experimental infection of chinchillas was confirmed by both scanning electron microscopy and confocal microscopy (Ehrlich *et al*., 2002). All animals with effusions had evidence of biofilms. Development of *Pseudomonas aeruginosa* biofilms in the middle ear of experimentally infected cynomolgus monkeys has also been observed (Dohar *et al*., 2005). Therefore, it is reasonable to suggest that the ability of *M. bovis* to form biofilms may be directly relevant to otitis media in calves.

Other Microbial Factors That Might Contribute to *M. bovis* Virulence

There are several other biological properties of mycoplasmas that have been implicated as virulence determinants. Mycoplasmas compete with host cells for nutrients and biosynthetic precursors, and can therefore disrupt host cell maintenance and function (Baseman and Tully, 1997). After cytadherence, many mycoplasmal species generate enzymes such as

phospholipases, as well as other products such as hydrogen peroxide and superoxide radicals, which may damage host cells (Baseman and Tully, 1997; Minion, 2002). However, much of the host cell damage and resulting clinical manifestations in mycoplasmal infections are due to the host immune reaction and inflammatory responses rather than direct toxic effects of mycoplasmal products (Rosengarten *et al*., 2000; Jones and Simecka, 2003).

Several toxins have been identified in mycoplasmas, including the neurotoxin associated with *Mycoplasma neurolyticum* (Tully, 1981) and the most recent description of community-acquired respiratory disease syndrome (CARDS) toxin in *Mycoplasma pneumoniae* (Kannan and Baseman, 2006). There is one report of a 73 kD polysaccharide toxin in *M. bovis* (Geary *et al*., 1981). The polysaccharide component was present in association with a membrane glycoprotein and when injected intradermally into guinea pigs increased vascular permeability, activated complement, and resulted in a massive recruitment of eosinophils into the dermis. Infusion of large amounts of the polysaccharide into the udder induced clinical mastitis and lesions consistent with mycoplasmal mastitis. However, this polysaccharide has not been further characterized. Phenotypic classification of *M. bovis* isolates based on the presence or absence of *in vitro* cytotoxic activity has been reported, but cytotoxic strains have not been fully characterized and the relevance of this phenotype with respect to virulence potential is unknown (Rosenbusch, 1996a; Rosenbusch, 1996b).

Bovine Immunology: Relevant Background Information

Lymphocyte Subpopulations in Cattle

There are three major bovine lymphocyte subpopulations: B cells, T cells expressing the αβ T cell receptor, and T cells expressing the γδ T cell receptor. As in other species, lymphocytes within the αβ T cell population coexpress either CD4 (T helper [Th] cells) and are

MHC class-II restricted, or express CD8 (cytotoxic/suppressor T cells) and are MHC class-I restricted. Some cells within the γδ T cell population also coexpress CD8 (MacHugh *et al*., 1997). T cells have important effector functions and are pivotal in the regulation of the nature and intensity of an immune response. Th cells produce cytokines in response to recognition of an antigen-MHC complex on antigen presenting cells (APC). By secreting particular cytokines, Th cells play a vital role in activation of B cells, other T cells, macrophages and various other cells that participate in the immune response (Sordillo *et al*., 1997). $CD8^+$ T cells are uniquely equipped to recognize and kill bacterial or viral infected cells, as well as tumor cells, parasites and some free bacteria. They are also very important modulators of immune and inflammatory responses through the production of cytokines.

In laboratory rodents, Th cells can be divided into distinct Th1 and Th2 subpopulations; Th1 cells secrete cytokines such as IFN-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-β that are associated with inflammatory responses, whereas Th2 cells secrete cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 that mediate humoral responses (Jones and Simecka, 2003). IFN-γ and IL-4 are the classical cytokines used to indicate Th1 and Th2 immune responses, respectively. In rodents, the T cell response is often polarized to a Th1 or Th2 response, with large increases in one cytokine and the T cells that produce it, and a corresponding low production of the opposing cytokine (Jones and Simecka, 2003). In cattle, however, this clear division of immune response phenotypes is less evident, and strongly polarized cytokine profiles are rarely observed (Brown *et al*., 1998c). However, highly skewed immunoglobulin isotype expression patterns occur in many cattle diseases, especially in chronic infections, with IgG_1 responses being driven by IL-4 and IgG_2 responses driven by IFN-γ (Brown *et al*., 1998c). These types of responses have been applied in cattle to indicate Th1 versus Th2 polarization, with IgG_1

indicating a Th2 response, and IgG_2 indicating a Th1 response. In addition, ratios of IL-4 and IFN-γ have been used to determine Th1 versus Th2 responsiveness in cattle (Brown *et al*., 1998d; Vanden Bush and Rosenbusch, 2003; Miao *et al*., 2004). Thus, although Th1/Th2 responses may not be well defined in cattle, some polarization of the immune response does occur and can be defined by IgG_1/IgG_2 or IL-4/IFN-γ ratios.

Ruminants have a relatively higher percentage of γδ T cells compared with other species (Hein and Mackay, 1991). The percentage of the circulating mononuclear cell population that expresses the γδ T cell receptor is approximately 10-15% in adult cattle, and up to 40% in neonatal calves (Wilson *et al*., 1996; Kampen *et al*., 2006). Bovine γδ T cells can be divided into subpopulations that differ in terms of tissue distribution and function (Wyatt *et al*., 1994; Wyatt *et al*., 1996; Wilson *et al*., 1998). The largest subpopulation within the circulation expresses the surface molecule WC1 (Workshop Cluster 1). $WC1^+$ γδ T cells are $CD3^+$ but do not express CD2, CD4, or CD8 (MacHugh *et al*., 1997). Between 65 and 90% of circulating γδ T cells are $WC1^+$, and this percentage is not affected by age (Blumerman *et al*., 2006; Kampen *et al*., 2006). $WC1^+$ T cells are also found in the white pulp of the spleen, outer cortex of peripheral lymph nodes, mucosal associated lymphoid tissue (MALT), epithelial layers of the gut and respiratory tract, skin, and sites of inflammation (Clevers *et al*., 1990; Wilson *et al*., 1999). In contrast, $WC1^-$ γδ T cells, which do express CD2 and CD8, represent a small percentage of the circulating γδ T cell population (MacHugh *et al*., 1997). $WC1^-$ $CD8^+$ γδ T cells comprise a large percentage of the γδ T cells in some tissues including the red pulp of the spleen and in the healthy mammary gland, uterus and other mucosal epithelial sites (e.g. lamina propria of the gut) (MacHugh *et al*., 1997; Hedges *et al*., 2003).

The role that γδ T cells play in immune responses is poorly understood, but they appear to have broad effector and regulatory functions and are involved in many aspects of the bovine immune response to pathogens (Pollock and Welsh, 2002). γδ T cells can recognize non-protein antigens such as bacterial carbohydrates as well as classical protein antigens, and may therefore have unique roles in immune responses to unconventional antigens (Pollock and Welsh, 2002). Gene expression and microarray data suggest that the $WC1^+$ T cells are primarily an inflammatory cell population with some subsets expressing IFN-γ, whereas $WC1^-$ γδ T cells have regulatory functions and promote quiescence (Hedges *et al*., 2003; Rogers *et al*., 2005). Within the $WC1^+$ T cell population, distinct subsets of γδ T cells exist that may perform different functions (Rogers *et al*., 2006; Price *et al*., 2007). $WC1^+$ T cell subsets in peripheral sites contribute to early production of IFN-γ during infection with intracellular and extracellular pathogens, and are thought to be important in linking the innate and adaptive immune responses (Price *et al*., 2007). In addition, subsets of circulating $WC1^+$ T cells express surface molecules that allow them to home efficiently to sites of inflammation, whereas $WC1^-$ γδ T cells do not express these molecules and are not recruited to sites of inflammation (Wilson *et al*., 2002). In very young calves, γδ T cells appear to have a predominantly dampening effect on antibody responses and on antigen-specific and mitogen-stimulated responses of other T cells (Howard *et al*., 1989). Calves that have been depleted of $WC1^+$ γδ T cells have reduced non-specific production of IFN-γ, greater mucosal and systemic antibody responses to antigens, and increased tendency to Th2-biased responses (Taylor *et al*., 1995; Kennedy *et al*., 2002; Rogers *et al*., 2005). Thus, bovine γδ T cells are likely to be important in early immune responses to a broad range of antigens, and distinct γδ T cell subsets are likely to have unique functions in these immune responses.

Lymphocyte subpopulations in the lungs or BAL fluid from normal cattle have been described (Mathy *et al*., 1997; McBride *et al*., 1997). Mathy *et al*., (1997) examined lymphocyte subpopulations in lungs of adult cattle. T cells predominated over B cells in BAL and lung parenchyma cell populations, and $CD8^+$ T cells predominated over $CD4^+$ T cells. γδ T cells made up approximately 9% of the lymphocyte populations. Most $CD4^+$ and $CD8^+$ T cells expressed high amounts of the activation marker CD44, as did B cells from BAL fluid. The authors did not report on the $WC1^+$ subpopulation, but other investigators found that $WC1^+$ γδ T cells are present within bronchial lymph nodes of normal adult cattle (Cassidy *et al*., 2001; Sopp and Howard, 2001). In addition, in the lungs of healthy 8-month-old cattle, small numbers of $WC1^+$ γδ T cells were resident in bronchial submucosa and interalveolar septae (Cassidy *et al*., 2001). Further, 1-year-old calves experimentally inoculated with *M. haemolytica* had a substantial increase in the percentage of γδ T cells in BAL fluid by 7 days post-infection (McBride *et al*., 1999). Although their function within the lung is unknown, the presence of γδ T cells is consistent with a role in the early response to lung infection.

Although limited information is available, lymphocyte subpopulations in the peripheral lymphoid tissues of the respiratory tract in cattle have been described. Lymphocyte subpopulations in palatine and pharyngeal tonsils of healthy adult cattle have been reported (Rebelatto *et al*., 2000). Populations at both tonsil sites were similar, and consisted of approximately 3% γδ T cells, 2% $WC1^+$ γδ T cells, 15% $CD4^+$ T cells and 7% $CD8^+$ T cells, with the remainder being B and other mononuclear cells. In 10-day-old calves, γδ T cells comprised approximately 8% of the mononuclear cell population in bronchial lymph nodes and 15% in lungs, although there was large calf-to-calf variation (McInnes *et al*., 1999). In adult cattle, $WC1^+$ γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells comprised 5%, 30% and 11% of bronchial

lymph node mononuclear cells, respectively (Sopp and Howard, 2001). These and other studies have shown that γδ T cells are resident in the lymphoid tissues of the URT and LRT and therefore could be expected to play a role in early immune responses at these sites.

Anatomical Barriers and Innate Defenses of the Bovine Respiratory Tract

In the URT, defenses against infection are mainly in the form of physical and mechanical barriers to invading microbes, as well as in the specific antibacterial substances secreted onto the mucosal surface (Dungworth, 1993; Ellis, 2001). The structural design of the respiratory tract means that almost all inhaled particles are trapped in the nasal turbinates, trachea and bronchi, with subsequent removal by mucociliary clearance before the trapped particles reach the alveoli. The mucosa of the URT and airways is also coated with mucus that contains non-specific and specific antimicrobial factors such as lysozyme, surfactants, and pathogen-specific immunoglobulins. The importance of the mucociliary apparatus in lung defense in cattle is illustrated by the fact that damage to the structural integrity of the mucociliary system by viral agents is strongly associated with increased risk of secondary bacterial pneumonia (Ames, 1997; Kapil and Basaraba, 1997).

Alveolar macrophages, neutrophils, natural killer (NK) cells, and mast cells are involved in innate immune defenses of the LRT. In addition, epithelial cells contribute to innate defenses through the secretion of pro-inflammatory cytokines and chemokines (Kruger and Baier, 1997; Ackermann and Brogden, 2000; Yang *et al*., 2002). Alveolar macrophages are probably the most important cell in initial response to infectious agents (Ellis, 2001). Alveolar macrophages can be activated through a variety of pathways by contact with pathogens or their products; opsonized particles are generally more effective activators of alveolar macrophages than are unopsonized particles (Howard and Taylor, 1983). Activated alveolar macrophages secrete substances such as

IL-8 that are chemotactic for neutrophils and other macrophages as well as pro-inflammatory acute-phase cytokines such as IL-1, IL-6 and TNF-α (Caswell *et al*., 1998). In addition, activated alveolar macrophages also display increased phagocytic capacity and bactericidal ability. As in other body systems, acute phase cytokines activate endothelial cells of blood vessels, initiating a cascade of events that results in leakage of serum factors including complement into the lung. Acute phase cytokines also potentiate expression of adhesion molecules to allow trafficking of leukocytes into affected lung (Caswell *et al*., 1998; Ackermann and Brogden, 2000). Neutrophils attracted to the site participate in phagocytosis and killing of pathogens, and are also involved in exacerbation of inflammatory responses in several bovine respiratory diseases (Ackermann and Brogden, 2000; Ellis, 2001).

Major functions of NK cells are to kill tumor- or virus-infected cells, but they may also play a role in the initial response to infection in the bovine lung. In other species, NK cells responding to early lung infection activate macrophages by secretion of pro-inflammatory cytokines including IFN-γ and TNF-α; NK cells also secrete a variety of chemotactic factors (Curtis, 2005). Although NK cells are associated with protective responses against IBR infection in cattle (Ellis, 2001), little work has been done to define the role of NK cells in responses of the bovine respiratory tract to this or other pathogens.

Mast cells are located in the submucosa of the URT and LRT. Large numbers of mast cells are present along the respiratory tract of adult cattle, but numbers of mast cells in neonatal calves are more limited (Chen *et al*., 1990; Ackermann and Brogden, 2000; Ramirez-Romero *et al*., 2000). In other species, mast cells contribute to non-specific immune responses to bacterial pathogens through a variety of pathogen- and inflammatory-associated stimuli, in addition to their well-recognized role in IgE-mediated allergic reactions (Brandtzaeg *et al*., 1996; Boyce,

2003). Although the role of mast cells in bovine respiratory disease has not been well studied, mast cell degranulation at the site of infection has been shown to contribute to the acute inflammatory response to M. *haemolytica* inoculation in young calves (Ackermann and Brogden, 2000). The role of mast cells in bovine respiratory disease is also receiving increasing attention as being important in the immunopathogenesis of BRSV and *H. somni* infections (Jolly *et al*., 2004; Gershwin *et al*., 2005).

Adaptive Immune Responses of the Bovine Respiratory Tract

The URT, including the pharyngeal and palatine tonsils, contains organized MALT. Interaction of cells of the immune system with potential pathogens of the respiratory system occurs at the MALT sites. Primary interactions occur between activated APCs, especially dendritic cells, and lymphocytes in tonsils and in draining lymph nodes. For respiratory pathogens, the importance of the URT as a site of immune induction is emphasized by studies where inoculation of an antigen into the URT results in specific antibody in both nasal and BAL fluids, but inoculation directly into the LRT only results in specific antibody in the lung. This can be important for disease protection; intranasal immunization of cattle against *M. haemolytica* resulted in protective immune responses to aerosol challenge, whereas intratracheal immunization did not (Jericho *et al*., 1990). In adult animals, IgA is the predominant antibody isotype secreted in the URT, although in calves other isotypes including IgG may be equally or more important; this is discussed under neonates, below.

The contribution of immune responses to protection from, or exacerbation of, clinical disease caused by a number of viral and bacterial bovine respiratory pathogens has been widely studied. Both cell-mediated and antibody responses to viral respiratory pathogens are associated with reduced clinical disease (Ellis, 2001; Endsley *et al*., 2002; Woolums *et al*., 2003; Ellis *et al*., 2007). However, cellular responses may also contribute to pathology in infected cattle (Ellis,

2001; Gershwin *et al*., 2005). For some pathogens, local cell-mediated immunity, mediated by $CD8^+$ T cells, is thought to be the critical protective immunological mechanism (Ellis, 2001). Protection against the bacterial pathogens *M. haemolytica*, *P. multocida,* and *H. somni* is associated with high concentrations of antibody (maternal or endogenous) to various virulence determinants, particularly outer membrane proteins involved in iron acquisition, as well as the leukotoxin of *M. haemolytica* (Mosier, 1997; Potter *et al*., 1999; Ackermann and Brogden, 2000). Thus effective protection is pathogen-dependent and may involve one or more arms of the immune system.

Although specific immune responses confer protective immunity to many pathogens, some pathogen-specific immune responses are receiving increasing attention for their roles in exacerbation of respiratory disease in cattle. For example, lung lesions due to BRSV infections are thought to have a significant immunopathological component resulting from the stimulation of a strong Th2-biased immune response, production of IL-4, and substantial amounts of IgE (Gershwin *et al*., 2000). In lung infection with *M. haemolytica*, immune complex deposition within alveolar walls and the subsequent inflammatory response is thought to contribute to pathology of this disease (McBride *et al*., 1999; Ackermann and Brogden, 2000). Similarly, host immune responses are a major contributor to mycoplasmal disease in cattle and will be discussed in more detail below.

Immunology of the Neonatal Calf

Although calves are born with a competent immune system, they are immunonaive and many aspects of the immune system are developmentally immature (Barrington and Parish, 2001). This functional immaturity of the immune system is considered a major factor in determining the increased susceptibility to bacterial and viral infections observed during the first few months of life (Barrington and Parish, 2001). Colonization of the URT of dairy calves with

M. bovis often occurs within the first few weeks of life, with the peak incidence of clinical disease at around a month of age. During this period, the immune system of the young calf is undergoing rapid changes associated with maturation (Barrington and Parish, 2001; Nonnecke *et al*., 2003; Foote *et al*., 2005a). Therefore, age-specific features of the immune system are likely to be important in determining the susceptibility or resistance of the young dairy calf to *M. bovis*-associated disease. Vaccine strategies that target young calves may need to be tailored specifically to this age group.

Influence of Colostrum

Because the bovine syndesmochorial placenta does not permit passive transfer of maternal antibody *in utero*, calves are born essentially agammaglobulinemic and rely on maternal antibodies absorbed from colostrum for disease protection in the first few months of life (Davis and Drackley, 1998). More than 80% of the immunoglobulin in bovine colostrum is IgG_1; the remainder is mostly IgG_2 and IgA (Davis and Drackley, 1998). Fresh colostrum also contains large numbers of viable leukocytes as well as factors involved in non-specific immune defenses (Park *et al*., 1992; Barrington and Parish, 2001). Selected populations of functional T cells are transferred into colostrum and readily cross the neonatal intestinal barrier and become distributed systemically (Liebler-Tenorio *et al*., 2002; Reber *et al*., 2006). In the neonatal calf, maternal antibodies, lymphocytes and other factors modulate immune responses, especially B cell responses (Barrington and Parish, 2001; Endsley *et al*., 2003; Reber *et al*., 2005; Prgomet *et al*., 2007). The functions of maternal T cells in the neonatal calf have been partially defined *in vitro*; maternal $CD4^+$ T cells are thought to stimulate immune responses in newborns by secretion of cytokines while $CD8^+$ T cells are thought to have mainly an immunosuppressive or dampening effect on the neonatal immune response (Riedel-Caspari and Schmidt, 1991a; Riedel-Caspari and Schmidt, 1991b; Barrington and Parish, 2001). How long maternal T cells survive in the calf and

their long term effects have not been determined. The contribution of maternal lymphocytes to immune responses in the respiratory tract of newborn calves is unknown, although maternal lymphocytes do play a role in neonatal resistance to some enteric pathogens (Archambault *et al*., 1988; Riedel-Caspari, 1993). B cells are also transferred into colostrum, although their primary role is believed to be synthesis of dimeric IgA within mammary secretions (Barrington and Parish, 2001).

The half-life of colostral antibody in calves is 11.5 to 16 days (Sasaki *et al*., 1976; Davis and Drackley, 1998), and the majority of passively acquired antibody is cleared by transfer across the mucosal epithelia, where it is functional and helps prevent infections (Besser *et al*., 1988a; Besser *et al*., 1988b). Transfer of maternal IgG from serum to nasal secretions has been demonstrated in young lambs (Wells *et al*., 1975). In calves, most of the work in this area has involved study of receptor-mediated transcytosis of IgG into the intestinal lumen (Besser *et al*., 1988a; Besser *et al*., 1988b), and there are limited data defining the mechanisms by which maternal immunoglobulin is involved in protection of the respiratory tract. However, there is a strong association between failure of passive transfer of maternal antibody and increased risk and severity of respiratory disease in young calves, leaving little doubt that maternal immunoglobulin does play an important role in protecting the LRT from disease in the neonatal calf (Thomas and Swann, 1973; Williams *et al*., 1975; Davidson *et al*., 1981; Blom, 1982; Corbeil *et al*., 1984; Van Donkersgoed *et al*., 1993; Donovan *et al*., 1998a).

Aside from the obvious benefits in protecting the calf from infectious disease, colostral antibody also has potent immunomodulatory effects and can prevent the development of an active humoral immune response to certain antigens (Riedel-Caspari and Schmidt, 1991b; Ellis *et al*., 1996; Barrington and Parish, 2001; Ellis *et al*., 2001). This has particular relevance to the

development of effective vaccines for use in neonatal calves. However, at least with some antigens, an anamnestic response in the face of maternal antibody can occur after second exposure even without a measurable humoral response after the first exposure (Menanteau-Horta *et al*., 1985; Ellis *et al*., 1996). Age-matched colostrum-deprived calves as well as neonatal calves euthanized immediately after birth have increased numbers of IgG_1- and IgG_2-secreting cells in lymph nodes as compared with colostrum-fed calves (Aldridge *et al*., 1998); thus feeding of colostrum actually depletes the numbers of IgG_1- and IgG_2-secreting cells in lymph nodes. This effect does not require the presence of viable maternal leukocytes and is thought to be mediated by antibody or other soluble factors in colostrum. Isotype-specific depletion of antibody-secreting cells represents one mechanism by which colostrum down-regulates humoral capacity in newborn calves. Colostrum also modulates cell-mediated immune responses in calves, and peripheral blood lymphocytes in colostrum-fed calves have lower blastogenic responses to T cell mitogens than do colostrum-deprived calves (Clover and Zarkower, 1980).

Innate Immune Responses in Neonatal Calves

Despite the fact that the innate immune system is of primary importance in protection from disease during the first few months of life, there are limited data on the functional capacity of innate defenses in neonatal calves. The total number of neutrophils in peripheral blood is higher in newborn calves than in adult cattle, and gradually decreases over the first 2 months of life (Kampen *et al*., 2006; Mohri *et al*., 2007). Results of *in vitro* studies of the functional maturity of neutrophils in newborn calves are conflicting (Hauser *et al*., 1986; Menge *et al*., 1998; Kampen *et al*., 2006). In a recent study, Kampen *et al*., (2006) reported that *in vitro* phagocytosis, respiratory burst, and bactericidal activity was intact and functional in neutrophils from 1-week-old calves. Another major cell of the innate immune system, NK cells, comprise a greater percentage of the total lymphocyte population in calves (< 6 months of age) than in adult

cattle (Kulberg *et al*., 2004), but NK cell functions specific to young calves have not been defined.

Alveolar macrophages are a major cell type contributing to innate defenses of the LRT, including defenses against mycoplasmal infections (Cartner *et al*., 1998). The proportion of alveolar macrophages in BAL fluid is similar for calves at 1 week of age and for adults (Pringle *et al*., 1988; Yeo *et al*., 1993), but *in vitro* phagocytic capacity was reported to be markedly reduced in calves less than 3 weeks of age (Yeo *et al*., 1993). Alveolar macrophages of young calves also have impaired secretion of neutrophil chemotactic factors compared with adult cattle (Lu *et al*., 1996). This implies that the alveolar macrophages in calves are functionally immature even though their numbers are equivalent to those found in immunocompetent adults. Reduced phagocytosis, decreased secretion of cytokines and chemotactic factors, and/or lowered bactericidal activity in neonates compared with adults has been reported for alveolar macrophages in other species including humans, rhesus monkeys, horses, pigs, rats, and sheep (Weiss *et al*., 1986; Liu *et al*., 1987; D'Ambola *et al*., 1988; Kurland *et al*., 1988; Grigg *et al*., 1999; du Manoir *et al*., 2002; Goldman *et al*., 2004).

Adaptive Immune Responses in Neonatal Calves

The initial site of immune system interactions with respiratory pathogens in the URT is MALT. Palatine and pharyngeal tonsils are not fully developed in the neonatal calf and do not attain a mature MALT structure until approximately 2 months of age (Schuh and Oliphant, 1992; Manesse *et al*., 1998). In 3 week-old calves, T and B cell dependent areas in the tonsils are not well-differentiated, with few germinal centers and few WC1+ γδ T cells as compared with tonsils of 2-month-old calves. Numbers of T and B cells are much less than in mature tonsils. Maturation of MALT in the URT is thought to be triggered by exposure to antigens over the first

few weeks of life (Manesse *et al*., 1998). The impact of the apparent immaturity of the neonatal calf tonsils has not been studied, but lower numbers of B and T cells at these sites could be expected to limit the number of antigens in the URT that the calf is able to respond to early in life.

The circulating lymphocyte population in young calves differs significantly from that of adult cattle. Calves have higher absolute numbers of lymphocytes in peripheral blood than do adult cattle (Kulberg *et al*., 2004), but calves have a much lower proportion of circulating B cells (Senogles *et al*., 1978; Nonnecke *et al*., 1999; Kampen *et al*., 2006) and a much higher proportion of γδ T cells (Wilson *et al*., 1996; Nonnecke *et al*., 1999; Kampen *et al*., 2006). Composition of the circulating lymphocyte population changes gradually over the first few months of life, and by 3 to 4 months the relative proportions of various lymphocyte populations are similar to those of adult cattle (Nonnecke *et al*., 1999; Nonnecke *et al*., 2005; Kampen *et al*., 2006). The relative proportion of peripheral blood mononuclear cells (PBMC) reported to be γδ T cells is 35-40% in the first week of life, decreasing to approximately 25% at one month of age (Wilson *et al*., 1996). The decrease in the relative proportion of γδT cells that occurs over the first few months of life is due to an increase in the absolute numbers of other lymphocyte subsets, mainly $CD4^+$ T cells and B cells, rather than a decrease in the absolute γδT cell numbers (Kampen *et al*., 2006). The absolute number and proportion of $CD4^+$ T cells in healthy calves increases in the first few weeks of life, whereas there is little difference between calves and adults in the number or relative proportion of $CD8^+$ T cells (Kampen *et al*., 2006; Foote *et al*., 2007).

Perhaps the most obvious difference between the lymphocyte populations of calves and adults is that young calves have markedly lower numbers and relative proportions of circulating

B cells than do adults (Senogles *et al*., 1978; Nagahata *et al*., 1991; Nonnecke *et al*., 2003; Kampen *et al*., 2006). For example, the relative proportion of PBMC that were B cells expressing the maturation marker CD21 was very low in the first week of life (4%) and then increased gradually to 6 months of age (30%) (Kampen *et al*., 2006). Other investigators have reported that B cell numbers reach adult levels by a month of age (Senogles *et al*., 1978; Nagahata *et al*., 1991). Endogenous antibody production is measurable as early as a few days of age (Barrington and Parish, 2001), but expression of some isotypes and/or allotypes of antibody is delayed for weeks to months after birth (Corbeil *et al*., 1997). Overall, humoral antibody responses and *in vitro* responses of B cells to stimulation are markedly less in neonatal calves than in adult cattle (Nagahata *et al*., 1991; Barrington and Parish, 2001; Nonnecke *et al*., 2003).

Immunoglobulin secreted into the lumen of the respiratory tract helps prevent adhesion of pathogens to host cells and acts as an opsonin for phagocytic cells (Daniele, 1990; Brandtzaeg *et al*., 1996). In mature animals, most immunoglobulin on mucosal surfaces is secretory IgA, but other isotypes may predominate in young animals (Sheoran *et al*., 2000). BAL fluid from 2-week-old calves contains a higher proportion of IgG_2 compared to serum, suggesting that local selective transfer of IgG_2 occurs in the LRT of calves (Pringle *et al*., 1988). The ratio of IgG/IgA and of IgG_1/IgG_2 were found to be 12:1 and 1.3:1, respectively in BAL fluid from 2-week-old calves (Pringle *et al*., 1988), although the ratio of IgG/IgA in BAL of young calves has varied among reports, probably due to differences in sampling technique (Walker *et al*., 1980; Wilkie and Markham, 1981). Plasma cells secreting IgG_2 do not appear in the respiratory tract of calves until after the second week of life (Allan *et al*., 1979), so IgG_2 present in BAL fluid of younger calves is likely to be derived from distant lymphoid tissues or from maternal immunoglobulin. In the intestinal tract, IgM is the predominant endogenous antibody present in the first few weeks of

life (Logan and Pearson, 1978; Heckert *et al*., 1991), but there is little data on the levels of IgM in respiratory secretions of young calves.

The number of antigens to which the calf can produce an adaptive immune response is limited at birth compared with mature cattle, and calves respond to specific antigens at different times early in life. Some antigens may elicit an antibody response at birth, whereas others may not elicit a response until weeks or months of age (Barrington and Parish, 2001). The mechanisms by which this occurs are poorly understood, but several factors may contribute to the limited immune response observed in the newborn calf. The low numbers of functional B cells in neonatal calves and the suppression of humoral responses by colostrum have already been discussed. In addition, T cells of neonatal calves are hypo-responsive in activation (Nonnecke *et al*., 2003; Foote *et al*., 2005a) and homing mechanisms (Foote *et al*., 2005a) when compared to those of older calves and adults. Relative to the mitogen induced responses of T cells from adult cattle, T cells from neonatal calves show decreased proliferative capacity ($CD4^+$ cells), delayed increase in the expression of the IL-2 receptor (CD25) associated with activation ($CD4^+$ cells), no expression of the adhesion molecule CD44 associated with leukocyte trafficking to sites of inflammation ($CD4^+$ and γδT cells), and no decrease in expression of the lymph node homing receptor, CD62L ($CD4^+$, $CD8^+$ and γδT cells). However, by 8 weeks of age mitogen-induced and antigen-specific responses are similar to those of adult cattle, indicating that T cell function matures rapidly during the first few weeks of life (Foote *et al*., 2005a; Foote *et al*., 2005b).

The neonates of many species have a decreased capacity to produce cytokines, especially those associated with Th1 responses. There is a tendency to a Th2-biased immune response characterized by a predominance of IL-4 and IgG_1 in neonatal response to antigens (Adkins,

2000; Siegrist, 2000). This Th2 bias is also observed in calves, and the capacity of PBMCs from neonatal calves to produce IFN-γ is substantially less than that of adult cattle (Nonnecke *et al*., 2003). However, neonates, including calves, are capable of producing a Th1-biased response when exposed to potent inducers of such responses such as purified proteins from *Mycobacterium bovis* (Adkins, 1999; Ota *et al*., 2002; Nonnecke *et al*., 2005).

The nutritional status of the neonatal calf affects immune responses. Feeding calves at a high plane of nutrition is associated with reduced viability of circulating T cell populations (Foote *et al*., 2007) as well as reduced mitogen-induced proliferative responses of T cells (Foote *et al*., 2005a). The effects of protein-energy malnutrition on the neonatal calf immune system is unknown, but in other species malnutrition and weight loss are associated with defects in cell-mediated immunity, antibody production, cytokine production, and phagocytic function (Chandra, 2002). Although the mechanism by which nutrition influences immune function in the neonate is unknown, it is clear that nutrition during the pre-weaning period can have a major impact on the rate of maturity of the immune system in the young calf.

Summary of the Neonatal Calf Immune Response

In summary, components from all arms of the immune system (local and systemic, innate and adaptive) of the neonatal calf differ substantially from that of adult cattle; these components undergo rapid immunological maturation during the first few months of life. The young calf initially can respond to only a limited number of antigens and this repertoire of antigens increases gradually over time. Humoral immune responses, in particular, are suppressed during the first few weeks of life, especially in colostrum-fed calves. The relative proportion of T cell subsets, the intensity of their response to antigen, and the types of cytokines that are secreted all differ substantially in neonates as compared with adults. Importantly, neonates tend to produce a

Th2 polarized immune response. The relative immaturity of innate and adaptive immune responses likely contributes to the increased susceptibility to infectious disease that is observed in young calves. The immaturity of adaptive immune responses in young calves also has important implications for development of effective vaccines for use in neonates.

Immunology of the Eustachian Tube and Middle Ear

Otitis media can occur in all age groups, but young animals and human infants are at greatest risk. Although there is little published on age-related anatomical changes in the middle ear and eustachian tubes of cattle, anatomical features that vary between adults and infants such as the length and the angle of the eustachian tube are thought to contribute to susceptibility to otitis media (Bluestone, 1996). Because of inefficient eustachian tube opening, infants are more likely than adults to develop negative pressure in the middle ear which can increase the risk of entry of nasopharyngeal fluids and bacteria (Bluestone, 1996). However, the major factor determining age-related susceptibility to otitis media is the functional immaturity of the immune system in neonates and high susceptibility to viral and secondary bacterial infections of the URT observed in this age group (Giebink, 1994; Bakaletz, 1995; Chonmaitree and Heikkinen, 1997; Adkins, 2000; Barrington and Parish, 2001). The age at which colonization of the nasopharynx or tonsils first occurs affects the risk of developing otitis media. For example, infants that are first colonized in the nasopharynx with *Streptococcus pneumoniae, H. influenzae* or *Moraxella catarrhalis* before 3 months of age have increased risk and severity of otitis media compared with infants who are first colonized after 3 months of age (Faden *et al*., 1997). Colonization of the nasopharynx with bacterial pathogens within the first week of life is associated with extremely high rates of otitis media (Leach *et al*., 1994). Interestingly, a similar pattern is observed in animals. Age-related susceptibility to otitis media is also observed in *M. hyorhinis* infections of piglets and *M. bovis* infections of calves, although age-specific factors contributing

to susceptibility in these species have not been determined (Morita *et al*., 1995; Friis *et al*., 2002). These findings suggest that the ability to delay colonization by only a few weeks might have a dramatic impact on susceptibility to *M. bovis*-associated otitis media in calves.

Pathogens that cause otitis media in children are able to colonize and cause a local inflammatory response in the nasopharyngeal tonsils (adenoids). The adenoids may act as a nidus of infection, seeding the distal end of the eustachian tube, which is in close physical proximity (Rynnel-Dagoo and Freijd, 1988; Kiroglu *et al*., 1998). In fact, surgical removal of the adenoids is often effective at curing older children with chronic or recurrent otitis media (Rynnel-Dagoo and Freijd, 1988; Paradise *et al*., 1999; Rosenfeld *et al*., 2004). The pharyngeal tonsil in cattle is the anatomical equivalent of the adenoids (Schuh and Oliphant, 1992). However, its role in colonization of the URT and subsequent seeding of the eustachian tubes has not been addressed in calves.

For all species studied, the middle ear and eustachian tube are lined by a respiratory epithelium. The mucosa of the eustachian tube and parts of the middle ear consists of ciliated epithelial cells and mucous-secreting cells. Cilia beat in a coordinated fashion to clear fluid, bacteria and other particles from the middle ear to the nasopharynx, as well as to prevent entry of pathogens into the middle ear (Bluestone, 1996). Cells within the eustachian tube mucosa also secrete non-specific antibacterial substances such as surfactant proteins that may be important in protection against pathogens (Lim *et al*., 1987; Paananen *et al*., 2001). Increased mucus production by cells of the eustachian tube is stimulated by the presence of inflammatory mediators (Lim *et al*., 1987). Viral and bacterial infections of the nasopharynx and eustachian tube are associated with damage to the eustachian tube epithelium and disruption of ciliary function (Miyamoto and Bakaletz, 1997; Chonmaitree, 2000; Heikkinen and Chonmaitree,

2003). In addition, the inflammation associated with these infections, along with other causes of inflammation of nasopharyngeal mucosa such as allergic disease, can cause physical obstruction of the eustachian tube (Lim *et al*., 1987; Bluestone, 1996). In fact, eustachian tube dysfunction is thought to be the most important risk factor for the development of otitis media (Bluestone, 1996).

In animal models of human otitis, macrophages are the primary cells responding to infection of the middle ear during the acute phase of otitis media (Bakaletz *et al*., 1987; Takahashi *et al*., 1992). Impaired function of alveolar macrophages is described in neonatal calves (Yeo *et al*., 1993; Lu *et al*., 1996), but whether macrophages of the middle ear are likewise suppressed in young calves has not been determined. Substantial numbers of mast cells are also present in the middle ear of rodents and humans, mainly located adjacent to blood vessels in the lamina propria (Brandtzaeg *et al*., 1996). Although no definitive data are available, it is generally assumed that mast cells play a role in inflammation; whether there are similar numbers of mast cells in the middle ears of young calves has not been reported.

The middle ear and eustachian tubes contain MALT that is involved in the production of a localized specific immune response to bacterial and viral agents (Ogra, 2000). However, MALT is typically not found in healthy children less than 1 month of age, and this may be a factor in the increased susceptibility to otitis media in this very young age group (Kamimura *et al*., 2000). Both B cells and T cells are present in the MALT of the middle ear during otitis media, but data regarding the cell-mediated immune response in the middle ear are limited (Ogra, 2000). In healthy rats, IgA can be detected in the mucosa of the eustachian tubes but in relatively small amounts; in the healthy, uninfected middle ear almost no antibody-secreting cells are present (Watanabe *et al*., 1992). During otitis media, however, large amounts of IgA are

detected in the eustachian tube, and IgA, IgG, and IgM are all detected in the middle ear (Svinhufvud *et al*., 1992). In young children with otitis media, a strong local IgA response can occur in the nasopharynx without a detectable systemic antibody response (Virolainen *et al*., 1995; Nieminen *et al*., 1996).

Passive protection against bacterial otitis media in infants is provided by feeding breast milk containing high amounts of pathogen-specific IgA, which prevents adherence to and colonization of the pharyngeal mucosa (Hanson *et al*., 1984; Duffy *et al*., 1997). Similarly, passive transfer with IgA has been effective in limiting colonization of the nasopharynx and preventing clinical disease in animal models of *H. influenzae* otitis media (Kennedy *et al*., 2000). Interestingly, while complete eradication of *H. influenzae* from the nasopharynx was highly effective at preventing otitis media, reduction of the bacterial load in the nasopharynx to below a critical threshold level appeared similarly effective (Kennedy *et al*., 2000).

In humans and in animal models of human otitis media, viral infection of the URT is a major predisposing factor to bacterial otitis media. In pigs, no specific viruses have been identified in association with *M. hyorhinis*-induced otitis media (Morita *et al*., 1995; Friis *et al*., 2002). However, *M. hyorhinis* itself causes eustachitis and it may therefore induce the eustachian tube dysfunction that is an important factor leading to development of otitis media (Morita *et al*., 1999). Whether viral infections play a role in *M. bovis*-associated otitis media in cattle has not been established; unlike some mycoplasmal pathogens, *M. bovis* did not cause marked disruption of ciliary activity in tracheal organ cultures (Howard *et al*., 1987b).

Immune Responses to Mycoplasmal Infections, with a Focus on *M. bovis*

Mycoplasmal respiratory infections are characterized by an initial inflammatory response triggered by interactions of mycoplasmas with cells of the respiratory tract. Frequently, the host is unable to clear the infection and the mycoplasma persists despite an active immune response

(Fernald, 1982; Cartner *et al*., 1998; Razin *et al*., 1998). These features mean that virtually all aspects of the host immune system are involved in responses to mycoplasmal infections, and immune responses critically affect the level of mycoplasmal infection and the progression of disease. Innate responses and humoral immunity are the major contributors to defense against mycoplasmal respiratory infections, whereas cell-mediated immunity is less important in protection (Cartner *et al*., 1998; Jones and Simecka, 2003; Woolard *et al*., 2005). However, much of the pathology and resulting clinical manifestations that occur in mycoplasmal diseases are an effect of the host immune response rather than a direct effect of the mycoplasmas themselves. Cell-mediated immunity likely plays a major role in these immunopathological responses (Rottem and Naot, 1998; Rosengarten *et al*., 2000; Jones and Simecka, 2003).

Interactions between mycoplasmal pathogens and their hosts are much more complex than might be expected from the small genome, structural simplicity and limited biosynthetic capacity of mycoplasmas. Mycoplasmas can induce a broad range of immunomodulatory events by direct effects on macrophages, neutrophils, and lymphocytes, and by indirect effects through induction of cytokine secretion from these and other cells such as epithelial cells (Baseman and Tully, 1997; Rosengarten *et al*., 2000). The complicated relationship between mycoplasmas and their hosts means that many aspects of these interactions are poorly understood, even for the host-pathogen relationships for which there is a large body of research data. For *M. bovis* infections, very little is known about the host and microbial factors that contribute to development of disease or to the production of an effective immune response.

Innate Immune Responses to Mycoplasmal Infections

Innate immune responses are critical in the early phase of mycoplasmal respiratory infections for clearance of the microorganism and control of infection (Cartner *et al*., 1998; Hickman-Davis, 2002). Macrophages, and alveolar macrophages in particular, are the most

important cells in innate defense of the respiratory tract. However, other cells including neutrophils, NK cells and epithelial cells play important roles in initial responses to mycoplasmal infections and, in some cases, contribute to detrimental host responses (Cartner *et al*., 1998; Hickman-Davis, 2002).

The importance of alveolar macrophages in control of mycoplasmal respiratory infections is illustrated by comparing strains of mice that are genetically resistant or susceptible to *M. pulmonis*-induced lung disease (Parker *et al*., 1987; Hickman-Davis *et al*., 1997; Cartner *et al*., 1998). This difference in disease susceptibility is due to enhanced clearance of *M. pulmonis* from the lungs of resistant, compared with susceptible, strains of mice. Clearance from the lungs in resistant mice occurs early in the course of infection, before any influx of inflammatory cells, suggesting that resident alveolar macrophages are responsible (Parker *et al*., 1987). Consistent with this hypothesis, mice of the same resistant or susceptible genetic backgrounds but which lacked the ability to produce antibody or T cell responses retained the differences in pulmonary clearance of mycoplasma (Cartner *et al*., 1998). Depletion of alveolar macrophages in resistant strains of mice resulted in a dramatic increase in the numbers of *M. pulmonis* in the lung and in the severity of disease, whereas depletion of alveolar macrophages in susceptible strains had minimal effect, confirming the essential role of alveolar macrophages in mycoplasmal killing during early lung infection (Hickman-Davis *et al*., 1997).

Alveolar macrophages are often unable to engulf and kill mycoplasmas without opsonization (Howard and Taylor, 1983; Hickman-Davis, 2002). Various opsonins have been identified as important in this role, including specific antibodies, complement and surfactant proteins (Bredt *et al*., 1977; Howard and Taylor, 1979; Howard and Taylor, 1983; Hickman-Davis, 2002). Opsonization with specific antibody was required *in vitro* for killing of *M. bovis* by

macrophages (Howard *et al*., 1976). IgG_2 was a superior opsonin to IgG_1, but both isotypes could mediate these interactions (Howard, 1984). Whether other opsonins, such as surfactant proteins, are important in defense against *M. bovis* has not been determined.

In addition to opsonization, alveolar macrophages also require activation for efficient phagocytosis and killing of some mycoplasmas (Hickman-Davis, 2002). As well as displaying enhanced killing abilities, activated macrophages secrete large amounts of pro-inflammatory cytokines and recruit neutrophils and other immune cells to the site of infection (Razin *et al*., 1998). Many mycoplasmal pathogens, including *M. bovis*, are potent activators of alveolar macrophages (Jungi *et al*., 1996; Rottem and Naot, 1998). Detrimental host inflammatory responses have been attributed to excessive TNF-α production by alveolar macrophages in mycoplasmal infections (Faulkner *et al*., 1995), including *M. bovis* (Rosenbusch, 2001). What benefit stimulation of an exuberant inflammatory response has to the survival of mycoplasmal pathogens is unclear, but induction of TNF-α is not a feature of nonpathogenic mycoplasmal species. The bovine mycoplasmal pathogens *M. bovis*, *Mycoplasma dispar* and *M. mycoides*, subsp. *mycoides* biotype SC are all potent *in vitro* stimulators of TNF-α production by bovine alveolar macrophages, but the non-pathogenic species *M. bovirhinis* and *Acholeplasma laidlawii* do not trigger this response (Jungi *et al*., 1996).

Following macrophage activation and expression of pro-inflammatory cytokines and chemoattractants, neutrophils are recruited to sites of inflammation. In fact, neutrophils are often the most abundant immune cell early in mycoplasma-associated respiratory disease, and they may remain relatively abundant even in chronic disease. The extent of neutrophil recruitment is often directly correlated with the severity of disease. Neutrophils are a prominent cell type in the lungs, middle ear, and joints of *M. bovis* infected calves (Adegboye *et al*., 1995a; Rodriguez *et*

al., 1996; Clark, 2002; Shahriar *et al*., 2002; Maeda *et al*., 2003; Khodakaram-Tafti and Lopez, 2004; Lamm *et al*., 2004; Gagea *et al*., 2006). Even in calves without clinical lung disease, the presence of *M. bovis* is associated with increased numbers of neutrophils in BAL fluid (Allen *et al*., 1992b). Widespread activation of macrophages by pathogenic mycoplasmas can result in excessive recruitment of neutrophils to sites of infection, with subsequent release of large amounts of inflammatory mediators that are associated with increased disease severity (Xu *et al*., 2006b). *In vitro* studies showed that bovine neutrophils are able to kill opsonized *M. bovis*, but this interaction requires the presence of IgG_2; IgG_1 was not an effective opsonin (Howard, 1984). Despite their ability to kill opsonized mycoplasmas *in vitro*, the overall contribution of neutrophils to clearance of mycoplasmas *in vivo* is unknown. Thomas *et al*., (1991) showed that unopsonized *M. bovis* can adhere to the surface of neutrophils without being ingested, and that adherent viable or non-viable *M. bovis* cells inhibit respiratory burst activity. This ability to suppress neutrophil function, coupled with the fact that young calves produce very little IgG_2, which is required, at least *in vitro*, for neutrophil-mediated killing of *M. bovis*, may mean that neutrophils are not particularly effective in the clearance of *M. bovis* in young calves.

In addition to interactions with macrophages and neutrophils, mycoplasmas can interact with other cell types such as epithelial cells (Seya *et al*., 2002). In fact, interactions between mycoplasmas and other cells are probably critical in the initiation of an inflammatory response. Mycoplasmas have been shown to stimulate both nasal epithelial cells (Kazachkov *et al*., 2002) and type II epithelial cells in the lung (Kruger and Baier, 1997) to produce IL-8 and other neutrophil chemoattractants. *Mycoplasma bovis* activates bovine lung microvascular endothelial cells to express cell surface molecules specific for mononuclear cell and neutrophil transmigration (Lu and Rosenbusch, 2004).

Little is known about the role of NK cells in mycoplasmal respiratory disease; they are recruited to sites of inflammation in the initial stages of infection by chemoattractants released from macrophages. Large amounts of IFN-γ are secreted by NK cells, as well as by other cells such as γδ T cells. IFN-γ is thought to be important for the activation of macrophages during the initiation of the inflammatory response, and may have other protective or potentially pathologic roles in mycoplasmal disease (Lai *et al*., 1990b; Woolard *et al*., 2005). Like NK cells, little is known about the role of mast cells in mycoplasmal disease. Recent studies (Xu *et al*., 2006a) using mast-cell deficient mice indicate that mast cells may be important in innate immune containment and clearance of *M. pulmonis* infection in mice. Potential roles for mast cells in bovine mycoplasmal disease have not been reported.

To summarize, innate immune responses are very important in the early clearance of mycoplasmas from the lung. In particular, alveolar macrophages are essential in the early response to infection. However, inappropriate activation of alveolar macrophages by mycoplasmas may promote an excessive inflammatory response. Little is known about the innate responses specific to *M. bovis* infections in the lungs of calves, or in other sites including the middle ear, mammary gland and joints of affected cattle. Given the ability of *M. bovis* to modulate responses of macrophages and neutrophils *in vitro*, together with the relative scarcity of effective opsonins and the functional immaturity of macrophages in young calves, it is reasonable to conclude that impaired innate responses are likely to contribute to the increased susceptibility to *M. bovis* infections that is observed in this age group.

Adaptive Immune Responses to Mycoplasmal Infections

Adaptive immune responses to mycoplasmal infections play important roles in determining the progression of disease. Adaptive responses can clearly be beneficial in clearing

or controlling mycoplasmal infections, but they also can be ineffective and can be major contributors to the severity of disease (Rottem and Naot, 1998; Rosengarten *et al*., 2000; Jones and Simecka, 2003). Despite a substantial body of work examining adaptive responses to mycoplasmal infections, the optimal immune responses for protection and the types of responses contributing to disease remain poorly defined.

The fact that adaptive immune responses can protect from disease is illustrated by examples of successful vaccination against some mycoplasmal infections (Taylor *et al*., 1977; Cassell and Davis, 1978; Howard *et al*., 1987a; Thacker *et al*., 2000; Kyriakis *et al*., 2001). However, immunity after vaccination or infection is often short-lived. For example, after inoculation of the mammary gland with *M. bovis*, cows were resistant to subsequent re-challenge after 2 months in both previously infected and non-infected quarters; at 6 months, cows were generally resistant to infection only in the previously challenged quarter, and at one year all quarters were susceptible (Bennett and Jasper, 1978a). Prior infection with *M. bovis* seems to protect cows from developing the severe clinical mycoplasmal mastitis that is typically observed on primary infection; most re-infections result in subclinical or very mild clinical disease (Bennett and Jasper, 1978b). Thus, adaptive immune responses that are in place at the time of mycoplasmal exposure do appear to contribute to the control or prevention of new mycoplasmal infections.

Adaptive immune responses are frequently ineffective at eliminating established mycoplasmal infections, and mycoplasmas are often able to persist in the face of an intense response (Fernald, 1982; Cartner *et al*., 1998; Razin *et al*., 1998; Rosengarten *et al*., 2000). Ongoing, ineffective immune responses result in the chronic inflammation that is associated with many mycoplasmal diseases (Cartner *et al*., 1998; Rosengarten *et al*., 2000; Jones and Simecka,

2003). Exactly how mycoplasmas manage to avoid clearance by the host is not well understood. However, mycoplasmas exhibit the ability to induce a broad range of immunomodulatory events (Baseman and Tully, 1997) that may induce ineffective responses. In addition, variation of surface antigens may help mycoplasmas to avoid clearance mediated by adaptive immune responses (Rosengarten *et al*., 2000).

Adaptive responses also play an important role in immunopathologic disease. Cellular responses in mycoplasmal infections are characterized by large accumulations of lymphocytes (Simecka *et al*., 1992), suggesting that lymphocyte activation and recruitment to sites of mycoplasmal infection are important in the development of pathology. Autoimmune reactions also contribute to some mycoplasmal respiratory diseases (Kitazawa *et al*., 1998; Wilson *et al*., 2007), but have not been identified in disease caused by *M. bovis*.

Humoral Immune Responses to *M. bovis* in Cattle

Experimental infection of cattle with *M. bovis* usually elicits a strong humoral immune response. Specific serum immunoglobulin is detectable as early as 6 days (IgM) to 10 days (IgG) after experimental inoculation of *M. bovis* into the respiratory tract of calves (Brank *et al*., 1999; Le Grand *et al*., 2002). Humoral responses to *M. bovis* in calves are characterized by high levels of IgG_1 (Howard and Gourlay, 1983; Vanden Bush and Rosenbusch, 2003). Very little IgG_2 is produced in calves infected at 12 weeks of age (Vanden Bush and Rosenbusch, 2003), suggesting that the response to *M. bovis* respiratory infection in calves has a Th2-bias. In addition, the younger the calf, the higher the ratio of IgG_1 to IgG_2 produced in response to *M. bovis* infection (Howard and Gourlay, 1983), consistent with the delay in IgG_2 production observed in very young animals (Adkins, 2000; Siegrist, 2000).

In cattle infected with non-mycoplasmal pathogens, both IgG and IgA are important in immune responses of the LRT, and IgA is important in the URT (Mosier, 1997; Potter *et al*.,

1999; Ackermann and Brogden, 2000); both compartments contribute significantly to antibody responses. Although data are limited, these types of local responses seem to also occur in *M. bovis* infections of calves. In one study, IgG_1-producing plasma cells predominated in the lungs at 2 weeks after *M. bovis* inoculation, accompanied by smaller numbers of IgM, IgG_2 and IgA producing cells. By 4 weeks, a significant increase in the number of IgG_2 producing cells was observed (Howard *et al*., 1987c). These studies used 4- to 6-week-old calves, and the distribution of antibody-producing cells in the respiratory tract of other age groups with *M. bovis* infection has not been reported. In the URT, IgG_1 producing cells were observed in the submucosa of the trachea, and IgA producing cells were abundant in the trachea and nasal cavity of infected calves (Howard *et al*., 1987c). These findings are consistent with the distribution of immunoglobulin isotypes found in nasal lavage and BAL fluids after *M. bovis* infection (Howard *et al*., 1980). In calves with experimentally-induced *M. bovis* arthritis, titers of IgG_1, IgG_2 and IgM are similar in both serum and joint fluid, consistent with leakage of serum proteins into affected joints. However, IgA concentrations in joint fluid are greater than those in serum, indicating some local production of IgA during *M. bovis* arthritis (Chima *et al*., 1981). Local humoral responses in calves with *M. bovis*-associated otitis media have not been reported.

In contrast with the humoral response observed after experimental *M. bovis* infection of calves, responses in naturally infected calves are more variable. Virtala *et al*., (2000) reported that only 57% of 75 pneumonic dairy calves less than 3 months of age in which *M. bovis* was isolated from tracheal wash samples had a 4-fold or greater increase in *M. bovis* serum antibody titers by IHA. The authors concluded that a rise in titer on paired serum samples was not a good predictor of *M. bovis*-associated respiratory disease, possibly due to the presence of maternal antibody. Maternal antibody is associated with suppression of humoral responses to specific

antigens, but vaccination of young calves with killed *M. bovis* or respiratory challenge with live *M. bovis* in the face of maternal antibody usually elicits a detectable humoral response (Howard and Gourlay, 1983). It is reasonable to hypothesize that mild or superficial infections of the respiratory tract fail to elicit a systemic antibody response in colostrum-fed calves, whereas more significant challenges or systemic presentation of antigen usually do elicit such a response. Other investigators have also failed to find a correlation between serum antibody titers and the presence *M. bovis* in the LRT of naturally-infected individual animals (Rosendal and Martin, 1986; Martin *et al*., 1989). However, on a group level, seroconversion has been predictive of *M. bovis*-associated respiratory disease (Martin *et al*., 1990; Tschopp *et al*., 2001). Specific serum immunoglobulin concentrations remain elevated for months to years after an immune response to clinical *M. bovis*-associated disease (Le Grand *et al*., 2001; Nicholas and Ayling, 2003).

In adult cows, inoculation of the mammary gland with *M. bovis* results in a classical early serum IgM response followed by IgG as the response matures. Peak serum titers occur 6 to 8 weeks after experimental infection (Bennett and Jasper, 1980). Both IgG_1 and IgG_2 are produced by infected cows (Boothby *et al*., 1987), suggesting that the immune response to intramammary infection in mature animals is less Th2-biased than that of young animals with respiratory tract infections. In the mammary gland, local IgG_1, IgG_2 and IgA responses to *M. bovis* mastitis are observed (Bennett and Jasper, 1980; Bennett and Jasper, 1978b; Boothby *et al*., 1987).

In summary, it appears that specific antibody to *M. bovis* is generally present in respiratory secretions of *M. bovis*-infected calves. Systemic humoral responses are also often present, but subclinical infections of the respiratory tract may not generate a detectable serum antibody response.

Function of Humoral Responses to Mycoplasmal Infections

Together with innate immune responses, humoral responses are probably the most important in protection from mycoplasmal infections. Systemic humoral responses are particularly important in preventing disseminated mycoplasmal infections such as arthritis (Cartner *et al*., 1998), and local humoral responses provide important opsonins to the cells of the innate immune system to aid in clearance of mycoplasmas (Howard and Taylor, 1983). Animals and humans with humoral deficiencies initially develop mycoplasma-induced lung disease that is of similar severity to that of immunocompetent hosts. However, immunodeficient hosts typically go on to develop chronic pneumonia and disseminated disease such as arthritis or meningitis, while these events occur less frequently in immunocompetent hosts (Taylor-Robinson *et al*., 1980; Berglof *et al*., 1997). Consistent with these observations, pre-existing serum IgG titers to *M. bovis* are correlated with protection from arthritis by intravenous or aerosol challenge (Chima *et al*., 1981; Nicholas *et al*., 2002).

The role of humoral responses in protection from mycoplasmal infections is also illustrated by the fact that passive transfer of antibody can prevent disseminated mycoplasmal infections. Passive transfer of antibody in immunodeficient mice prevents the development of mycoplasmal arthritis (Cartner *et al*., 1998), and has also been associated with protection from some mycoplasmal respiratory pathogens (Taylor and Taylor-Robinson, 1977; Barile *et al*., 1988; Rautiainen and Wallgren, 2001). The role of passively transferred antibody in protection from *M. bovis*-associated disease has not been evaluated in controlled challenge studies. Limited data from field studies do not support a protective role for maternal antibody against *M. bovis* infections. In one study of 325 colostrum-fed dairy calves, there was no significant association between *M. bovis*-specific serum antibody titers in the first 2 weeks of life and occurrence of pneumonia (Van Donkersgoed *et al*., 1993). Likewise, Brown *et al*., (1998a) did not find an

association between *M. bovis*-specific serum antibody concentrations at 7 days of age and occurrence of *M. bovis*-associated disease in 50 Holstein calves. Administration of large volumes of hyperimmune serum against *M. bovis* to calves at the same time as or following intranasal inoculation of *M. bovis* had no effect on the severity of respiratory disease (Brys and Pfutzner, 1989).

Antibody present at the site of infection may be more important for protection from *M. bovis* infections than is systemic antibody. Concentrations of antibodies in serum after experimental induction of *M. bovis* mastitis did not differentiate between cows susceptible or resistant to reinfection of the mammary gland, but concentrations of IgA and IgG in milk from glands resistant to reinfection were higher than those in susceptible glands (Bennett and Jasper, 1978a; Bennett and Jasper, 1978b). In addition, the daily production of total IgG and IgA during peak infection was greater in mammary glands that were able to resolve the infection than in glands that remained chronically infected (Bennett and Jasper, 1980). In studies where vaccination resulted in some protection from *M. bovis*-associated respiratory disease, both serum antibody titers (Nicholas *et al*., 2002), and IgG concentrations in BAL fluids (Howard *et al*., 1980) have been correlated with disease protection.

The Vsps are the preferred targets of the humoral immune response in *M. bovis* infections (Brank *et al*., 1999; Rosengarten *et al*., 2000), although other *M. bovis* surface lipoproteins also elicit antibody responses (Behrens *et al*., 1996; Robino *et al*., 2005). However, the specific surface molecules of *M. bovis* involved in eliciting protective humoral responses have not been defined.

In summary, humoral immune responses, especially local antibody responses at the site of infection, appear to be important in protection from *M. bovis* infections. Conversely, strong

humoral responses often develop during *M. bovis*-associated clinical disease but fail to clear *M. bovis* from the host. The tendency towards an IgG_1 dominated humoral response in calves may not be optimal for clearance of *M. bovis*, given that IgG_2 is a superior opsonin for macrophage- and neutrophil-mediated killing of *M. bovis* (Howard, 1984). More work is needed in calves to better define the humoral responses that are most efficient at *M. bovis* clearance as well as responses that protect from new infections.

The Role of T Cell Responses to Mycoplasmal Infections

Over 30 years ago, histopathological similarities between perivascular cellular infiltrates observed in *M. pneumoniae* infections and the lesions of cutaneous delayed hypersensitivity reactions led to the suggestion that cell-mediated mechanisms might be contributing to mycoplasmal disease (Fernald *et al*., 1972). It is now widely accepted that mycoplasmal respiratory infections have substantial immunopathological components, characterized in part by large accumulations of lymphocytes in affected areas of the respiratory tract (Simecka *et al*., 1992). Lymphocyte aggregation is not as marked in *M. bovis* infections (Rosenbusch, 2001) as with some mycoplasmal infections in other hosts, but lymphocytes are still a substantial contributor to lesions in *M. bovis*-associated disease. Both B and T cells accumulate in the lungs of affected calves (Howard *et al*., 1987c), in the joints of calves with mycoplasmal arthritis (Gourlay *et al*., 1976; Adegboye *et al*., 1996; Gagea *et al*., 2006) and in the mammary glands of cows with *M. bovis* mastitis (Bennett and Jasper, 1977a; Seffner and Pfutzner, 1980). These findings suggest that lymphocyte activation and recruitment to sites of *M. bovis* infection are important in the development of pathology. However, there are only limited data describing the lymphocyte populations that contribute to these responses in cattle, and virtually no data specifically from neonatal calves.

Because of their essential role as regulators of the immune system, T cells play a pivotal role in the development of protective responses as well as in host mediated immunopathogenesis. T cells are a major component of the mononuclear infiltrates observed in the lungs and draining lymph nodes of mycoplasma infected hosts (Davis *et al*., 1982; Rodriguez *et al*., 1996; Rodriguez *et al*., 2001; Jones and Simecka, 2003). Studies of *M. pulmonis* disease in mice suggest that T cells are of limited importance in initial responses to mycoplasmal infections. Both T cell deficient mice and severe combined immunodeficiency mice develop less severe lung disease than their immunocompetent counterparts (Keystone *et al*., 1980; Cartner *et al*., 1998). These effects are independent of mycoplasmal numbers in the lungs. Reconstitution of immunodeficient mice with naive T cells restores the severity of respiratory disease to the level observed in immunocompetent mice (Cartner *et al*., 1998). These studies indicate that T cells are unlikely to play a major role in early control of mycoplasmal infections but instead are associated with regulating detrimental host inflammatory responses. However, T cells do contribute to the establishment of humoral responses that, as discussed earlier, are beneficial in control of mycoplasmal infections.

Much of the current understanding of T cell responses in mycoplasmal infections is based on studies of *M. pulmonis* infections in resistant and susceptible strains of mice. In susceptible strains of mice, increases in both $CD4^+$ and, to a lesser extent, $CD8^+$ T cells occur in the lungs and draining lymph nodes during infection with *M. pulmonis.* These major T cell subsets have opposing regulatory roles in the progression of mycoplasmal lung disease. *In vivo* depletion of $CD4^+$ cells results in reduced lung lesions in infected mice, but depletion of $CD8^+$ cells results in dramatically more severe lung lesions; these changes are not associated with changes in mycoplasma numbers in the lungs. Therefore, $CD8^+$ T cells are involved in dampening of the

inflammatory reaction in mycoplasmal lung disease, whereas $CD4^+$ T cells contribute to disease pathology (Jones *et al*., 2002). The interaction between these cell types has a major impact on the outcome of mycoplasmal respiratory disease.

The role of γδ T cells in mycoplasmal respiratory disease has not been clearly defined, but they appear to be important in the pathogenesis of murine mycoplasma infection. In mice, γδ T cell numbers in the lungs increase early in infection and return to basal levels by day 14 (J. W. Simecka, personal communication). Knockout mice unable to produce γδ T cells develop significantly less severe *M. pulmonis*-associated disease than immunocompetent mice, despite similar numbers of mycoplasmas in the lungs of both groups, suggesting that γδ T cells play a role in the development of inflammatory lesions (J. W. Simecka, personal communication). γδ T cells are thought to contribute to IFN-γ secretion early in the infection process, and therefore play a role in macrophage activation and initiation of the host response, but other roles of γδ T cells in mycoplasmal infections are poorly defined. In calves, γδ T cells are a major lymphocyte population and have been shown to play a role in other infectious diseases. Thus, there is a clear need to determine whether γδ T cells respond to *M. bovis* infection in cattle and if they contribute to the regulatory network involved in the generation of immunity and pathologic responses against the mycoplasma.

T cell subsets in the lungs of calves with *M. bovis* infection have not been defined. In a study of *M. bovis* infection in 3-month-old goat kids, intratracheal inoculation resulted in clinical respiratory disease and pathology similar to that reported for calves (Rodriguez *et al*., 2000). T cells predominated in lymphoid accumulations in the lungs at 14 and 21 days post infection, and $CD4^+$ T cells were a greater contributor to these lesions than were $CD8^+$ T cells. Although

this study was conducted in a different host species, it suggests that activation of $CD4^+$ T cells plays a prominent role in *M. bovis* infections.

Cytokine and T Helper Subset Responses to Mycoplasmal Infections

Respiratory mycoplasmal infections are characterized by production of pro-inflammatory cytokines and associated lung inflammation (Faulkner *et al*., 1995; Narita *et al*., 2000; Sun *et al*., 2006). Mycoplasmas also induce cytokines that can down-regulate inflammatory responses *in vitro* and in animal models (Sun *et al*., 2006). The intensity of the inflammatory response following mycoplasma infection is driven by the balance of cytokines produced (Chambaud *et al*., 1999). IFN-γ is produced early in infection by a variety of cells, including NK cells, γδ T cells and others, resulting in macrophage activation and in the promotion of Th1 responses. In contrast, IL-4 promotes Th2 cell maturation, IgE responses, and is important in maintenance of humoral mucosal responses, which are an important contributor to protection from mycoplasmal disease. The immune response in the lungs of mice infected with *M. pulmonis* is characterized by both IL-4 and IFN-γ responses, with IFN-γ predominating at 14 days post-infection (Jones *et al*., 2002). These findings are consistent with a mixed Th1-Th2 response in mice with mycoplasmal lung disease. Experiments using IFN-γ knockout mice found that the presence of IFN-γ early in infection is important for innate clearance of mycoplasmas. Infected knockout mice had higher mycoplasmal numbers in the lungs and increased severity of lung lesions compared with immunocompetent mice (Woolard *et al*., 2004). Similarly, T-bet deficient mice, which are unable to produce IFN-γ as well as producing very strong Th2 cytokine responses, develop much more severe mycoplasmal lung disease than do immunocompetent mice (Bakshi *et al*., 2006).

Little is known about the cytokine environment or the Th subsets present in the lungs of calves with mycoplasmal disease. However, PBMC responses, serum cytokine and serum

antibody responses were characterized in 12-week-old calves infected by combined intratracheal and intranasal inoculation with *M. bovis* (Vanden Bush and Rosenbusch, 2003). At 21 days after inoculation, PBMCs from *M. bovis* infected calves exhibited antigen-specific proliferative responses *in vitro*. In addition, $CD4^+$, $CD8^+$ and γδ T cells all exhibited higher *in vitro* activation (CD25 expression) in response to *M. bovis* antigens than did cells from uninfected control calves. The PBMCs from infected animals secreted IFN-γ and IL4 in response to *in vitro* stimulation with *M. bovis* antigen. Intracellular staining of stimulated cells revealed approximately equal numbers of IFN-γ and IL-4-secreting cells. There was a strong IgG_1 humoral response, and little IgG_2 was present in the serum of infected calves. These findings indicate that calves infected with *M. bovis* produce a mixed Th1-Th2 systemic cytokine response, although the lack of IgG_2 production is consistent with a Th2-biased response. Studies in *M. pulmonis*-infected mice have demonstrated that the Th profile can differ between compartments of the immune system (Jones *et al*., 2001; Jones *et al*., 2002), and so whether the above findings using PBMCs of *M. bovis*-infected calves are representative of the immune environment at the site of infection is unknown.

Recruitment of T Cells in Mycoplasmal Infections

Lymphocytes are recruited to sites of mycoplasmal infection by chemokines released from cells of the innate immune system. In a recent study, microarray analysis of cytokine and chemokine expression in the lungs of genetically resistant and susceptible strains of mice during *M. pulmonis* infection was examined (Sun *et al*., 2006). Pro-inflammatory cytokines and a number of chemokines were produced in susceptible, but not resistant, mice and the degree of cytokine expression was correlated with the severity of disease. The expression of two potent chemokines for monocytes and lymphocytes, macrophage inflammatory protein-1β, and monocyte chemoattractant protein-2, was markedly up regulated during mycoplasmal disease.

These chemokines were preferentially associated with lesions within the lungs of infected mice, and cells producing these chemokines were physically associated with clusters of $CD4^+$ T cells that expressed receptors for these proteins. Thus, it may be that chemotactic factors released at sites of mycoplasmal infection in the lung are largely responsible for recruitment of lymphocytes, and thereby determine the severity and type of inflammatory response. This is in contrast to previous hypotheses that local non-specific proliferation of lymphocytes by *M. pulmonis* mitogens is responsible for the observed lymphocyte accumulation (Naot *et al*., 1984; Davis *et al*., 1985). *M. bovis* has not displayed lymphocytes mitogenic potential *in vitro*, so it may be that a similar mechanism of lymphocyte recruitment occurs in infected calves.

Immunomodulatory Effects of *M. bovis* on Bovine Lymphocytes

Several studies have demonstrated immunomodulatory effects of *M. bovis* on cell-mediated immune responses. Thomas *et al*., (1990), reported that *M. bovis* suppresses bovine PBMC responses to the mitogen phytohemagglutinin *in vitro.* Earlier studies had found that lymphocytes from calves immunized with killed *M. bovis* antigens (and no adjuvant) have reduced proliferative responses in *M. bovis*-specific and mitogen-induced *in vitro* assays (Bennett and Jasper, 1977b). Similar findings were reported for lymphocytes from cows that had recovered from *M. bovis* mastitis, although uninfected controls were not included for comparison (Bennett and Jasper, 1978b). Supernatant from *M. bovis* cultures has also been reported to suppress *in vitro* lymphocyte proliferation (Bennett and Jasper, 1977b). Consistent with this observation, a 26 kD peptide homologous to the C-terminal region of the *M. bovis* surface lipoprotein Vsp L and present in culture supernatant inhibited mitogen-induced *in vitro* proliferation of bovine lymphocytes (Vanden Bush and Rosenbusch, 2003). The recombinant peptide was recognized by sera from calves with naturally-occurring *M. bovis* infections,

suggesting that the protein is expressed *in vivo*. Whether it is shed *in vivo* or is surface bound is unknown.

M. bovis has been reported to induce apoptosis of bovine lymphocytes *in vitro* (Vanden Bush and Rosenbusch, 2002); this action was inhibited by treatment with chloramphenicol, indicating that *M. bovis* protein production is necessary for the induction of programmed lymphocyte death. The *in vivo* significance, the extent to which induction of apoptosis occurs, and the cell type(s) targeted are unknown, but induction of apoptosis in a particular lymphocyte subset could be another mechanism by which *M. bovis* modulates the host immune response.

Hypersensitivity Responses to *M. bovis* Infections

An interesting finding from early experimental infection studies of calves with mycoplasmal respiratory disease and cows with *M. bovis* mastitis was the presence of acute and, in some cases, delayed type hypersensitivity reactions to intradermal injection of *M. bovis* antigen (Bennett *et al*., 1977; Bennett and Jasper, 1978b; Boothby *et al*., 1988). Maximal inflammatory responses at skin test sites were reported to occur within the first 4 hours after injection, and skin reactions resolved rapidly (Bennett and Jasper, 1978b) or persisted at close to maximal levels for more than 72 hours (Boothby *et al*., 1988). Animal-to-animal variation was been reported; pronounced skin sensitivity test responses to *M. bovis* antigens were present in some cows that had recovered from *M. bovis* mastitis, but not in others. Further, skin test results did not differentiate between cows susceptible or resistant to re-infection of the mammary gland (Bennett and Jasper, 1978a). These observations could indicate that hypersensitivity responses contribute to development of pathology in some animals during *M. bovis* infections. However, the antigens involved in these dermal responses need to be better defined. Serum IgE levels in *M. bovis* infected animals have not been reported. IgE-mediated responses have been implicated as important in the pathogenesis of *M. pneumoniae* infections in atopic humans (Yano *et al*.,

1994; Seggev *et al*., 1996; Stelmach *et al*., 2005). Acute and delayed type hypersensitivity responses to intradermal administration of mycoplasmal antigens have also been reported with *M. mycoides* subsp. *mycoides* biotype SC and *M. pneumoniae* infections (Windsor *et al*., 1974; Yano *et al*., 1994).

Protective Immunity to *M. bovis*

Relevant Experiences with Mycoplasmal Vaccines for Diseases Other Than *M. bovis*

Vaccination that results in reduced severity of disease is possible for a number of mycoplasmal pathogens (Taylor *et al*., 1977; Cassell and Davis, 1978; Whithear, 1996; Maes *et al*., 1998; Thacker *et al*., 2000; Dawson *et al*., 2002; Dedieu *et al*., 2005), including *M. bovis* (Howard *et al*., 1987a; Stott *et al*., 1987; Nicholas *et al*., 2002). However, vaccination rarely prevents establishment of infection or shedding of mycoplasmas (Cassell and Davis, 1978; Howard *et al*., 1980; Thacker *et al*., 2000; Nicholas *et al*., 2002). Furthermore, vaccination can result in harmful exacerbation of immune responses (Boothby *et al*., 1986b; Thiaucourt *et al*., 2003). Positive or negative host responses to mycoplasmal vaccination seem difficult to predict; vaccines will appear efficacious in some studies and some individuals, but not others. These findings are not surprising given the complex nature of host-mycoplasmal relationships, and are likely to be at least partly attributable to the fact that immune responses to mycoplasmas are strongly influenced by genetics and other host-related factors (Simecka *et al*., 1987; Parker *et al*., 1989; Shahriar *et al*., 2002). The frequent switching of dominant surface antigen expression in many mycoplasmas is another factor that may influence vaccine efficacy.

Most mycoplasmal vaccines in use today are administered systemically. However, studies of *M. pulmonis* infection in mice have shown that the nasal route of immunization can protect from mycoplasmal disease (Lai *et al*., 1990a), and is superior to systemic immunization in generating mucosal IgA responses in both the URT and LRT (Taylor and Howard 1980; Hodge

and Simecka 2002). As well as protecting from clinical disease, nasal immunization can reduce mycoplasmal colonization of the URT (Taylor and Howard 1980; Hodge and Simecka 2002). However, mucosal adjuvants may be required to achieve these effects (Hodge and Simecka 2002), and some of these adjutants have been associated with development of adverse inflammatory responses to mycoplasmal antigens (Simecka *et al*., 2000). Together, these data suggest that mucosal targeting of vaccines against mycoplasmal respiratory pathogens may be more effective than current systemic approaches, but further work is needed to identify appropriate mucosal adjuvants for mycoplasmal vaccines.

Despite the limitations of current vaccines, a number of commercially successful vaccines for mycoplasmal diseases of livestock are in use throughout the world. Most current mycoplasmal vaccines are either live attenuated or inactivated preparations of whole cells. Subunit or recombinant protein vaccines have been largely unsuccessful to date, although newer technologies are resulting in experimental vaccines and delivery systems that may prove to be efficacious against some mycoplasmal diseases (Barry *et al*., 1995; Abusugra and Morein, 1999; March *et al*., 2006).

Perhaps the most widely used mycoplasmal vaccines are *Mycoplasma hyopneumoniae* bacterins in pigs. *Mycoplasma hyopneumonia*e is a pathogen contributing to the porcine respiratory disease complex, a world-wide disease that causes substantial economic losses in the grower-finisher phase of pig production (Pfutzner and Blaha, 1995). A number of field efficacy trials, as well as experimental infection studies, have found that vaccination against *M. hyopneumoniae* is associated with reduced rates of clinical disease, reduced treatment costs, improved feed efficiency and improved weight gain (Le Grand and Kobisch, 1996; Maes *et al*., 1998; Maes *et al*., 1999; Okada *et al*., 1999; Bouwkamp *et al*., 2000; Thacker *et al*., 2000;

Kyriakis *et al*., 2001; Dawson *et al*., 2002), although it must be pointed out that several of these trials were industry-sponsored. Vaccine-induced immunity in pigs is associated with increased levels of *M. hyopneumoniae*-specific IgG and IgA in BAL fluid (Boettcher *et al*., 2002), and increased IFN-γ production and reduced TNF-α production in lungs (Thacker *et al*., 2000). Pigs are first vaccinated as early as 7 days of age, indicating that in some hosts, neonates can be successfully immunized against mycoplasmas. Passive transfer of specific antibodies in the colostrum of vaccinated sows occurs, and has been associated with reduced prevalence of *M. hyopneumoniae* in piglets (Ruiz *et al*., 2003; Kristensen *et al*., 2004). Although these vaccines are associated with reductions in clinical disease, they do not prevent colonization of the URT or shedding of *M. hyopneumoniae* (Meyns *et al*., 2006). To address these issues, several experimental *M. hyopneumoniae* vaccines targeted to the mucosal immune system have been reported (Fagan *et al*., 2001; Shimoji *et al*., 2002; Lin *et al*., 2003), but further work is required to determine their field efficacy and potential benefits over the current vaccines. Inactivated mycoplasmal vaccines are also used in other livestock species, including vaccines against *M. agalactiae* in sheep, a pathogen that is closely related to *M. bovis*. However, little data are available on the efficacy of these vaccines.

Vaccines against the important avian respiratory pathogens *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are widely used in commercial poultry production. In contrast to the killed bacterins used for *M. hyopneumoniae* in pigs, attenuated live strains of *M. gallisepticum* and *M. synoviae* are used to vaccinate poultry (Whithear, 1996; Papazisi *et al*., 2002). They are administered by mucosal routes, including in drinking water, by aerosol or by eye drop. These strains colonize the URT, displacing endemic strains in infected flocks and stimulating mucosal cellular and humoral immune responses against future virulent challenges (Whithear, 1996). A

number of studies have demonstrated these vaccines to be efficacious in reducing losses due to clinical and subclinical mycoplasmal disease (Markham *et al*., 1998a; Markham *et al*., 1998b; Barbour *et al*., 2000; Biro *et al*., 2005; Feberwee *et al*., 2006; Jones *et al*., 2006). However, problems do occur with these vaccines, including inherent virulence of some vaccine strains and failure to establish infection in the URT or to stimulate long-term immunity in other strains (Whithear, 1996).

Live attenuated mycoplasmal vaccines are also used for the control of contagious bovine pleuropneumonia (CBPP) caused by *M. mycoides* subsp. *mycoides* biotype SC in endemically-infected sub-Saharan Africa (Thiaucourt *et al*., 1998). Vaccines are injected subcutaneously and stimulate short-lived serum antibody responses; protection is associated with induction of a mucosal Th1-biased response (Dedieu *et al*., 2005). The vaccines do not prevent colonization of vaccinated animals (Thiaucourt *et al*., 1998). CBPP vaccines are typically administered to susceptible cattle in regions surrounding an outbreak, but have only limited efficacy in containing these outbreaks (Thiaucourt *et al*., 2004). Several strains of varying degrees of attenuation have been used (Dyson and Smith, 1975). Unfortunately, the more virulent vaccine strain that provides better protection against CBPP has a high rate of serious, and sometimes fatal, side effects including severe hypersensitivity reactions and reversion to virulence (Mbulu *et al*., 2004; Thiaucourt *et al*., 2004). Other experimental vaccines for CBPP have not been successful; inactivated vaccines have often resulted in exacerbation of clinical disease in challenge studies (Gourlay, 1975). A recent study described a bacteriophage DNA vaccine for *M. mycoides* subsp. *mycoides* biotype SC that was effective in a mouse-challenge model, but this approach has not yet been applied in the natural host (March *et al*., 2006).

From these studies of mycoplasmal vaccines in use today, it can be concluded that some vaccines provide disease protection, but that virtually none are able to prevent chronic infection of the host and shedding of mycoplasmas. In addition, deleterious effects of vaccination are often reported. More sophisticated approaches to vaccine development and delivery, as well as a better understanding of the host immune response in mycoplasmal diseases are clearly required.

Vaccination Against *M. bovis*

A number of attempts to vaccinate cattle against *M. bovis* mastitis have been reported, but have been largely unsuccessful. In one series of studies evaluating the effect of vaccination on susceptibility to *M. bovis* mastitis, cows were vaccinated five times at 2 week intervals during the dry period with killed *M. bovis*; the first three doses were administered subcutaneously in Freund's complete adjuvant (FCA), and the last two doses without adjuvant by intramammary infusion (Boothby *et al*., 1986a; Boothby *et al*., 1986b; Boothby *et al*., 1987). One week after calving, vaccinated and control cows were experimentally challenged in two of four quarters with live *M. bovis*. All challenged quarters became infected, developed clinical mastitis, and had a drastic (greater than 85%) loss of milk production. Inflammatory responses occurred earlier and were more severe in vaccinated cows. Vaccinated cows cleared *M. bovis* from the milk earlier than unvaccinated cows, but inflammation persisted. In addition, vaccination did not protect from quarter to quarter spread of *M. bovis*. Serum antibody titers to IgM, IgG_1 and IgG_2, and milk whey titers for IgG_1 were higher prior to challenge in vaccinated compared to control cows. After challenge, *M. bovis*-specific IgA, IgG_1 and IgG_2 were elevated in milk whey of both vaccinated and control cows, suggesting that intramammary exposure to live organisms was necessary to elicit a local, specific IgA response.

A number of vaccines for prevention of *M. bovis*-associated disease in calves have been evaluated in experimental challenge studies and field trials. Many of these have demonstrated

that vaccination can offer some protection from clinical mycoplasmal disease in calves. For example, in an experimental study, (Chima *et al*., 1980), 1- to 5-month-old beef calves were vaccinated subcutaneously with live *M. bovis*, intraperitoneally with live *M. bovis*, or subcutaneously with a formalin-inactivated bacterin. Two boosters were given at 10 day intervals and animals were challenged by intravenous inoculation of *M. bovis*. Clinical arthritis was seen in 100% of non-vaccinated as compared with 13% of vaccinated calves, and lesion severity was decreased in those vaccinated calves that did get arthritis.

In a study of an apparently efficacious vaccine in young calves, Nicholas *et al*., (2002) vaccinated 3-week-old dairy calves with a single dose of saponin-inactivated bacterin. Calves received an aerosol challenge with live *M. bovis* 3 weeks after vaccination. Vaccinated calves had fewer numbers of *M. bovis* at colonized sites, fewer numbers of body sites colonized by *M. bovis,* and reduced severity and incidence of clinical disease and lesions compared with control calves. There was also a significant decrease in body weight gain in control calves compared with vaccinates. Additionally, no vaccinated calves and two of seven control calves developed arthritis. Vaccinated calves produced a strong IgG response prior to challenge, but IgG subtypes were not reported. No adverse events associated with vaccination were reported.

A killed vaccine against four bovine respiratory pathogens (BRSV, PI_3, *M. bovis,* and *M. dispar*) was evaluated for protection against naturally-occurring respiratory disease in beef calves (Howard *et al*., 1987a; Stott *et al*., 1987). Calves were vaccinated subcutaneously and received two boosters at 3 week intervals. In one study (Stott *et al*., 1987), three batches of beef calves aged 12, 7 and 3 weeks at the time of first vaccination were used, and calves were followed for 6 months. Respiratory disease occurred in a significantly higher ($P < 0.05$) proportion of the control calves (27%) compared with the vaccinates (16.3%). In a second study

(Howard *et al*., 1987a) using the same vaccination protocol, *M. bovis* and BRSV were implicated in outbreaks of respiratory disease during the trial period. Morbidity due to respiratory disease was significantly reduced in vaccinated calves (25%) compared with controls (32%), and mortality in the vaccinated group was similarly reduced (2% and 9% for vaccinates and controls, respectively). No adverse effects of vaccination were noted.

In a report of *M. bovis* vaccination of feedlot cattle (Urbaneck *et al*., 2000), a bacterin consisting of autogenous formalin-inactivated strains of *M. bovis* and *M. haemolytica* was used in 3,000 cattle at arrival. The feedlot had a history of *M. bovis*-associated clinical disease. The vaccine was reported to be efficacious for the prevention of respiratory disease in newly introduced cattle, but, unfortunately, comparisons were made to an historical control group. No adverse effects of vaccination were noted.

Despite the promise shown in some of the studies discussed above, other vaccine trials have been less successful. Rosenbusch (1998) vaccinated 2-month-old dairy calves with a formalin-inactivated bacterin prepared from two strains of *M. bovis*; calves received a single booster at 3 weeks post-vaccination. Calves were challenged by transthoracic inoculation of *M. bovis*. Vaccination exacerbated disease, with four of five vaccinated calves and one of five control calves developing severe respiratory disease. A similar exacerbation of disease was seen in calves vaccinated with partially purified membrane proteins from *M. bovis*; increased clinical disease and pathology following aerosol challenge was greater in vaccinated calves than in controls (Bryson *et al*., 1999).

Mycoplasma bovis vaccine antigens have been shown to exert some of the immuno-modulatory effects that are observed with live *M. bovis*, and these effects can be altered by the presence of specific adjuvants. For example, lymphocytes from calves inoculated subcutaneously

with killed *M. bovis* had reduced mitogen-induced and antigen-specific lymphoproliferative responses *in vitro*, while those inoculated with killed *M. bovis* in FCA exhibited increased responses (Bennett and Jasper, 1977b). Calves given the vaccine in FCA also developed higher serum antibody titers against *M. bovis*, and much greater immediate and delayed cutaneous hypersensitivity responses to *M bovis* antigens than did calves given the unadjuvanated vaccine.

Even where *M. bovis* vaccines have been associated with clinical benefits, they often fail to induce an immune response that clears infection (Chima *et al*., 1980; Nicholas *et al*., 2002). For example, intramuscular injection with formalin-killed *M. bovis* with adjuvant followed after 14 days by intratracheal inoculation with killed organisms without adjuvant resulted in reduced *M. bovis* in the lungs compared to control calves after intratracheal challenge, but significant numbers of mycoplasmas were still present in vaccinated calves (Howard *et al*., 1980). Induction of protective immune responses against *M. bovis* by vaccination is also complex. For example, in the aforementioned study (Howard *et al*., 1980), a vaccination protocol of three subcutaneous injections also induced protective responses, but two intramuscular or two intratracheal inoculations did not. In these studies, the number of *M. bovis* isolated from the lungs of calves was negatively correlated with IgG concentrations in BAL fluid, and different vaccination regimens were more or less effective at inducing an IgG response in the respiratory tract.

Despite very limited data on the field efficacy of *M. bovis* vaccines, several bacterin-based vaccines for *M. bovis* are licensed for marketing in the U.S. Currently, one vaccine is licensed for reducing the duration and severity of mycoplasmal mastitis in adult dairy cattle (Mycomune®; Biomune, Lenexa, KS). At least two vaccines are licensed for prevention of *M. bovis*-associated respiratory disease in cattle. One product (Myco-B Bac™; Texas Vet. Labs, Inc., San Angelo, TX), is aimed at stocker and feeder cattle. Another product (Pulmo-Guard™

MbP; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) is licensed for vaccination of cattle older than 45 days of age and is primarily marketed to the beef industry. The technical bulletin for Pulmo-Guard™ (Boehringer Ingelheim, 2003) describes two experimental challenge trials using 4- to 6-week-old Holstein calves, each trial using 10 vaccinated and five control animals. Calves were vaccinated twice, 2 weeks apart by subcutaneous injection. The company reports that vaccinated calves had reduced gross lung lesion scores, higher *M. bovis*-specific serum IgG_1 and IgG_2 titers, and higher levels of *M. bovis*-specific IgA in BAL fluid compared with controls. In addition to these vaccines, a number of U.S. companies are licensed to produce custom autogenous bacterins using strains of *M. bovis* isolated from the target herds. To the best of the author's knowledge, no controlled, peer-evaluated efficacy studies of any of the above commercial or autogenous bacterins have been reported. To date, no commercial *M. bovis* vaccines are labeled for use in young dairy calves in North America. The lack of well-designed, independent efficacy studies that include a valid control group, blinding of evaluators, adequate power, clinically relevant outcomes, and are conducted in an appropriate age group is a major gap in our understanding of the true potential value of currently available vaccines as a management strategy to control *M. bovis* infection.

In conclusion, vaccination against *M. bovis* is possible, but vaccines reported to date do not prevent colonization of the URT with *M. bovis*. Vaccination can also induce harmful effects. Probably the biggest challenge for the development of vaccines for use in dairy calves is the early age at which these calves often become infected. Achieving a protective immune response in young calves prior to challenge may be very difficult. New approaches, including investigation of passive transfer, the mucosal route of immunization and development of more sophisticated vaccines and delivery systems are needed. Also, appropriate field efficacy studies

of the available commercial and autogenous vaccines in North American dairy calf production systems are urgently needed.

Experimental Infection with *M. bovis* in Calves

A number of experimental models have been used to study *M. bovis* infection in calves. Various routes of inoculation have been employed, including inhalation of aerosolized bacteria, intranasal, intra- or transtracheal, endobronchial, transthoracic, intravenous, intraarticular or subcutaneous inoculation, as well as combinations of these routes (Chima *et al*., 1980; Howard *et al*., 1980; Pfutzner *et al*., 1983a; Ryan *et al*., 1983; Gourlay and Houghton, 1985; Lopez *et al*., 1986; Brys *et al*., 1989; Gourlay *et al*., 1989b; Nicholas *et al*., 2002; Vanden Bush and Rosenbusch, 2003). Although useful in the study of events associated with *M. bovis* infection at particular body sites, none of these models mimic the ingestion of *M. bovis*-contaminated milk, a major route of infection in young calves (Bennett and Jasper, 1977c; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). In addition, most experimental infection studies have been conducted in calves that are at least 2 weeks of age, whereas natural colonization with *M. bovis* often occurs in younger calves (Brown *et al*., 1998a; Stipkovits *et al*., 2000). Although neonatal calves are immunocompetent, their immune system responds differently to many antigens than that of older calves (Barrington and Parish, 2001), so selection of an appropriate age group is likely to be important for a model to accurately mimic natural disease. Importantly, the experimental models previously used to study *M. bovis* infection in calves did not induce clinical otitis media, which is a newly emerging disease in young calves. An experimental infection model to study the events that occur in the URT of young calves after exposure to *M. bovis* in milk, particularly those factors leading to the dissemination of infection and the development of otitis media and LRT disease, would be invaluable.

Summary and Critical Gaps in Knowledge

Mycoplasma bovis has emerged as an important pathogen of young dairy calves. A variety of clinical diseases are associated with *M. bovis* infections of calves, including respiratory disease, otitis media, arthritis and some other less common presentations. Clinical disease associated with *M. bovis* is often chronic, debilitating and poorly responsive to antimicrobial therapy, and current management strategies often fail to control clinical mycoplasmal disease. Thus, there is a critical need to develop better preventive, control and treatment strategies for *M. bovis*-associated disease in young calves. Improvements in these areas are hampered by a lack of understanding of the epidemiology of *M. bovis* infections in young calves and of the host-pathogen interactions involved in the establishment of infection and development of clinical disease. A number of critical gaps in knowledge need to be addressed:

- Other than the feeding of *M. bovis*-contaminated milk, few specific risk factors for *M. bovis* infections in young calves have been identified. In addition, risk factors associated with dissemination of *M. bovis* from the URT to the LRT and with clinical disease expression are poorly understood. Clearly, well designed epidemiological studies of *M. bovis* in infected calf-rearing facilities are required to establish risk factors and provide guidance for dairy producers to prevent and control disease. In addition, long-term epidemiological studies would be helpful to determine the impact of *M. bovis* infection in young calves on the risk of URT or mammary gland infection with *M. bovis* as adults. Prevalence estimates for *M. bovis*-associated disease in U.S. dairy calves have not been published and would be useful in determining the true extent of this problem and in estimating associated losses.

- Because effective biosecurity is probably one of the best ways to prevent *M. bovis* infections, studies to define the optimal diagnostic tests for determining the *M. bovis* infection status of young calves need to be conducted.

- Current treatment measures need to be critically evaluated. Controlled clinical trials evaluating the efficacy of particular therapeutic and metaphylactic antibiotic regimens for clinical disease in U.S. dairy calves are needed. In addition, the safety and efficacy of myringotomy and irrigation of the middle ear in calves with otitis media needs to be assessed.

- The role of passive transfer of maternal antibodies in *M. bovis*-associated disease needs to be defined from both epidemiological and immunological perspectives.

- Research into the microbial factors involved in the ability of *M. bovis* to colonize, persist, and cause disease in the host is ongoing, but many critical gaps in knowledge remain. This field will likely be greatly assisted by the *M. bovis* genome sequencing projects that are currently nearing completion. One factor in particular that needs to be addressed is to define whether specific surface antigens of *M. bovis* are involved in protective versus immunopathological responses.

- Current understanding of the pathogenesis of otitis media in young calves is extremely limited. Although current data from field and pathology studies indicate that *M. bovis* does cause otitis media in calves, experimental infection studies are required to fulfill Koch's postulates and to better define the host-pathogen interactions leading to this disease. In particular, the route of infection with otitis media needs to be defined. Whether other agents, such as viruses, increase the risk of mycoplasmal otitis media in calves also needs to be determined.

- The immune response to *M. bovis* infections appears to be complex. A much better understanding of the immune responses of young calves to *M. bovis* is needed. In particular, responses that contribute to development of disease or production of an effective immune response need to be determined; this knowledge may lead to improved vaccines against *M. bovis* infections. Specifically, the local innate and adaptive immune responses to *M. bovis* that are important at sites of infection in the URT, LRT and middle ear of young calves need to be defined. The lymphocyte populations and cytokines involved in these responses at the sites of infection also need to be determined. The role, if any, of hypersensitivity responses and IgE in *M. bovis*-associated disease needs to be investigated.

- Experimental models that mimic naturally occurring disease as closely as possible may improve our understanding of *M. bovis* infections in calves. Models that utilize the appropriate age group and a natural route of infection, so as to accurately represent events involved in establishment of URT infection, dissemination of *M. bovis* to other sites and development of clinical otitis media and LRT would be invaluable.

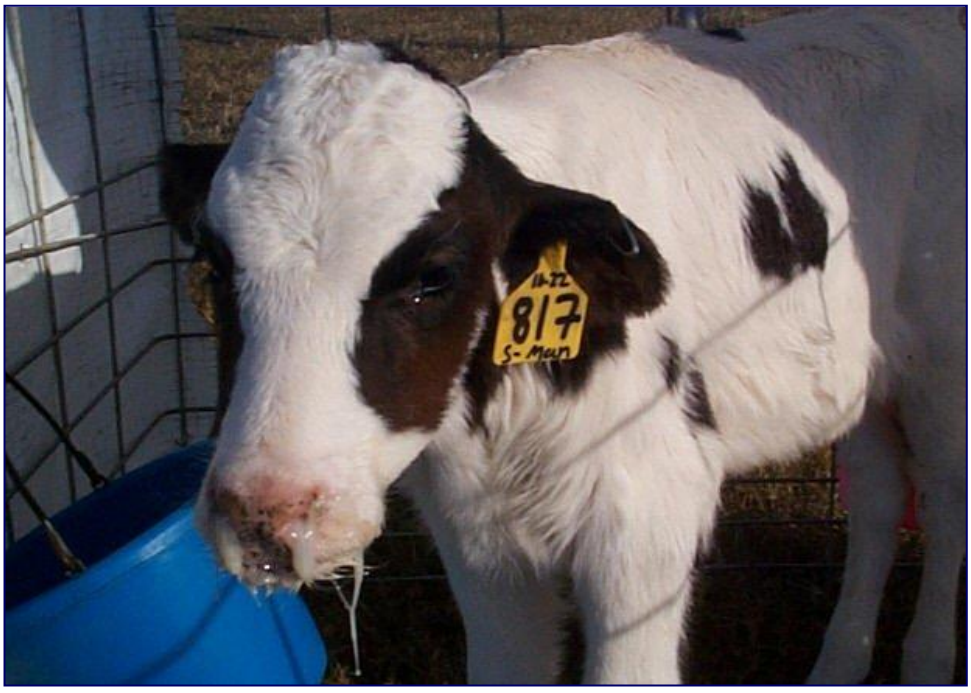
- In experimental challenge and field studies, efficacy of vaccination against *M. bovis* has been variable. Although some vaccines have reduced clinical disease, they do not prevent colonization and shedding; some have been associated with exacerbation of clinical disease. More sophisticated approaches to vaccine development and delivery systems and a better understanding of host immune response in mycoplasmal diseases would likely lead to improved vaccine strategies. A better understanding of the immunology of the neonatal calf, especially with respect to ability to respond to different antigens, the types of responses that are produced, and modulation of these responses by mucosal and systemic adjuvants may improve our ability to produce efficacious vaccines, if, indeed, vaccination of the very young calf against *M. bovis* is possible. The efficacy of the mucosal route for immunization of young calves against *M. bovis* needs to be evaluated in a relevant experimental infection model. In addition to research into new vaccination strategies, critical evaluation of currently marketed *M. bovis* vaccines for use in young calves in well designed, independent efficacy studies that include a valid control group, blinding, adequate power, relevant clinical outcomes and that are conducted in an appropriate age

group are clearly required. The lack of such studies is a major gap in understanding the potential of currently available vaccines as a management strategy to control *M. bovis* infections in young calves.

Overall Goals of Study

The overall goal of these studies was to address key deficiencies in the current knowledge of *M. bovis*-associated disease in young calves. Ultimately, these studies may lead to the development of improved preventative or control strategies for *M. bovis*. Because there is a lack of data on the efficacy of currently available *M. bovis* vaccines, especially in young calves, we conducted a field trial to determine the efficacy of a commercial vaccine for the prevention of *M. bovis*-associated disease in this age group. In addition to this field trial, the major focus of the studies presented here was to improve our knowledge of the local immune response to *M. bovis* in the respiratory tract of young dairy calves. This second main objective involved development of a reproducible model of *M. bovis* infection of the URT that closely mimicked natural infection in young dairy calves. This model was then used to define the lymphocyte responses generated along the respiratory tract during infection with *M. bovis*.

A



B

Figure 1-1. Clinical manifestations of *Mycoplasma bovis*-associated respiratory disease. A) Calf with purulent nasal discharge as well as a right ear droop. B) Calf with purulent nasal discharge.

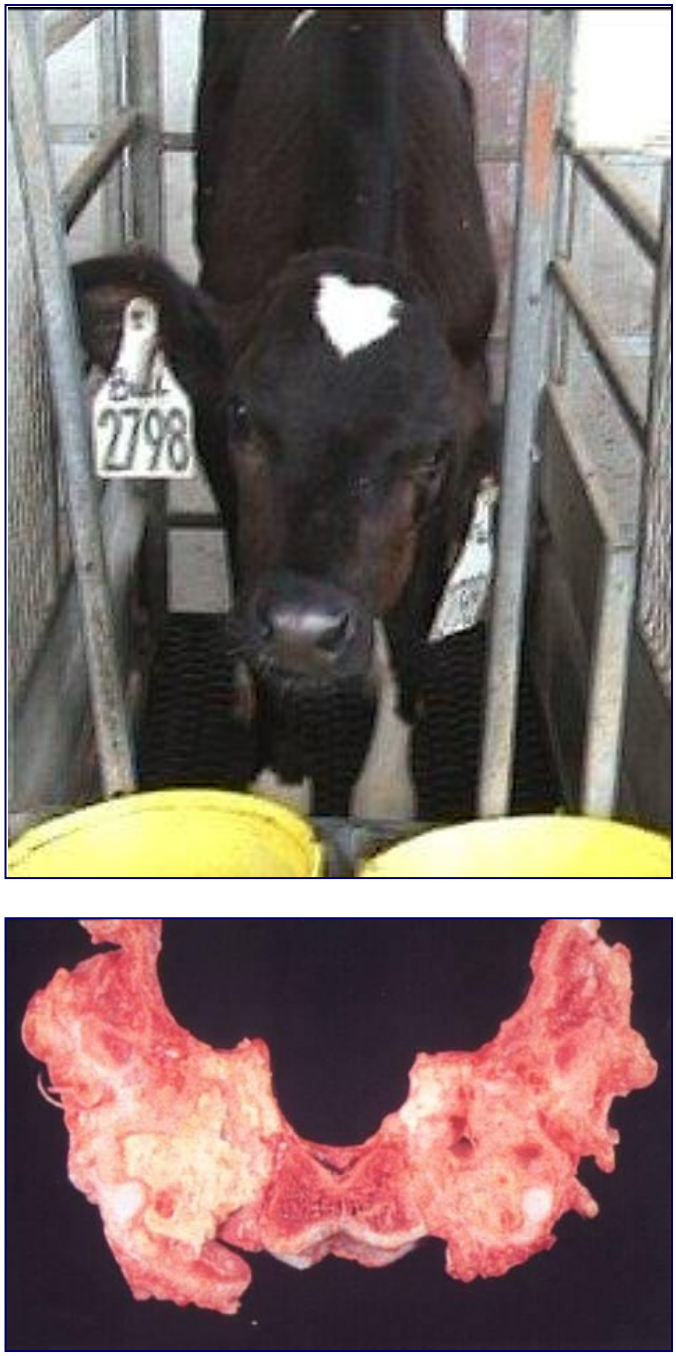
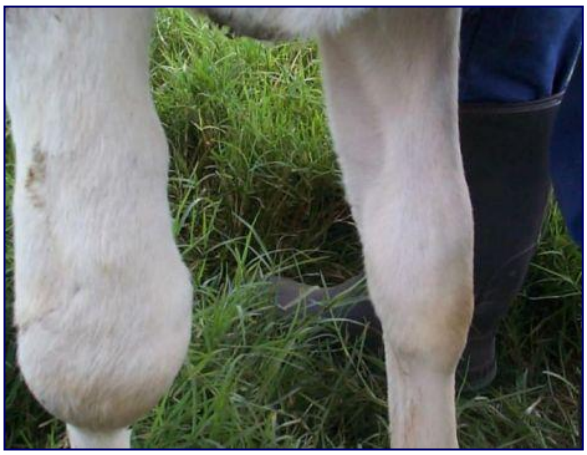


Figure 1-2. Clinical manifestations and macroscopic lesions of *Mycoplasma bovis*-associated otitis media. A) Calf with left ear droop and epiphora. B) Transverse section of skull at the level of the tympanic bullae. Bullae are impacted with caseous exudate, especially on the left side.



A

B

C

Figure 1-3. Clinical manifestations and macroscopic lesions of *Mycoplasma bovis*-associated arthritis and tenosynovitis. A) Swollen right carpal joint and proximal forelimb due to *M. bovis* arthritis and tenosynovitis. B) Flexed carpal joint with an incision into the extensor tendons containing purulent exudate, as well as dermal necrosis over the carpal joint. C) Incision into the dorsal aspect of the carpal joint containing copious purulent exudate.

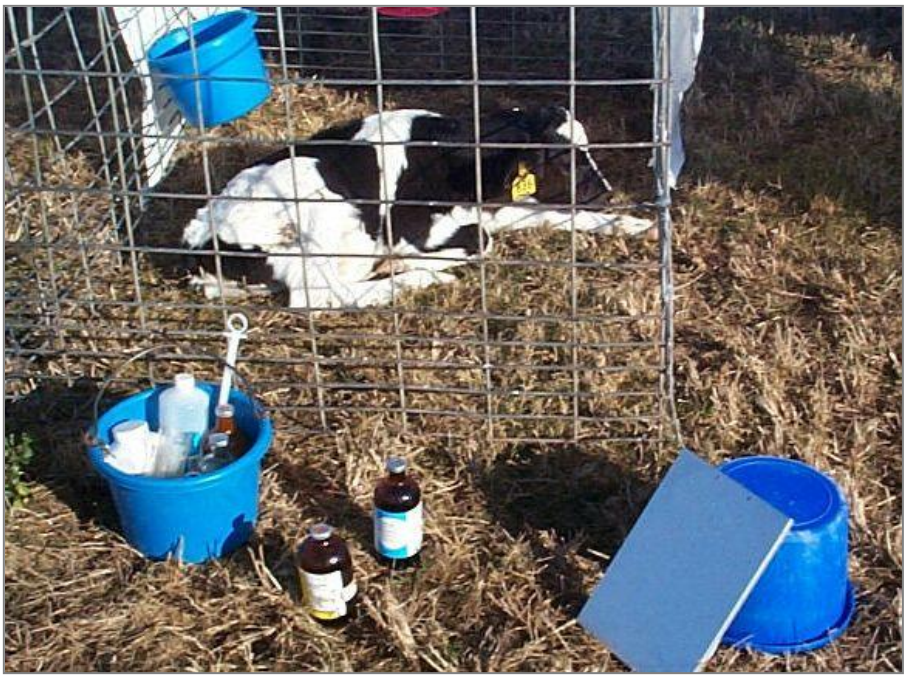


Figure 1-4. Substantial economic costs are incurred for treatment and management of calves with *Mycoplasma bovis*-associated disease.

Figure 1-5. Ingestion of milk contaminated with *Mycoplasma bovis* is a primary route of transmission in pre-weaned calves.

CHAPTER 2
FIELD EVALUATION OF A *Mycoplasma bovis* BACTERIN IN YOUNG DAIRY CALVES

Introduction

Mycoplasma bovis is distributed world-wide and is a significant pathogen of adult dairy cows as well as intensively reared beef and dairy calves (Brown *et al*., 1998a; Stipkovits *et al*., 2001; Thomas *et al*., 2002a; Fox *et al*., 2003; Gonzalez and Wilson, 2003; Nicholas and Ayling, 2003; Lamm *et al*., 2004; Gagea *et al*., 2006). Clinical manifestations include mastitis, respiratory disease, otitis media, polyarthritis, and tenosynovitis (Adegboye *et al*., 1996; Brown *et al*., 1998a; Step and Kirkpatrick, 2001a; Step and Kirkpatrick, 2001b; Nicholas and Ayling, 2003). Although the pathogenicity of *M. bovis* is well-established, the disease patterns associated with the microorganism are variable. Outbreaks can be acute with substantive morbidity and mortality or manifest as endemic disease with sporadic cases (Rodriguez *et al*., 1996; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000; Nicholas and Ayling, 2003; Gagea *et al*., 2006). In addition to its role as a primary pathogen, *M. bovis* also exhibits synergism with other pathogens in the bovine respiratory disease complex (Houghton and Gourlay, 1983; Gourlay and Houghton, 1985; Lopez *et al*., 1986; Thomas *et al*., 1986; Virtala *et al*., 1996b; Shahriar *et al*., 2000; Poumarat *et al*., 2001; Gagea *et al*., 2006).

In the past decade, *M. bovis* has emerged as an important cause of respiratory disease, otitis media and arthritis in pre-weaned calves (Brown *et al*., 1998a; Stipkovits *et al*., 2000; Stipkovits *et al*., 2001; Nicholas and Ayling, 2003; Lamm *et al*., 2004). Onset of clinical disease occurs between 2 and 6 weeks of age. The disease is chronic and poorly responsive to antibiotic therapy (Gourlay *et al*., 1989a; Allen *et al*., 1992a; Adegboye *et al*., 1995a; Apley and Fajt, 1998; Shahriar *et al*., 2000; Stipkovits *et al*., 2000; Gagea *et al*., 2006). In herds with clinical *M. bovis* disease, a high prevalence of upper respiratory tract (URT) colonization occurs in

healthy calves, suggesting that only a subset of calves develop clinical disease (Bennett and Jasper, 1977c; Springer *et al*., 1982; Allen *et al*., 1992a; ter Laak *et al*., 1992a; Brown *et al*., 1998a; Mettifogo *et al*., 1998). Morbidity and mortality occurs as a result of respiratory infection, otitis media, and arthritis, acting alone or in concert. Respiratory infection occurs when *M. bovis* spreads from the URT to the lower respiratory tract. Otitis media occurs when *M. bovis* spreads to the middle ear, probably via the eustachian tube. Respiratory disease and otitis can present independently, together, or sequentially. Arthritis occurs as a result of hematogenous spread with localization in joints, usually as sequelae to respiratory disease. Multiple joints are often affected, and mortality is frequently observed in arthritic calves.

Ingestion of contaminated milk, especially unpasteurized waste milk, has been identified as an important primary route of transmission of *M. bovis* to young calves (Pfutzner and Schimmel, 1985; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). The role of colostrum in transmission is less well-established in dairy calves, but is known to be important in small ruminant mycoplasmal disease (DaMassa *et al*., 1983). Once infection has established, aerosol droplet and direct contact probably play an important role in calf-to-calf transmission (Jasper *et al*., 1974; Bennett and Jasper, 1977c; Nicholas and Ayling, 2003). The economic consequences of infection are primarily associated with intensive treatment of affected calves coupled with culling of animals that are unresponsive to therapy (Nicholas and Ayling, 2003). Control of *M. bovis* infection in calves focuses on removal of identified risk factors for acquisition of *M. bovis.* Removal of infected milk from the diet by pasteurization or feeding of milk replacer has been successfully applied to reduce infection (Pfutzner and Meeser, 1986; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000; Stabel *et al*., 2004); breaks in pasteurization have been associated with subsequent infection outbreaks. Management practices

to reduce stocking density and improve ventilation are examples of changes that can reduce undifferentiated respiratory disease and are often recommended for *M. bovis* control (Ames, 1997; Rosenbusch, 2001; Step and Kirkpatrick, 2001a). Similarly, control of other pathogens that are involved in the bovine respiratory disease complex is likely to reduce *M. bovis* infections. At the level of the calf, management techniques that improve general immune function, such as improving nutritional status and minimizing environmental stress, have been suggested as beneficial (Rosenbusch, 2001; Step and Kirkpatrick, 2001a). Vaccination is a potential strategy to control infection, but as discussed in Chapter 1 and briefly summarized below, efforts to develop efficacious vaccines have been problematic.

Mycoplasma bovis vaccines have afforded some protection from respiratory disease in European field trials (Howard *et al*., 1987a; Stott *et al*., 1987; Urbaneck *et al*., 2000). Other vaccines have been efficacious against respiratory disease (Howard *et al*., 1980; Nicholas *et al*., 2002), and arthritis (Chima *et al*., 1980; Chima *et al*., 1981; Nicholas *et al*., 2002) in experimental challenge studies. Importantly, in some cases vaccination has significantly exacerbated clinical disease (Rosenbusch, 1998; Bryson *et al*., 1999). Most vaccine studies have been performed in calves that are older than the age at which colonization with *M. bovis* is often first observed (Stipkovits *et al*., 1993; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000; Stipkovits *et al*., 2001). There are several commercial *M. bovis* vaccines currently marketed in the U.S., as well as a number of companies that manufacture autogenous *M. bovis* bacterins. However, none are licensed for use in young dairy calves, and, to the best of the author's knowledge, no independent studies have been published on their efficacy. Thus, there is a critical gap in the knowledge of vaccine strategy and efficacy for protection of the young dairy calf that is at risk for otitis media, pneumonia, and arthritis.

In order to address the lack of knowledge on the efficacy of currently available vaccines in young calves, we conducted a field trial using a commercial *M. bovis* bacterin that was approved for use in feeder and stocker calves. The objective of this field trial was to determine the efficacy of this commercially produced *M. bovis* bacterin for the prevention of *M. bovis*-associated disease (respiratory disease, otitis media, arthritis) and mortality in dairy calves from birth to 90 days of age. Additional objectives were to compare vaccinated and placebo-treated calves with respect to 1) weight gain from birth to 90 days of age, 2) rates of nasal colonization by *M. bovis*, and 3) *M. bovis*-specific serum immunoglobulin (Ig) concentrations.

Methods

Study Populations

We studied 373 Holstein heifers in three Florida herds using a randomized field trial design. The reference population for this study was heifer calves in Florida dairy herds with endemic *M. bovis* infection. The study unit was a Holstein heifer calf clustered in one of three herds in north-central Florida. Herds were selected based on their willingness to participate and on a history of mycoplasma-associated disease in calves. According to calf health records, at least 15% of calves were treated for respiratory disease, otitis media and/or arthritis during each of the 2 years preceding the study.

Herd A, containing approximately 500 lactating cows, was the University of Florida Institute of Food and Agricultural Sciences Dairy Research Unit. Calves were bedded on sand in individual hutches placed approximately 1 m apart, in an open-sided barn (Figure 2-1A). Calves were fed unpasteurized bulk tank milk. Calves received a modified live virus (MLV) intranasal vaccine against parainfluenza virus type 3 (PI_3) virus and infectious bovine rhinotracheitis virus (IBR) in the first week of life. An intramuscular MLV vaccine against IBR, PI_3, bovine respiratory syncytial virus (BRSV) and bovine viral diarrhea virus (BVDV) types 1 and 2 was

administered at 2, 6 and 8 weeks of age. A 7-way clostridial vaccine was administered at 2 and 6 weeks of age. Calves were weaned at approximately 6 weeks of age and turned out into group pens at approximately 8 weeks of age.

Herd B was a commercial herd of approximately 750 lactating cows. The majority of calves were housed in individual elevated metal crates in a concrete-floored open-sided barn (Figure 2-1B), with some calves housed on grass in individual hutches. Calves housed in metal crates had nose-to-nose contact with neighboring calves. The calf feeding protocol varied during the study period and included milk replacer and unpasteurized or pasteurized waste milk. The vaccination protocol was similar to that described for Herd A. Calves were weaned at 6 to 8 weeks of age and turned out into group pens at 8 to 10 weeks of age.

Herd C was a commercial herd of approximately 1,000 lactating cows. Calves were housed on grass in individual hutches placed at least 1 m apart (Figure 2-1C). Calves were primarily fed pasteurized waste milk, supplemented with milk replacer when necessary. Several failures of pasteurization were documented during the study period. Calves received an oral bolus containing antibodies against bovine coronavirus and *Escherichia coli* at the time of colostrum feeding (First Defense, Portland, ME). The vaccination protocol for MLV intranasal PI_3/IBR and clostridial vaccines was similar to that described for Herd A. An intramuscular MLV vaccine against PI_3, IBR, BVDV types 1 and 2, and BRSV was administered at 4 and 8 weeks of age. Calves were weaned at 6 to 8 weeks of age and turned out into group pens at 8 to 10 weeks of age.

Study Design

All Holstein heifer calves that were born during the study period and were considered healthy by the producer at 3 days of age were enrolled in the study. The enrollment period extended from March to December, 2002. Calves were assigned to either a vaccinated or a

control group based on ear tag numbers, with odd numbers assigned to one group and even numbers to the other group. Assignment of odd and even numbers to groups was decided on a per farm basis by a coin flip. A 1 ml dose of a heat-inactivated, single strain, *M. bovis* bacterin in proprietary oil-based adjuvant that had a conditional license for use in U.S. feeder and stocker calves (Texas Vet Lab, Inc.) or a sterile vaccine vehicle (control group) was administered subcutaneously in the neck at 3 days and 2 weeks of age. A 2 ml booster dose was administered at 5 weeks of age. Vaccine or placebo boosters were not administered to calves that were sick at 2 weeks of age; however, if the calf recovered within 5 days, then the booster was administered at 3 weeks of age. Calves that failed to recover within 5 days remained in the study but were coded as "booster 1 missed". A similar protocol was followed for calves that were sick at the time of their 5 week booster. The bacterin and placebo were prepared and coded by the vaccine manufacturer. Investigators and farm personnel were blinded throughout data collection and analysis. Data recorded for each calf included date of birth, ear-tag number, group allocation, dates of vaccine/placebo administration, and date of weaning. The dates of administration of any preventative treatments or other vaccines were recorded for each calf.

The primary outcomes of interest were treatment for respiratory disease, otitis media, and arthritis, as well as mortality attributed to these diseases. Calves were followed until 90 days of age, and all treatment for clinical disease was recorded by farm personnel using standardized case definitions (Table 2-1). Sick calves were treated as per normal farm protocols. For each clinically-ill calf, farm personnel recorded the type and dose of antimicrobial, the date(s) of treatment, and the reason for treatment. Whenever a calf died, farm personnel recorded the cause of death if this was obvious. In most cases, cause of death was verified by field necropsy performed by the investigators. Study personnel visited each of the dairies at least once a week to

collect calf health data, monitor compliance, and collect samples. Because passive transfer of colostral immunoglobulins can influence the immune response to vaccination or to infectious agents, blood was collected from all calves between 2 and 9 days of age for the measurement of total serum protein concentration.

A subset of calves from Herds A (n=40) and B (n=60) was studied more intensively. These calves were weighed at birth and approximately 90 days of age. Weight gain from birth to 90 days was expressed in kg/day. Nasal swabs (Figure 2-2A) and blood samples (Figure 2-2B) were collected weekly until 8 weeks of age and then at 90 days of age. Serum was analyzed for *M. bovis*-specific IgA, IgM, IgG_1 and IgG_2 by enzyme-linked immunosorbent assay (ELISA). Swabs were cultured to detect nasal colonization with *M. bovis*.

Collection and Processing of Nasal Swabs

Prior to collecting nasal swabs, gross debris was wiped from the external nares using sterile gauze. A sterile rayon-tipped swab with a polyurethane plastic shaft (BBL™ CultureSwab™ Liquid Stuart Medium, BD, Franklin Lakes, NJ) was inserted into the ventral nasal meatus to a depth of approximately 4 inches. Swabs were kept on ice during transport and were processed within 6 hr of collection. Each swab was used to streak the surface of modified Frey's agar. All mycoplasma cultures were performed in modified Frey's broth and agar medium containing 2.25% (wt./vol.) Mycoplasma broth base (Frey) (BD Diagnostic Systems, Sparks, MD), 0.02% (wt./vol.) DNA from herring sperm, 20% (vol./vol.) horse serum, 10% (vol./vol.) fresh yeast extract, 0.5% (wt./vol.) glucose, and supplemented with 100,000 U/l each of penicillin G and polymixin B and 65 mg/l of cefoperazone, with the final pH adjusted to 7.6 to 7.8. Plates were incubated at 37°C in 5% CO_2 and examined at 2, 4, 7 and 10 days for mycoplasmal growth. Colonies with typical *M. bovis* morphology were plugged into broth, incubated at 37°C for

48 hours and stored at -80°C until they could be confirmed as *M. bovis* by polymerase chain reaction (PCR). Samples were confirmed as *M. bovis* by PCR amplification of the *uvr*C gene.

To prepare samples for PCR, 500 µl of broth culture was thawed at room temperature then pelleted by centrifugation at 14,000 rpm at 4°C for 1 hr. The supernatant was discarded and the pellet resuspended in 20 µl of lysis buffer (100 mM tris [hydroxymethyl] aminomethane, pH 7.5 with 0.05% [vol./vol.] Tween 20 and 6.5 mM dithiothreitol). Samples were incubated at 99°C for 20 min then cooled to 20°C. 5 µl of clarified sample was used as the DNA template in the PCR. As a positive control, broth was inoculated with the *M. bovis* type strain (ATCC 27368) and processed with nasal isolates. Sterile water was used as a negative control template.

Mycoplasma bovis was identified by PCR of the housekeeping gene *uvr*C (Subramaniam *et al*., 1998). PCR reactions were carried out in a total volume of 50 µl containing 5 µl of template, 2.5 U *Taq* DNA polymerase (Promega Corporation, Madison, WI), 3 µl of 25 mM $MgCl_2$ (final concentration 2.0 mM, Promega), 5 µl of 10X reaction buffer (final concentration 50 mM KCl, 10 mM Tris pH 9.0, 0.1% [vol./vol.] Triton X-100, Promega), 2 µl of a mixture of equal parts 10 mM deoxyribonucleotide triphosphates, 1 µl of each primer (final amount 20 pmol, commercially synthesized), and 32.75 µl of sterile, purified DEPC-treated water. The primers used in the PCR were Mbo*uvr*C2-L (5′-TTACGCAAGAGAATGCTTCA-3′) and Mbo*uvr*C2-R (5′-TAGGAAAGCACCCTATTGAT-3′), corresponding to bases 362 to 381 and 1988 to 1969 in the *uvr*C coding sequence (Genbank AF003959), respectively. The PCR cycling conditions were initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, polymerization at 72°C for 60 sec, and a final extension for 10 min at 72°C. PCR products were analyzed by electrophoresis at 110 V for 1 hr in 1.5% agarose gels and visualized by staining with ethidium bromide.

The ELISA Procedure

Blood samples were allowed to clot after collection, and then serum was harvested by centrifugation and stored at -80ºC. Whole-cell lysate antigen (Schumacher *et al*., 1993) was prepared from a 1 liter culture of *M. bovis* type strain PG45 grown at 37°C in modified Frey's broth. The protein concentration was determined using a colorimetric assay (Bio-Rad, Hercules, CA) and adjusted to 100 μg/ml. The antigen was stored in aliquots at -80°C and thawed at room temperature when required. The ELISA procedure was optimized using standard methodology. Microtiter plates (Maxisorb F96, Nunc, Kamstrup, Denmark) were coated with 20 μg per well of antigen in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% (wt./vol.) NaN_3 (PBS/A), and incubated overnight at 4°C. Plates were then washed three times with PBS/A containing 0.05% (vol./vol.) Tween 20 (PBS/T) using an automated plate washer (ELx405 Auto Plate Washer, BioTek Instruments, Inc., Winooski, VT), blocked with 300 μl per well of blocking buffer (PBS/T containing 1% [wt./vol.] egg albumin), and stored at 4°C for a minimum of 24 hr or until needed. Sera were diluted (1:100 for IgG_1 assay; 1:50 for IgM and IgG_2 assays; 1:25 for IgA assay) in blocking buffer and 50 μl of the diluted serum was added to duplicate wells; plates were incubated at room temperature for 1 hr. Plates were washed as described above and 50 μl of goat anti-bovine isotype conjugated to alkaline phosphatase (Bethyl Laboratories Inc., Montgomery, TX) and diluted to 1:1,000 in blocking buffer was added to each well. Plates were incubated at room temperature for 2 hr and then washed as described above. 100 μl of 0.1% (wt./vol.) p-nitrophenol phosphate was added to each well and plates were incubated in the dark at room temperature for 1 hr. The optical density (OD) in each well was read at a wavelength of 405 nm using an automated plate reader (ELx808 Ultra Microplate Reader, BioTek Instruments, Inc., Winooski, VT). For each microtiter plate, the blank was the

mean value for two wells coated with antigen and incubated with the conjugated secondary antibody and substrate only. The blank OD value was subtracted from each sample well, and mean values for each pair of duplicate tests calculated.

A pool of sera from 20 calves with naturally occurring mycoplasmal disease and high *M. bovis*-specific titers were included on each plate as a positive control; the negative control was a pool of serum collected from the same 20 calves prior to ingestion of their first colostrum meal. The cutoff for a positive titer was the average OD value (minus the blank) for the negative control sera plus two standard deviations, established over ten assay runs. The highest dilution of the test serum that gave an average OD value higher than the cutoff was defined as the titer for that sample. Within-batch and between-batch assay variability was assessed by using the Youden plot graphic method (Jeffcoate, 1982). The ELISA values obtained for the lowest, middle and highest dilution of the control serum included on each plate were used to establish target values and control limits to be used for monitoring the consistency of the assay (ten batches). The values obtained at the beginning of a series of assays were plotted against the values obtained for the same standards at the end of the series. If values for the pooled sera deviated more than 10% from target values, the assay was repeated.

Field Necropsy

A standard field necropsy was performed by one of the study veterinarians on most calves that died or had to be euthanized during the study. Calves were examined to determine the cause of death and specifically determine the involvement of *M. bovis* associated pathology. All necropsies included culture of swabs of the palatine tonsils, tympanic bullae and primary bronchi for mycoplasmas. Additionally, if the animal had previously been diagnosed with respiratory disease, arthritis or otitis media, or if any macroscopic lung pathology was observed, appropriate samples were collected from the lesion site(s) to determine the involvement of *M. bovis* as well

as other viral and bacterial respiratory pathogens. Further samples were collected when deemed necessary to determine the cause of death by the veterinarian performing the necropsy. All swabs and fresh tissue samples were transported on ice to the laboratory as soon as possible and were processed within 24 hr after collection. When tissue samples for fixation were collected, they were placed into containers of 10% buffered formalin and submitted to the Diagnostic Pathology Service, College of Veterinary Medicine, University of Florida. Samples then were embedded in paraffin and sections (5 μm) stained with hematoxylin and eosin. Histopathology was read by diagnostic pathologists without knowledge of experimental treatment groups. In addition to culture for mycoplasmas (described under nasal swabs, above), swabs for aerobic microbiological culture were processed and isolates identified using routine clinical bacteriological methods. These methods were focused on identifying bacterial pathogens of the respiratory tract other than mycoplasmas, particularly *Arcanobacterium pyogenes, Histophilus somni, Mannheimia haemolytica* and *Pasteurella multocida* as well as pathogens that may cause septicemia and associated sequelae in young calves. Additional diagnostic testing was performed as requested by the veterinarian who conducted the necropsy based on the presumptive diagnosis and any macroscopic pathology. Samples were submitted to the Florida State Diagnostic Laboratory (Kissimmee, FL) for detection of bovine respiratory viruses when indicated.

Sample Size

Morbidity due to *M. bovis* was the major outcome of interest and was therefore used to calculate sample size. At the time the study was initiated, health records indicated that the incidence of respiratory disease, otitis media and/or arthritis in the study herds was at least 15%. We hypothesized that a reduction in incidence to 5% would be biologically and economically significant. Using these values together with 95% confidence and 80% power, and taking into

consideration an attrition rate of approximately 10%, the calculated sample size was 180 calves per group.

For the secondary outcomes of interest from a subset of calves in Herds A and B, we hypothesized that a reduction in the nasal colonization rate from 50% to 20% would be biologically significant. Using these values together with the parameters outlined above, the calculated sample size was 50 calves per group.

Statistical Methods

Calves were excluded from analyses if clinical signs referable to other organ systems occurred concurrently with respiratory disease, otitis media or arthritis, with the exception of diarrhea without fever of less than 7 days duration. Categorical variables were compared among groups using Chi-square tests; data were analyzed for effects of herd and passive transfer status by Stratified Mantel-Haenszel analysis. Simple continuous variables were compared among groups using t-tests, and ELISA data were analyzed using repeated measures ANOVA. Analyses were performed using commercial statistical software (SPSS 12.0, SPSS Inc, Chicago IL).

Results

Between March and December, 2002, 328 calves from Herds B and C (166 and 162 calves, respectively) were enrolled and were eligible for inclusion in the study (Table 2-2). Despite a history of *M. bovis* infection, Herd A did not experience any *M. bovis*-associated disease during the study and therefore is excluded from some analyses, but data from this herd are included where relevant.

The incidence risk for clinical respiratory disease, otitis media, and arthritis was assessed from birth to 90 days of age (Table 2-3). *Mycoplasma bovis*-associated respiratory disease and otitis media were major contributors to calf disease in Herds B and C. One case of arthritis was observed in Herd B, and none were observed in Herd C. Herd A had a much lower overall

mortality risk (0.02) than did Herds B (0.13) and C (0.10); *M. bovis*-associated mortality in Herd B accounted for the majority of the mortality risk (0.10 vs. 0.13 overall).

The baseline data for vaccinated and control animals are shown in Table 2-4. Vaccinated and control groups had equivalent levels of post-colostral total serum protein. A small percentage of calves did not receive their second vaccine due to illness. In Herd B, no control calves missed the third vaccine as opposed to 9% of the vaccine group that missed this vaccination ($P = 0.005$).

Vaccination did not influence the age of first treatment for either otitis media or respiratory disease (Table 2-5). Similarly, vaccination neither reduced overall *M. bovis*-associated morbidity nor altered the temporal expression of disease in either herd (Table 2-6). Morbidity specifically associated with respiratory disease also was not affected by vaccination (Table 2-7). The incidence of otitis media was higher ($P = 0.004$) in vaccinated calves than in control calves in Herd B, but no differences in the incidence of otitis media between groups were observed in Herd C (Table 2-8). There was no difference between vaccinated and control calves in Herd B in the age of first treatment for otitis media (data not shown). There was no significant difference between vaccinated and control calves with respect to mortality (Table 2-9). For Herds A and B, weight gain was monitored from birth to 90 days of age; no significant difference was observed in average daily gain between groups in Herd A. Similarly, no significant difference was observed in average daily gain between vaccinated (0.48 ± 0.18 kg/day, n=27) and control (0.54 ± 0.11 kg/day, n=29) calves in Herd B, where endemic *M. bovis* disease was present.

Nasal colonization was not affected by vaccination; in Herd B where endemic *M. bovis* disease was present, the mean percentage (± SEM) of calves with *M. bovis*-positive nasal swabs at each sampling time was 81.4 ± 8.2% for vaccinated calves and 75.8 ± 6.7% in control calves.

The average number of sampling times that *M. bovis* was recovered from each calf was also not affected by vaccination. However the temporal pattern of colonization observed in calves from Herd A (no mycoplasmal disease) was quite different from that observed in calves in Herd B (significant mycoplasmal disease; Figure 2-3). Calves in Herd A had minimal to no nasal shedding of *M. bovis* during the pre-weaning period. Calves in Herd A were moved out of individual hutches into group pens after the 8 week samples were collected; at the next sampling period (12 weeks of age), the level of nasal colonization was similar to that in the herd that experienced *M. bovis*-associated disease. In Herd B, calves were shedding *M. bovis* as early as 1 week of age, and by 3 weeks of age over 70% of calves were colonized in the URT. This level of colonization was maintained throughout the sampling period.

The serum antibody subclass response in a subset of calves in Herds A and B was assessed by ELISA. No significant differences between vaccinated and control calves were found in either Herd A or B for IgA (Figure 2-4), IgM (Figure 2-5), or IgG_2 (Figure 2-6). However, vaccination did induce a serum IgG_1 response (Figure 2-7). Significant differences ($P < 0.05$) between vaccinated and control groups were first evident at 7 weeks of age in Herd A (no endemic disease) and at 12 weeks of age in Herd B (endemic disease). We then assessed if there was an association between Ig subclass response and morbidity in calves from Herd B. There was no association of any Ig subclass response with morbidity, nasal colonization rate, or weight gain.

Interestingly, there was no significant association between post-colostral total serum protein concentrations and the incidence or duration of treatment for respiratory disease or otitis media in Herds B and C (data not shown). Similarly, the incidence of *M. bovis*-specific calf mortality in Herds B and C was not associated with post-colostral total serum protein

concentrations (data not shown). There was also no association between *M. bovis*-specific IgA, IgM, IgG_1 or IgG_2 post-colostral serum titers and either morbidity or mortality in a subset of calves in Herd B (data not shown).

Discussion

The commercial *M. bovis* bacterin tested in this trial was not efficacious in prevention of either *M. bovis*-associated respiratory disease or otitis media in pre-weaned calves in two north-central Florida herds with endemic *M. bovis* disease. The response to vaccination was herd-dependent, and a higher rate of otitis media was associated with vaccination in one herd.

Other investigators have reported some protection from mycoplasmal respiratory disease by subcutaneous vaccination of calves with killed whole cell bacterins (Howard *et al*., 1980; Howard *et al*., 1997; Nicholas *et al*., 2002). In a study of an apparently efficacious vaccine in young calves, Nicholas *et al*., (2002) vaccinated 3-week-old dairy calves with a single dose of saponin-inactivated bacterin. Calves received an aerosol challenge with live *M. bovis* 3 weeks after vaccination. Vaccinated calves had fewer numbers of *M. bovis* at colonized sites, fewer body sites colonized by *M. bovis,* and reduced severity and incidence of clinical disease and lesions as compared to control calves. There was also a significant decrease in body weight gain in control calves compared with vaccinates. Additionally, no vaccinated calves and two of seven control calves developed arthritis. Vaccinated calves produced a strong IgG response prior to challenge, but IgG subtypes were not reported. No adverse events associated with vaccination were reported.

A killed vaccine against four bovine respiratory pathogens (BRSV, PI_3, *M. bovis*, and *M. dispar*) was evaluated for protection against naturally-occurring respiratory disease in beef calves (Howard *et al*., 1987a; Stott *et al*., 1987). Calves were vaccinated subcutaneously and received two boosters at 3 week intervals. In one study (Stott *et al*., 1987), three groups of beef

calves aged 12, 7 and 3 weeks at the time of first vaccination were used, and calves were followed for 6 months. Respiratory disease occurred in a significantly higher ($P < 0.05$) proportion of the control calves (27%) compared with the vaccinates (16.3%). In a second study (Howard *et al*., 1987a) using the same vaccination protocol, *M. bovis* and BRSV were implicated in outbreaks of respiratory disease during the trial period. Morbidity due to respiratory disease was significantly reduced in vaccinated calves (25%) compared with controls (32%), and mortality in the vaccinated group was similarly reduced (2% and 9% for vaccinates and controls, respectively). No adverse effects of vaccination were noted.

There are a number of key differences between the studies reported above and our study that may have influenced vaccine efficacy. Firstly, the strain of bacteria, the antigen concentration, the method of bacterial inactivation and the adjuvant used are all factors that exert significant effects on the efficacy of bacterial vaccines, although there are limited data on how these affect *M. bovis* vaccines in particular. Although some of these data are not reported in the above studies, and some are not available for our vaccine (e.g. the adjuvant used is proprietary), it is likely that all these factors varied significantly between our study and those listed above. Secondly, calves in the above studies were first vaccinated at a substantially older age than the calves in our study. As discussed in Chapter 1, immune responses of the newborn calf have unique characteristics and undergo rapid changes during the first few weeks of life (Barrington and Parish, 2001). Vaccination at 3, 14 and 35 days of age (as was performed in our study) may not elicit the same type of immune response as vaccination at 3 weeks of age (as in the Nicholson *et al*., 2002 study, above). Our vaccination protocol was chosen based on a) protocols that were being applied on dairies in Florida, and b) the early age of infection that had been observed in previous studies (Brown *et al*., 1998a). Thirdly, calves in endemically-infected herds

in our study became colonized at a very early age, meaning that infection was likely well established before a vaccine-induced immune response could develop. As discussed in Chapter 1, adaptive immune responses that develop after infection are very inefficient at clearing mycoplasmal infections and often result in detrimental chronic inflammatory responses. Lastly, the challenge load of *M. bovis* that calves are exposed to can affect the efficacy of vaccination. Given the high incidence of clinical mycoplasmal disease and the early age of colonization observed in our endemically-infected herds, the level of *M. bovis* challenge that calves were exposed to may have been significantly greater than that of the calves in other vaccine studies (Howard *et al*., 1980; Howard *et al*., 1997; Nicholas *et al*., 2002).

Vaccinated calves in one herd in our study had a greater risk of otitis media than did control calves. The risk of otitis media in control calves in Herd B seemed substantially less than that in Herd C, but examination of calf health records from previous years in Herd B showed that the risk of otitis media observed in control calves was similar to that which had been historically present (data not shown). Therefore, vaccination seemed to exacerbate clinical otitis media in this herd. There are other reports of exacerbation of clinical disease following *M. bovis* vaccination (Boothby *et al*., 1987; Rosenbusch, 1998; Bryson *et al*., 1999). However, the immune mechanisms associated with adverse outcomes after *M. bovis* vaccination have not been determined.

Vaccination of calves did stimulate a systemic humoral immune response, with an increase in serum IgG_1 being detectable after the third vaccination. A tendency towards Th2-biased IgG_1-dominated humoral responses has also been reported after infection of calves with *M. bovis* (Howard *et al*., 1987c; Vanden Bush and Rosenbusch, 2003). As IgG_2 is a much more effective opsonin for phagocytosis of *M. bovis* than is IgG_1, it is not surprising that an IgG_1

response is ineffective for control of *M. bovis* respiratory infections (Howard *et al*., 1976). It is somewhat puzzling that a humoral response to infection was not obvious in control calves in Herd B where there was a high incidence of *M. bovis*-associated disease. Statistical comparison of IgG_1 responses in control groups in Herds A and B was not conducted. However, it appears that in the control group in Herd A, post-colostral IgG_1 antibody levels continued to decline throughout the study period (see Figure 2-7), whereas in the control group in Herd B, they did not decline after 7 weeks of age. This result may reflect continued stimulation of the immune response as a result of the endemic nature of *M. bovis* in this herd. Other investigators have also noted a poor correlation between serum antibody responses and *M. bovis* infection in individual calves during the first 3 months of life (Virtala *et al*., 2000). However, *M. bovis* infection can result in local mucosal antibody responses without eliciting a substantial systemic humoral response (Howard *et al*., 1980).

The vaccine used in our study was ineffective at preventing URT colonization with *M. bovis* in calves, even when colonization occurred after a humoral immune response was well established. Calves in Herd A were not colonized until between 8 and 12 weeks of age, whereas a significant increase in serum IgG_1 responses was evident by 7 weeks of age. This is consistent with other reports on *M. bovis* vaccines; even where *M. bovis* vaccines have been associated with clinical benefits, they typically fail to induce an immune response that prevents URT infection (Chima *et al*., 1980; Nicholas *et al*., 2002). As discussed in Chapter 1, protection from URT colonization and from clinical respiratory tract disease is better correlated with local mucosal immune responses than with serum antibody titers.

Post-colostral total serum protein concentrations or *M. bovis*-specific antibody levels were not associated with protection from *M. bovis*-associated disease in calves in this study.

However, as colostrum was not pasteurized on this farm, it is possible that some colostrum containing high antibody concentrations to *M. bovis* may have come from cows with intramammary infection and therefore may also have contained live *M. bovis*. This could certainly mask any protective effect of passive transfer when assessed on a herd level. Further studies are required to determine the efficacy of passive transfer for prevention of *M. bovis*-associated disease in a controlled setting.

To the best of the author's knowledge this is the first controlled, independent efficacy study of any of the *M. bovis* vaccines available in North America. The response to vaccination was herd-dependent, and a higher rate of otitis media was associated with vaccination in one herd. The vaccine did stimulate a systemic IgG_1 response that was detectable after the third vaccination. However, most clinical disease occurred prior to this adaptive humoral immune response. Pre-weaned calves in endemically-infected herds were colonized with *M. bovis* at a very young age, and it is likely that this represents the greatest impediment to successful vaccination in this age group. Whether vaccination may be efficacious at preventing clinical disease in older calves was not evaluated in this study. In conclusion, vaccination was not efficacious in preventing *M. bovis*-associated disease in pre-weaned calves in two endemically-infected Florida dairy herds, nor was it effective at preventing colonization of the URT in older calves in a third dairy herd. New approaches to immune protection of young calves from *M. bovis* infections, including controlled studies investigating the efficacy of passive transfer and the mucosal route of immunization, are needed.

A

B

C

Figure 2-1. Calf housing conditions for the three study farms. A) Herd A. B) Herd B. C) Herd C.

Table 2-1. Clinical definitions of disease used by calf producers during this study.

Disease	Clinical definition
Scours	Diarrhea plus rectal temperature of < 103.5º F
Scours with fever	Diarrhea plus rectal temperature of ≥ 103.5º F
Digestive	Clinical signs attributable to alimentary tract disease, other than scours
Fever of unknown cause	Fever in the absence of specific clinical signs
Respiratory disease	Fever (rectal temperature ≥ 103.5º F) plus increased respiratory rate or effort and/or coughing and/or nasal discharge
Otitis media	Ear droop and/or evidence of ear pain (head shaking, scratching or rubbing ear)
Arthritis	Lameness attributable to painful distention of any joint
Navel infection	Enlarged umbilical stalk that is non-reducible on palpation, confirmed by a veterinarian at next visit
Other	Clinical signs described by farm personnel or veterinarian.

A

B

Figure 2-2. Sampling of a subset of calves in Herds A and B. A) Collection of nasal swabs. B) Collection of blood from the jugular vein.

Table 2-2. Summary of calves enrolled in vaccine field efficacy study.

Herd	Vaccinated	Control	Exclusions*	Total
A	21	20	0	41
B	81	85	2	168
C	82	80	2	164
All Herds	184	185	4	373

* In Herd B, one calf was excluded because of concurrent disease and one calf was excluded because of a booster was inadvertently missed; in Herd C, one calf was excluded because of concurrent disease and one calf was excluded because it received the wrong booster.

Table 2-3. Incidence risk for *Mycoplasma bovis*-associated disease and mortality between 3 and 90 days of age in calves in the three study herds.

	Herd A	Herd B	Herd C
Disease			
All *M bovis*-associated	0.00	0.55	0.74
Otitis media	0.00	0.22	0.35
Respiratory disease	0.00	0.48	0.69
Arthritis	0.00	0.04	0.00
Other	0.07	0.15	0.19
Mortality			
M. bovis-associated	0.00	0.10	0.03
All causes	0.02	0.13	0.10

Table 2-4. Baseline data for calves in Herds B and C.

	Vaccinated (*n*=163)	Control (*n*=165)	‡*P*
Herds B+C			
†TSP (g/dl)	5.84 ± 0.73	5.76 ± 0.58	ns
†† No 2nd vaccine	9/157 (6%)	7/157 (4%)	ns
††No 3rd vaccine	10/153 (7%)	8/153 (5%)	ns
Herd B			
††No 3rd vaccine	7/77 (9%)	0/82 (0%)	0.005

†TSP = total serum protein; Results are expressed as mean ± standard deviation.
††Results are expressed as number of calves that missed the vaccine/total number of calves eligible for that vaccine, percentage is given in parentheses.
‡ns = no significant difference

Table 2-5. The age at which calves in Herds B and C received their first treatment for otitis media or respiratory disease.

	Age in days at first treatment		
Disease	Vaccinated	Control	*P*
Otitis media			
Herd B	37 ± 16	37 ± 17	ns
Herd C	27 ± 10	24 ± 9	ns
Herd B+C	32 ± 13	31 ± 13	ns
Respiratory disease			
Herd B	30 ± 13	33 ± 14	ns
Herd C	20 ± 17	21 ± 17	ns
Herd B+C	24 ± 15	25 ± 16	ns

Results are expressed as mean age in days ± standard deviation; ns = no significant difference

Table 2-6. Temporal expression of *Mycoplasma bovis*-associated disease in vaccinated and control calves in Herds B and C

Age	Herd	Vaccinated (%)	Control (%)	*P*
4 weeks	B	19/81 (23)	17/85 (20)	ns
	C	46/82 (56)	46/80 (58)	ns
	B+C	65/163 (40)	63/165 (38)	ns
8 weeks	B	42/81 (52)	38/85 (45)	ns
	C	57/82 (70)	53/80 (66)	ns
	B+C	99/163 (61)	91/165 (55)	ns
12 weeks	B	44/81 (54)	40/85 (47)	ns
	C	58/82 (71)	55/80 (69)	ns
	B+C	102/163 (63)	95/165 (58)	ns

Results are expressed as the number of calves receiving their first therapeutic intervention by 4, 8, or 12 weeks of age/total number of calves. ns = no significant difference.

Table 2-7. Morbidity due to respiratory disease in vaccinated and control calves.

	Vaccinated (%)	Control (%)	*P*
Herd B			
Respiratory only	18/81 (22)	26/85 (31)	ns
All Respiratory	38/81 (47)	36/85 (42)	ns
Herd C			
Respiratory only	35/82 (43)	25/80 (31)	ns
All Respiratory	55/82 (67)	51/80 (64)	ns
Herd B + C			
All Respiratory	93/163 (57)	87/165 (53)	ns

Results are expressed as the number of calves with respiratory disease/total number of calves in that group. "All Respiratory" includes calves that were treated for respiratory disease alone or for respiratory disease and otitis media or arthritis. ns = no significant difference.

Table 2-8. Morbidity due to otitis media in vaccinated and control calves.

	Vaccinated (%)	Control (%)	*P*
Herd B			
Otitis media only	4/81 (5)	1/85 (1)	ns
Otitis media + Respiratory	20/81 (25)	9/85 (11)	0.017
All otitis media	24/8 (30)	10/85 (12)	0.004
Herd C			
Otitis media only	3/82 (4)	4/80 (5)	ns
Otitis media + Respiratory	20/82 (24)	26/80 (33)	ns
All otitis media	23/82 (28)	30/80 (38)	ns

Results are expressed as the number of calves with otitis media/total number of calves in that group. ns = no significant difference.

Table 2-9. Overall and *Mycoplasma bovis*-associated mortality in vaccinated and control calves.

	Vaccinated (%)	Control (%)	*P*
All mortality			
Herd B	11/81 (14)	10/85 (12)	ns
Herd C	6/82 (7)	11/80 (13)	ns
Herd B+C	17/163 (10)	21/165 (13)	ns
M. bovis-associated			
Herd B	9/81 (11)	7/85 (8)	ns
Herd C	1/82 (1)	4/80 (5)	ns
Herd B+C	10/163 (6)	11/165 (7)	ns

Results are expressed as the number of calves that died/total number of calves in that group. ns = no significant difference.

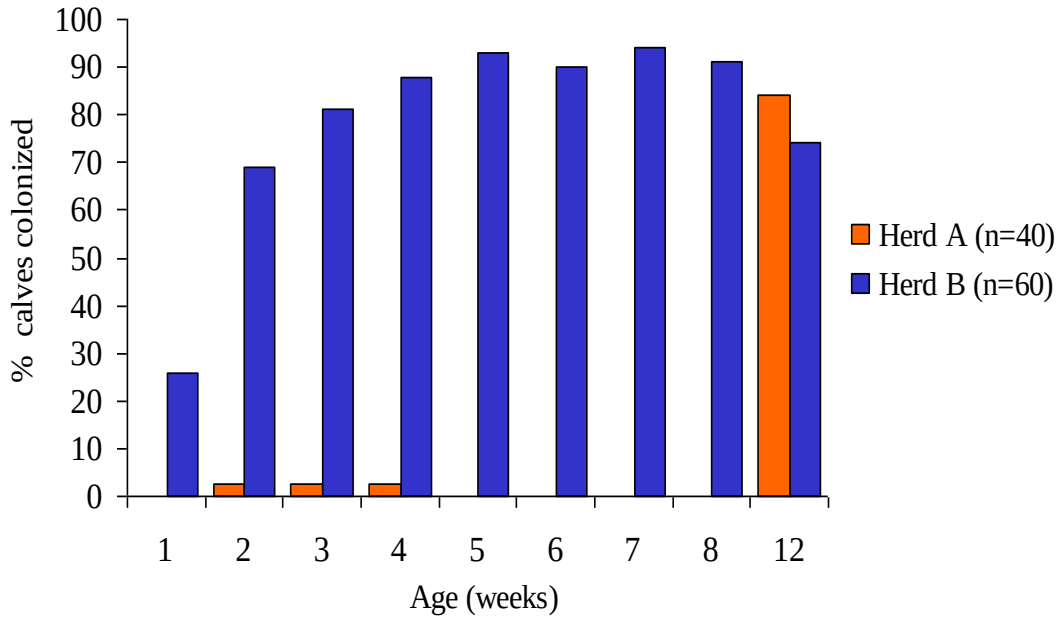


Figure 2-3. Temporal pattern of nasal colonization of calves by *Mycoplasma bovis* in Herds A and B.

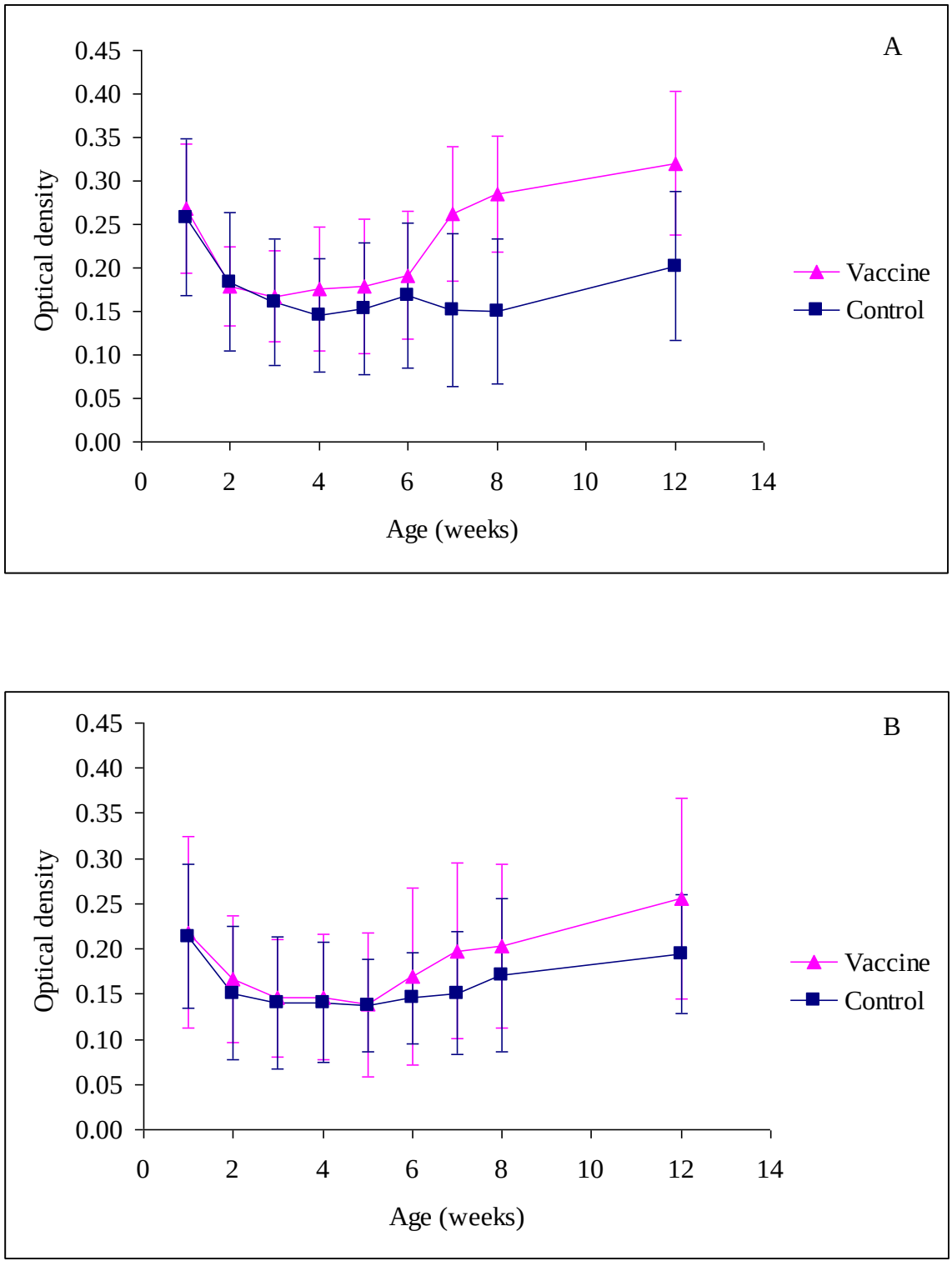


Figure 2-4. Immunoglobulin A response in vaccinated and control calves. A) Herd A. B) Herd B.

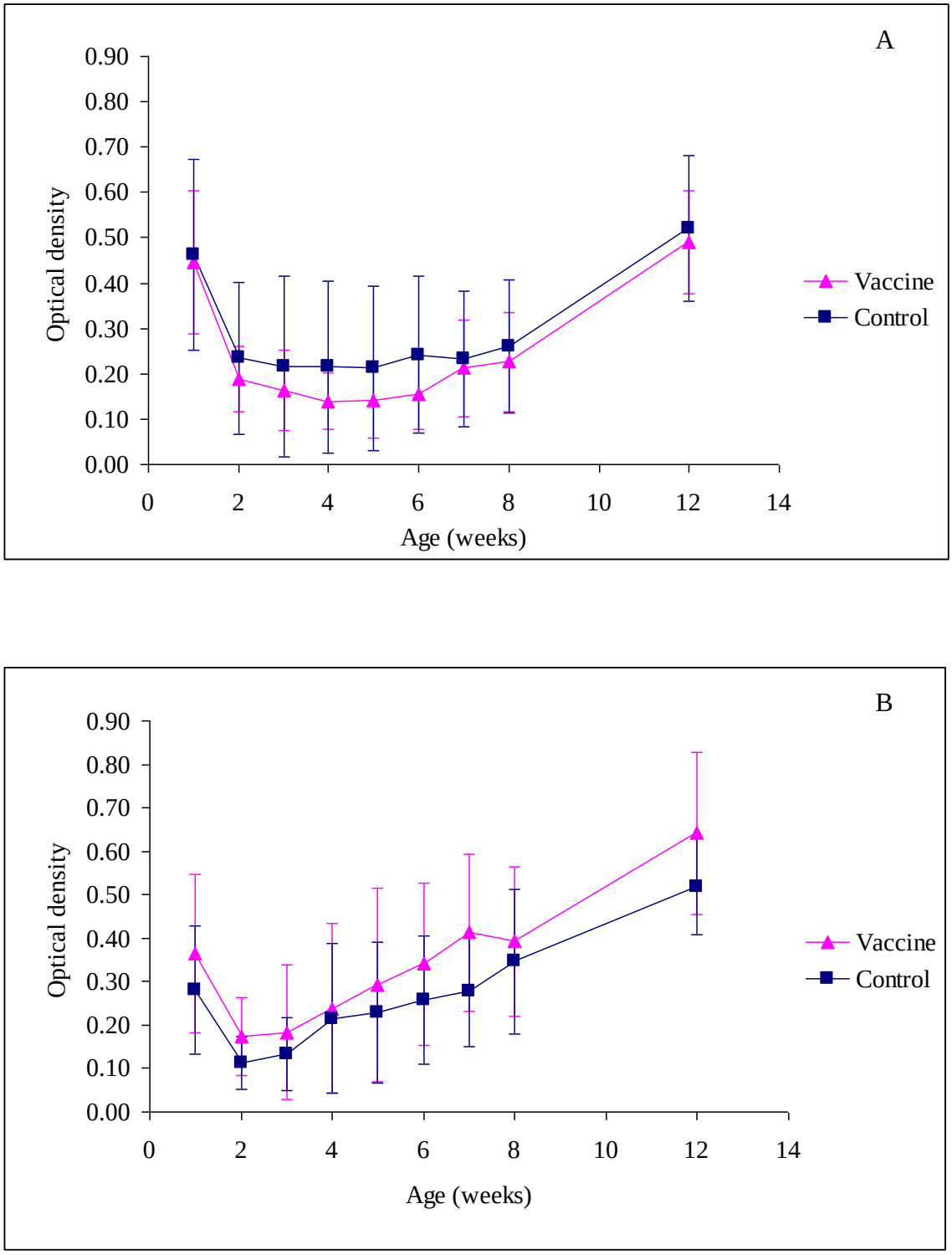


Figure 2-5. Immunoglobulin M response in vaccinated and control calves. A) Herd A. B) Herd B.

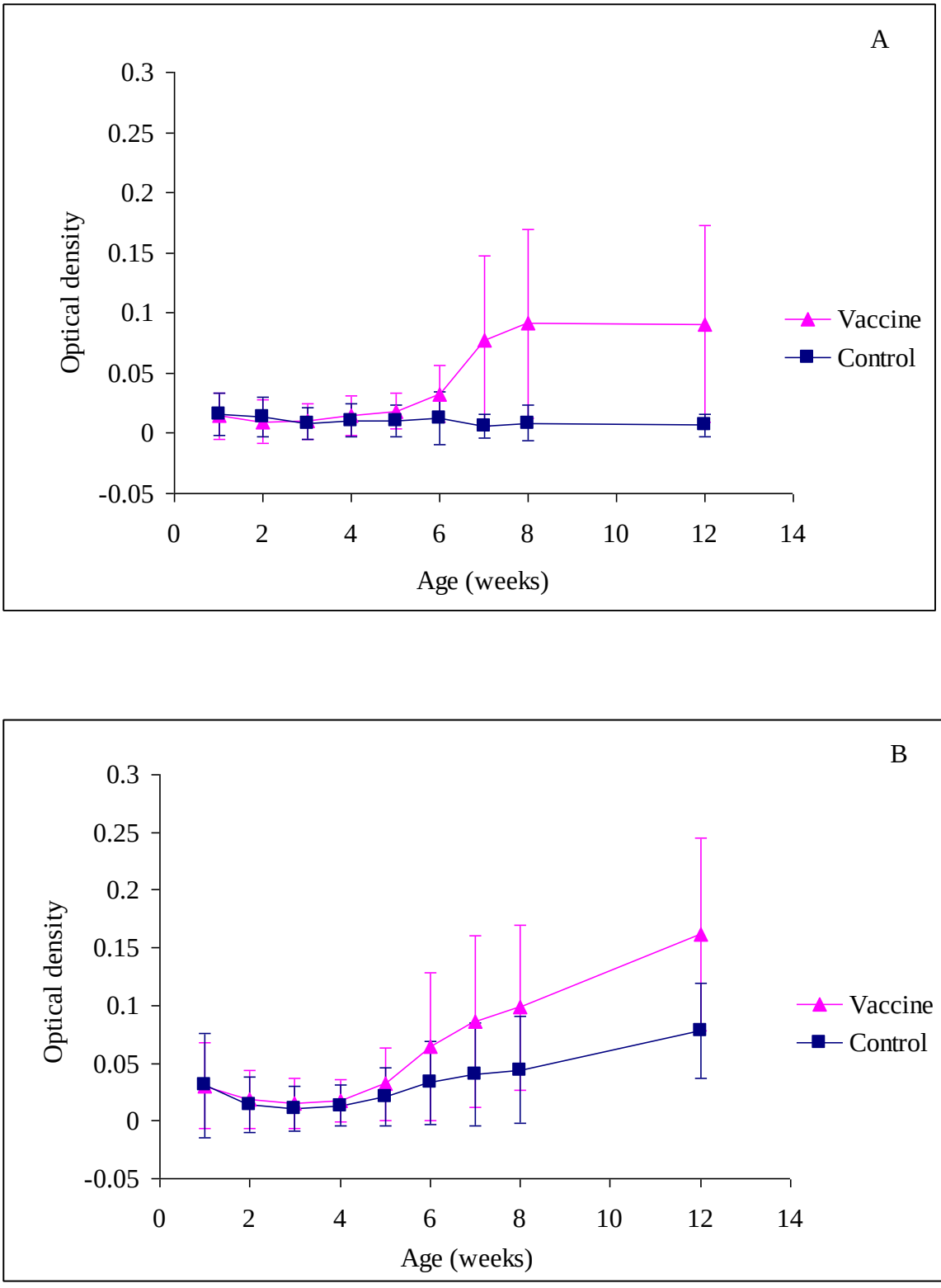


Figure 2-6. Immunoglobulin G_2 response in vaccinated and control calves. A) Herd A. B) Herd B.

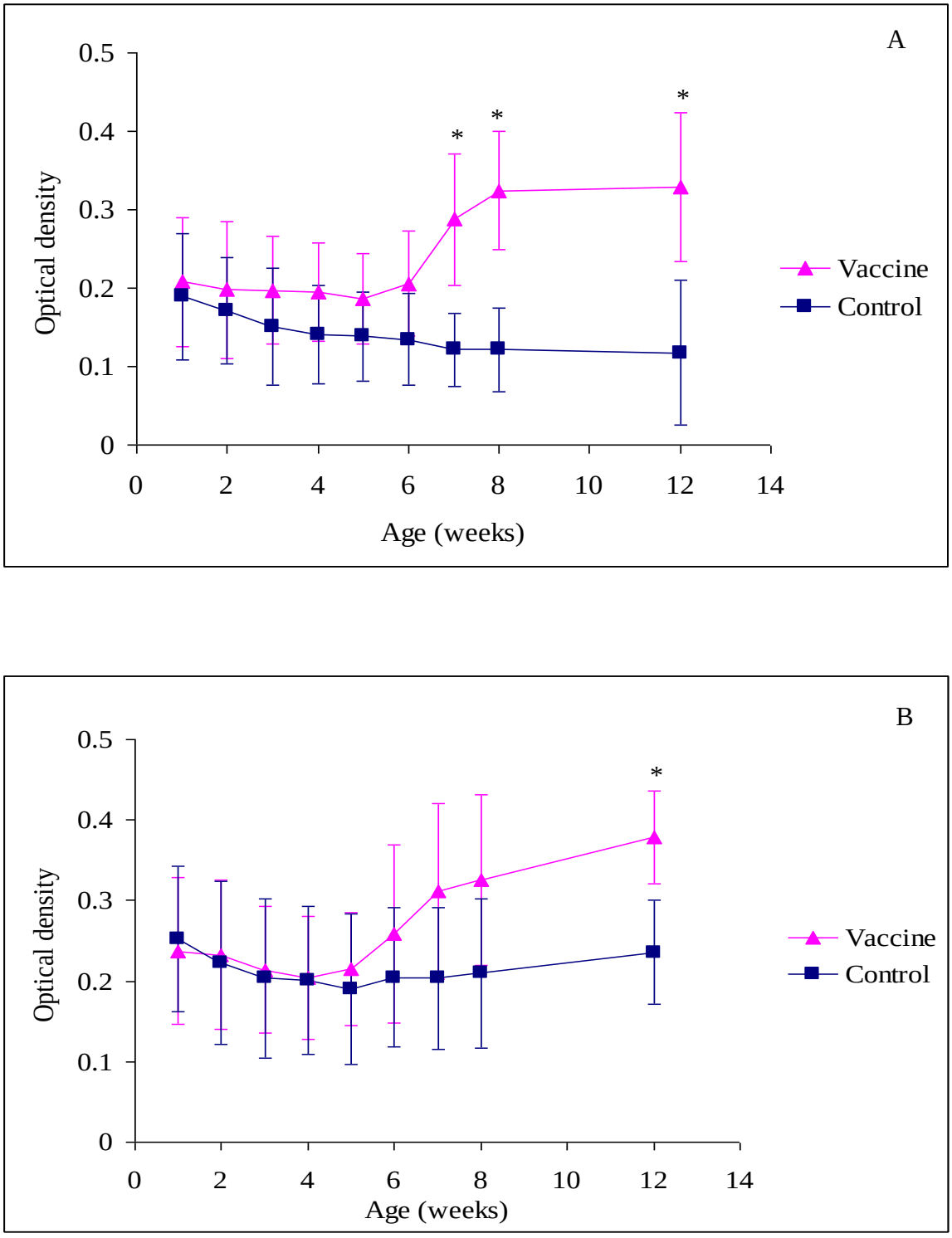


Figure 2-7. Immunoglobulin G_1 response in vaccinated and control calves. A) Herd A. B) Herd B. *Asterisks indicates time points at which vaccinated and control groups were statistically different ($P < 0.05$).

CHAPTER 3
ORAL INOCULATION OF DAIRY CALVES WITH *Mycoplasma bovis* RESULTS IN RESPIRATORY TRACT INFECTION AND OTITIS MEDIA: ESTABLISHMENT OF A MODEL OF AN EMERGING PROBLEM

Introduction

Mycoplasma bovis has emerged in recent years as a widespread and important etiologic agent of otitis media, respiratory disease and arthritis in intensively-reared pre-weaned dairy calves. Clinical disease caused by *M. bovis* tends to be chronic, debilitating and unresponsive to antimicrobial therapy (Gourlay *et al*., 1989a; Adegboye *et al*., 1995a; Apley and Fajt, 1998; Pollock *et al*., 2000; Stipkovits *et al*., 2000; Rosenbusch, 2001; Shahriar *et al*., 2002). Disease outbreaks with high morbidity rates occur (Gourlay *et al*., 1989a; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000; Stipkovits *et al*., 2001) and can be economically devastating for the affected farm. An absence of efficacious vaccines for use in young calves combined with a poor response to therapeutic agents means that this disease is often very difficult to control once established in a herd. Therefore, there is a critical need to develop improved preventative or control measures for *M. bovis*-associated calf disease.

Ingestion of milk or colostrum from cows shedding *M. bovis* from the mammary gland is considered to be a major means of transmission to young dairy calves (Bennett and Jasper, 1977c; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000), although direct contact with infected animals and secondary transmission through fomites are also likely to be important. Following exposure by any of these routes, the upper respiratory tract (URT) appears to be the initial site of colonization (Bennett and Jasper, 1977c; Brys and Pfutzner, 1989), and this colonization precedes the development of clinical disease. However, as with many other pathogens which inhabit mucosal surfaces, colonization alone does not necessarily result in the development of clinical disease, and *M. bovis* is frequently isolated from the nasal passages of

apparently healthy cattle (ter Laak *et al*., 1992a; Brown *et al*., 1998a; Mettifogo *et al*., 1998; Maeda *et al*., 2003).

The host and microbial factors that contribute to development of disease after colonization of the URT by *M. bovis* are poorly understood. A defined experimental animal model that mimics naturally occurring disease would facilitate understanding of these factors. In addition such a model could be applied in the development of optimal vaccine approaches against *M. bovis* as well as efficacy testing of new treatments and vaccines for use in young dairy calves. A number of experimental models have been used to study *M. bovis* infection in calves. Various routes of inoculation have been employed, including inhalation of aerosolized bacteria, intranasal, intra- or transtracheal, endobronchial, transthoracic, intravenous, intraarticular or subcutaneous inoculation, as well as combinations of these routes (Chima *et al*., 1980; Howard *et al*., 1980; Pfutzner *et al*., 1983a; Ryan *et al*., 1983; Gourlay and Houghton, 1985; Lopez *et al*., 1986; Brys *et al*., 1989; Gourlay *et al*., 1989b; Nicholas *et al*., 2002; Vanden Bush and Rosenbusch, 2003). Although useful in the study of events associated with *M. bovis* infection at particular body sites, none of these models mimic the ingestion of *M. bovis*-contaminated milk, a major route of infection in young calves (Bennett and Jasper, 1977c; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). In addition, most experimental infection studies have been conducted in calves that are at least 2 weeks of age, whereas natural infection often occurs in younger calves (Brown *et al*., 1998a; Stipkovits *et al*., 2000). Although neonatal calves are immunocompetent, their immune system responds differently to many antigens than does that of older calves (Barrington and Parish, 2001), so selection of an appropriate age group is likely to be important for a model to accurately mimic natural disease. Importantly, the experimental models previously used to study *M. bovis* infection in calves did not induce clinical otitis media,

which is a newly emerging disease in young calves. An experimental infection model to study the events that occur in the URT of young calves after exposure to *M. bovis* in milk, particularly those factors leading to the dissemination of infection and the development of otitis media and lower respiratory tract (LRT) disease would be invaluable.

The goal of this study was to develop a reproducible model of *M. bovis* infection of the URT that closely mimicked natural infection, and to compare this model with a transtracheal inoculation approach. Because the very young calf presents some special challenges with respect to vaccine development and treatment of clinical disease, we chose to focus our studies on this age group. To best mimic a natural route of infection, we infected calves by feeding milk-replacer inoculated with a field strain of *M. bovis*. This model consistently resulted in colonization of the URT and eustachian (auditory) tubes and caused otitis media in 37% of calves by two weeks post-infection. The model is suitable for use in further studies to define local and systemic immune responses to *M. bovis* infection of the URT and to evaluate new therapeutic or preventative strategies against *M. bovis*-associated disease.

Methods

Calves

All animal work was approved by the University of Florida (UF) Institutional Animal Care and Use Committee. Healthy male Holstein calves were obtained from the UF Dairy Research Unit, where no clinical mycoplasmal disease had been observed in calves for 2 years preceding the study. Calves were removed from the cow at birth before suckling could occur. In the first 12 hr of life, calves were fed two doses of a mycoplasma-free colostrum-replacement product formulated from a spray-dried bovine serum (Acquire™, APC Inc., Ames, IA). Calves were weighed, ear-tagged and given one oral dose of a commercial product containing antibody against F5-piliated *E. coli* (Bovine Ecolizer, Novartis Animal Health U.S., Inc., Greensboro,

NC). Serum and nasal swabs were collected prior to initial colostrum replacer feeding and at approximately 48 hr of age. Total serum protein was measured at 48 hr of age as an estimate of passive transfer of immunoglobulin (Ig). Nasal swabs were cultured to detect mycoplasmas and other URT pathogens. At 1 to 4 days of age, calves were transported to UF research facilities where they were housed in individual stalls with no direct contact between animals. Calves were maintained on non-medicated milk replacer and had free-choice access to non-medicated starter pellets and fresh water. Calves that developed uncomplicated diarrhea (diarrhea without fever) in the pre-enrollment period were given oral electrolytes (Enterolyte HE™, Pfizer, Kalamazoo, MI) as needed to replace fluid and electrolyte losses. Calves that developed other clinical signs of disease were not enrolled in the study. Control and infected groups were housed in different rooms.

Strain of *M. bovis* and Experimental Infection

Mycoplasma bovis F1, confirmed as *M. bovis* by 16S *rRNA* gene sequencing (data not shown), is a field strain isolated from a lung abscess in a calf with severe fibrinous bronchopneumonia. The source herd had experienced high morbidity rates due to *M. bovis*-associated pneumonia and otitis media in pre-weaned dairy calves in the two years prior to the isolate being obtained. A second passage culture of *M. bovis* F1 in Frey's broth was stored in aliquots at -80°C and used for all infection studies.

All calves were inoculated between 7 and 11 days of age (Day 0 = day of inoculation). For the oral inoculation groups, calves received a total dose of $2.9 \pm 2.5 \times 10^{10}$ colony forming units (CFU) of *M. bovis* F1 (infected group, n=8) or an identical volume of sterile Frey's broth (control group, n=4) over three consecutive feedings in a 24 hr period. At each feeding, an aliquot of *M. bovis* F1 was thawed at room temperature, mixed with two pints of milk replacer at

35 to 37˚C in a bucket and immediately fed to the calf. The remaining volume of the calf's milk was then added to the bucket and fed.

For the transtracheal inoculation groups, calves received a single inoculum ($3.8 \pm 1.1 \times 10^9$ CFU/ml) of *M. bovis* F1 in sterile, endotoxin-free isotonic saline (Abbott Laboratories, Chicago, IL; infected group, n=5) or an identical volume of saline (control group, n=4). Approximately two hr prior to inoculation, an aliquot of *M. bovis* F1 was thawed at room temperature, pelleted, washed twice, and resuspended in 20 ml of saline. Each calf was sedated with xylazine hydrochloride, and the inoculum delivered at the level of the tracheal bifurcation using a commercial transtracheal wash kit (MILA International, Inc., Florence, KY).

Clinical Monitoring and Sample Collection

A complete physical examination was performed or supervised by a veterinarian on each calf at approximately the same time each day, and data were recorded using standardized forms. Calves were also observed a second time during the day for clinical abnormalities. Due to biosecurity protocols in the housing facility, the examiners were not blinded as to calf infection status.

A clinical scoring system was developed in which calves were scored in four categories: Behavior (0: normal behavior, gets up when approached; 1: depressed or dull, must be stimulated to get up; 2: gets up only with assistance) rectal temperature (0: < 103°F; 1:103 to 104.9°F; 2: $\geq$105°F), clinical signs of otitis media (0: no clinical signs of otitis media; 1: occasional head-shaking and/or scratching ears, ear droop evident at rest; 2: occasional head-shaking and/or scratching ears, ear droop evident continuously; 3: frequent head-shaking or ear scratching, pronounced ear droop) and clinical signs of respiratory disease (0: none of the following clinical abnormalities: cough, mucopurulent nasal discharge, abnormal breath sounds on thoracic

auscultation, tachypnea (> 60 breaths/min) or dyspnea; 1: one of the above clinical abnormalities; 2: two of the above clinical abnormalities; 3: three or more of the above clinical abnormalities). The scores for each of the four categories were summed each day to give a maximum overall daily clinical score of 10.

Swabs of the left and right nares (BBL™ CultureSwab™ Liquid Stuart Medium, BD, Franklin Lakes, NJ) were collected at 0, 3, 7 and 14 days post-infection for mycoplasma culture. Blood samples were collected by jugular venipuncture at the same times for mycoplasma culture (all time points) and for assay of *M. bovis*-specific serum IgG titers by enzyme-linked immunosorbent assay (ELISA) (days 0 and 14).

Collection of Tissues

Calves were euthanized 14 days after the first inoculation of *M. bovis*, or earlier if criteria for euthanasia were met. One calf had to be euthanized at 10 days post-infection; samples were collected immediately prior to euthanasia from this animal. At necropsy, each of the six major lung lobes, the spleen, the tracheobronchial lymph nodes (TBLN) and the medial and lateral retropharyngeal lymph nodes (MRPLN and LRPLN) were examined for gross lesions and samples collected for culture and histopathology. Lymph nodes were weighed prior to sample collection. Each lung lobe was weighed and photographed; digital photographs were later used to calculate the percentage of each lobe affected with visible lesions. Mean lung lobe weights of control calves were used to calculate the ratio of each lobe to the total lung mass, and these figures used to determine the total percentage of visibly affected lung for each calf (Jericho and Langford, 1982). For mycoplasmal culture, approximately 300 mg of tissue was collected aseptically from a standard site in each lung lobe and the spleen. In addition, the cut surface of each of these tissues was swabbed. Swabs were collected aseptically from the mucosal or synovial surfaces of the palatine tonsils, trachea, primary bronchi, carpal and stifle joints, and

from the cut surface of the MRPLN, LRPLN and TBLN. Samples also were collected from each of these tissues for histopathology. Spinal fluid was aspirated from the atlanto-occipital space for culture. The exterior and cut surfaces of the tissues described above as well as all other major organs were examined for gross abnormalities.

Samples from the nasopharynx, eustachian tubes and tympanic bullae were collected after removal of the brain and bisection of the skull. The brain, meninges, nasal passages and sinuses were examined for gross lesions. Swabs of the mucosal surface of the pharyngeal tonsil (Schuh and Oliphant, 1992) and nasal mucosa were collected for culture, and tissue collected for histopathology. After collection of swabs from the distal eustachian tubes via the nasopharyngeal openings, the external ear canal, tympanic bulla and the eustachian tube were removed using a reticulating saw. The distal portion of the eustachian tube was removed for histopathology. A small (4×4mm) section of bone was removed aseptically from the most ventral aspect of the tympanic bulla. Any exudate present within the bulla was aspirated for culture, and the tympanic mucosa was swabbed.

Histopathology

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax and sections (5 μm) stained with hematoxylin and eosin. The ear was fixed in 10% neutral buffered formalin, subsequently band-sawed through a line incorporating the rostral margins of the insertion of the stylohyoid bone and the external auditory meatus, then trimmed and decalcified for 24 hr prior to embedding. Histopathology was read in a blinded fashion without knowledge of experimental groups or gross pathologic findings. Histopathology of the eustachian tubes, nasal mucosa, tonsils, trachea, primary bronchi and lymph nodes was graded on a scale from 1 (minimal to no lesions and/or lymphoid hyperplasia) to 3 (most severe lesions

and or lymphoid hyperplasia). In addition, tissues were graded with respect to numbers of plasma cells present on a scale from 1 (minimal or no plasma cells) to 4 (large numbers of plasma cells). Histopathology of the tympanic bullae and lungs was graded from 1 (minimal to no lesions) to 5 (most severe lesions), and lungs were also graded with respect to the degree of lymphoid infiltration and hyperplasia of bronchial-associated lymphoid tissue from 1 (minimal to no lymphoid hyperplasia) to 4 (marked and widespread lymphoid hyperplasia).

Microbiology

All mycoplasma cultures were performed in modified Frey's broth and agar medium containing 2.25% (wt/vol) Mycoplasma broth base (Frey) (BD Diagnostic Systems, Sparks, MD), 0.02% (wt/vol) DNA from herring sperm, 20% (vol/vol) horse serum, 10% (vol/vol) fresh yeast extract, 0.5% (wt/vol) glucose and supplemented with 100,000 U/l each of penicillin G and polymixin B and 65 mg/l of cefoperazone, with the final pH adjusted to 7.6 to 7.8. For culture of blood samples, 5 ml of blood collected into tubes containing sodium citrate was inoculated into 45 ml of broth within 15 min after collection and subcultured onto agar at the time of broth inoculation and after 48 hr incubation at 37ºC. Swabs were streaked on agar plates within 30 min after collection and also used to inoculate broth, from which ten-fold serial dilutions were plated (20 μl) in duplicate on agar. When present, the volume of exudate aspirated from the tympanic bulla was measured, a specified volume inoculated into broth and serial dilutions plated as described. Spinal fluid was inoculated into broth and subcultured onto agar plates. Tissues collected for quantitative culture were weighed and minced in broth from which ten-fold serial dilutions were plated (20 μl) in duplicate on agar.

Plates were incubated in 5% CO_2 at 37ºC and examined at 2, 3, 5, 7 and 10 days for the presence of mycoplasmal colonies. Colonies with typical morphology were classified as positive

pending polymerase chain reaction (PCR) confirmation. Two to eight single isolated colonies were inoculated into separate aliquots of broth, incubated for 24 hours at 37ºC, and then stored at -80ºC. In addition, when plates were positive for mycoplasmal growth, the original broth dilutions were subcultured on agar to check viability and stored at -80ºC. Isolates were identified as *M. bovis* based on PCR of the 16S*rRNA* gene (Mattsson *et al*., 1991). DNA fingerprinting by insertion sequence (IS) typing was performed (see below) to ensure that recovered isolates originated from the F1 strain used for inoculation.

Results for streaked plates were recorded as positive or negative for mycoplasmal growth. Semiquantitative culture results for swabbed tissues were expressed as Log_{10} of the highest dilution that yielded mycoplasmal colonies. When only the undiluted broth was positive, results were assigned a Log_{10} value of 0.5. Quantitative culture results were expressed as CFU/g of tissue or CFU/ml of exudate. Whole lung culture data represents an average of the six standardized lung sites sampled and was calculated by summing the CFU isolated from all lung sites and dividing this by the total weight of lung tissue sampled.

In addition to culturing for mycoplasma, swabs of the nares, palatine tonsils, trachea, lungs and tympanic bullae were processed using routine clinical bacteriological methods to identify other potential bacterial pathogens of the respiratory tract. Appropriate samples were submitted to the Florida State Diagnostic Laboratory for diagnosis of bovine respiratory syncytial virus, bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, and parainfluenza-3 virus.

Insertion Sequence Typing

Stored *M. bovis* broth cultures representing a single-colony expansion from each positive site were thawed at room temperature. The inoculating isolate (*M. bovis* F1) was used as the reference for hybridization profiles and the PG45 type strain of *M. bovis* used for probe

synthesis. Cells were harvested by centrifugation at 1500 × g for 30 min, rinsed twice with sterile PBS and centrifuged at 10,000 × g for 15 min. Genomic DNA extractions were performed using UltraClean Microbial DNA Isolation Kits (MO BIO, Carlsbad, CA) as per the manufacturer's protocol. DNA was quantified spectrophotometrically and stored at -80ºC. Probes for the insertion sequences IS*Mbov*2 and IS*Mbov*3 (Miles *et al*., 2005) were synthesized by PCR in a 50 µL reaction mixture which contained a final concentration of 40 ng of *M. bovis* PG45 template DNA, 0.8 µM each of the forward and reverse primers, 1.5 mM $MgCl_2$, 200 µM of each dNTP with 70 µM DIG-11-dUTP nucleotide mix and 3.5 U *Taq* enzyme (PCR DIG Probe Synthesis Kit, Roche Applied Science, Penzberg, Germany). The primers used were IS*Mbov*2-F (GGTAAATCTAGTTCGAAGATG), IS*Mbov*2-R (GGGTAAACAGAACTTGCAAC), IS*Mbov*3-F (CAGGAAATGTTACTGATTCA) and IS*Mbov*3-R (TTGTTTGCTTCCAGCTTTCA) (Miles *et al*., 2005). PCR conditions for probe synthesis were 3 min denaturation at 95°C followed by 30 cycles of denaturation for 30 sec at 95°, annealing for 30 sec at 55°C for IS*Mbov*2 or 50°C for IS*Mbov*3, extension for 1 min at 68°C, and a 5 min final extension at 68°C.

Genomic DNA (4.5 to 5 µg) was digested with 20 U *Eco*RI (New England Bio Labs, Inc., Ipswich, MA) overnight at 37°C in a final volume of 30 µL. The resulting fragments were separated on 0.8% agarose gel by electrophoresis at 20 V for 18 to 20 hr. The ensuing gel was depurinated and denatured then transferred to a nylon membrane (Nytran SPC, pore size 0.45 µm, Whatman, Inc., Florham Park NJ) using a vacuum blotting apparatus for 90 min following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). After transfer, the membrane was probed overnight at 65°C. The IS*Mbov*2 probe was diluted to 1.5 µg/ml of hybridization buffer (DIG Easy Hyb, Roche Applied Science, Penzberg, Germany)

per 100 cm^2 membrane and the IS*Mbov*3 probe was diluted to 2.0 µg/ml of hybridization buffer per 100 cm^2 membrane. After probing, the membrane was washed twice with low stringency buffer (2X NaCl sodium citrate [SSC], 0.1% sodium dodecyl sulfate [SDS]) at room temperature for 5 min with shaking and twice with high stringency buffer (0.5X SSC, 0.1% SDS) at 65°C for 15 min with shaking. Blocking, washing and detection were performed using DIG Wash and Block Buffer Set and DIG Nucleic Acid Detection Kit as per the manufacturer's protocols (Roche Applied Science, Penzberg, Germany). Hybridization profiles were recorded digitally (FluorChem 8900, Alpha Innotech, San Leandro, CA) and examined for differences in banding patterns between the F1 isolate used for inoculation and isolates recovered from tissues at necropsy.

The ELISA Procedure

Blood samples were allowed to clot after collection, and then serum was harvested by centrifugation and stored at -80ºC. Serum end-point titers of *M. bovis*-specific IgG were determined using ELISA. Whole-cell lysate antigen (Schumacher *et al*., 1993) was prepared from a 1 liter culture of *M. bovis* type strain PG45 grown at 37°C in Frey's broth. The protein concentration was determined using a colorimetric assay (Bio-Rad, Hercules, CA) and adjusted to 100 µg/ml. The antigen was stored in aliquots at -80°C and thawed at room temperature when required. The ELISA procedure was optimized using standard methodology. Microtiter plates (Maxisorb F96, Nunc, Kamstrup, Denmark) were coated with 20 µg per well of antigen in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and (wt./vol.) NaN_3 (PBS/A), and incubated overnight at 4°C. Plates were then washed three times with PBS/A containing 0.05% (vol./vol.) Tween 20 (PBS/T) using an automated plate washer (ELx405 Auto Plate Washer, BioTek Instruments, Inc., Winooski, VT), blocked with 300 µl per well of blocking

buffer (PBS/T containing 1% [wt./vol.] egg albumin), and stored at 4°C for a minimum of 24 hr or until needed. Two-fold serial dilutions of serum were made in blocking buffer and 50 μl of each dilution added to duplicate wells; plates were incubated at room temperature for 1 hr. The highest serum dilution tested was 1:8,196. Plates were washed as described above and 50 μl of goat anti-bovine IgG conjugated to alkaline phosphatase (Bethyl Laboratories Inc., Montgomery, TX) and diluted to 1:1,000 in blocking buffer was added to each well. Plates were incubated at room temperature for 2 hr and then washed as described above. 100 μl of 0.1% (wt./vol.) p-nitrophenol phosphate was added to each well and plates incubated in the dark at room temperature for 1 hr. The optical density (OD) in each well was read at a wavelength of 405 nm using an automated plate reader (ELx808 Ultra Microplate Reader, BioTek Instruments, Inc., Winooski, VT). For each microtiter plate, the blank was the mean value for two wells coated with antigen and incubated with the conjugated secondary antibody and substrate only. The blank OD value was subtracted from each sample well, and mean values for each pair of duplicate tests calculated.

Two-fold serial dilutions of a pool of sera from 20 calves with naturally occurring mycoplasmal disease and high *M. bovis*-specific IgG titers were included on each plate as a positive control, as well as a 1:2 dilution of a negative control pool of serum collected from the same 20 calves prior to ingestion of their first colostrum meal. The cutoff for a positive titer was the average OD value (minus the blank) for the negative control sera plus two standard deviations, established over ten assay runs. The highest dilution of the test serum that gave an average OD value higher than the cutoff was defined as the titer for that sample. Within-batch and between-batch assay variability was assessed by using the Youden plot graphic method (Jeffcoate, 1982). The ELISA values obtained for the lowest, middle and highest dilution of the

control serum included on each plate were used to establish target values and control limits to be used for monitoring the consistency of the assay (ten batches). The values obtained at the beginning of a series of assays were plotted against the values obtained for the same standards at the end of the series. If values for the pooled sera deviated more than 10% from target values, the assay was repeated.

Statistical Analysis

Continuous variables (total serum protein concentrations, bodyweight, % of lung with macroscopic lesions, serum IgG titers) were compared between groups using ANOVA or repeated measures ANOVA (IgG titers). Tukey's tests were applied to post-hoc comparisons. Ordinal variables (lesion scores, daily clinical scores) were analyzed using Kruskal-Wallace ANOVA or Friedman Test, as appropriate. Correlations between numbers of mycoplasmas isolated at various body sites were examined using Pearson's correlation analyses. A *P* value of 0.05 or less was considered statistically significant, with the exception of the overall significance levels in ANOVA, where a *P* value of 0.1 was considered significant. Preliminary analyses performed on data from the oral and transtracheal control groups determined that there were no statistical differences between the 2 groups for any outcome variable. Data from the 2 control groups were then pooled for the main analyses to increase statistical power. Analyses were performed using commercial statistical analyses packages (SPSS 12.0, SPSS Inc, Chicago IL and SAS/STAT, SAS institute, Inc., Cary NC).

Results

Oral Inoculation of Calves and Development of Clinical Disease

Oral inoculation of calves resulted in development of clinical disease. Prior to infection, calves were monitored for their health and serology. Post-colostral total serum protein concentrations, pre- and post-colostral *M. bovis*-specific serum antibody titers, and bodyweight

on day 0 did not vary between infected and control calves (data not shown). Most calves were treated with oral electrolytes for uncomplicated calf diarrhea during the pre-enrollment period, and there was no significant difference in the number of calves treated among groups. No mycoplasmas or other respiratory tract pathogens were isolated from calves prior to enrollment, and the calves were in good health at the time of infection.

Eight calves were inoculated orally, and five calves were inoculated transtracheally with *M. bovis* F1. As controls, four calves per route were inoculated with sterile carrier (Frey's broth or sterile saline for the orally or transtracheally inoculated groups, respectively). The clinical status of each calf was monitored twice a day. Three of the eight (37%) calves infected by the oral route developed clinical signs of otitis media. These clinical signs were first observed on 7, 9 or 13 days post-infection, depending on the individual calf. Affected calves developed unilateral or bilateral ear droops, occasional head-shaking and were mildly depressed or lethargic. Two of the three calves developed ptosis. Calves with otitis media were febrile (rectal temperature > 103°F) on the day prior to (n=2) or on the day (n=1) that an ear droop was first observed. In contrast to orally inoculated calves, clinical signs of otitis media were not observed in any of the calves inoculated by the transtracheal route.

Six of the eight (75%) calves infected by the oral route exhibited clinical signs of LRT disease. In most cases, clinical signs were transient and mild. However, two of the calves with otitis media developed more serious LRT disease (mucopurulent nasal discharge, coughing, intermittent tachypnea and dyspnea and persistent abnormalities of breath sounds on auscultation), and one was euthanized at 10 days post-inoculation due to increasing severity of clinical disease including persistent fever. None of the orally inoculated control calves exhibited clinical signs of respiratory disease. Within the transtracheally inoculated group, four of the five

infected calves and two of the four control calves exhibited transient tachypnea and/or abnormal breath sounds on auscultation in the first few days after inoculation. During the second week of the study, control calves were all clinically normal, whereas three of five (60%) transtracheally infected calves exhibited mild and transient clinical signs of respiratory disease (tachypnea, abnormal breath sounds on auscultation, mucopurulent nasal discharge). Clinical signs of arthritis were not observed in calves inoculated by oral or transtracheal routes. There was no statistically significant difference among groups in median daily clinical scores. However, calves that received *M. bovis* via the oral route tended ($P = 0.06$) to have more days when a daily clinical score of > 2 was present than transtracheally inoculated or control calves (Figure 3-1).

Colonization of the Upper Respiratory Tract

The URT, and in particular, the tonsils, was a major site of colonization by *M. bovis*. *Mycoplasma bovis* was isolated from both palatine and pharyngeal tonsils of all inoculated calves at necropsy (Figure 3-2A and B). Colonization at these sites tended to be heavier in orally than transtracheally inoculated calves. Similarly, *M. bovis* was isolated from the eustachian tubes of calves inoculated by either route, but the CFU recovered from the eustachian tubes were up to 10^5 times higher in orally than transtracheally inoculated calves (Figure 3-2C and D). Eustachian tube colonization was bilateral in all cases except one. Large numbers (10^5 to 10^9 CFU/ml) of *M. bovis* were isolated from both tympanic bullae of all three calves with clinical signs of otitis media (Figure 3-2C). Once *M. bovis* colonization of the bullae occurred, the CFU levels were the highest achieved at any body site. *M. bovis* was not isolated from the bullae of orally inoculated calves without clinical signs of otitis media or from any of the transtracheally inoculated calves. In addition, *M. bovis* was isolated from the MRPLN of all except one of the infected calves (Figure 3-2A and B). It is notable that despite heavy colonization of tonsils, *M. bovis* was only isolated from nasal swabs of two calves over the complete course of the study (Figure 3-2A and

B), and then only on day 14 post-infection. No mycoplasmas were recovered from control calves, nor were other bacterial or viral pathogens of the respiratory tract isolated from any calf. All mycoplasmal isolates recovered from infected calves were confirmed to be *M. bovis* by PCR sequencing of the 16S *rRNA* gene (data not shown) and had IS hybridization profiles consistent with that of the F1 isolate used for inoculation (data not shown).

Isolation of *M. bovis* from Lungs and Clinical Signs of Respiratory Disease

Isolation of *M. bovis* from the lungs was associated with clinical signs of respiratory disease in calves inoculated by the oral route. Four of the eight (50%) orally inoculated calves were colonized in at least one LRT site (trachea, bronchi, TBLN and/or lung) at necropsy (Figure 3-2E to H). However, colonization was most extensive in the two calves that had more severe clinical signs of respiratory disease, and importantly, these were the only orally inoculated calves in which *M. bovis* was isolated from the lungs (Figure 3-2G and H). In contrast, *M. bovis* was isolated from the lungs of four of five (80%) transtracheally inoculated calves (Figure 3-2G and H), but these calves only exhibited transient and mild clinical signs of respiratory disease. *M. bovis* appeared to have been cleared from the LRT in one of the transtracheally inoculated calves and was only recovered from URT sites (both tonsil sites and the MRPLN).

Colonization of the Tonsil and Development of Disease

The level of tonsil colonization was associated with the development of disease. The Log_{10} CFU of *M. bovis* isolated from the pharyngeal tonsils was positively correlated with that isolated from both the left ($R^2 = 0.74$, $P = 0.004$) and right ($R^2 = 0.72$, $P = 0.005$) eustachian tubes (Figure 3-3). In addition, the severity of lesions in the eustachian tube and in the tympanic bullae was positively correlated with the number of mycoplasma isolated from the same site (J. Powe, F. P. Maunsell, J. W. Simecka and M. B. Brown, submitted for publication). Similarly,

there was a trend ($P = 0.11$) for the number of *M. bovis* isolated from the palatine tonsils to be positively correlated with that isolated from the lungs (data not shown).

Gross and Histopathologic Lesions

Experimentally inoculated calves had gross and/or histopathologic lesions typical of *M. bovis* infection. In nasal passages, there was little evidence of inflammation or other pathologies in either orally or transtracheally inoculated calves, and histopathologic scores of nasal passages were no different than those from control calves. There was also no difference among groups in histopathologic scores of pharyngeal tonsils. Histopathologic scores of the palatine tonsils of infected calves tended to be higher than those of control calves, but these differences were not statistically significant ($P = 0.1$).

All three calves with clinical signs of otitis media had bilateral suppurative otitis media at necropsy (Figure 3-4). There was an obvious purulent discharge from the nasopharyngeal opening of the eustachian tube from one of these calves, but other gross abnormalities of the URT were not observed. As indicated above, these calves with otitis media were inoculated orally with *M. bovis,* and the pathology is fully characterized in a companion paper (J. Powe, F. P. Maunsell, J. W. Simecka and M. B. Brown, submitted for publication). Importantly, there was no evidence of eustachitis or middle ear disease in transtracheally inoculated calves.

There were histopathologic changes in the lymph nodes draining the URT of calves after mycoplasma inoculation. In orally inoculated calves, histopathologic scores for both MRPLN and LPRLN were higher ($P < 0.05$) than those of control calves (Figure 3-5A and B). Lymphoid hyperplasia, edema and focal areas of necrosis and suppurative lymphadenitis were observed in these lymph nodes (Figure 3-6). Transtracheally infected calves tended to have higher scores than control calves (Figure 3-5A and B) but these differences were not statistically significant. As part of the overall histopathologic score, lymphoid tissues were graded based on the number

of plasma cells present. Plasma cell subscores for MRPLN and LRPLN were significantly higher in infected calves when compared with control calves, regardless of the route of inoculation (Figure 3-5C and D).

In the LRT, neither orally nor transtracheally inoculated calves had significant gross or histopathologic lesions in tracheal or primary bronchial mucosa. Focal areas of consolidation and pneumonic lesions were observed in the lungs of four of eight (50%) orally inoculated and four of five (80%) transtracheally inoculated calves (Figure 3-7), although there was no statistically significant difference in the percentage of visibly affected lung among groups. In contrast, histopathologic lung lesion scores differed among groups ($P < 0.05$); calves infected by the transtracheal route had higher lung lesion scores when compared with control calves (Figure 3-8A). Calves from which *M. bovis* was isolated from the lung had focal areas of suppurative or non-suppurative broncho- or bronchointerstitial pneumonia, sometimes with foci of coagulative necrosis surrounded by a mixed inflammatory cell population (Figure 3-8D). These calves also had areas of bronchiolitis with peribronchial infiltration of lymphocytes, plasma cells and macrophages, often accompanied by suppurative bronchial exudates. The bronchiolar changes were mostly limited to small airways. Subscores for lymphoid hyperplasia in lungs were significantly higher ($P < 0.05$) in both orally and transtracheally inoculated calves than in control calves (Figure 3-8B). In contrast to the findings for the lymph nodes in the URT, histopathological scores or plasma cell subscores for tracheobronchial lymph nodes did not vary significantly among groups (data not shown).

Immunoglobulin Response

Orally infected calves exhibited a *M. bovis*-specific serum IgG response. It is important to note that some *M. bovis*-specific Ig was passively transferred via the colostrum substitute fed to the calves, and, therefore colostral-derived antibody to *M. bovis* was detected at day 0. While

the titer of passively-acquired serum IgG in control calves either remained static or declined over the study period, the titer of *M. bovis*-specific serum IgG in infected calves remained stable or increased (Figure 3-9), indicating an active immune response to infection in these animals. When the fold-change in *M. bovis* specific serum antibody titers between days 0 and 14 of the study were compared among groups, calves in the orally infected group had a significant fold-increase in IgG ($P = 0.049$), compared with the control group. Calves infected by the oral route exhibited a stronger serum IgG response than calves infected by the transtracheal route, although there were individual calf variations.

Discussion

We have definitively demonstrated that bucket nursing of milk containing *M. bovis* does result in colonization of the URT of young calves and can cause clinical disease. Despite the fact that ingestion of *M. bovis*-contaminated milk or colostrum is thought to be a major route of natural infection in young calves (Bennett and Jasper, 1977c; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000), experimental infection by this route has not previously been reported. Other investigators have reported that calves allowed to nurse cows with *M. bovis* mastitis develop URT colonization and/or *M. bovis*-associated clinical disease (Stalheim and Page, 1975; Bennett and Jasper, 1977c), and colonization of the URT by *M. bovis* occurs more frequently in calves fed infected milk than in those fed uninfected milk (Bennett and Jasper, 1977c). Reports of a strong temporal association between the feeding of milk containing *M. bovis* and disease outbreaks have added support to the hypothesis that contaminated milk is a means by which infection is introduced into a group of calves (Dechant and Donovan, 1995; Walz *et al*., 1997), although direct contact with infected animals and secondary transmission through respiratory aerosols or fomites are also likely to play a significant role in calf-to-calf spread. Because our model mimics an important natural route of *M. bovis* infection, it will facilitate studies of host

and pathogen-related events that are relevant to natural infection in young calves, especially those events involved in colonization of the URT and dissemination from the URT to the LRT and/or middle ear. In addition, experimental verification that calves fed contaminated milk do become colonized with *M. bovis* lends support to control measures aimed at eliminating *M. bovis* contaminated milk and colostrum from calf diets.

Using the oral route of inoculation, our model resulted in colonization of the eustachian tubes with *M. bovis* in seven of eight inoculated calves, with otitis media developing in 37% of calves by 2 weeks post-infection. The clinical signs of otitis media observed in naturally infected calves include fever, anorexia, listlessness, ear pain evidenced by head shaking and scratching at or rubbing ears, epiphora, and ear droop and other signs of facial nerve paralysis (Walz *et al*., 1997; Brown *et al*., 1998a; Maeda *et al*., 2003; Francoz *et al*., 2004). In some cases, purulent discharge from the ear canal is observed following rupture of the tympanic membrane (Walz *et al*., 1997; Francoz *et al*., 2004). Secondary complications such as otitis interna (Lamm *et al*., 2004) and meningitis (Francoz *et al*., 2004) can occur. In addition, calves with *M. bovis*-induced otitis media often have concurrent pneumonia (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). With the exception of rupture of the tympanic membrane and secondary complications of otitis media, all of the clinical signs reported in natural infections were observed in our experimentally infected animals, providing support for the validity of the oral route of infection in our model system. The more severe sequelae, which were not observed in calves in this study, are most frequently associated with chronic otitis media, and under our protocol, calves were euthanized prior to reaching this stage of disease. Otitis media in our experimentally infected calves was shown to be histopathologically similar to natural disease and was likely due to an ascending infection through the eustachian tubes (J. Powe, F. P. Maunsell,

J. W. Simecka and M. B. Brown, submitted for publication). Thus, the clinical signs and pathology observed in our study is consistent with that described for naturally occurring *M. bovis*-induced otitis media in young calves.

Clinical signs of LRT disease were observed in six of eight calves inoculated by the oral route. Although clinical signs were mainly transient and mild, two calves developed more serious LRT disease and these were the only calves from which *M. bovis* was recovered from the lung at necropsy. Histopathological lesions in these calves were consistent with other reports of naturally occurring and experimentally induced *M. bovis* pneumonia (Rodriguez *et al*., 1996; Maeda *et al*., 2003). Interestingly, there was little gross or histopathological evidence of tracheitis or lesions involving large bronchi in inoculated calves, even when *M. bovis* was recovered from these sites. Large airway lesions are reported to accompany field cases of mycoplasmal pneumonia (Dungworth, 1993), although it may be that these lesions do not develop until later in the course of disease, that they require the presence of other pathogens, or that they are not a prominent feature of *M. bovis* infection in this age animal.

Variable disease expression is a key feature of mycoplasmal infections in general (Rosengarten *et al*., 2000), including *M. bovis* (Gourlay and Houghton, 1985; Allen *et al*., 1991). Consistent with this feature of the naturally occurring disease, 37% of the orally inoculated calves in our model developed clinical otitis media and 75% exhibited mild, transient clinical signs of respiratory disease. Although inoculation of a larger dose of *M. bovis* may have resulted in increased severity or incidence of clinical disease and/or pathology, we selected our inoculum dose to be biologically relevant in order to mimic naturally occurring disease as closely as possible. The dose utilized in our model was a total of $2.9 \pm 2.5 \times 10^{10}$ CFU over 3 milk feedings, which equated to approximately 10^6 CFU/ml of milk replacer. Concentrations of

M. bovis in mammary secretions of infected cows vary markedly, but are frequently 10^6 CFU/ml or greater (Jasper, 1981; Fox and Gay, 1993). In pilot studies, we found that repeated exposure to 10^6 CFU of *M. bovis* per ml of milk was necessary to achieve colonization of the URT in all inoculated calves (data not shown).

Variation in disease expression may also have been influenced by genetic variability among calves; this is a potential disadvantage of using an outbred host in an experimental infection model. However, there are currently no suitable alternative inbred laboratory animals in which to model *M. bovis* infection of young calves. An additional factor that is likely to have contributed to the variable disease expression observed in our study is that calves were euthanized on or prior to 14 days after infection. This infection period was chosen because we are primarily interested in studying the immunological events that occur early in infection. However, several calves without otitis media did have colonization of the eustachian tubes at necropsy, but with lower CFU of *M. bovis* recovered than those with otitis media. Given the direct correlation between CFU in tonsils, eustachian tubes and the bullae, it is likely that a longer infection period would result in increased numbers of *M. bovis* in these sites with a concomitant increased rate of otitis media in this model. Increasing the length of the infection period may also result in an increase in the rate and severity of LRT disease, although further studies would be required to determine this.

The URT, and in particular, the tonsilar mucosa was an important site of colonization following oral inoculation of *M. bovis* in this study. In fact, both the palatine and pharyngeal tonsils of all inoculated calves were colonized with *M. bovis* at the time of necropsy. Our findings are consistent with studies of naturally occurring *M. bovis* infection in calves that have suggested that the URT is the initial site of colonization (Bennett and Jasper, 1977c; Brys *et al.*,

1989). We found that the number of *M. bovis* recovered from the tonsils was correlated with the presence of clinical disease. It is intriguing to hypothesize that control strategies specifically aimed at limiting growth of *M. bovis* in tonsils may be effective in preventing clinical disease, and this oral inoculation model could be applied to evaluate such potential control measures. Additionally, we found that the tonsils of infected calves can be heavily colonized with *M. bovis* without the microorganism being recovered from deep nasal swabs, suggesting that, although more technically challenging to obtain, tonsil swabs may be a better choice for determining the true *M. bovis* colonization status of an animal. Our tonsil swabs were obtained at necropsy which eliminated the difficulties of obtaining good access to the sampling site; further studies will be required in live animals of various ages to determine the usefulness of tonsil swabs for determining the *M. bovis* status of an animal in a clinical setting. Once colonization of the URT occurs, a variety of factors are likely to influence dissemination from this site and development of disease; these may include virulence factors expressed *in vivo* by *M. bovis*, the host immune response, the frequency and dose of exposure, the presence of other pathogens and various environmental factors. The use of our model to obtain a better understanding of these factors may lead to improved preventative strategies against this disease.

Calves inoculated with *M. bovis* by either the oral or transtracheal routes exhibited local and systemic immune responses to infection. Both transtracheally and orally inoculated calves had increased lymphoid hyperplasia in the lymph nodes of the URT and in the lungs when compared with control calves, consistent with an active response to infection. Calves inoculated by the oral route had a higher serum IgG response than calves infected by the transtracheal route, despite the presence of significant histopathological lung lesions in the latter group. Although there did appear to be a trend for serum IgG titers to *M. bovis* to increase over the 14 day

infection period in transtracheally inoculated calves (Figure 3-9), they were not statistically different from the control group. The difference in IgG response between orally and transtracheally inoculated calves may reflect differences in inoculation dose between the two groups, or could indicate that a *M. bovis* infection of the URT stimulates a stronger humoral response than one which originates primarily in the LRT.

Calves infected transtracheally did become colonized in the URT, presumably by ciliary transport of organisms up the trachea. However, the numbers of *M. bovis* recovered from URT sites were usually fewer than for orally inoculated calves, and transtracheally inoculated calves did not develop otitis media. These findings indicate that oral inoculation is better suited to study of events occurring during *M. bovis* infection of the URT and middle ear than a model where *M. bovis* is inoculated directly into the LRT. It was also interesting that transtracheally inoculated calves had significantly greater lung lesion scores than orally inoculated calves, despite exhibiting only mild and transient clinical signs of LRT disease. *M. bovis* was recovered from the lung of four of the five transtracheally inoculated calves and only two of the eight orally inoculated calves, suggesting that if lung colonization within 14 days of inoculation is the goal of a particular experimental inoculation procedure then transtracheal route may be a better choice. However, the host and pathogen events involved in dissemination from URT sites to the lung are likely to be important in naturally occurring disease and may not be present in a model where *M. bovis* is inoculated directly into the LRT.

In summary, we have developed a reproducible model of *M. bovis* infection of the URT that closely mimics naturally occurring *M. bovis* infections in young (pre-weaned) calves. There are important differences between very young calves and older cattle in terms of their immune environment and the occurrence of middle ear infections. Therefore, an infection model that uses

a clinically relevant age group, especially when considering events leading to middle ear infection, is likely to be critical when studying this emerging disease problem. Our study also has direct clinical relevance by definitively demonstrating for the first time that calves consistently become infected when they ingest *M. bovis* contaminated milk, and that calves can be colonized heavily in the tonsils without *M. bovis* being detected on nasal swabs. The oral inoculation model that we have presented here is particularly suited to the study of host-pathogen interactions during initial colonization of the tonsils, expansion of infection and dissemination to the LRT and middle ear. In addition, the model could be used to investigate potential new preventative or control strategies, especially those aimed at limiting colonization of the tonsils and/or spread to the middle ear.

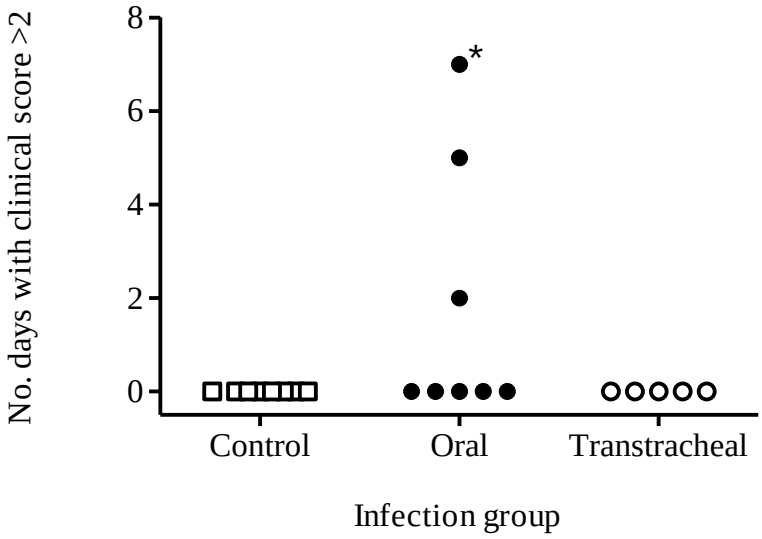


Figure 3-1. Number of days that calves had a daily clinical score of > 2. Calves were followed for 14 days post-inoculation or until they reached criteria for euthanasia (*One calf was euthanized at 10 days post-inoculation). The maximum daily clinical score was 10. Calves were inoculated with sterile carrier (controls, *n*=8) or with *Mycoplasma bovis* by oral (*n*=8) or transtracheal (*n*=5) routes.

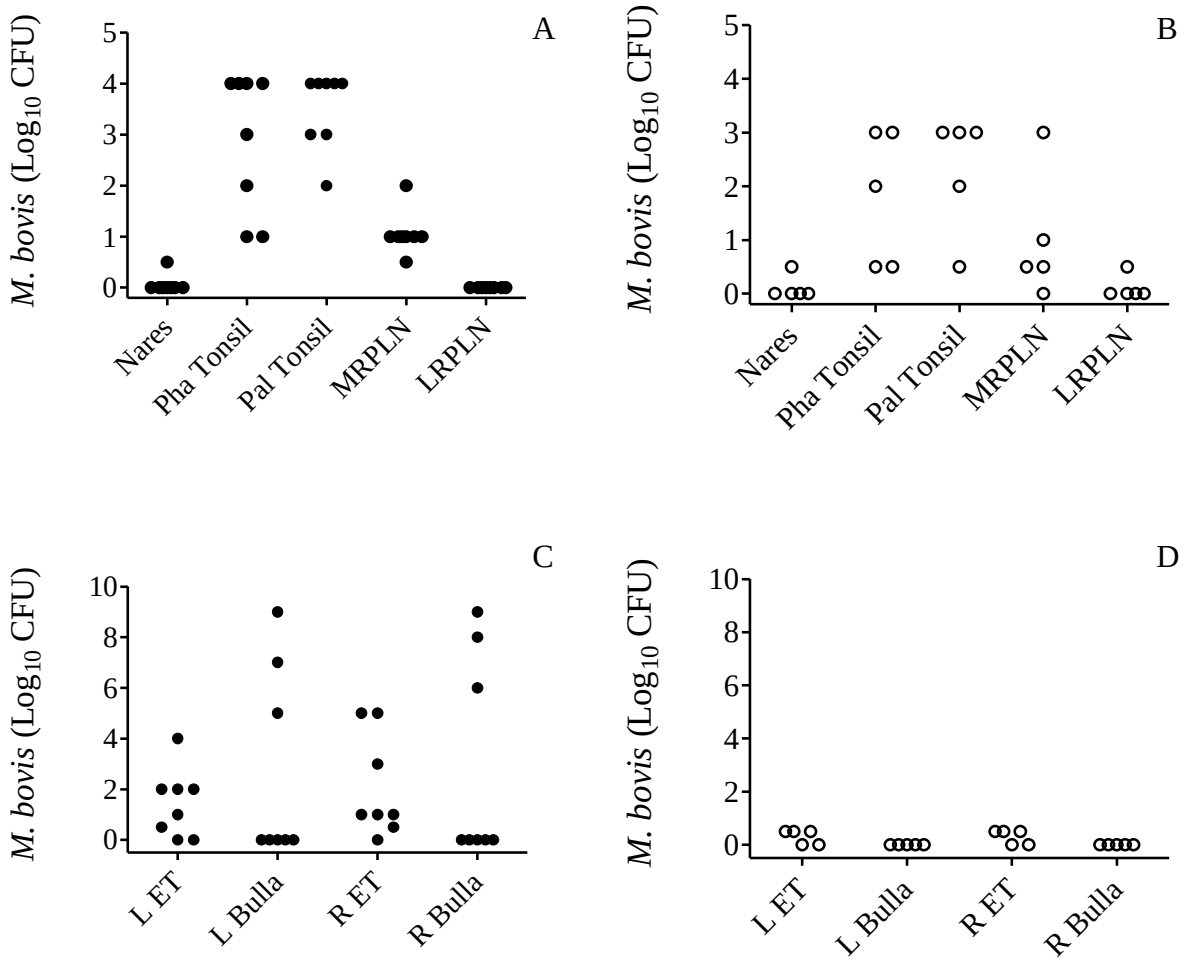


Figure 3-2. The number of *Mycoplasma bovis* recovered at necropsy. A) *M. bovis* recovered from upper respiratory tract sites (URT) for calves inoculated by the oral (*n*=8) route. B) *M. bovis* recovered from URT sites for calves inoculated by the transtracheal (*n*=5) route. C) *M. bovis* recovered from middle ear sites for calves inoculated by the oral route. D) *M. bovis* recovered from middle ear sites for calves inoculated by the transtracheal route. E) *M. bovis* recovered from lower respiratory tract (LRT) sites of calves inoculated by the oral route. F) *M. bovis* recovered from LRT sites of calves inoculated by the transtracheal route. G) *M. bovis* recovered from the lungs of calves in the oral and transtracheal inoculation groups. H) The number of lung sites from which *M. bovis* was recovered out of a total of 6 standard sites which were cultured (1 site per lung lobe) for calves in the oral and transtracheal inoculation groups. Semiquantitative culture results are expressed as Log_{10} of the highest dilution that yielded mycoplasmal colonies. When only the undiluted broth was positive, results were assigned a Log_{10} value of 0.5. Quantitative culture results are expressed as colony forming units (CFU)/g of tissue (lung) or CFU/ml of exudate (tympanic bullae). No mycoplasmas were recovered from control calves (data not shown). Pha Tonsil = pharyngeal tonsil,

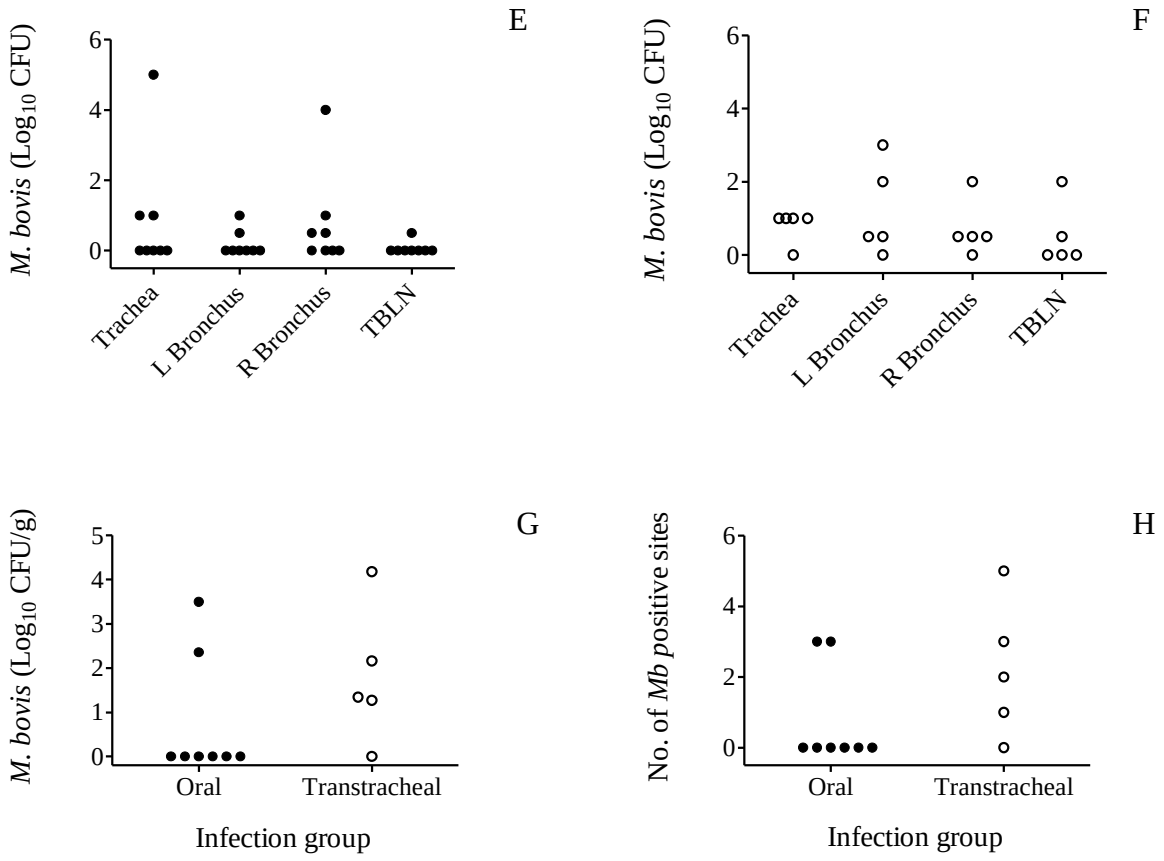


Figure 3-2. (continued). Pal Tonsil = palatine tonsil, MRPLN=medial retropharyngeal lymph nodes, LRPLN = lateral retropharyngeal lymph nodes, L ET = left eustachian tube, R ET = right eustachian tube, L Bulla = left tympanic bulla, R Bulla = right tympanic bulla, L Bronchus = left primary bronchus, R Bronchus = right primary bronchus, TBLN = tracheobronchial lymph nodes, No. = number, *Mb* = *Mycoplasma bovis*.

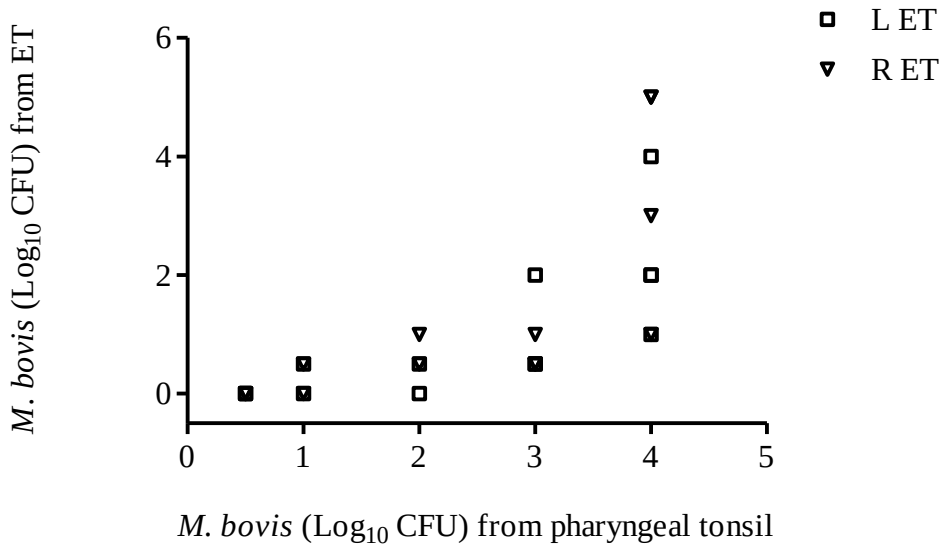


Figure 3-3. Relationship between the number of *Mycoplasma bovis* recovered from the left and right eustachian tubes (L ET and R ET, respectively) and pharyngeal tonsils in calves inoculated with *M. bovis* by either the oral (*n*=8) or transtracheal (*n*=5) routes. Culture results are expressed as Log_{10} of the highest dilution that yielded mycoplasmal colonies. When only the undiluted broth was positive, results were assigned a Log_{10} value of 0.5. No mycoplasmas were recovered from carrier inoculated control calves (data not shown).

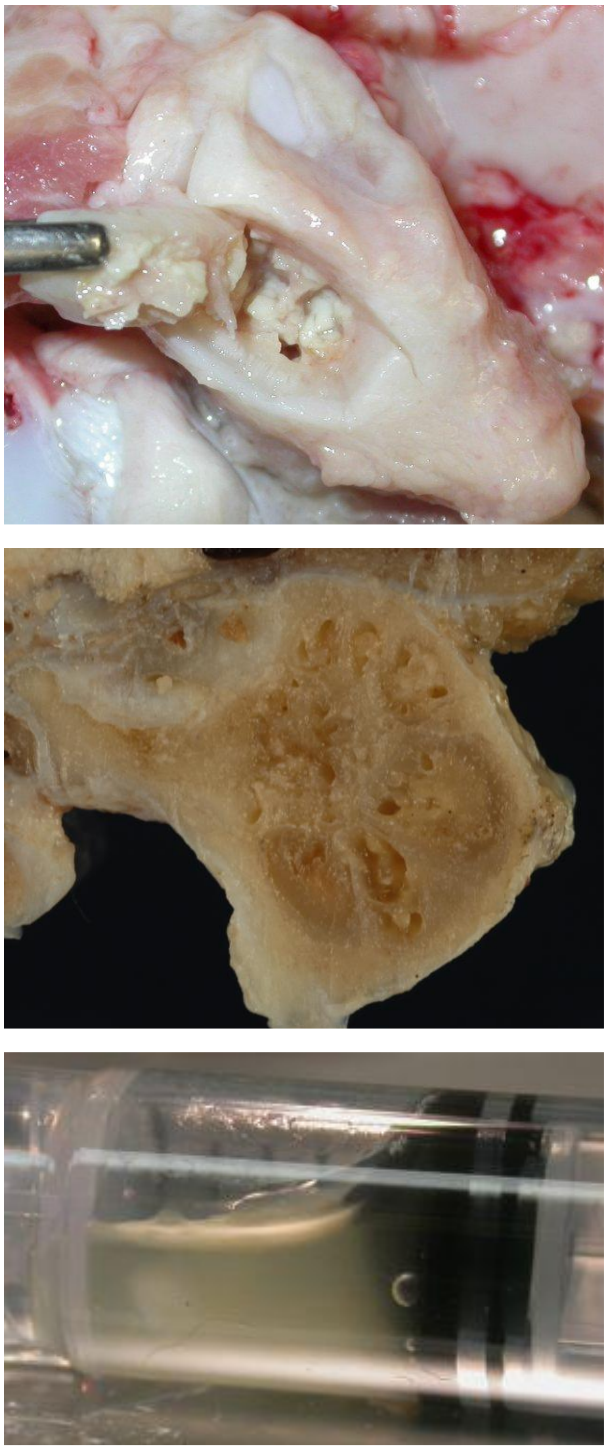


Figure 3-4. Macroscopic lesions of otitis media in calves orally inoculated with *Mycoplasma bovis*. A) Ventral aspect of the tympanic bulla reflected to reveal exudate. B) Caseous exudate within a sagittal section of the tympanic bulla after tissue fixation. C) Syringe containing suppurative exudate aspirated from the tympanic bulla.

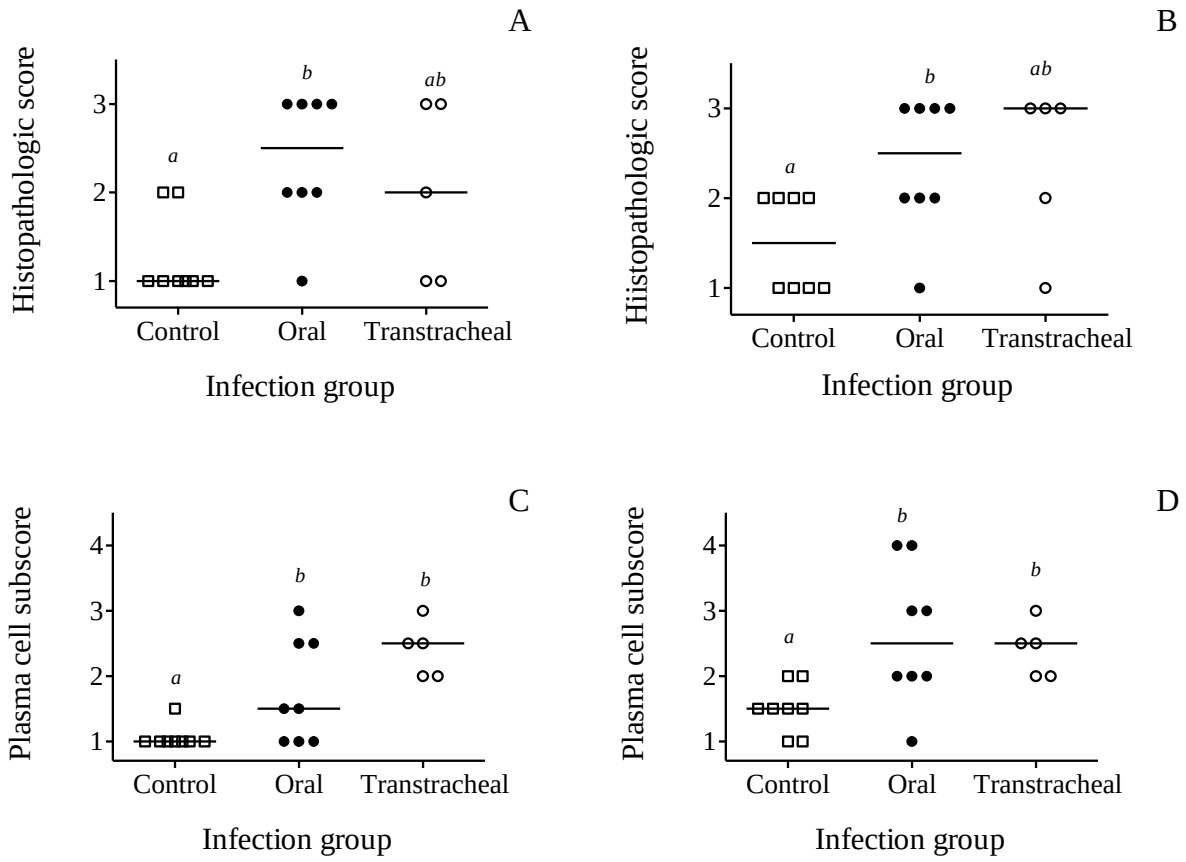


Figure 3-5. Histopathology of retropharyngeal lymph nodes from control calves (n=8) or calves inoculated with *Mycoplasma bovis* by oral (n=8) or transtracheal (n=5) routes. A) Histopathological lesion scores of medial retropharyngeal lymph nodes (MRPLN). B) Histopathological lesion scores of lateral retropharyngeal lymph nodes (LRPLN). C) Plasma cell subscores of MRPLN. D) Plasma cell subscores of LRPLN. Samples were collected at necropsy (14 days post-infection except for one calf inoculated with *M. bovis* by the oral route which had to be euthanized at 10 days post-infection). Tissues were graded on a scale from 1 (minimal or no lesions or lymphoid hyperplasia) to 3 (severe lesions and/or marked lymphoid hyperplasia) and from 1 (few plasma cells) to 4 (large numbers of plasma cells) for histopathologic scores and plasma cell subscores, respectively. Data are represented as scores for individual calves with the median value for the group indicated by a horizontal line. [ab]Superscript letters at the top of each data column indicate significant ($P < 0.05$) differences between groups.

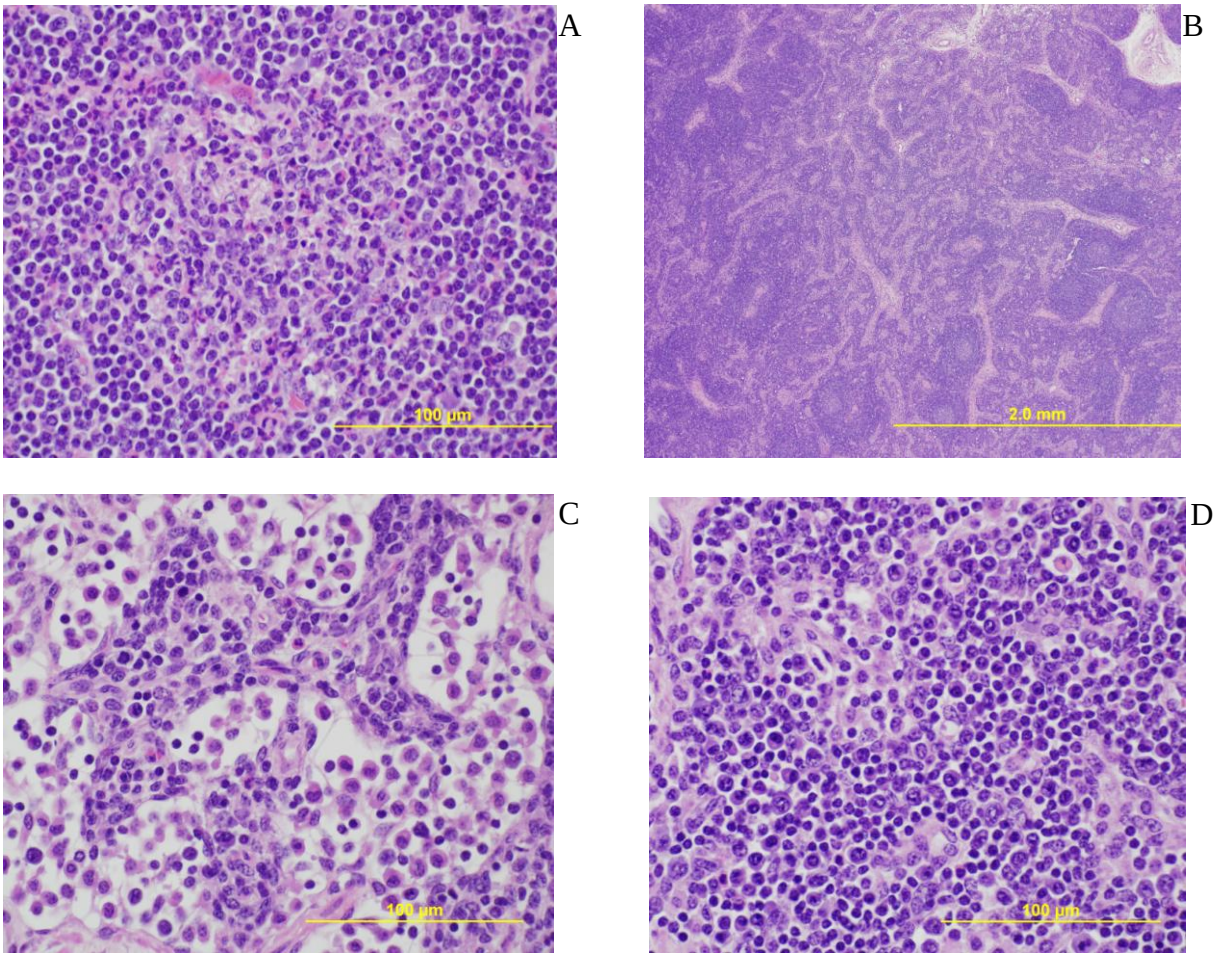


Figure 3-6. Representative histopathological findings in retropharyngeal lymph nodes of calves inoculated with sterile carrier (controls) or with *Mycoplasma bovis* by oral or transtracheal routes. A) Medial retropharyngeal lymph node of an infected calf. Scattered small accumulations of neutrophils can be seen in this region of cortex, indicative of focal lymphadenitis. Magnification ×60. B) Lateral retropharyngeal lymph node (LRPLN) of an infected calf with lymphoid hyperplasia. Magnification ×4. C) LRPLN of a control calf. Medullary sinuses contain scattered small lymphocytes. Magnification ×60. D) LRPLN of an infected calf. Medullary sinuses contain large numbers of plasma cells and small and large lymphocytes. Magnification ×60.

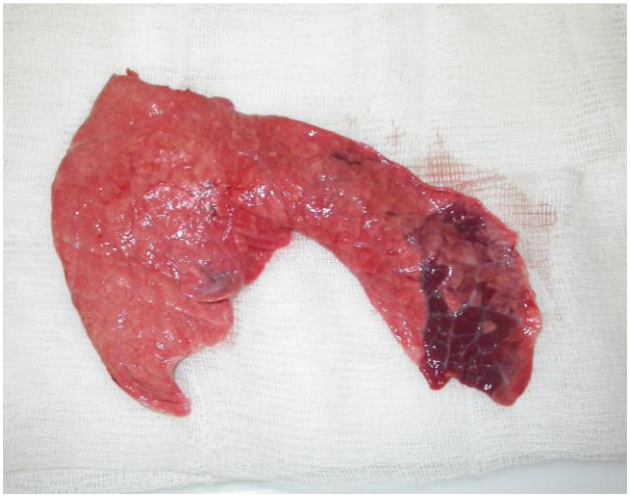


Figure 3-7. Representative macroscopic lung lesion in a calf experimentally infected with *Mycoplasma bovis* by the oral route.

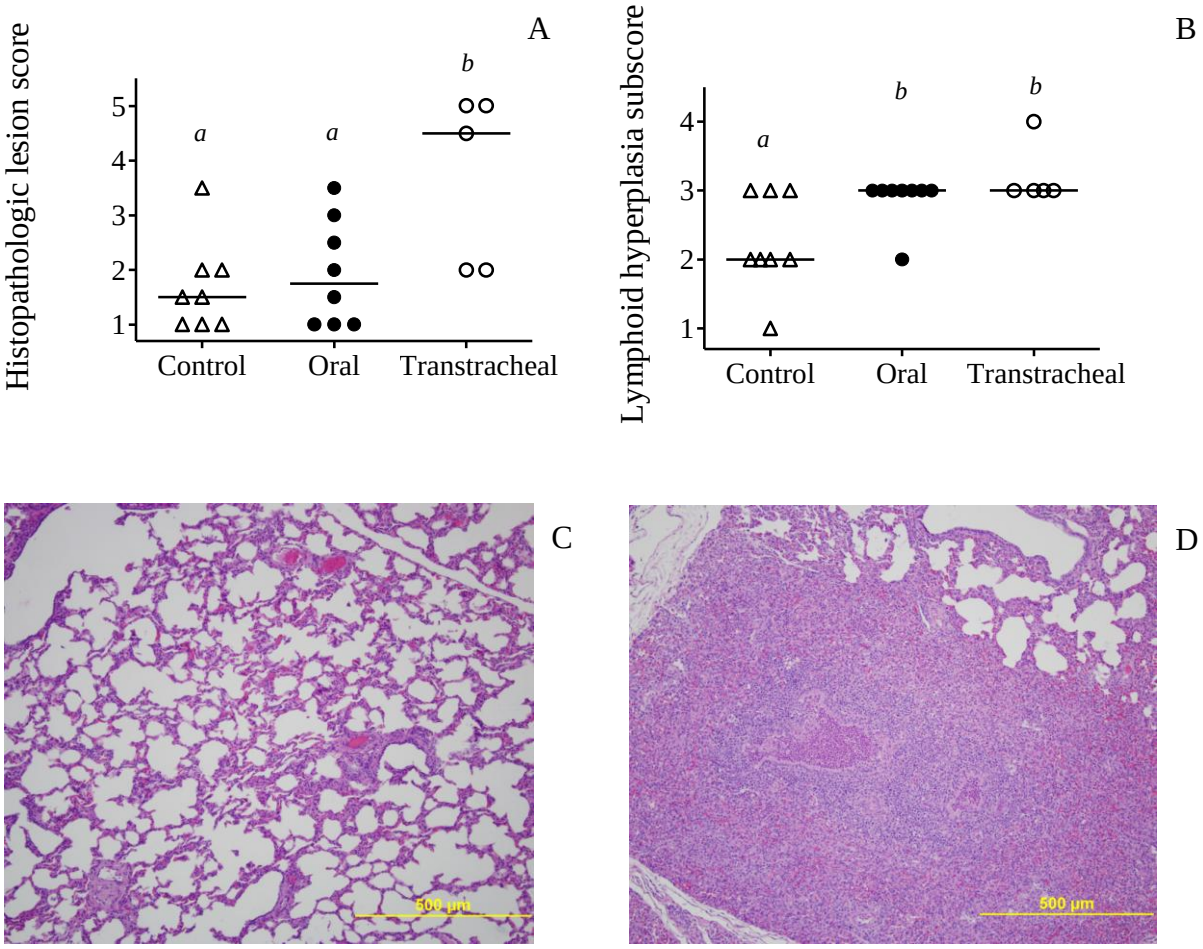


Figure 3-8. Histopathological findings in the lungs of calves inoculated with sterile carrier (controls, *n*=8) or with *Mycoplasma bovis* by oral (*n*=8) or transtracheal (*n*=5) routes. Samples were collected at necropsy (14 days post-infection except for one calf inoculated with *M. bovis* by the oral route which had to be euthanized at 10 days post-infection). A) Overall histopathological lesion scores. B) Subscores for lymphoid hyperplasia. Data in A and B are represented as scores for individual calves with the median value indicated by a horizontal line. Tissues were graded on a scale from 1 (minimal or no lesions) to 5 (most severe lesions) and from 1 (no lymphoid hyperplasia) to 4 (marked lymphoid hyperplasia) for lesion scores and lymphoid hyperplasia subscores, respectively. [ab]Superscript letters indicate significant ($P < 0.05$) differences between groups. C) Representative histopathologic appearance of a lung section with a lesion score of 1 (control calf). D) Representative histopathologic appearance of a lung section with a lesion score of 5 (orally inoculated calf from which *M. bovis* was recovered from the lung). Magnification ×10.

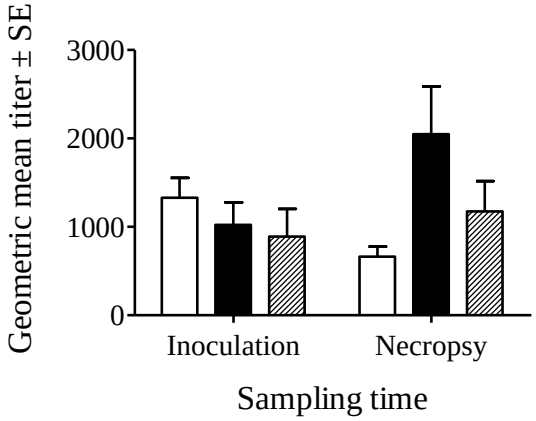


Figure 3-9. Geometric mean end-point titers for *Mycoplasma bovis*-specific serum IgG. Calves were experimentally inoculated with sterile carrier (*n*=8, solid white bars) or with *M. bovis* by the oral (*n*=8, solid black bars) or transtracheal (*n*=5, hatched bars) routes. Geometric mean end-point titers are shown at the time of inoculation (day 0) and at necropsy (14 days post infection, except for one calf inoculated with *M. bovis* by the oral route which was had to be euthanized at 10 days post-infection).

CHAPTER 4
IMMUNE RESPONSES IN THE RESPIRATORY TRACT OF CALVES INFECTED WITH *Mycoplasma bovis*

Introduction

Mycoplasma bovis is an important contributor to morbidity and mortality in pre-weaned dairy calves, causing respiratory disease, otitis media and arthritis as well as some other less common clinical manifestations (Stipkovits *et al*., 2001; Nicholas and Ayling, 2003; Francoz *et al*., 2004; Lamm *et al*., 2004). In addition, *M. bovis* causes chronic respiratory disease and arthritis in stocker and feeder cattle (Haines *et al*., 2001; Thomas *et al*., 2002a; Gagea *et al*., 2006) and is a major mastitis pathogen in adult dairy cattle (Jasper, 1981; Fox *et al*., 2003; Gonzalez and Wilson, 2003). One of the major routes of transmission of *M. bovis* to young calves is thought to be ingestion of contaminated milk from cows with *M. bovis* mastitis (Stalheim and Page, 1975; Pfutzner and Schimmel, 1985; Walz *et al*., 1997; Brown *et al*., 1998a; Butler *et al*., 2000). Calf-to-calf transmission via aerosols, fomites and direct contact are also likely to be important (Jasper *et al*., 1974; Bennett and Jasper, 1977c; Tschopp *et al*., 2001; Nicholas and Ayling, 2003). Regardless of the route of exposure, *M. bovis* first colonizes the upper respiratory tract (URT) (Bennett and Jasper, 1977c; Pfutzner and Sachse, 1996). Colonization often occurs very early in life; during some *M. bovis*-associated disease outbreaks the majority of calves have been infected before 2 weeks of age (Brown *et al*., 1998a; Stipkovits *et al*., 2000). Infection may remain localized, or *M. bovis* can disseminate to the lower respiratory tract (LRT), middle ear, joints and/or other body sites where it may cause clinical disease. Factors controlling dissemination of *M. bovis* from the URT and clinical disease expression are unknown.

Immune responses to *M. bovis* infections in young calves are poorly defined, despite the fact that immunologic responses probably have the greatest impact on the progression of

mycoplasmal disease. Innate responses and mucosal antibody responses are critical for early clearance and control of mycoplasmal infections (Cartner *et al*., 1998; Hickman-Davis, 2002). Effective killing of *M. bovis* by phagocytes requires the presence of mucosal antibody, particularly IgG_2 (Howard, 1984). Early mucosal and serum antibody responses in young calves infected with *M. bovis* are characterized by high levels of IgG_1 and little IgG_2, which is unlikely to be optimal for clearance of the infection (Howard *et al*., 1980; Howard and Gourlay, 1983; Howard *et al*., 1987c; Vanden Bush and Rosenbusch, 2003). Although antibody responses in *M. bovis* infections have been defined, there is only limited data describing the lymphocyte populations that contribute to adaptive immune responses in cattle, and virtually no data is available from neonatal calves. Advances in the development of vaccines or other strategies to prevent *M. bovis*-associated disease in young dairy calves are likely require a better understanding of the immune response to *M. bovis* in this age group. In particular, the local immune responses generated at the site of *M. bovis* infection, as well as immune system events leading to dissemination of infection, need to be defined in young calves.

Adaptive immune responses can protect from mycoplasmal respiratory infections (Taylor *et al*., 1977; Cassell and Davis, 1978; Whithear, 1996; Thacker *et al*., 2000; Kyriakis *et al*., 2001; Dedieu *et al*., 2005), including *M. bovis* (Howard *et al*., 1987a; Nicholas *et al*., 2002). However, immunity is often short-lived, and animals are susceptible to repeated infections (Bennett and Jasper, 1978b). Vaccination against *M. bovis*, *Mycoplasma hyopneumoniae* and *Mycoplasma pulmonis* confers only partial protection from disease, as organisms are easily isolated from challenged animals (Cassell and Davis, 1978; Howard *et al*., 1980; Thacker *et al*., 2000; Nicholas *et al*., 2002). Although adaptive responses that are present prior to challenge may afford some degree of protection, responses that develop after mycoplasmal infection often fail

to clear the organisms or prevent clinical disease. In fact, adaptive immune responses also contribute to the development of disease.

Many mycoplasmal respiratory diseases are clearly immunopathologic. One of the consistent characteristics of respiratory disease caused by a wide variety of mycoplasmal species is the large accumulation of lymphoid cells along the respiratory tract, independent of the host species (Simecka *et al*., 1992; Rodriguez *et al*., 1996). Both B and T cells accumulate in lungs of affected calves (Howard *et al*., 1987c), in joints of calves with mycoplasmal arthritis (Gourlay *et al*., 1976; Adegboye *et al*., 1996; Gagea *et al*., 2006) and in the mammary glands of cows with *M. bovis* mastitis (Bennett and Jasper, 1977a; Seffner and Pfutzner, 1980). These findings, together with similar findings in other host species (Jones and Simecka, 2003), suggest that lymphocyte activation and recruitment to sites of mycoplasmal infection are important in the development of pathology. Probably the best evidence for an immunopathologic response comes from studies in laboratory rats and mice infected with *M. pulmonis*, where the number of T cells recovered from the lungs is correlated with the severity of disease (Davis *et al*., 1982; Jones *et al*., 2002). Immunodeficient (T cell deficient and severe combined immunodeficiency) mice or T cell deficient hamsters develop significantly less severe mycoplasmal respiratory disease than their immunocompetent counterparts (Keystone *et al*., 1980; Cartner *et al*., 1998). Importantly, these changes in severity occur with little effect on the number of mycoplasmas in the lungs of infected animals. Thus, the severity of mycoplasmal respiratory diseases is increased by an intact T cell response. A similar phenomenon may occur in *M. bovis* infections; the accumulation of lymphocytes at sites of infection together with reports of enhanced disease severity after immunization for *M. bovis* (Rosenbusch, 1998; Bryson *et al*., 1999) are certainly consistent with the development of immunopathologic responses.

Specific T cell subsets have been associated with protective or immunopathologic responses in *M. pulmonis* respiratory disease. In mice infected with *M. pulmonis,* $CD4^+$ T cells are the major population contributing to lymphoid accumulations in the lungs and lymphoid tissues of the LRT, and *in vivo* depletion of $CD4^+$ T cells results in reduced severity of *M. pulmonis*-induced pulmonary lesions but has no effect on the numbers of mycoplasmas in the lungs (Jones *et al*., 2002). Thus, $CD4^+$ T cells appear to exacerbate the severity of mycoplasmal respiratory disease rather than resolving the infection. $CD8^+$ T cells also contribute to the T cell responses in mycoplasmal respiratory infections, but to a lesser extent than $CD4^+$ T cells (Jones *et al*., 2002). $CD8^+$ T cells appear to play an immunomodulatory role in mycoplasmal disease. Strains of rats that are resistant to *M. pulmonis* infections have a higher $CD8^+$:$CD4^+$ T cell ratio in their lungs than do susceptible strains of rats (Davis *et al*., 1985). *In vivo* depletion of $CD8^+$ T cells in mice results in a dramatic increase in the severity of mycoplasmal respiratory disease that is independent of mycoplasma numbers in the lungs. Thus $CD8^+$ T cells play a significant role in modulating the inflammatory response against *M. pulmonis* lung infections. The interaction between $CD8^+$ and $CD4^+$ T cells is thought to have a major impact on the outcome of *M. pulmonis* respiratory disease.

The T cell subsets involved in protective and immunopathologic responses in the lungs of calves with *M. bovis* infection have not been defined. However, in goat kids experimentally infected with *M. bovis,* T cells predominated in lymphoid accumulations in the lungs, and $CD4^+$ T cells were a greater contributor to these lesions than were $CD8^+$ T cells (Rodriguez *et al*., 2000). Although conducted in a different host species, the clinical disease and pathology were similar to that reported for *M. bovis* infection of calves. These findings suggest that activation of

$CD4^+$ T cells plays a prominent role in *M. bovis* infections, similar to findings reported for *M. pulmonis* disease in mice.

γδ T cells are a major component of the bovine lymphoid population and can comprise up to 40% of the circulating mononuclear cell population in young calves (Wilson *et al*., 1996; Kampen *et al*., 2006). Distinct subpopulations of γδ T cells are present in calves and differ in terms of their tissue distribution and function (Wyatt *et al*., 1994; Wyatt *et al*., 1996; Wilson *et al*., 1998). The presence or absence of the surface molecule WC1 can be used to divide bovine γδ T cells into two major subpopulations. $WC1^+$ γδ T cells are $CD3^+$ but do not express CD2, CD4, or CD8 (MacHugh *et al*., 1997). Between 65 and 90% of circulating γδ T cells are $WC1^+$ (Blumerman *et al*., 2006; Kampen *et al*., 2006). $WC1^+$ T cells are also found in the white pulp of the spleen, outer cortex of peripheral lymph nodes, mucosal associated lymphoid tissue, epithelial layers of the gut and respiratory tract, and in skin (Clevers *et al*., 1990; Wilson *et al*., 1999). In contrast, $WC1^-$ γδ T cells, which do express CD2 and CD8, represent a small percentage of the circulating γδ T cell population (MacHugh *et al*., 1997), and a large percentage of the γδ T cells in some tissues including the red pulp of the spleen and many mucosal epithelial sites (Hedges *et al*., 2003). γδ T cells are thought to be important in early immune responses to a broad range of antigens, and distinct γδ T cell subsets are likely to have unique functions in these immune responses (Pollock and Welsh, 2002). In calves, $WC1^+$ T cells contribute to early production of interferon-γ during infection (Price *et al*., 2006) and are the major γδ T cell population recruited to sites of inflammation (Wilson *et al*., 2002). Although the role of γδ T cells in *M. bovis* infections has not been determined, preliminary data suggests that γδ T cells contribute to the pathogenesis of murine mycoplasmal respiratory disease (J.W. Simecka, personal communication).

The broad, long-term objective of our studies is to determine the immune and inflammatory responses that impact the pathogenesis of and foster protection from bovine mycoplasmal respiratory disease. Based on studies of mycoplasmal disease in laboratory rodents, we hypothesize that the balance between beneficial and detrimental host responses during *M. bovis* respiratory disease is a function of distinct T cell populations. The primary objective of the work presented here was to characterize the B and T lymphocyte populations generated in the URT and LRT of neonatal calves infected with *M. bovis*. As described in Chapter 3, we have defined a model for *M. bovis* infection in young calves that uses feeding of *M. bovis* in milk as the means of inoculation. This model mimics natural infection of calves and results in consistent colonization of the URT. Additionally, the model results in clinical disease expression in a subset of infected calves by 2 weeks post-inoculation. In the current study, we use this model to define the local lymphocyte responses to *M. bovis* infection in young calves and compare these findings with calves infected by transtracheal inoculation.

Materials and Methods

Calves

The calves used for this study have been described in Chapter 3. Briefly, mycoplasma-free male Holstein calves were obtained at birth and were fed two doses of a mycoplasma-free colostrum-replacement product (Acquire™, APC Inc., Ames, IA). Calves were maintained on non-medicated milk replacer and had access to non-medicated calf starter pellets and fresh water at all times. All procedures were conducted with the approval of the University of Florida (UF) Institutional Animal Care and Use Committee.

Strain of *M. bovis* and Experimental Infection

Mycoplasma bovis F1, confirmed as *M. bovis* by PCR and 16S rRNA sequence, is a field strain isolated from a lung abscess in a calf with severe fibrinopurulent pneumonia and pleuritis. A second passage culture was stored in aliquots at -80°C and used for all infection studies.

The overall infection design is shown in Figure 4-1, and the details of the experimental infections are provided in Chapter 3. Briefly, calves were experimentally infected between 7 and 11 days of age with an oral dose of *M. bovis* F1 (total dose $2.9 \pm 2.5 \times 10^{10}$ colony forming units [CFU], infected group, *n*=8) or an identical volume of sterile modified Frey's broth (control group, *n*=4) at each of three consecutive feedings over a 24 hr period. The inoculum was added to milk replacer and bucket fed to calves. A second group of calves were infected via transtracheal inoculation of a single dose of 3×10^9 CFU of *M. bovis* F1 in 20 ml of PBS (infected group, *n*=5), or sterile PBS only (control group, *n*=4). A complete physical examination was performed daily and calves were scored as to the presence of clinical signs of mycoplasmal disease; the scoring system is described in Chapter 3.

At 0, 3, and 7 days post-infection, nasal swabs and blood were obtained for mycoplasmal culture. Serum was collected for determination of specific immunoglobulin (Ig) subclass responses to *M. bovis*. Blood was collected for determination of T cell populations by immunofluorescent cell staining, and additional blood was submitted to the UF Clinical Pathology Laboratory for total and differential leukocyte counts and measurement of plasma fibrinogen and total protein concentrations using standard methodology.

Calves were euthanized at 14 days post infection, with the exception of one orally infected calf that had to be euthanized 10 days post-infection due to severity of clinical disease. At 14 days post-infection, calves underwent full necropsy protocols. The collection of samples

for culture and histopathology, and the assessment of gross lesions were as described in Chapter 3. Histopathology of the eustachian (auditory) tubes, nasal mucosa, tonsils, trachea, primary bronchi and lymph nodes was graded on a scale from 1 (minimal to no lesions and/or lymphoid hyperplasia) to 3 (most severe lesions and or lymphoid hyperplasia). In addition, tissues were graded with respect to numbers of plasma cells present on a scale from 1 (minimal or no plasma cells) to 4 (large numbers of plasma cells). Histopathology of the tympanic bullae and lungs was graded from 1 (minimal to no lesions) to 5 (most severe lesions), and lungs were also graded with respect to the degree of lymphoid infiltration and hyperplasia of bronchial-associated lymphoid tissue from 1 (minimal to no lymphoid hyperplasia) to 4 (marked and widespread lymphoid hyperplasia). All scoring was done in a blinded fashion and the coding system was broken for final data analysis.

Preparation of Mononuclear Cells from Blood and Tissues

To examine the changes in B and T lymphocyte populations in calves after infection with *M. bovis*, mononuclear cells were isolated from blood samples collected at 0, 3, and 7 days post-infection, and from lungs, tracheobronchial lymph nodes (TBLN), lateral retropharyngeal lymph nodes (LRPLN), medial retropharyngeal lymph nodes (MRPLN), palatine tonsils, peripheral blood and spleen samples at necropsy. Weights of whole organs and tissue samples were recorded prior to processing. Heparinized blood was diluted 1:1 in Hank's balanced salt solution (HBSS) and, using standard techniques, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Lymph node and spleen mononuclear cells were isolated by teasing in HBSS, followed by centrifugation. Spleen preparations were treated with ACK (ammonium chloride potassium) lysis buffer (Quality Biological Inc., Gaithersburg, MD) to lyse erythrocytes then washed twice in HBSS. Pulmonary mononuclear cells were prepared from at least 20 g of lung tissue pooled from two

sites in each of the six lung lobes. Lung was finely chopped in RPMI-1640 medium containing 1% (vol./vol.) 1M Hepes solution (Sigma-Aldrich, St. Louis, MO), 1% (vol./vol.) Cellgro Antibiotic-Antimycotic solution (Mediatech Inc., Herndon, VA), 1% (vol./vol.) L-glutamine, 10% (vol./vol.) gamma-free equine serum, 300 U/ml of DNase (Worthington Biochemical Corporation, Lakewood, NJ) and 300 U/ml Type IV collagenase (Worthington Biochemical Corporation, Lakewood, NJ). Lung preparations were incubated for 1.5 hr at 37°C and were vigorously pipetted every 20 to 30 min during the incubation period. Cells were separated from debris by pouring through mesh and centrifuged over Histopaque-1077. Cells from the interface were washed once in RPMI-1640 medium. Cells harvested from all sites were counted and re-suspended in RPMI-1640 medium at the appropriate concentrations for the assays described below.

Immunofluorescent Characterization of T Cell Populations

The proportions of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $WC1^+$ γδ T cells in mononuclear cell populations were determined using immunofluorescent staining and flow cytometry. The monoclonal antibody (mAb) clones recognizing various bovine lymphocyte surface molecules used were as follows: MM1A (CD3; VMRD Inc., Pullman, WA), CC88 (CD4; Serotech Inc., Raleigh, NC), CC63 (CD8; Serotech Inc., Raleigh, NC) and IL-A29 (WC1; VMRD Inc., Pullman, WA). For detection, mAb were conjugated directly with fluorescein isothiocyante (FITC) or phycoerythrin (PE) or were detected using a secondary FITC-conjugated anti-mouse Ig (Southern Biotechnology Associates Inc., Birmingham, AL). Monoclonal antibodies in staining buffer (PBS without calcium and magnesium containing 3% [vol./vol.] gamma-free equine serum and 0.05% [vol./vol.] Tween 20) were added to 1×10^6 mononuclear cells per tube at a final concentration of 5 μg/ml. Cells were incubated on ice for 30 min and

washed once in staining buffer. When required, the secondary antibody was added to cell preparations and samples were incubated on ice for a further 30 min then washed once in staining buffer. Stained cell preparations were fixed in 2% paraformaldehyde for 30 min, then resuspended in staining buffer and stored at 4°C for a maximum of 18 hr prior to flow cytometry.

The samples were measured with a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Mountain View, CA) using standard methodology. Lymphocyte gates and detector voltages were set using unstained cells from each tissue. Data were collected from 10,000 cells per sample and the gate was set for lymphocytes. Analysis was performed with FCS Express™ software (De Novo Software, Thornhill, Ontario, CA). The percentage of gated cells positive for each cell surface marker combination was determined.

ELIspot Assay

To determine if the distribution of B cell responses correspond to the changes in T cell populations, we developed an enzyme-linked immunospot (ELIspot) assay (Simecka *et al*., 1991) to monitor the number of *M. bovis*-specific antibody forming cells (AFC) along the respiratory tract. The assay was optimized using *M. bovis* immunized mice, followed by testing in infected calves. The number of cells producing *M. bovis*-specific antibody (IgM, IgG and IgA) were determined. A crude preparation of *M. bovis* F1 membranes was used as antigen in the ELIspot assay and was prepared as previously described (Jones *et al*., 2002). Ninety-six well ELIspot plates were coated with antigen at a concentration of 5 μg/ml, incubated at 4°C overnight, washed three times in PBS and blocked with PBS containing 10% (vol./vol.) gamma-free equine serum. Three concentrations of mononuclear cells (10^5, 10^4, 10^3 cells/ml) were prepared for each tissue to be analyzed, and 100 μl of cell suspension was added to each well. Each sample was analyzed in triplicate. Plates were incubated overnight in 5% carbon dioxide at 37°C, then

washed three times in PBS containing 0.05% (vol./vol.) Tween 20. Polyclonal antibodies to bovine IgG, IgM and IgA conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX) were diluted 1:2,000 in PBS containing 0.05% (vol./vol.) Tween 20 and 1% (vol./vol.) gamma-free equine serum. Primary antibodies were added to wells and plates were incubated at 4°C overnight. Plates were washed three times in PBS containing 0.05% (vol./vol.) Tween 20, and avidin-peroxidase diluted 1:1,000 in PBS containing 0.05% (vol./vol.) Tween 20 was added. Plates were incubated at room temperature for 2 hr, then washed three times in PBS containing 0.05% (vol./vol.) Tween 20. Spots were developed using the chromogenic substrate 3-amino-9-ethylcarbazole (AEC), plates were washed in water and then air dried. Spots were counted manually under a stereomicroscope. Results were expressed as the number of IgG, IgM or IgA AFC for the entire lymph node, per gram of lung or tonsil tissue, or per milliliter of blood.

The ELISA Procedure

Blood samples were allowed to clot after collection, and then serum was harvested by centrifugation and stored at -80ºC. Serum end-point titers of *M. bovis*-specific IgG_1, IgG_2, IgM, and IgA were determined. All secondary antibodies were polyclonal, conjugated to alkaline phosphatase (Bethyl Laboratories Inc., Montgomery, TX), and were used at a 1:1,000 dilution. All other methods, incubation times, concentrations, controls and data acquisition were the same as described for the IgG enzyme linked immunosorbent assay (ELISA) in Chapter 3. All serum samples were tested in duplicate.

Nasal lavage was performed at necropsy with 40 ml of sterile PBS. Recovered lavage fluids were centrifuged at 400 × *g* for 10 min, the cell fraction discarded and the supernatant stored at -80ºC. *Mycoplasma bovis*-specific mucosal IgA, IgG, IgG_1, IgG_2, and IgM responses in

undiluted nasal lavage fluids were determined using an ELISA as described above. Total amount of each isotype in nasal lavage fluids were calculated by coating microtiter plates (Maxisorb F96, Nunc, Kamstrup, Denmark) with triplicates of serial 10-fold dilutions of lavage fluid in blocking buffer (0.01 M sodium phosphate buffer [pH 7.2] containing 0.15 M NaCl, 0.02% NaN_3, 0.05% Tween 20 and 1% egg albumin). Serial dilutions of a standard bovine serum containing defined concentrations of immunoglobulins (Bethyl Laboratories Inc., Montgomery, TX) were included on the plate. Plates were incubated at 4°C overnight, and isotypes were detected with secondary antibodies as described for the standard ELISA. The concentration of each isotype in nasal lavage samples was calculated from the curve created from the standard bovine serum. Results for *M. bovis*-specific antibody levels were expressed as a ratio of the optical density (OD) units to the concentration of total antibody for that isotype in the sample.

Statistical Analyses

Continuous variables were compared among groups using ANOVA or repeated measures ANOVA. Tukey's tests were applied to post-hoc comparisons. Ordinal variables were analyzed using Kruskal-Wallace ANOVA or Friedman Test, as appropriate. A *P* value of 0.05 or less was considered statistically significant, with the exception of the overall significance levels in ANOVA, where a *P* value of 0.1 was considered significant. Preliminary analyses performed on data from the oral and transtracheal control groups determined that there were no statistical differences between the two groups for any outcome variable. Data from the two control groups were then pooled for the main analyses to increase statistical power. Analyses were performed using commercial statistical analyses packages (SPSS 12.0, SPSS Inc, Chicago IL and SAS/STAT, SAS institute, Inc., Cary NC).

Results

Isolation of *M. bovis* from Experimentally Infected Calves

Details on the isolation of *M. bovis* from various body sites are presented in Chapter 3 (see Figure 3-2) and will be briefly summarized here. All inoculated calves became colonized in the URT, regardless of the route of infection. In the orally inoculated group (*n*=8), mycoplasmas were isolated from the palatine tonsils, pharyngeal tonsils and MRPLN of all calves, from the eustachian tubes of seven calves, from both tympanic bulla of three calves, and from the LRT of two calves. In calves inoculated by the transtracheal route (*n*=5), *M. bovis* was recovered from the palatine and pharyngeal tonsils of all calves, from the MRPLN of four calves, from the eustachian tubes of three calves, and from the LRT of four calves. There were no mycoplasmas isolated from any of the control calves.

Clinical Disease and Pathology in Experimentally Infected Calves

Details on the clinical disease and pathology observed in inoculated calves are presented in Chapter 3 and will be briefly summarized here. Clinical evidence of otitis media was observed in three of the eight orally inoculated calves and none of the control calves. Transient and mild clinical signs of LRT disease were observed in most of the orally inoculated calves and none of the control calves. Two calves with otitis media developed more severe respiratory disease; one calf with severe clinical signs of disease was euthanized at 10 days post-infection. Transtracheal inoculation of *M. bovis* resulted in mild clinical signs of LRT disease in four of five infected calves, and no clinical signs of otitis media.

Experimentally inoculated calves had gross and/or histopathological lesions typical of *M. bovis* infection. Three of eight orally infected calves had suppurative otitis media. No gross lesions of the URT were observed in calves that were inoculated by the transtracheal route. Increased histopathological scores, lymphoid hyperplasia and increased numbers of plasma cells

were observed in the URT lymph nodes of infected calves (see Chapter 3, and Figures 3-4 and 3-5 for more detail). Lymphoplasmacytic infiltrates were also observed in the tympanic mucosa of calves with otitis media, and lymphocytes, plasma cells and histiocytes were present diffusely or in large dense aggregates in the lamina propria of eustachian tubes from which *M. bovis* was isolated (J. Powe, F. P. Maunsell, J. W. Simecka and M. B. Brown, submitted for publication). Calves inoculated by the oral route had significantly ($P = 0.041$) heavier LRPLN at necropsy than did control calves or calves inoculated by the transtracheal route (Figure 4-2), but weights of MRPLN did not differ among groups.

Lymphocyte accumulations were also a key feature of histopathology observed in the lungs. The lungs of calves infected by either route had significantly ($P < 0.05$) greater lymphoid hyperplasia compared with lungs of control calves (data shown in Chapter 3; see Figure 3-8 for details). In addition, in calves from which *M. bovis* was isolated from the lung, lymphocytes were a prominent component of the peribronchiolar and parenchymal cellular infiltrate in sites of lung pathology. In contrast to the findings for the lymph nodes in the URT, histopathologic scores or plasma cell subscores for TBLN did not vary significantly among groups (data not shown). However, TBLN of transtracheally inoculated calves were significantly ($P = 0.049$) heavier than those of control calves or calves inoculated by the oral route (Figure 4-2).

Complete Blood Counts and T Cell Responses in Peripheral Blood and Spleen

There were no significant differences among groups at any of the sampling times in total leukocyte, lymphocyte, segmented neutrophil or monocyte counts or in the relative proportions of these cells in peripheral blood (data not shown). Likewise, there were no significant differences among groups in total plasma protein concentration or plasma fibrinogen concentration at any of the sampling times (data not shown). There were also no differences

among groups in the percentages of $CD4^+$, $CD8^+$ or $WC1^+$ γδ T cells in mononuclear cells from peripheral blood at any of the sampling times, or from spleen collected at necropsy (Figure 4-3).

T Cell Populations in the URT and LRT

Both $CD4^+$ and $CD8^+$ T cell responses were observed in the URT of calves at 14 days after *M. bovis* infection. $CD4^+$ T helper (Th) cells were the major responding T cell population in the tonsils of orally inoculated calves, whereas the proportion of $CD8^+$ T cells was increased in the MRPLN (Figure 4-3). The relative proportion of $CD4^+$ and $CD8^+$ T cells in the URT of transtracheally inoculated calves were lower than those observed in orally inoculated calves. There was a tendency ($P = 0.089$) for an increase in the percentage of $CD4^+$ T cells in the tonsils of the transtracheally inoculated group compared with control calves, but responses in the URT lymph nodes were no different than those of control calves. The percentage of $WC1^+$ γδ T cells in URT tissues at necropsy was not affected by infection via either route as there were no differences among groups (Figure 4-3). In a limited number of cases, low cell recovery from specific tissues and some sample loss precluded complete analysis.

There was little difference between calves infected with *M. bovis* and control calves in the relative proportion of $CD4^+$, $CD8^+$ and $WC1^+$ γδ T cells in the TBLN or lungs (Figure 4-3). $CD4^+$ Th cell responses in the TBLN of calves inoculated by the transtracheal route were highly variable and ranged from 24% to 60% (Figure 4-3).

B cell and Antibody Responses

The URT was the major site of mycoplasma-specific B cell responses in orally inoculated calves (Figure 4-4). Using the *M. bovis*-specific ELIspot assay, the numbers of mycoplasma-specific AFC were determined in URT (LRPLN and MRPLN) and LRT (TBLN) lymph nodes, lungs, peripheral blood and spleen samples. Not all tissues were analyzed for every calf due to

low cell recovery from some tissues and some sample loss. As shown in Figure 4-4, *M. bovis*-specific B cell responses were observed in both the URT and LRT of orally inoculated calves. However, the URT had a much greater increase in the number of mycoplasma-specific AFC (IgM, IgG and IgA) than did the LRT. Calves infected by the transtracheal route had significantly ($P < 0.05$) higher numbers of IgG and IgA AFC in LRT sites when compared with orally inoculated and control calves (Figure 4-4). *Mycoplasma bovis*-specific B cell responses were also observed in the URT lymph nodes of calves inoculated by the transtracheal route, but responses were of a lesser magnitude than those observed for orally inoculated calves.

In agreement with the ELIspot data, orally inoculated calves had increased levels of *M. bovis*-specific IgA in nasal lavage fluids, compared with control or transtracheally inoculated groups (Figure 4-5). Orally inoculated calves also tended ($P = 0.11$) to have higher levels of *M. bovis*-specific IgG_1 in nasal lavage fluids than did control or transtracheally inoculated calves, although this response was highly variable. Specific *M. bovis* IgG_2 responses were not observed in nasal lavage fluids of orally or transtracheally inoculated calves. Orally inoculated calves had higher concentrations of total IgM in nasal lavage fluids than did other groups (Figure 4-5), whereas transtracheally inoculated calves had statistically higher ($P = 0.02$) concentrations of total IgG_2 in nasal lavage fluids than did control or orally inoculated calves (Figure 4-5).

Calves with otitis media tended to have higher *M. bovis*-specific IgG_1 responses and higher total IgM responses in the URT than did orally inoculated calves without otitis media; total and *M. bovis* IgG_2 and IgA responses were similar for calves with and without otitis media (Figure 4-6). Calves with otitis media also tended ($P = 0.15$) to have higher *M. bovis*-specific IgG_1:IgG_2 ratios in nasal lavage fluids than did other study calves, although there was inadequate statistical power to detect significant differences (Figure 4-6).

Experimentally infected calves exhibited a *M. bovis*-specific serum Ig response that was evident 14 days after infection (Figure 4-7). Antibody to *M. bovis* was detected at day 0, indicating passive transfer of specific Ig via the colostrum substitute fed to the calves. While the titer of passively-acquired serum IgG_1 in control calves remained static over the study period, the titer of *M. bovis*-specific serum IgG_1 in infected calves remained stable or increased, suggesting an active immune response to infection in these animals. There was a large amount of variation in individual titers in all groups, and any differences in *M. bovis*-specific serum IgG_1 titers among groups over the course of the study were not statistically significant. When the fold-change in *M. bovis*-specific serum antibody titers between days 0 and 14 of the study were compared among groups, calves in the orally infected group had a significant fold-increase in IgG_1 ($P < 0.001$) compared with the control group. Transtracheally infected calves also had a significant fold-increase in IgG_1 titers ($P = 0.015$), when compared with the control group. In addition to the IgG_1 response, a trend for an increase in serum IgA titers is apparent in infected calves over the course of the study, but no significant differences were detected among groups. Serum IgM responses followed a similar pattern as the serum IgG_1 and IgA, but responses were more marked, with the titer of *M. bovis*-specific serum IgM being significantly higher ($P = 0.01$) in orally infected calves than in transtracheally infected or control calves at necropsy. In contrast with the orally infected group, no differences were detected in serum IgM responses between the transtracheally inoculated group and the control group. Low post-colostral IgG_2 titers to *M. bovis* were observed in both control and infected calves, and no serum IgG_2 response was observed to *M. bovis* infection. Overall, calves infected by the oral route exhibited a greater serum antibody response than calves infected by the transtracheal route, although there were individual calf variations.

Discussion

Accumulation of lymphoid cells at the local site of infection was a key feature of the histopathological findings in calves inoculated with *M. bovis* by the oral or transtracheal routes. Draining lymph nodes at the major sites of respiratory tract infection were enlarged, supportive of a local immune response at those sites. In calves inoculated by a natural route through feeding of *M. bovis*-inoculated milk replacer, the URT was the major site of T cell immune responses, and both $CD4^+$ and $CD8^+$ T cell responses were observed in URT lymphoid tissues. Thus the major site of T cell responses corresponded to the major site of infection in orally inoculated calves. Our findings are consistent with the development of predominantly an URT disease and an accompanying local immune response after oral inoculation. Other investigators have reported that $CD4^+$ and $CD8^+$ T cells in PBMC of calves experimentally inoculated with *M. bo*vis exhibited higher *in vitro* activation (CD25 expression) in response to *M. bovis* antigens than did cells from uninfected control calves, suggesting that these cell populations are responding to infection (Vanden Bush and Rosenbusch, 2003). Our results indicate that in local lymphoid tissues, both $CD4^+$ and $CD8^+$ T cells are responding during the early stages of *M. bovis* infection by a natural route. Thus, both of the major T cell populations are likely to contribute to the immune responses in the URT, similar to studies in murine mycoplasma respiratory disease (Jones *et al*., 2002).

In contrast with calves inoculated by the oral route, significant changes in the relative proportions of T cell subpopulations were not observed in calves inoculated by the transtracheal route, despite a significant increase in the weight of TBLN and a substantial contribution of lymphocytes to lung lesions in transtracheally inoculated calves. There are several possible explanations for these data: expansion of lymphoid populations could have occurred without changes to the relative proportions of the three major T cell subpopulations, our small sample

size may have precluded finding significant differences if such differences did exist, or the lymphoid expansion may have been due to other cell populations (e.g. B cells, NK cells). Other investigators (Rodriguez *et al*., 2000) found that $CD4^+$ T cells were the major T cell population contributing to *M. bovis*-induced lung lesions in infected goat kids by immunohistochemical staining of affected lung, but the relative proportions of T cell subpopulations in infected and control kids were not reported.

There was no significant change in $WC1^+$ γδ T cell populations in either URT or LRT tissues of infected calves, suggesting that this population does not undergo preferential expansion during early *M. bovis* infection. Other investigators have shown that in young calves, $WC1^+$ γδ T cells are recruited to sites of epithelial inflammation (Wilson *et al*., 2002) and contribute to local immune responses in other respiratory diseases (Price *et al*., 2006). No substantial recruitment of $WC1^+$ γδ T cells was observed in lungs or palatine tonsils of infected calves. We did not examine T cell responses within the epithelium of the URT, so whether $WC1^+$ γδ T cells contribute to *M. bovis* responses at the level of the epithelium was not determined.

The presence of large numbers of plasma cells at the local site of *M. bovis* infection was a prominent feature of the histopathological findings in calves inoculated by the oral or transtracheal routes. Consistent with this finding, we observed that local B cell responses in the respiratory tract of calves experimentally inoculated with *M. bovis* corresponded to the site of infection. The URT was the major site of *M. bovis*-specific B cell and mucosal IgA responses in calves inoculated by the oral route, while the LRT was the major site of B cell responses in transtracheally inoculated calves. Calves infected by the transtracheal route did have significant B cell responses in the URT lymph nodes, which was not surprising given that all calves became colonized with *M. bovis* in the URT. However, responses were not as marked as those for orally

infected calves, corresponding well with the level of colonization observed in the two infection groups.

Interestingly, calves inoculated by the oral route had significantly higher numbers of mycoplasma-specific AFC in the LRT than uninfected calves. This suggests either the beginnings of an adaptive immune response developing in the lungs or, more likely, immune cells from the URT are migrating to other tissues. In support of tissue migration, occasional mycoplasma-specific AFC were found in the blood and spleen of orally inoculated calves (data not shown), demonstrating the presence of circulating cells.

Taken together, our data demonstrate that B cells responses, similar to changes in T cell populations, are preferentially found at the site of infection and are likely to play a role in disease progression. There are also indications that the B cell responses in the URT may augment responses in the lung and other tissues.

The ratio of IgG_1:IgG_2 is often used to indicate Th1- or Th2-biased responses in cattle (Brown *et al*., 1998c). Calves with otitis media tended ($P = 0.15$) to have higher local *M. bovis*-specific IgG_1:IgG_2 ratios that did other calves inoculated with *M. bovis* by the oral route, suggesting that these calves had a more Th2-biased response than did calves without otitis media. Three of four calves with the highest *M. bovis*-specific IgG_1:IgG_2 ratios had otitis media, and the fourth calf had the highest level of eustachian tube colonization without concurrent otitis media of any calf in the study (data not shown). In addition, the one transtracheally inoculated calf that cleared *M. bovis* infection from the LRT was the calf with the lowest *M. bovis*-specific IgG_1:IgG_2 ratio; this calf also had the highest total IgG_2 concentration in nasal lavage fluids of any calf in the study (0.512 μg/ml). Although the data was obtained from a relatively small number of animals, these data support the idea that a local Th1-biased (IgG_2) antibody response

may preferable to a Th2-biased response in clearing or controlling mycoplasmal infections. Thus, more extensive studies to define the role of Th1 local responses in protection are warranted.

The MRPLN and LRPLN of calves infected with *M. bovis* by the oral route showed different immune responses. No differences between groups were observed in the weight of MRLPN, whereas LRPLN of infected calves were heavier than those of control calves. There were significant increases in the proportion of $CD8^+$ T cells and in B cell responses in both lymph nodes, but B cell responses were much more marked in the LRPLN than in the MRPLN. Both lymph nodes drain the oro- and nasopharynx as well as the middle ear, but the MRPLN receives a greater proportion of lymph drainage from the nasopharynx including the pharyngeal tonsils, while the LRPLN receives a greater proportion of lymph from the oropharynx including the palatine tonsils (Pasquini, 1983). Our findings may reflect differences in the level of *M. bovis* colonization within the drainage field of each lymph node, or could be consistent with different roles for the MRPLN and LRPLN in *M. bovis* infections. In any event, future studies should take into account the fact that considerable differences can exist in the immune responses within these two lymph nodes.

Our findings support the hypothesis that local and systemic immune responses generated using the transtracheal approach differ from those generated after oral inoculation. Overall, our data support the idea that local immune responses within the respiratory tract are important in disease pathogenesis. Further comparison of immune responses generated after primary infection of the URT to those generated in the LRT will help to discern the relative contributions of these sites during mycoplasmal disease. These local responses will also be important considerations in the development of new vaccination strategies against *M. bovis*.

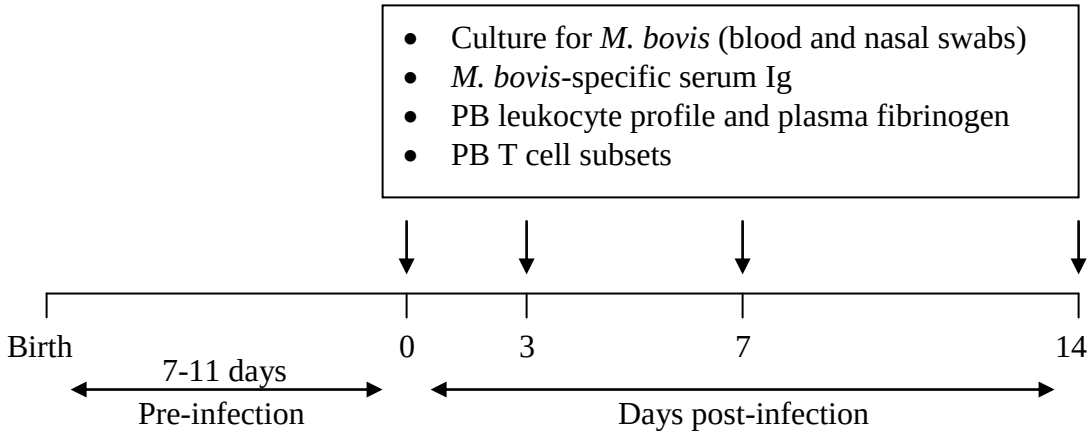


Figure 4-1. Overall experimental design for the infection study. Calves were infected between 7 and 11 days of life. Calves were sampled at infection (day 0), post-infection days 3 and 7, and at necropsy (post-infection day14, except for one calf inoculated with *Mycoplasma bovis* by the oral route which had to be euthanized at 10 days post-infection). Ig = immunoglobulin; PB = peripheral blood.

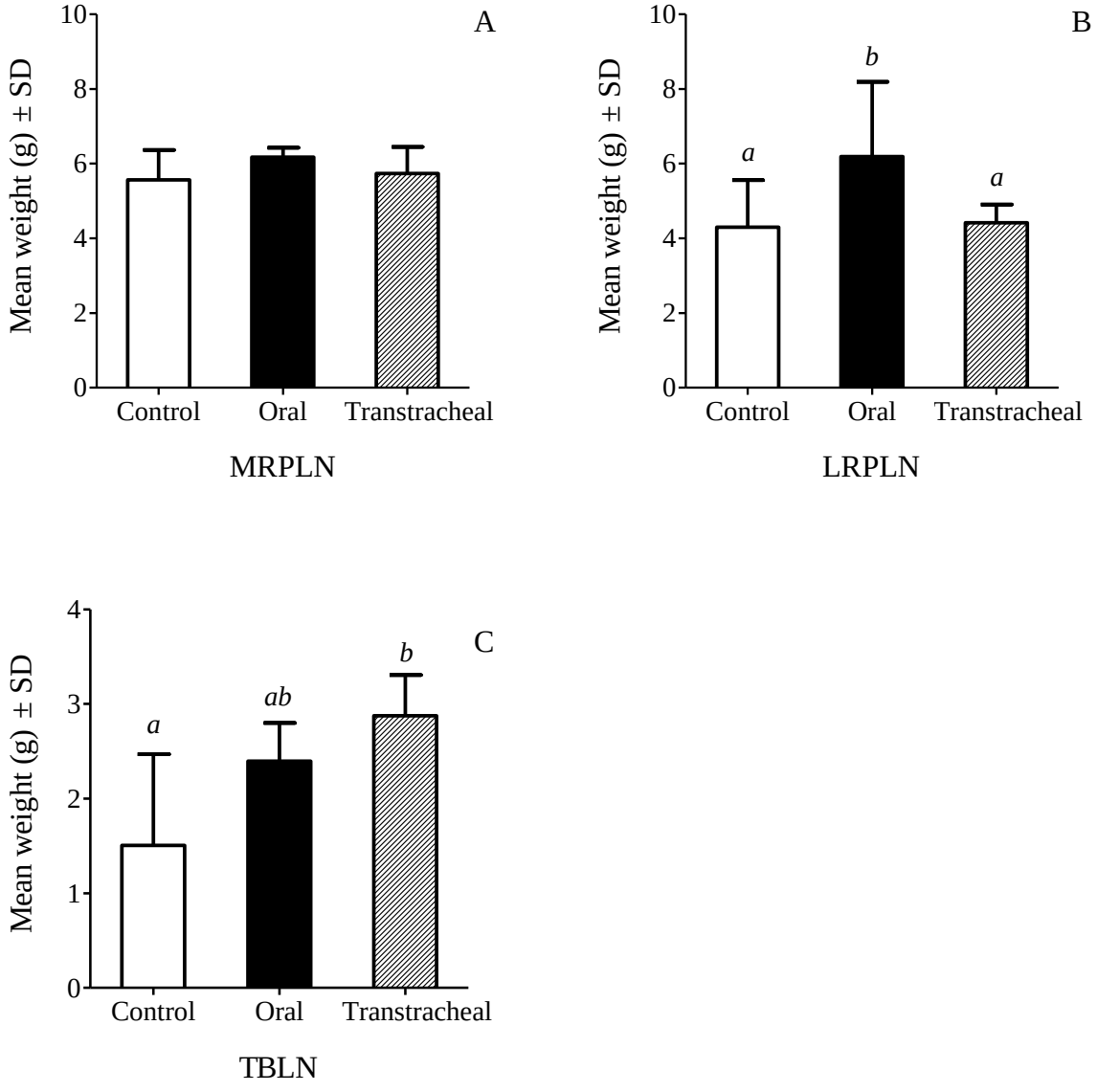


Figure 4- 2. Weights (mean ± SD) of upper and lower respiratory tract lymph nodes. A) Medial retropharyngeal lymph nodes (MRPLN). B) Lateral retropharyngeal lymph nodes (LRPLN). C) Tracheobronchial lymph nodes (TBLN). Weights for left and right retropharyngeal lymph nodes were combined for each calf. [ab]Superscript letters indicate significant ($P < 0.05$) differences between control calves (n=8), and calves inoculated with *Mycoplasma bovis* by the oral (n=8) or transtracheal (n=5) route at necropsy (14 days post infection except for one calf that was euthanized at 10 days post infection).

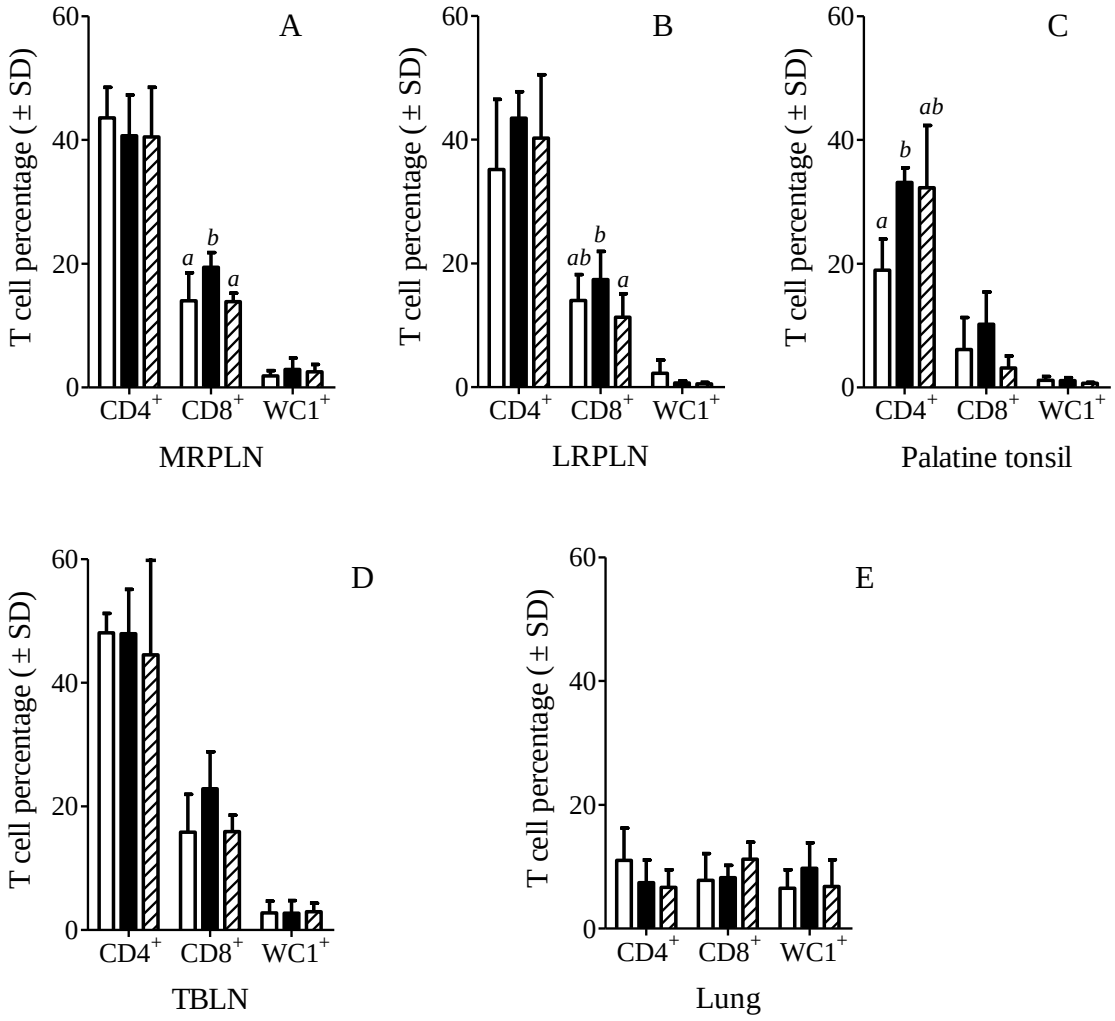


Figure 4-3. Relative percentages of $CD4^+$, $CD8^+$ and $WC1^+$ γδ T cells in mononuclear cells isolated from upper respiratory tract, lower respiratory tract, and systemic sites. A) Medial retropharyngeal lymph nodes (MRPLN). B) Lateral retropharyngeal lymph nodes (LRPLN) C) Palatine tonsil D) Tracheobronchial lymph nodes (TBLN) E) Lung. F) Peripheral blood. G) Spleen. Data are represented as the mean percentage ± SD of the mononuclear population isolated from tissues at necropsy (14 days post infection except for one calf that was euthanized at 10 days post infection) from control calves (white bars), and calves infected with *Mycoplasma bovis* by oral (black bars) or transtracheal (hatched bars) routes. ^{ab}Superscript letters indicate significant ($P < 0.05$) differences among groups for that tissue and cell population.

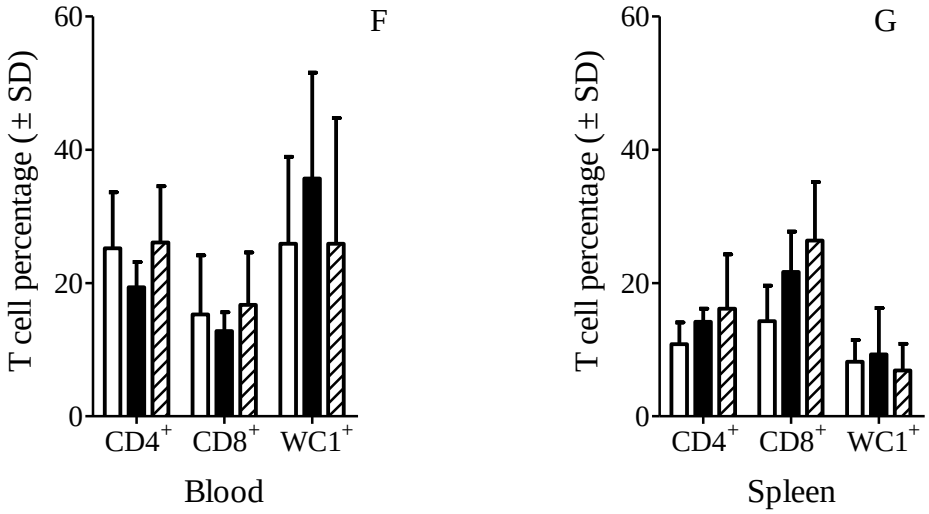


Figure 4-3. (continued).

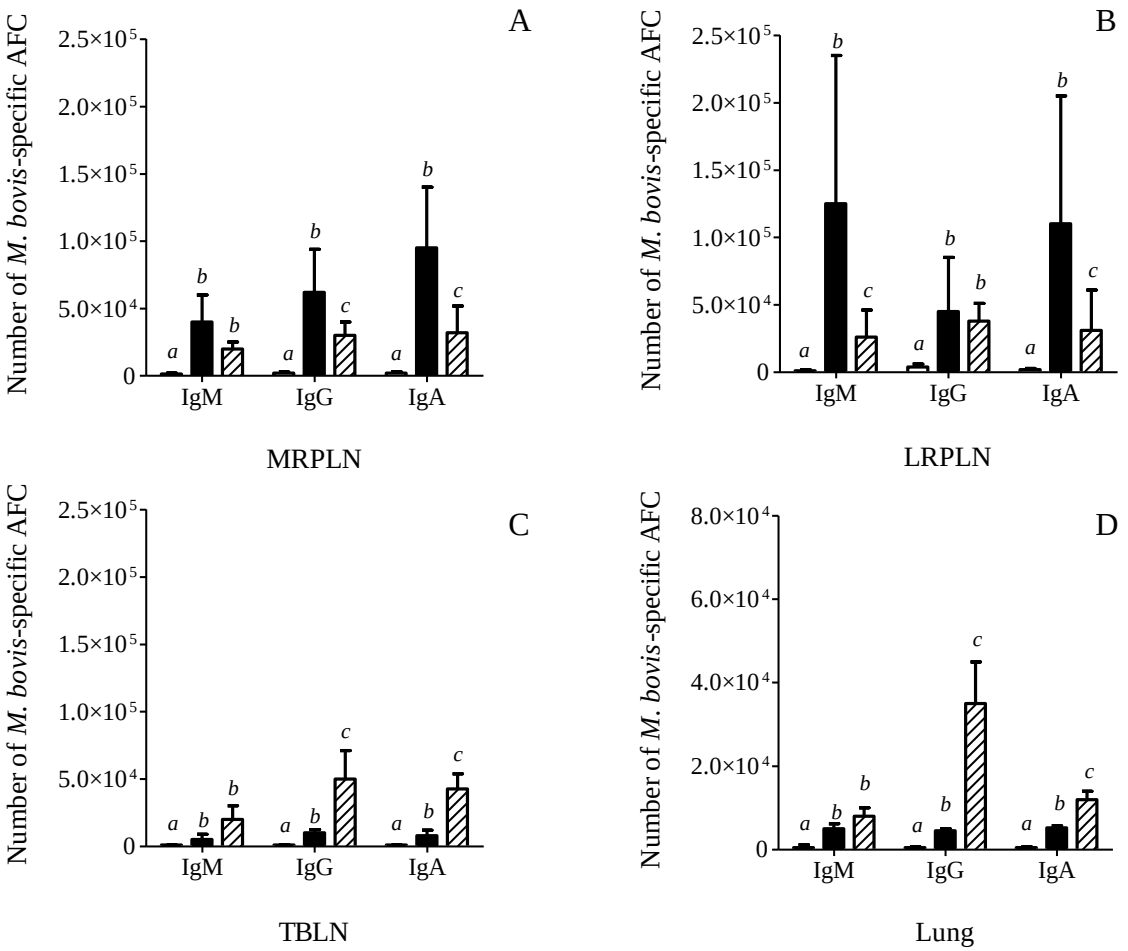


Figure 4-4. *Mycoplasma bovis*-specific B cell responses along the respiratory tract as determined by ELIspot assay. A) Medial retropharyngeal lymph nodes (MRPLN). B) Lateral retropharyngeal lymph nodes (LRPLN). C) Tracheobronchial lymph nodes (TBLN). D) Lung. The data for lymph nodes (A, B and C) are represented as the mean ± SD of the total number of cells within that tissue. Data for the lungs (D) are represented as the mean (± SD) of the number of cells/g of tissue. ELISpot assays were performed on tissue collected at necropsy (14 days post infection except for one calf that was euthanized at 10 days post infection) from control calves (n=5; white bars) and from calves inoculated by oral (n=4; black bars) or transtracheal (n=3; hatched bars) routes. [abc]Superscript letters indicate significant ($P < 0.05$) differences in class-specific responses between groups.

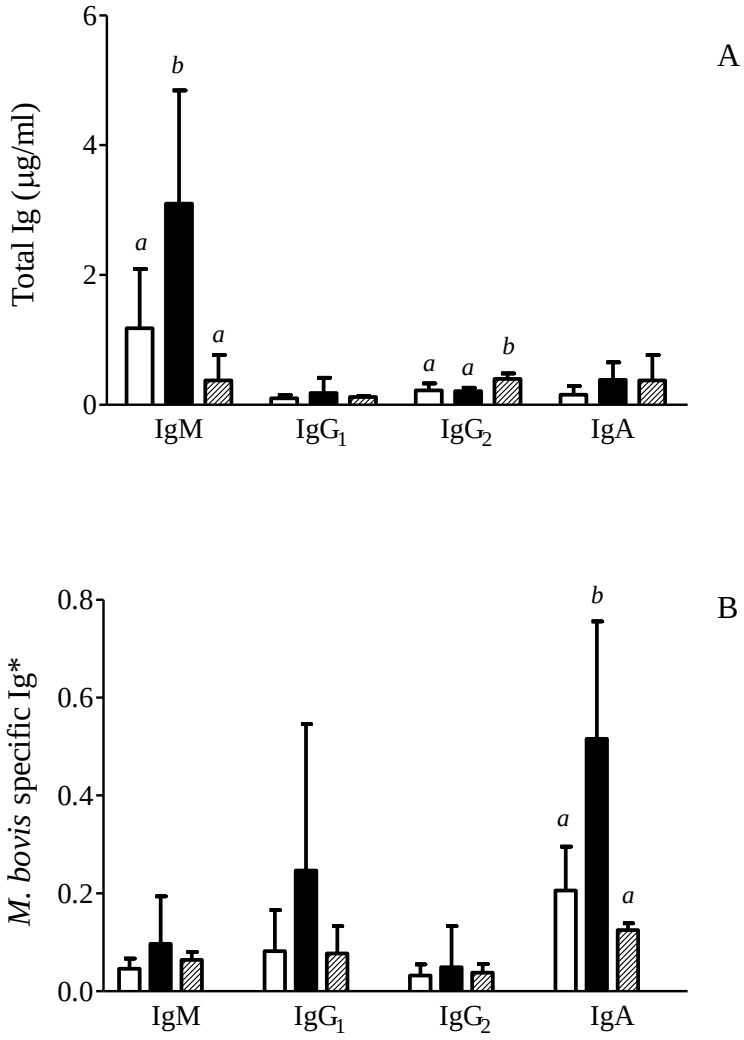


Figure 4-5. Mucosal antibody responses in the upper respiratory tract. Antibody responses were measured in nasal lavage fluids obtained from calves inoculated with *Mycoplasma bovis* by the oral (*n*=8, solid black bars) or transtracheal (*n*=5, hatched bars) route, and from control calves (*n*=6, solid white bars; samples were not collected on two control calves). Samples were collected at necropsy (14 days post infection, except for one calf inoculated with *M. bovis* by the oral route which had to be euthanized at 10 days post-infection). A) Concentration of class-specific total immunoglobulin (Ig) in nasal lavage fluid. B) *M. bovis*-specific antibody levels in nasal lavage fluid. *Optical density adjusted for the total amount of Ig, expressed as the optical density for *M. bovis*-specific Ig/total Ig in the sample for each isotype. [ab]Superscript letters indicate significant ($P < 0.05$) differences between groups.

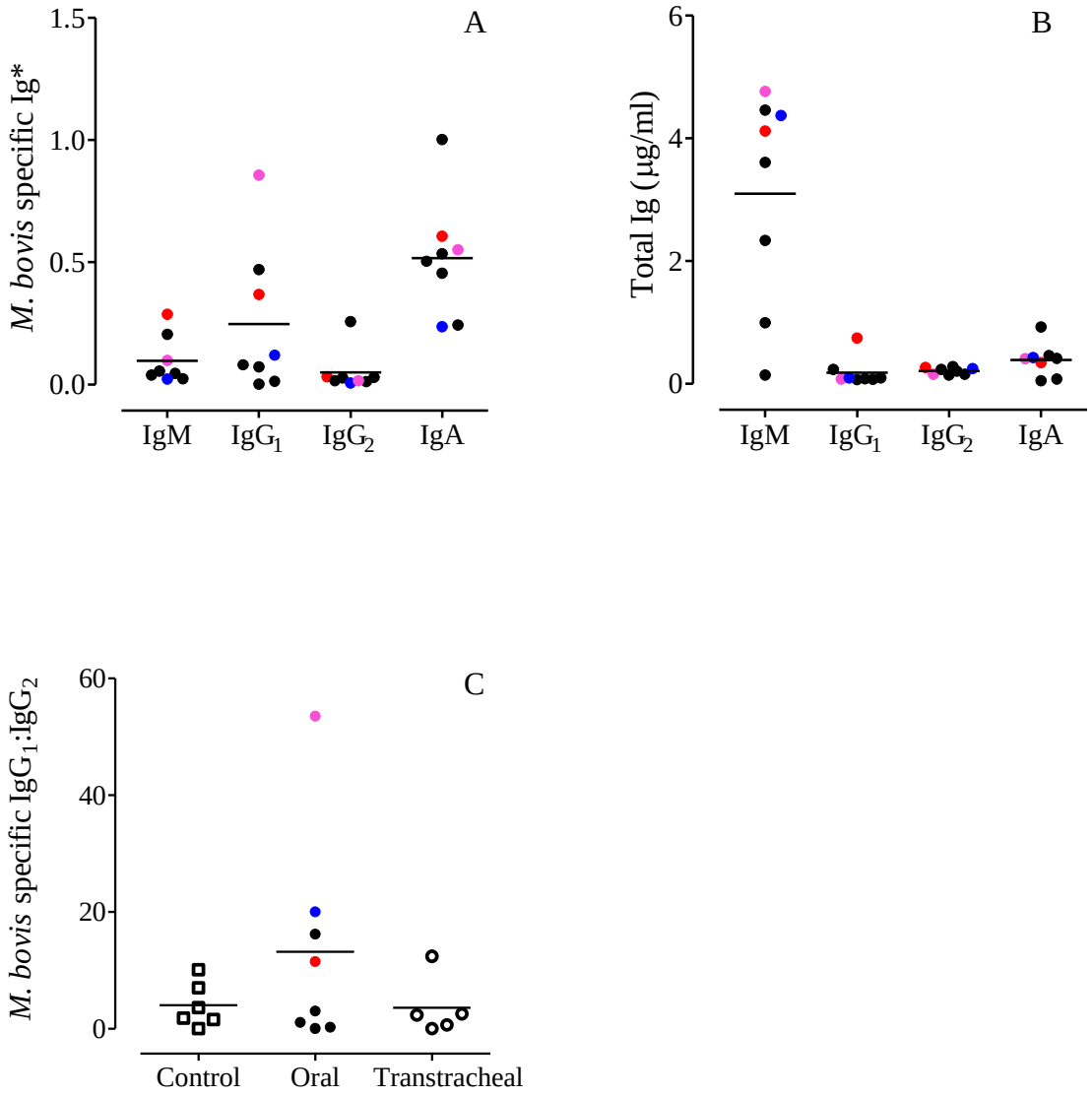


Figure 4-6. Mucosal antibody responses in the upper respiratory tract of individual calves with or without otitis media. A) *Mycoplasma bovis*-specific antibody levels in nasal lavage fluid of calves inoculated with *M. bovis* by the oral route (*n*=8). *Optical density adjusted for the total amount of immunoglobulin (Ig), expressed as the optical density for *M. bovis*-specific Ig/total Ig in the sample for each isotype. B) Total Ig (µg/ml) in nasal lavage fluids of calves inoculated with *M. bovis* by the oral route (*n*=8). C) The ratio of *M. bovis*-specific IgG_1 to IgG_2 in nasal lavage fluids of control calves (*n*=6) or calves inoculated with *M. bovis* by the oral (*n*=8) or transtracheal (*n*=5) route. Data points from the three calves with otitis media are highlighted in red, blue and pink, respectively, whereas data points from calves without otitis media are displayed in black and white. Nasal lavage samples were collected at necropsy (14 days post infection, except for one calf inoculated with *M. bovis* by the oral route which had to be euthanized at 10 days post-infection).

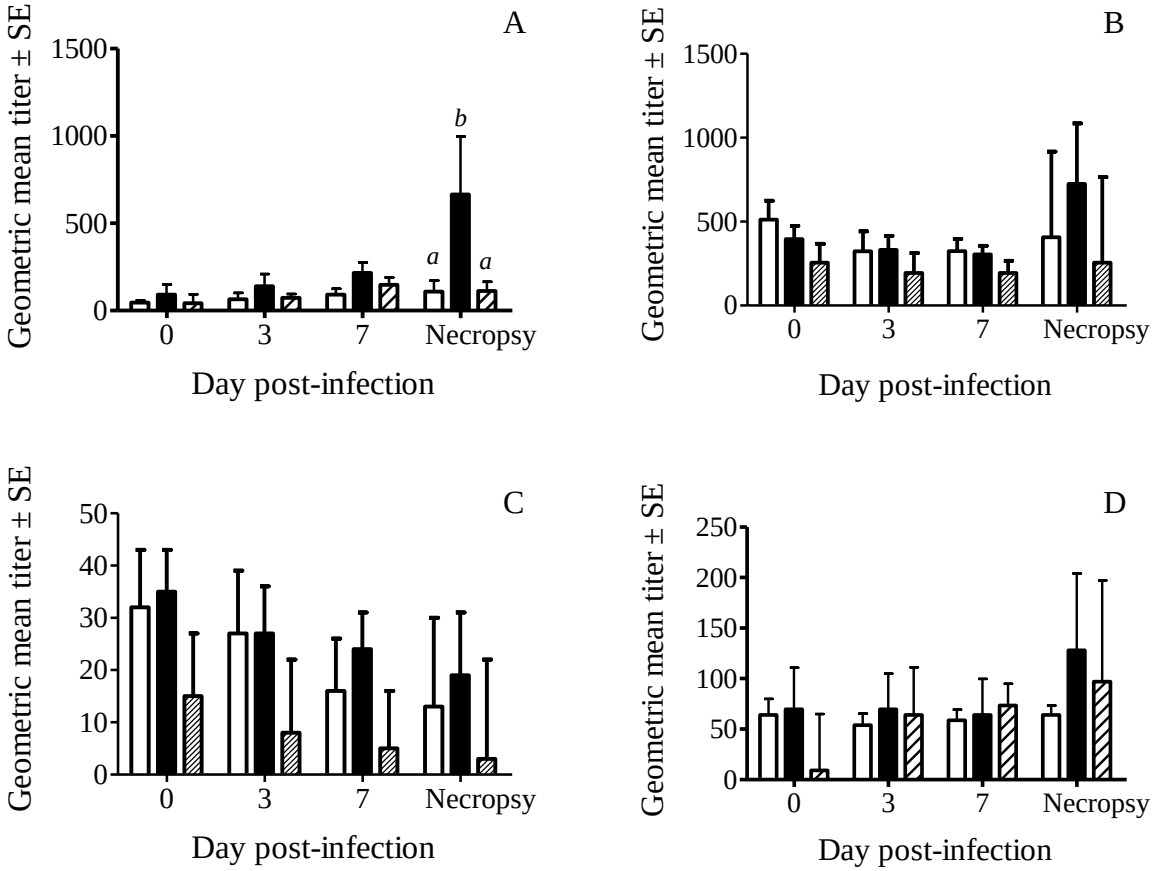


Figure 4-7. Geometric mean end-point titers for *Mycoplasma bovis*-specific serum immunoglobulin (Ig). A) IgM. B) IgG_1. C) IgG_2. D) IgA. Serum samples were collected at prior to inoculation (day 0), days 3 and 7 post-infection, and at necropsy (14 days post infection, except for one calf which had to be euthanized at 10 days post-infection) from calves experimentally inoculated with *M. bovis* by the oral (*n*=8; black bars) or transtracheal (*n*=5; hatched bars) routes, and from control calves (*n*=8; white bars). [ab]Superscript letters indicate significant ($P < 0.05$) differences among groups.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Mycoplasma bovis has emerged as an important pathogen of young dairy calves, and economic losses resulting from *M. bovis*-associated morbidity and mortality can be substantive. Clinical disease associated with *M. bovis* is often chronic, debilitating and poorly responsive to antimicrobial therapy, and current management strategies often fail to control disease. Further, most research has focused on adult cows with mastitis or on older calves with pneumonia, neglecting the young dairy calf population. Thus, there is a critical need to develop better preventive, control and treatment strategies for *M. bovis*-associated disease in young calves that are at high risk for both pneumonia and otitis media. Improvements in these areas are hampered by a lack of understanding of the epidemiology of *M. bovis* infections in young calves and of the host-pathogen interactions involved in the establishment of infection and the development of clinical disease. The overall goal of these studies was to address key deficiencies in the current knowledge of *M. bovis*-associated disease in young calves.

Field Efficacy of a Commercial *M. bovis* Bacterin in Young Dairy Calves

In experimental challenge and field studies, efficacy of vaccination against *M. bovis* has been variable in both adult cows and calves. Although some vaccines have reduced clinical disease in older calves (Chima *et al*., 1980; Howard *et al*., 1987a; Nicholas *et al*., 2002), they have not prevented colonization and shedding; indeed, some vaccines have been associated with exacerbation of clinical disease (Rosenbusch *et al*., 1998; Bryson *et al*., 1999). In addition to research into new vaccination strategies, critical evaluation of the currently marketed *M. bovis* vaccines in controlled, independent efficacy studies using an appropriate age group are clearly required. The lack of such studies has been a major gap in understanding the potential of currently available vaccines as a management strategy to control *M. bovis* infections in young

calves. In order to address this gap in knowledge, we conducted a field trial using a commercial *M. bovis* bacterin that was approved for use in feeder and stocker calves.

The major conclusions from the vaccine efficacy study were 1) that an *M. bovis* bacterin licensed for prevention of *M. bovis*-associated respiratory disease in stocker and feeder cattle was not efficacious in preventing disease in pre-weaned calves in two Florida dairy herds with endemic *M. bovis* disease, 2) vaccination was not efficacious at preventing colonization of the upper respiratory tract (URT) in older calves in a third dairy herd, and, 3) vaccination not only failed to protect young calves, but also exacerbated the rate of clinical otitis media in one herd.

In our study, we found that vaccination with a killed *M. bovis* bacterin at 3, 14 and 35 days of age did stimulate a systemic humoral immunoglobulin (Ig) G_1 response that was detectable after the third dose of vaccine. Little IgG_2 was produced, and as IgG_2 is a much more effective opsonin for phagocytosis of *M. bovis* than is IgG_1 (Howard, 1984), the vaccine-induced antibody response may have been less than optimal for effective clearance of *M. bovis* from the host. The observed IgG subclass responses were indicative of a Th2-biased humoral response and were similar to that reported after infection of older calves with *M. bovis* (Howard *et al*., 1987c; Vanden Bush and Rosenbusch, 2003). Importantly, we also determined that most calves in the herds with endemic *M. bovis*-associated disease were colonized before 3 weeks of age, meaning that infection was likely well established before a vaccine-induced immune response could develop. Adaptive immune responses that develop after infection are very inefficient at clearing mycoplasmal infections and often result in detrimental chronic inflammatory responses. Therefore, it is not surprising that vaccination failed to stimulate protective immune responses and that exacerbation of disease was observed in one herd.

Taken together, data from the field vaccine study provide compelling support for the concept that alternative approaches to protection of young calves from *M. bovis* infections are needed. Immune responses of the newborn calf have unique characteristics and undergo rapid changes during the first few weeks of life (Barrington and Parish, 2001) that may impact vaccine strategies and control measures. Therefore any new strategies need to be targeted specifically to this age group.

Establishment of an Experimental Model of *M. bovis* Infection and Immune Responses in the Respiratory Tract of Infected Neonatal Calves

A critical gap in knowledge is the availability of an experimental model of *M. bovis* infection in young calves that mimics naturally occurring disease. Therefore, the second main objective of the work presented in this dissertation was to develop a reproducible model of *M. bovis* infection of the URT that closely mimicked natural infection in young dairy calves and to use this model to define the immune responses generated along the respiratory tract during infection with *M. bovis*.

Current understanding of the pathogenesis of mycoplasmal infections in young calves is extremely limited. In our oral infection studies, we demonstrated for the first time that *M. bovis* consistently colonizes the eustachian (auditory) tubes of young calves, as well as the palatine and pharyngeal tonsils, after oropharyngeal exposure. We also demonstrated for the first time that inoculation with *M. bovis* can result in the development of otitis media in young calves. The high rate of pharyngeal tonsil and eustachian tube colonization observed in this study supports the idea that *M. bovis*-associated otitis media is a result of ascending infection from the pharyngeal tonsil via the eustachian tube, similar to otitis media caused by *Mycoplasma hyorhinis* in pigs (Morita *et al*., 1995; Morita *et al*., 1999). Therefore, generation of immunity in the URT, rather than systemic immunity, may be the most effective means of preventing otitis media. Because of

the anatomical proximity of the pharyngeal tonsil to the pharyngeal opening of the eustachian tubes, mycoplasma infection of the tonsils may increase the likelihood of eustachian tube colonization and eventual development of otitis media. Consistent with this hypothesis, we found that the level of tonsil colonization was correlated with the presence of clinical disease at necropsy. These findings suggest that strategies which prevent infection of the tonsils or limit replication at this site may be beneficial in preventing otitis media in calves.

Despite the fact that ingestion of *M. bovis*-contaminated milk is thought to be a major route of natural infection, experimental infection by this route has not previously been reported. In these experimental infection studies, we have verified that bucket-nursing of milk containing *M. bovis* does result in colonization of the URT of young calves and can cause clinical disease. This experimental verification that calves fed contaminated milk do become colonized with *M. bovis* lends support to control measures aimed at eliminating *M. bovis* contaminated milk and colostrum from calf diets.

Our model was used to compare the immune responses to *M. bovis* infection after oral inoculation with those observed after inoculation directly into the lower respiratory tract (LRT). Evaluation of humoral and cellular immune responses in the URT and LRT of calves inoculated by oral and transtracheal routes showed that the infection site corresponded to the distribution of immune responses. We demonstrated that the URT lymphoid tissues are major sites for immune responses after infection via a natural route and that both $CD4^+$ and $CD8^+$ T cells as well as mycoplasma-specific B cells responded to *M. bovis* infection of the URT. Similarly, when calves were inoculated directly into the LRT, substantial B cell responses were observed in the lungs and LRT lymph nodes. Overall, these data indicate that local immune responses within the respiratory tract are important in *M. bovis* infections.

Interestingly, calves with more severe clinical disease in this study had the highest IgG_1:IgG_2 ratios in nasal lavage fluids, while the one calf that cleared *M. bovis* from the LRT had the lowest IgG_1:IgG_2 ratio and the highest total IgG_2 concentration in nasal lavage fluids. Together with findings from other studies (Howard, 1984; Howard *et al*., 1987c; Vanden Bush and Rosenbusch, 2003) these data support the idea that a local Th1-biased (IgG_2) antibody response may be preferable to a Th2-biased response in clearing or controlling mycoplasmal infections in calves. Thus, more extensive studies to define the role of local Th1 responses in protection from *M. bovis*-associated disease are warranted.

In summary, we have developed a reproducible model of *M. bovis* infection of the URT that closely mimics naturally occurring *M. bovis* infections in neonatal calves. There are important differences between very young calves and older cattle in terms of their immune environment and the occurrence of middle ear infections. Therefore, an infection model that uses a clinically relevant age group, especially when considering events leading to middle ear disease, is likely to be critical when studying this emerging problem. The oral inoculation model that we have presented here is particularly suited to the study of host-pathogen interactions during initial colonization of the tonsils, expansion of infection and dissemination to the LRT and middle ear. In addition, the model could be used to investigate potential new preventive or control strategies, especially those aimed at limiting colonization of the tonsils and/or spread to the middle ear.

Implications for Control of *M. bovis* in Young Calves and Future Research Directions

There are many critical gaps in our knowledge of *M. bovis* infections in young calves that still need to be addressed. Clearly, well designed epidemiological studies of *M. bovis* in infected calf-rearing facilities are required to better establish risk factors and provide guidance for dairy producers to prevent and control disease. In addition, long term epidemiological studies would be helpful to determine the impact of *M. bovis* infections of young calves on the risk of URT or

mammary gland infection with *M. bovis* as adults. Prevalence estimates for *M. bovis*-associated disease in U.S. dairy calves have not been published and would be useful in determining the true extent of this problem and in estimating associated losses. In addition, current treatment measures need to be critically evaluated. Controlled clinical trials evaluating the efficacy of particular therapeutic and metaphylactic antibiotic regimens for clinical disease in U.S. dairy calves are needed, and the safety and efficacy of myringotomy in calves with otitis media needs to be assessed.

The results of our field vaccine efficacy trial provide compelling support for the concept that alternative approaches to immune protection of young calves from *M. bovis* infections are needed. More sophisticated approaches to vaccine development and delivery systems and a better understanding of host immune responses in mycoplasmal disease would likely lead to improved vaccination strategies. A better understanding of the immunology of the neonatal calf, especially with respect to ability to respond to different antigens, the types of responses that are produced, and modulation of these responses by mucosal and systemic adjuvants may improve our ability to produce efficacious vaccines, if, indeed, vaccination of the very young calf against *M. bovis* is possible.

The observations from the field trial on the nasal colonization patterns in herds with or without endemic *M. bovis*-associated disease strongly suggest that strategies which delay URT colonization until after the first few weeks of life may have a dramatic impact on susceptibility to *M. bovis*-associated disease in calves. Interestingly, studies of otitis media in humans have also shown that the age at which colonization of the nasopharynx or tonsils first occurs strongly affects the risk of developing otitis media, and that delaying colonization for a few weeks greatly reduces the incidence of clinical disease (Faden *et al*., 1997; Leach *et al*., 1994). In a study of

M. bovis-associated pneumonia in feedlot cattle, middle ear colonization with *M. bovis* was common but no evidence of otitis media was observed (Gagea *et al*., 2006), further supporting the idea that susceptibility to *M. bovis*-associated otitis media is largely age-related. In addition to age-related effects, studies in animal models of a human otitis media pathogen, *Haemophilus influenzae,* have indicated that there is a critical bacterial load in the nasopharynx below which otitis media does not usually occur, and that keeping bacterial levels lower than this threshold is effective in preventing otitis media (Kennedy *et al*., 2000). A similar situation may occur with *M. bovis* infections; in our experimental studies, the level of tonsil colonization was associated with the development of clinical disease. Our findings suggest that even if colonization of the URT cannot be prevented, strategies which limit the level of replication of *M. bovis* in the URT may be effective at preventing clinical disease. Strategies that could be evaluated for their efficacy in delaying URT colonization until calves are older, or limiting *M. bovis* replication in URT of young calves include metaphylactic antibiotic therapy, management practices to reduce environmental exposure to *M. bovis*, strategies to improve innate defenses in the respiratory tract, and alternative immunization strategies that improve local adaptive immune responses. In particular, based on our limited understanding of protective immune responses in mycoplasmal respiratory disease, immunization strategies that increase the presence of effective opsonins such as IgG_2 in the URT may be effective.

Local immunity along the respiratory tract plays a major role in resisting and controlling mycoplasma infection in a number of hosts, and our studies have demonstrated that locally generated immune responses are important in *M. bovis* infections. In addition, studies of murine mycoplasmal infections have shown that local immunization is more effective than systemic immunization for disease protection (Taylor and Howard, 1980; Hodge and Simecka, 2002).

Thus, further studies are warranted to determine if local immunization of the respiratory tract early in life will provide for effective protection against *M. bovis* infection in calves. To the best of the author's knowledge this route has been minimally investigated for protection against *M. bovis* infections. Local immunization of the mammary gland with killed *M. bovis* without adjuvant resulted in an IgG_1-dominated humoral response and exacerbation of mastitis in adult cows (Boothby *et al*., 1987). A combination of intramuscular (with Freund's complete adjuvant) and intratracheal (without adjuvant) inoculation with killed *M. bovis* was successful in reducing, but not preventing, lung colonization and clinical disease in challenged calves (Howard *et al*., 1980). However, intratracheal immunization with killed *M. bovis* alone failed to induce protective responses.

Careful evaluation of alternative methods of antigen formulation and use of mucosal adjuvants may identify strategies that result in a protective local response in the URT without exacerbation of clinical disease in young calves. The experimental infection model we have developed could be used in evaluating such strategies. Future studies are planned to determine whether the nasal route of immunization, using either killed or live *M. bovis* vaccines, can be used to generate local immunity against infection very early in life. More sophisticated systemic vaccine strategies such as those using subunit vaccines or DNA vaccines have not been particularly successful in protection against mycoplasmal infections in general, and none have been developed against *M. bovis*. In any case, any of these immunization alternatives requires an adaptive immune response and will likely be hampered by the early age at which colonization first occurs, unless concurrent strategies that delay colonization in young calves can also be developed and employed.

An alternative strategy that should be further investigated to prevent or limit *M. bovis* colonization is passive immunization of calves. Although passive transfer of *M. bovis*-specific colostral antibodies did not appear to affect the outcome of clinical disease in our field study, it is likely that some colostrum containing high antibody concentrations to *M. bovis* may have come from cows with intramammary infection and therefore may also have contained live *M. bovis*. This could certainly have masked any protective effect of passive transfer. Other data on passive immunization against *M. bovis* is extremely limited, and the role of passive transfer needs to be evaluated in a controlled setting. Even if colostral protection is not generally efficacious, it is possible that passive immunization with antibodies of specific isotypes (e.g. IgA or IgG_2) or antibodies that are directed to specific mycoplasmal antigens might limit or delay colonization of the URT. For example, potential target antigens might include putative virulence factors such as proteins involved in antigenic variation, adhesion, or biofilm formation.

In addition to understanding the host response to infection, it is also important to identify the virulence factors of *M. bovis*. Research into the microbial factors involved in the ability of *M. bovis* to colonize, persist, and cause disease in the host is ongoing, but many critical gaps in knowledge remain. This field will likely be greatly assisted by the *M. bovis* genome sequencing projects, including the *M. bovis* F1 genome project, that are currently nearing completion. One factor in particular that needs to be addressed is to define whether specific surface antigens of *M. bovis* are involved in protective versus immunopathological responses. Based on our studies, any microbial factors involved in colonization of the tonsils and other URT sites would also be key virulence determinants.

In summary, control and prevention of *M. bovis*-associated disease in young dairy calves presents a complex and challenging problem. Successful strategies will likely include a variety of

approaches, including practical on-farm management practices predicated on epidemiological risk factors, therapeutic modalities, modification and augmentation of the host immune response by mechanisms tailored to the specific needs of the neonatal calf, and an understanding of the microbial factors involved in acute and chronic *M. bovis* disease. Traditional vaccine strategies may prove difficult to implement in the young dairy calf, and new approaches to prevention will likely be required to protect this at risk age group. Our study was the first to critically assess the efficacy of a commercial vaccine in young calves; importantly the vaccine trial was performed under actual field conditions and on multiple farms. The results of the field trial served as an impetus to develop a reproducible model that mimicked natural disease and that could be used to address critical issue for improved vaccine development. Importantly, the development of this reproducible model will provide the foundation that may lead to the development of improved preventative or control strategies for *M. bovis* as well as provide a tool to assess the specific virulence factors of *M. bovis*.

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BIOGRAPHICAL SKETCH

Dr. Fiona P. Maunsell received her veterinary degree with honors in 1990 from the University of Melbourne in Victoria, Australia. She then worked in private practice in Newcastle, Australia until 1993. In 1995, Dr. Maunsell completed an internship in food animal medicine and surgery at the University of Illinois, and followed this with a 3-year residency program. She concurrently received a Master of Science degree from the University of Illinois in 1999. She is a Diplomate of the American College of Veterinary Internal Medicine (large animal internal medicine). After her residency, she joined the faculty in Food Animal Medicine and Surgery at the University of Illinois before moving to the University of Florida in 1999 for her doctoral research training.

Dr. Maunsell's clinical interests include diseases of dairy replacement heifers and metabolic and infectious diseases of cattle, particularly mastitis. Her research interests are focused on ruminant mycoplasmal infections, and, in particular, on *Mycoplasma bovis* infections of young calves. She was the recipient of the American Association of Bovine Practitioners Research Summaries Graduate Student Award, 36th Annual Convention of the American Association of Bovine Practitioners, Columbus, OH, 2003. Dr. Maunsell is a member of the following professional societies: American Association of Bovine Practitioners, American Dairy Science Association, American Veterinary Medical Association, Australian Veterinary Association, The Society of Phi Zeta (Honor Society of Veterinary Medicine), and the International Organization for Mycoplasmology