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MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGY OF *MYCOPLASMA BOVIS*

A Dissertation in

Pathobiology

by

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ABSTRACT

Mycoplasma bovis is an important pathogen causing pneumonia, mastitis, arthritis, conjunctivitis, otitis, genital infections, and septicemia in cattle. A molecular epidemiological analysis of *M. bovis* strains submitted to the Pennsylvania Animal Diagnostic Laboratory (PA-ADL) between December 1, 2007 and November 30, 2008 was conducted. During this period, *M. bovis* represented 63.6% of *Mycoplasma* species isolated from samples submitted to PA-ADL. The ability to separate isolates into clonally distinct groups showing strong genetic heterogeneity was demonstrated through the utilization of a novel technology, amplified fragment length polymorphism (AFLP). The results were consistent with the existence of at least two clonally distinct groups, although there was no clear geographical, month of isolation, or source origination relationship, indicating a currently unclassified characteristic is responsible for the strain heterogeneity. AFLP may serve as a valuable tool for molecular characterization of *M. bovis* strains from the United States.

Mycoplasma bovis isolates submitted to the PA-ADL (n=192) were tested for antimicrobial susceptibility to enrofloxacin, erythromycin, florfenicol, spectinomycin, ceftiofur, tetracycline, and oxytetracycline using a broth micodilution testing method which incorporated a redox color change reagent. The antimicrobials which were most effective for *M. bovis* using the broth microdilution method were florfenicol, enrofloxacin, and tetracycline with a minimum inhibitory concentration (MIC) range of 2 - 32 µg/mL, 0.1 - 3.2 µg/mL, and 0.05 - >12.8 µg/mL, respectively. A significant difference in the susceptibility levels between quarter milk and lung *M. bovis* isolates was found for spectinomycin. Using CLSI-approved breakpoints for other bovine respiratory disease pathogens it was determined that enrofloxacin, spectinomycin, ceftiofur, erythromycin, tetracycline, oxytetracycline, and florfenicol demonstrated resistance in 2.1%, 58.3%, 100%, 100%, 12.5%, 22.4%, and 11.4% of isolates, respectively.

MIC values of a subset of the *M. bovis* isolates (n=12) were tested using a novel side-scatter flow cytometric technique with concurrent staining using SYBR green I and propidium iodide. The most effective antimicrobials for the 12 isolates were determined to be enrofloxacin and florfenicol. Flow cytometry offers potential in clinical applications due to high throughput-capability, quick turn-around time, and the objective nature of interpreting results.

Based upon the increasing resistance patterns of *M. bovis* to several currently administered veterinary antimicrobials, novel small molecule compounds were screened for potential inhibition of *Mycoplasma bovis* growth and dose-dependent response was evaluated. The determination of inhibition of *M. bovis* growth in a milk environment using novel small molecule natural compounds was also studied. The data suggest that 32 of the 483 compounds tested were able to inhibit growth of *M. bovis* using a tetrazolium salt assay. Methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate demonstrated a dose response in the tetrazolium broth culture assay and at 3 hours and 24 hours in fresh quarter milk. Small molecule natural compounds offer the potential for prophylactic or therapeutic use on organic and natural farms as a viable alternative to traditional antimicrobial agents.

A longitudinal study was conducted to determine the cumulative incidence and prevalence of *M. bovis* in special-fed veal calves from 4 Pennsylvania herds. The period prevalence of *M. bovis* colonization was 90.5% for the nasal swabs and 38% for swabs collected at the bronchial bifurcation. A total of 90 lung lesions were identified from the 252 calves in the study. A total of 42% of the lung lesions collected were culture positive for *M. bovis*, while 37% of bronchi swabs were culture positive. Evidence suggests that *M. bovis*, in concert with other respiratory pathogens, is responsible for pneumonic lesions in veal calves. It was determined that

calves with a member of the *Pasteurellaceae* family cultured from a lung lesion was 10.8 (OR=10.7885; 95% CI 2.2245-52.3231) times more likely to also have *M. bovis* present in the lesion than *Pasteurellaceae* alone. The data suggest that nasal swab testing on special-fed veal farms can be used to screen calves for the presence of *M.* bovis, but are unlikely to be successful at determining exactly which calves are at increased risk of developing lung lesions.

A blinded, controlled trial of two commercially available *M. bovis* bacterin vaccines for the prevention of respiratory disease associated with *M. bovis* infection in special-fed veal calves was conducted. Vaccine A efficacy was compared to a placebo and vaccine B efficacy was compared to 0.9% sterile saline solution. Upper-respiratory tract colonization was not impacted by vaccination status. The presence of lung lesions was significantly reduced by treatment of vaccine A (p=0.0325), however lung lesions from tissues positive for *M. bovis* was not significantly reduced. Vaccine B was not shown to significantly reduce total lung lesions or *M. bovis*-specific lung lesions. There was no association between specific antibody concentrations and *M. bovis*-associated morbidity in the veal calves. Approximately 30% of all calves in the study were found to be seropositive for *M. bovis* upon acclimation to the barn, indicating a potential problem of maternal *M. bovis* in Pennsylvania. A detectable difference in the IL-1β and TNF-α level for vaccinated calves (vaccine A and vaccine B) was seen as compared to control calves. Under the field conditions of this study, vaccine efficacy for special-fed veal calves was determined to be 44% and less than 1% for vaccine A and vaccine B, respectively.

In summary, the findings presented indicate that *M. bovis* is an important public health concern in Pennsylvania. Although virulence specific factors or environmental factors contributing to the severity of *M. bovis*-associated disease have yet to be discovered, this work has provided a basis for continued investigation. Further understanding of the changing

epidemiology of *M. bovis* in Pennsylvania will provide valuable information for the design and implementation of *M. bovis* prevention and management strategies in the United States.

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Chapter 1

Introduction

1.1 Introduction

Mycoplasma bovis, discovered in 1961, is a well established pathogen of ruminants known to cause pneumonia, mastitis, conjunctivitis, otitis, arthritis and abortions. Bovine mastitis and pneumonia caused by *M. bovis* are complex multifactor diseases that are some of the most difficult bacterial infections to treat and control, accounting for significant economic and production loses in the beef and dairy industries. Although *M. bovis* is sufficient to cause disease, the major role it plays is in co-infections with *Mannheimia haemolytica, Pasteurella multocida, Arcanobacterium pyogenes,* and *Histophilus somni,* leading to high morbidity and mortality.

Mycoplasma species are inherently refractory to several groups of antimicrobials, such as beta lactams, due to the lack of a cell-wall. The poor ability to control disease outcomes using current antimicrobial therapies and reports of increasing levels of resistance to several antimicrobials has lead to a strong interest in potential vaccines and new therapeutic options. In order to improve prevention of *M. bovis* infections, use of vaccines may become more important especially as housing units increase animal density. Understanding the effectiveness and economic feasibility of currently available vaccines may help producers reduce incidence of disease.

There is an opportunity to begin to transition to the use of novel compounds with mycoplasmastatic or mycoplasmacidal activity to halt the spread of infection within an individual animal or throughout a herd. Ideal compound candidates would be applicable to the growing number of "organic" farms as well. Potential candidates suitable for both traditional and organic farms include natural small molecule compounds derived from plants, insects, etc.

Through molecular characterization and epidemiological analysis there is potential to assist in determining appropriate treatment or prevention methods to incorporate in Pennsylvania's dairy, beef, and veal markets.

1.2 Statement of the Problem

The unknown epidemiology and unique cellular characteristics of *Mycoplasma bovis* attribute to the difficulty selecting appropriate therapeutic and prophylactic options for controlling *M. bovis*-associated infections in Pennsylvania. It is increasingly necessary to develop susceptibility and typing methodologies that have the potential for robust, high throughput testing with an opportunity to distribute data between laboratories with ease and efficiency. It is crucial to understand the epidemiology and impact of current prophylactic options in order to appropriately implement susceptibility and typing methods.

1.3 Research Objectives

1. To conduct a molecular epidemiological analysis of *M. bovis* and characterize *M. bovis* isolates using AFLP fingerprinting in order to determine the phylogeny of all *M. bovis* isolated from various sample types submitted to the Pennsylvania Animal Diagnostic Laboratory (PA ADL) over a one year time period.
2. To conduct and evaluate antimicrobial susceptibility testing for *M. bovis* isolates collected from PA ADL for 7 veterinary approved antimicrobial agents using a broth microdilution method and flow cytometry.
3. To screen *M. bovis* for susceptibility to ~480 small molecule natural compounds.
4. To survey *M. bovis* and bovine respiratory disease (BRD) bacterial pathogen colonization and infection in the upper and lower respiratory tract in Pennsylvania veal calves.
5. To determine the efficacy of commercially available *M. bovis* vaccines in veal calves.

Chapter 2

Review of Literature

2.1 Mycoplasmas

In 1898, the agent for bovine pleuropneumonia (*Mycoplasma mycoides*) became the first mycoplasma cultivated (Nocard and Roux, 1898). Early research of mycoplasmas considered these new organisms as viruses or bacterial L-forms (Razin and Hayflick, 2010). Due to the classic "fried-egg" morphology (Figure 1.1) of mycoplasmas the confusion persisted until late 1960's (Razin and Hayflick, 2010).

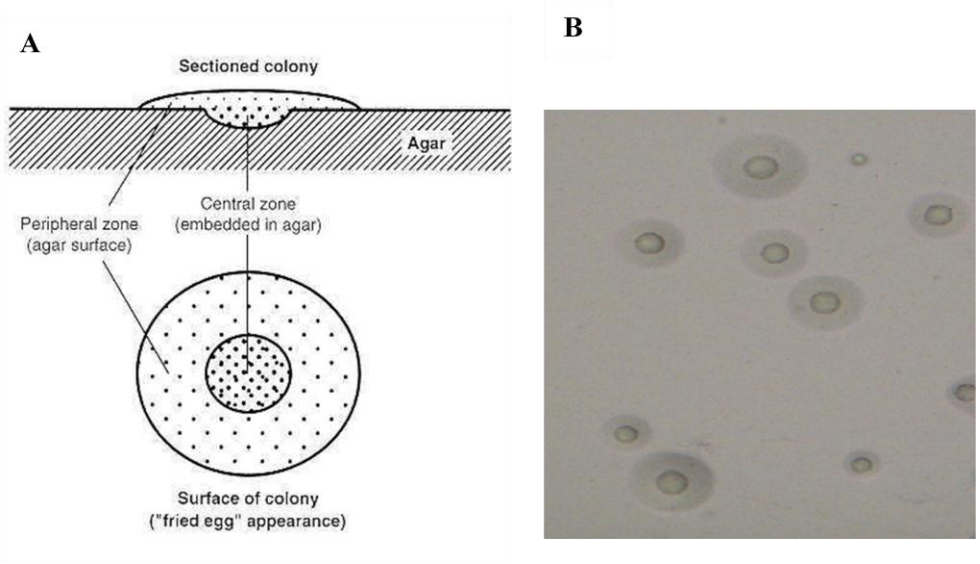

Figure 2.1 Morphology of *Mycoplasma.* A: Diagram of "fried-egg" morphology (Razin, 2002). B: *Mycoplasma bovis* on pleuropneumonia-like organism agar (photo by M. K. Soehnlen).

In fact mycoplasmas are the largest genus of the class *Mollicutes* (latin. *molli* -"soft" *cutis* - "skin") which is noted for the lack of a cell wall. *Mollicutes* are considered the smallest free-living organisms. Despite the ever expanding knowledge of the number and classification of species in the *Mollicutes*, the terms "mycoplasmas" and "mollicutes" are used often used interchangeably to refer to any species in the class. This may be in part due to the shared traits such as small size, extremely small genomes (0.58-0.138 Mbp), low G+C content (23-40mol%), and limited biosynthetic capacity (Weisburg et al., 1989; Poveda, 1999; Nicholas et al., 2008). In

truth there are nearly 200 species have been established in the class *Mollicutes* (Razin, 2006). The five families which make-up the class *Mollicutes* include *Mycoplasmataceae*, *Spiroplasmataceae, Acholeplasmataceae, Entomoplasmataceae,* and *Anaeroplasmatacea. Mollicutes* have been shown to infect or colonize humans, mammals, reptiles, fish, insects, and plants. The major characteristics and taxonomy of the class Mollicutes is shown in Table 1.1.

Despite these seemingly drastic differences as compared to other bacteria, *Mycoplasma* has been shown to replicate essentially the same as other prokaryotes (Bredt et al., 1973; Razin, 1978; Razin and Hayflick, 2010). However, in *Mycoplasma* species multinucleated filaments may form during binary fission due to cytoplasmic division sometimes lagging behind genome replication (Razin, 1978; Razin et al., 2010). The lack of a cell-wall means that *Mycoplasmas* will be Gram-negative. In spite of this, *Mollicutes* are most evolutionarily related to Gram-positive bacteria, namely, *Bacilli* and *Clostridia.* It is the 16S rRNA sequence studies that provided the most frequently used basis for the current phylogeny (Woese et al., 1980; Weisberg et al., 1989; Johansson et al., 1998; Johansson et al., 2002). Despite decades of discussion, it has been demonstrated that *Mycoplasma* branched from it's relatives due to degenerative or reductive evolution (Woese et al., 1985). The phylogenetic tree based upon the 16S rRNA sequences is provided in Figure 1.2. The clusters are particularly interesting since species that share the same host environment may be distantly related, such as *M. mycoides* subsp. *mycoides* small colony and *M. bovis. Mycoplasma bovis* is part of the hominis cluster while *M. mycoides* is part of the spiroplasma cluster. These cluster differences may have developed during another generation of genome reduction indicating the potential of the wall-less state arising from more than a single area of the *Eubacteria* tree (Weisberg et al., 1989).

Table 2.1 Major characteristics and taxonomy of the class Mollicutes

Classification	No. of recognized species	Genome size (kb)	Genome G+C (mol%)	Cholesterol requirement	Distinctive properties	Habitat
Mycoplasmataceae						
Genus I: *Mycoplasma*	107 (11)[a]	580–1350	23–40	Yes	Optimum growth 37°C	Humans and animals
Genus II: *Ureaplasma*	7	760–1170	27–30	Yes	Urease positive	Humans and animals
Entomoplasmataceae						
Genus I: *Entomoplasma*	6	790–1140	27–29	Yes	Optimum growth 30°C	Insects and plants
Genus II: Mesoplasma	12	870–1100	27–30	No	Optimum growth 30°C	Insects and plants
Spiroplasmataceae						
Genus I: *Spiroplasma*	34	780–2220	24–31	Yes	Helical filaments	Insects and plants
Acholeplasmataceae						
Genus I: *Acholeplasma*	14	1500–1650	26–36	No	Optimum growth 30–37°C	Animals and plant surfaces
*Anaeroplasma*taceae						
Genus I: Anaeroplasma	4	1500–1600	29–34	Yes	Obligate anaerobes, oxygen sensitive	Bovine-ovine rumen
Genus II: Asteroleplasma	1	1500	40	No		
Undefined taxonomic status						
Phytoplasma	ND[b]	530–1185	23–29	ND	Uncultured in vitro	Insects and plants

Abbreviation: ND, not determined.

[a]The number of Candidatus species is given in parentheses and includes the hemoplasmas (*Eperythrozoon* and *Haemobartonella*) recently transferred to the genus *Mycoplasma* (Neimark et al., 2001).

[b]The taxonomic status of the uncultured phytoplasmas has not been finally defined; seven Candidatus *Phytoplasma* spp. have so far been published.

Updated and modified from Razin et al. (1998).

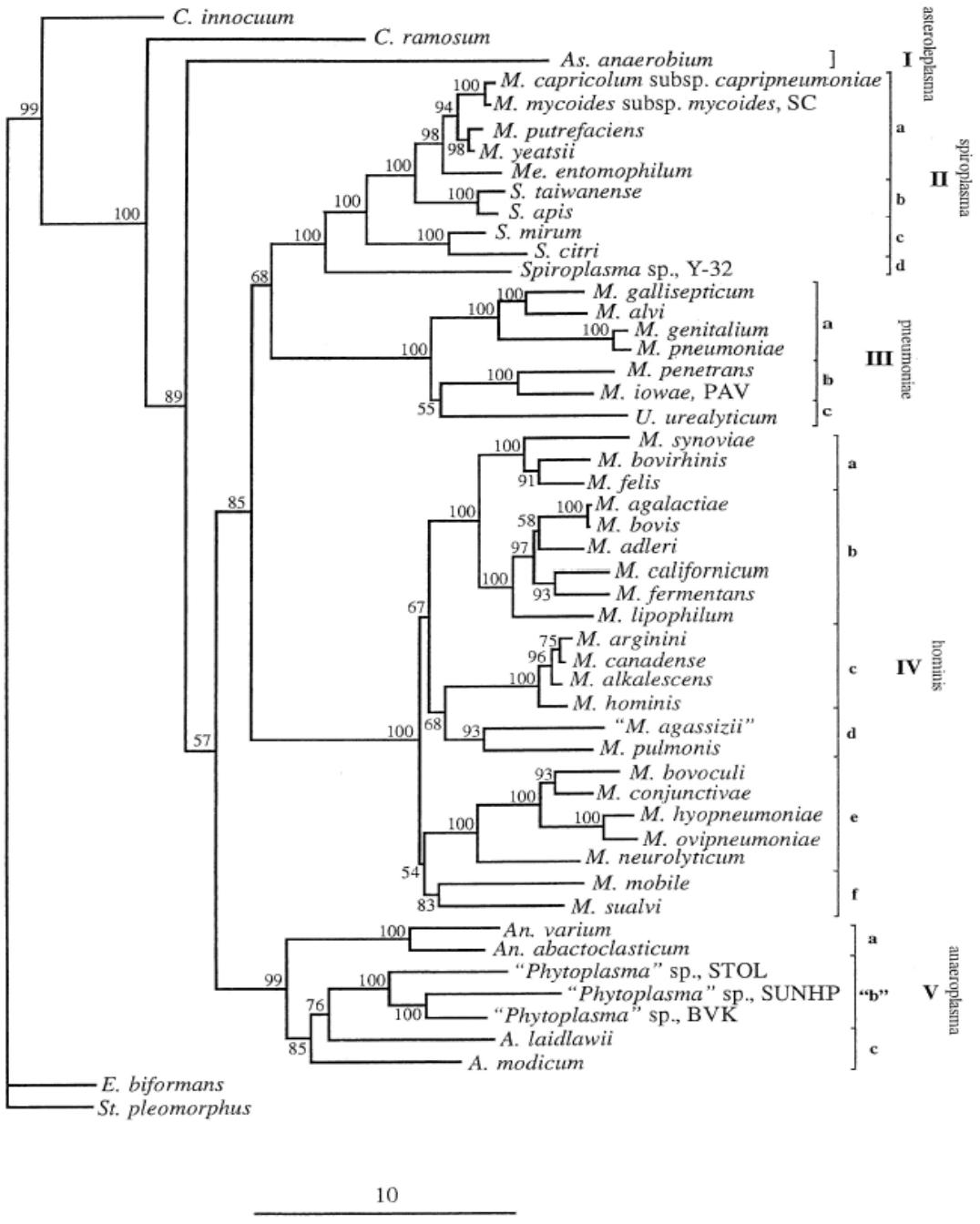

Figure 2.2 Phylogenetic tree of representing all mycoplasma groups and clusters based on 16S rRNA sequences. *Clostridium* and *Eubacterium* included as representatives of closely related genera. (Johansson et al., 1998; Razin, 2006)

2.2 Isolation and Detection Methods

There is difficulty in detecting and isolating due to the strict nutritional requirements, slow growth, and pH requirements of various *Mollicutes*. There is currently no single media formulation that is appropriate for the adequate growth of all *Mollicutes*. Most medium includes beef heart infusion, peptone, yeast extract, serum, and a variety of supplements (Razin and Hermann, 2002). Mycoplasmas require fatty acids, amino acids, vitamins and nucleic acid and lipid precursors from an exogenous source (Nicholas et al., 2008). There are also fermentative and non-fermentative *Mycoplasma* species. It is these differences between species that make diagnosis of infections due to a specific species challenging. Many times mycoplasma infections are considered "rule out" diagnoses. However, proper sample collection and transport may lessen the difficulties of isolation.

Sample collection should follow the minimum standards as other bacteria, namely, optimal recovery is from fresh samples and aseptic technique using sterile swabs pre-wetted in transport medium should be used whenever feasible (Nicholas et al., 2008). Transportation of samples to processing facility should occur as quickly as possible and preferably at 4°C. Tissue or organ samples that can not be processed upon arrival at the laboratory should be stored deep frozen or lyophilized (Nicholas et al., 2008). Lyophilized tissue is an excellent option for international transport of samples due to the difficulties keeping samples frozen and the numerous import regulations in most countries related to organisms capable of causing human, animal, and plant disease (Houshaymi et al., 2000; Nicholas et al., 2008).

The isolation of a *Mycoplasma* from samples may be performed on solid or liquid media. Bacterial contamination will be noted in broth as gross turbidity at 24 hours (Nicholas et al., 2008). Between 3 and 5 days the *Mycoplasma* growth will appear as opalescence and in some cases a fine film will appear on the media surface (Nicholas et al., 2008). Plates may be inspected at ~3 days under magnification for the presence of colonies with a "fried egg" appearance

(Nicholas et al., 2008). Although it is possible to subculture broth to broth using a 10% (v/v) inoculum, subculture from a plate is performed by excising a block of colonies with a sterile spatula or using the tip of a hypodermic needle or Pasteur pipette. The agar plug containing the colonies is either transferred into broth or carefully slid across a new plate (Nicholas et al., 2008).

Following isolation of the *Mycoplasma* identification using biochemical, serological, immunological, or molecular methods is performed. Biochemical and serological procedures should be used in tandem allowing for the identification of mixed cultures. However, serological screening is cumbersome and lack of readily available antisera presents a challenge for many diagnostic laboratories (Barber and Fabricant, 1971). Another issue to consider is that serology implies exposure and not disease when antibodies are found in the host animal (Fox et al., 2005). Dextrose fermentation has been shown to allow for the grouping of fermenting and non-fermenting for most species (Edward, 1954; Freundt, 1958; Tourtellote and Jacobs, 1960; Aluotto et al., 1970; Barber and Fabricant, 1971). Serology is effective in indicating exposure to *Mycoplasma* and is often regarded as more sensitive than culture alone in cases of previous antimicrobial treatment due to potential *in-vitro* growth inhibition (Nicholas and Ayling, 2003; Caswell and Archambault, 2008).The numerous serological tests available include hemagglutination, film inhibition, and indirect enzyme-linked immunosorbent assay (ELISA) (Nicholas and Ayling, 2003; Caswell and Archambault, 2008; Nicholas et al., 2008).

Antibody-based tests, such as sandwich ELISA using monoclonal antibodies, fluorescent antibody, antigen-capture ELISA, metabolic inhibition tests, dot immunobinding using polyclonal antsera, immunoperoxidase labeling, and immunobinding assays have all been used to successfully identify specific species (Gourlay et al., 1989; Infante Martinez et al., 1990; Pourmarat et al., 1991; Ball et al., 1994; Poveda, 1998; Infante et al., 2002; Flores-Guitierrez et al., 2004; Arcangioli et al., 2008). However, most of these assays are expensive, time and labor intensive, and require antisera, which are often not commercially available (Caswell and Archambault, 2008).

Modern molecular methods have begun to replace the serological, immunological, and biochemical detection methods. These methods include polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). The use of PCR primers targeting the 16S rRNA region is capable of differentiating many species of mycoplasma, but may mis-diagnose closely related species (Thomas et al., 2004). An example of this phenomenon is *M. bovis* and *M. agalactiae* which are identical in the 16S rRNA region. Speciation of closely related species occurs based upon other genes with known differences, such as the DNA repair gene, *uvr*C, which is used for *M. bovis* and *M. agalactiae* (Subramaniam et al., 1998; Thomas et al., 2004). There are currently a few important species that do not have species specific primers, such as the ruminant species *M. canadense*, *M. californicum,* and *M. verecundum* (Nicholas et al., 2008). The advantage of PCR testing procedures is the viability of cells is not necessary to determine the presence of *Mycoplasma*; meaning that live cultures or samples containing non-viable cultures may be identified (Fox et al., 2005). This may be especially valuable for samples that have to travel long distances to reach diagnostic laboratories. Additionally, DGGE has been successfully employed to detect and differentiate 67 *Mycoplasmas* of which 27 are of veterinary importance (McAuliffe et al., 2003; McAuliffe et al., 2005; Nicholas et al., 2008). Caution must be used when differentiating *M. canadense*, *M. bovis*, and *M. verecundum* due to the profile migration differences being close (McAuliffe et al., 2003; McAuliffe et al., 2005; Nicholas et al., 2008). The use of DGGE serves as a powerful tool since it may be used for detection, identification, and molecular epidemiology studies during outbreaks associated with one or multiple species of *Mycoplasma.*

Other methods have been successfully administered to identify *Mycoplasma* in tissue samples as well as for the determination of viable *Mycoplasma* cells which aids in the accuracy of testing. Paraffin-embedded tissues have also been successfully used with in-situ hybridization to detect *Mycoplasma* (Jacobsen et al., 2010). Immunohistochemistry may be used for antigen detection in fixed tissue samples (Haines and Chelack, 1991; Haines et al., 2001; Caswell and

Archambault, 2008). The difficulty quantifying viable *Mycoplasmas* has lead to the development of quantification methods such as opacity, hemagglutination, dry weight, pH change, protein production, cholesterol and glucose consumption, flow cytometry, and microtiter dilution methods (Feldner et al., 1979; Kahane et al., 1979; Fletcheret al., 1981; Snell, 1981; Albers et al., 1982; Assunca et al., 2006). The use of these quantifying methods have improved testing of *Mycoplasma* species by allowing for proper counts when performing procedures, such as antimicrobial susceptibility testing, novel drug screening, or virulence factor screening (Rosenbusch et al., 2005; Nicholas et al., 2008). The increasing awareness of *Mycoplasma*-associated disease and the molecular approaches for detection and classification indicate that more cases of *Mycoplasma*-associated disease are likely to be diagnosed in the future.

2.3 *Mycoplasma bovis*

Mycoplasma bovis is a contagious pathogen of cattle which has been associated with pneumonia, mastitis, conjunctivitis, otitis, arthritis, neurological disorders, abortions, seminal vesculitis, and possibly septicemia and meningitis (Hale et al., 1962; LaFaunce and McEntee, 1982; Pfutzner, 1990; Stipkovits et al., 1993; Kirby and Nicholas, 1996; Walz et al., 1997; Maeda et al., 2003; Lamm et al., 2004; Ayling et al., 2005; Fox et al., 2005; Francoz et al., 2005; Nicholas et al., 2008; Raedelli et al., 2008). *Mycoplasma bovis* is considered the most important mycoplasma pathogen of cattle in the United Sates, which has no reported cases of the causative agent of contagious bovine pleuropneumia, *M. mycoides* subsp. *mycoides* SC (Gourlay, 1981; Nicholas and Ayling, 2003). The first reported *M. bovis* case was from cattle with mastitis, isolated in the United States during the summer of 1961 (Hale et al., 1962). Due to the similarities with *Mycoplasma agalactiae*, the causative agent of contagious agalactiae in small ruminants; *M. bovis* was considered a subspecies for many years (Hale et al., 1962; Caswell and Archambault, 2008). Biochemical tests are insufficient for the differentiation of *M. bovis* and *M. agalactiae*, as

that neither ferments glucose, nor hydrolyzes arginine and both use lactate and pyruvate (Abu-Amero et al., 2000; Khan et al., 2005). Although, large ruminant host are generally assumed to be infected with *M. bovis,* the biochemical similarities require molecular testing to differentiate the species (Caswell and Archambault, 2008; Nicholas et al., 2008).

Mycoplasma bovis has been studied for nearly 50 years, yet the knowledge of how to control disease due to infection is limited. According to Caswell and Archambault (2008) the areas which have been reported as still having "substantial uncertainty" include the clinical impact of genotypic diversity, the presence of virulence factors effect upon tissue damage, mechanisms of immunity, and the role of co-infections in disease.

2.3.1 Pathogenesis

Despite often being considered only an opportunistic pathogen, *Mycoplasma bovis* has been shown to be capable of causing respiratory disease in gnotobiotic calves in the absence of viruses or other agents (Gourlay et al., 1975). Additionally, there is no evidence of environmental factors significantly influencing the course of disease (Jasper, 1974; Boughton, 1979; Jasper, 1982). The pathogenesis of *M. bovis* is reported to be dependent upon multiple factors including the synergistic effect of the bacterial and viral co-infections and the host immune response, indicating the complexity of infection (Fulton, 2009). The multitude of disease conditions associated with *M. bovis* infections means that many research studies have been conducted regarding the pathogenesis, but many questions are still unresolved.

Mycoplasma bovis is a highly invasive organism that is not limited to the initial area of colonization, which is often the respiratory tract (Minion, 2002). The bacteria are able to gain access to multiple organ systems quickly while surviving host immune responses (Minion, 2002). One possible mechanism for this is due to the reported ability of *M. bovis* to attach to peripheral blood mononuclear cells (PBMC) and erythrocytes (van der Merwe , et al., 2010). The

mechanism of *M. bovis* attachment to host cells is still unclear and controversial. One *in-vivo* study has suggested that *M. bovis* typically adheres to the bovine bronchiolar epithelial cell surfaces, but does not migrate intracellularly (Thomas et al., 1987). Other studies suggest that *M. bovis* may be found intracellularly in neutrophils, macrophages, and hepatocytes (Maeda et al, 2003; Srikumaran et al., 2007; Dyer et al., 2008; van der Merwe et al., 2010).

Some mechanisms of pathogenesis are common to *M. bovis* regardless of infection site while others have only been studied in specific organ systems. Adherence of *M. bovis* to host cells is a complex process which has been linked to reduced host immune response and virulence (Basseman et al., 1995; Minion, 2002). Adherence of *M. bovis* is known to be mediated by the family of variable surface proteins (Vsp) and other proteins (pMB67 and p26); although there are likely more undiscovered mediators (Sachse et al., 1996; Thomas et al., 2003a; Thomas et al., 2003b). The Vsps are involved in cytoadherence and are likely one of the most important ways that *M. bovis* evades host immune responses (Rosengarten et al., 1994).

Vsps lead to antigenic variation through phase switching, antigenic variation, and size variation (Behrens et al., 1994; Rosengarten et al., 1994). There are at least 13 members of the lipid-modified Vsp family which are each distinguishable using monoclonal antibodies directed at the unique antigenic epitopes (Rasberry and Rosenbusch, 1995; Lysnyansky et al., 2001). Each Vsp exhibits phase switching (ON-OFF) and size variation which allow for demonstration of significant variability among strains of *M. bovis* through involvement in DNA rearrangements, inversions, and recombination (Lysnansky et al, 1996; Beier et al., 1998; Lysnansky et al, 2001). The various Vsps are evenly distributed on the surface of *M. bovis* (Behrens et al., 1996a). The Vsp size variation is due to the repetitive sequences which can make up to 80% of the coding sequence (Lysnansky et al, 1999). Despite the differences between Vsp sequences the first 29 amino acids on the N-termus are conserved across all Vsps (Lysnansky et al, 1999). The size variations and phase switching are suggested to allow *M. bovis* the ability change the surface epitopes in order to evade the host immune response (Behrens et al., 1994; Rosengarten et al.,

1994; Behrens et al., 1996a; Lysnansky et al, 1996; Beier et al., 1998; Lysnansky et al, 1999; Lysnansky et al, 2001; Minion, 2002). Other common methods of host immune response evasion are the induced apoptosis of lymphocytes, widespread macrophage activation, and inhibition of the neutrophilic oxidative burst (Howard et al., 1987; Thomas et al., 1991; Jungi et al., 1996; Vanden Bush and Rosenbusch, 2002; Vanden Bush and Rosenbusch, 2004). Regarding the other proteins recognized as adhesins; p26 is sensitive to temperature, trypsin, and neuraminidase making it a poor choice for diagnostic potential or as a vaccine construct, but pMB67 also undergoes phase switching and size variation similar to Vsps and is not lipid modified and does not contain repetitive sequences (Behrens et al., 1996b; Sachse et al., 1996). Despite the possibilities of Vsps, pMB67, or even p26 as new diagnostic reagents or for use in vaccine constructs, relatively little is know about the effect they have in natural infections.

Lung invasion by *M. bovis* results in injury to the host cells and inflammation. It is possible for *M. bovis* to adhere to the bovine tracheobronchial epithelial cells leading to colonization in the lung, however, unlike other animal mycoplasmas this adherence does not lead to ciliostasis (Howard et al., 1987; Caswell et al., 2010). Following infection, *M. bovis* may continuously evade the host immune defenses causing chronic colonization in the lungs and the eventual dissemination to other organs, especially the joints and middle ear (Maunsell and Donovan, 2009; Caswell et al., 2010). It has been suggested that ascending infection of the Eustachian tube is the likely route of spread to the inner ear from the respiratory tract (Maunsell and Donovan, 2009). Meanwhile, it is believed that hematologic dissemination allows for the spread of infection to the joints (Thomas et al., 1986). Once *M. bovis* has been detected in a herd of calves, it is often detected from nasal swabs of most other calves within 7 days indicating the potential of speedy dissemination through a herd (Pfutzner, 1996).

It is known that *M. bovis* infection elicits a specific immune response of immunoglobulin (Ig) M and IgG in the serum and IgA in the nasal secretions and lung fluid (Caswell et al., 2010). *Mycoplasma bovis*-specific IgM is detectable at 7 days, detected at a maximum peak at 14 days,

and increasing for up to 63 days post-infection (Vanden Bush and Rosenbusch, 2003). It is the increased levels of IgG1 over the IgG2 isotype that suggests a T-helper (Th) 2 response, which is generally an antibody mediated response (Vanden Bush and Rosenbusch, 2003). The T-cell responses documented include CD8+ T cells, CD4+ T cells, and γδ T cells, but the role of these responses remains unknown (Vanden Bush and Rosenbusch, 2003).

Early mouse studies demonstrated that the phagocytosis of mycoplasmas by macrophages and polymorphonuclear leukocytes (PMNLs) occurs following the recruitment to infected areas by chemotaxis (Cassell et al., 1973; Marshall et al., 1995). Unfortunately, there have been many contradicting reports regarding the ability to navigate around the host immune responses contributing to the lack of knowledge regarding the mechanisms of *Mycoplasma* phagocytosis (Marshall et al., 1995). The studies of *M. bovis* do indicate that it is capable of resisting phagocytosis if it is unopsonized, however it has been suggested that this is not likely to impact the pathogenesis, and bovine macrophages are capable of specific opsonization (Howard et al., 1976; Marshall et al., 1995). Importantly it has been reported that the phagocytosis of other bacteria, such as *Escherichia coli*, by PMNLs is impaired by the presence of *M. bovis* (Howard and Taylor, 1983). Although there are currently no reports on the action of *M. bovis* on the secretatory functions of macrophages; other bovine mycoplasmas, such as *M. dispar* and *M. mycoides*, do suppress the production of TNF-α, IL-1 and glucose consumption (Almeida and Rosenbusch, 1991; Almeida et al., 1992). *Mycoplasma* may damage the phagocyte membrane through the production of oxygen radicals, which may also be pathogenicity factor (Kahne, 1984; Meier and Habermehl, 1991). The production of radicals may also disrupt the glutathione redox cycle which has been associated with the oxidative burst activity of neutrophils and the PMNLs NADPH oxidase generation (Kahne, 1984; Almagor et al., 1986; Paoletti et al., 1990; Thomas et al., 1991). Overall, phagocytosis of mycoplasmas may be avoided or impaired by; morphology, proteins (like Vsps), or capsular material; reduced integrity of the phagosome membrane or oxidative burst due to proteases, lipases, oxygen radicals, and phospholipases; production of

ammonia; or mycoplasmas may survive the phagocytosis and escape into the cytoplasm (Marshall et al., 1995). Although to date not all of these methods have been confirmed in *M. bovis* it has been suggested that most are likely (Marshall et al., 1995). Figure 2.3 depicts the stages which *Mycoplasma* may circumvent phagocytosis (Marshall et al., 1995).

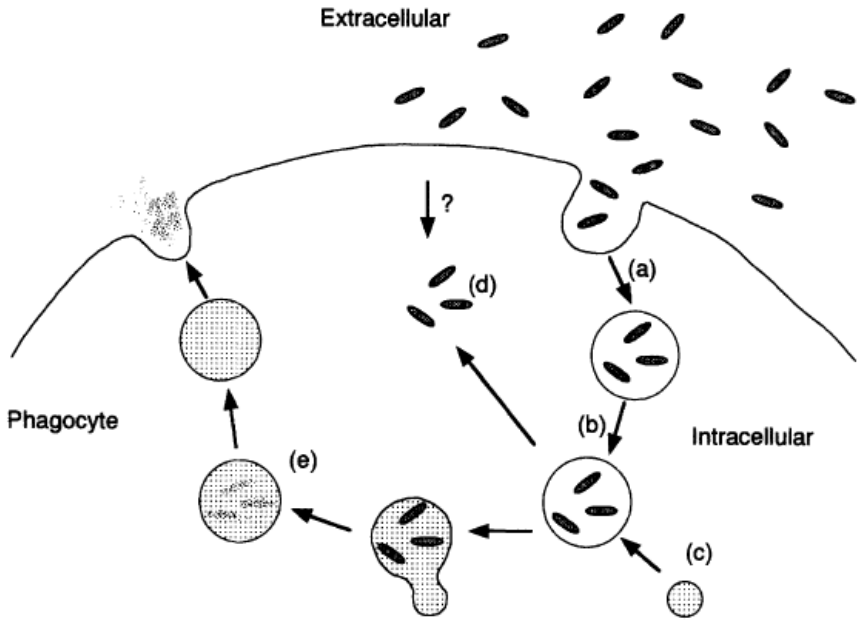

Figure 2.3 The stages of phagocytosis that might be circumvented by mycoplasmas.
(a) Attachment to and phagocytosis of mycoplasmas may be inhibited by the filamentous morphology, presence of antiphagocytic proteins or capsular material, binding of Fc portion of immunoglobulin, or localization of mycoplasmas between cilia and cytoplasmic processes making them inaccessible to phagocytes. (b) Integrity of the phagosome membrane, phagocyte metabolism and activation of the respiratory burst may be affected by mycoplasma proteases, lipases, and phospholipases and by oxygen radicals produced by mycoplasmas. (c) Phagosome-lysosome fusion may be impaired by the production of ammonia by mycoplasmas. (d) Mycoplasmas may survive phagocytosis and escape into the cytoplasm (mechanism unknown). (e) Mycoplasma survival following phagosome-lysosome fusion is unlikely. (Marshall et al., 1995)

Like several other *Mycoplasma* species, *M. bovis* is capable of producing biofilms (McAuliffe et al., 2006). Formation of a biofilm is strain-specific and likely associated with Vsp expression (McAuliffe et al., 2006). Research has shown that formation of biofilms by *M. bovis*

enables survival from heat shock and desiccation (McAuliffe et al., 2006). Biofilms are also known to impact antimicrobial resistance, however, *M. bovis* biofilm formation does not significantly alter minimum inhibitory concentration (MIC) values, but only the phenotypic effects at concentrations below the antimicrobial MIC levels (McAuliffe et al., 2006). It is still to be determined if the biofilms produced by *M. bovis* are only for survival in the environment or if they may play a role in immune response evasion as well (McAuliffe et al., 2006).

Mycoplasma bovis is considered the most important *Mycoplasma* associated with mastitis (Gourlay, 1981). Although many of the mechanisms of *M. bovis* pathogenesis are shared regardless of infection site, one of the hallmark pathological responses to *M. bovis* infection of the mammary gland is neutrophilic leukocytosis, followed by leucopenia (Jasper, 1987). The typical response observed is an outpouring of neutrophils and other cells into the milk leading to a purulent secretion (Jain et al., 1969; van der Molen and Grootgenhuis, 1979; Jasper, 1987). This response is noted for 10 to 14 days (Jain et al., 1969; van der Molen and Grootgenhuis, 1979; Jasper, 1987). In spite of the leukocytosis, high concentrations of *Mycoplasma* may be cultured without evidence of growth inhibition (Bennett and Jasper, 1978a). Interestingly, sterile leukocytosis of the mammary gland was not sufficient to kill *M. bovis* without specific antibody present (Brownie et al., 1979; Howard and Taylor, 1983). The effects of intracellular survival in neutrophils may indeed play a role in this phenomenon (van der Merwe et al., 2010). The spread of infection happens from lobule to lobule within a quarter with an accumulation of neutrophils (Hale, 1962; van der Molen and Grootgenhuis, 1979; Jasper, 1987). Four to six days post-infection the interstitial tissue will be invaded by neutrophils, macrophages, and other white blood cells (Kehoe et al., 1967). The milk ducts may then be permanently affected due to changes in the tissue (Kehose et al., 1967). In some cases a duct may be replaced by fibrosis or by persistent abscesses (Kohoe et al., 1967; Jasper, 1987). The exact effects of infection including severity, number of quarters infected, clinical duration of infection, shedding, and subclinical

manifestation are known to vary from animal to animal, but very few cows recover completely (Boughton and Wilson, 1978; Bushnell, 1984; Kauf et al., 2007).

Pathogenesis of *M. bovis* is a complex process that depends not only on the host responses, but also on the synergism with other bacteria and viruses, the environment, and factors that are likely strain specific. It will be necessary to continue research of *M. bovis* responses in natural infections in order to determine which factors are most important, but *in-vitro* studies may help determine the mechanisms at work.

2.3.2 Transmission and Risk of Disease

The first report regarding transmission of *M. bovis* was from a mastitis case of *M. bovis* (Hale, 1962). The initial belief that a reaction to penicillin was causing problems in the herd was disproved allowing for the experiments that showed *M. bovis* is transmissible in mastitic secretions and may spread from udder to udder in infected cows or from contaminated milking equipment (Hale et al., 1962; Gonzalez and Wilson, 2003). Generally, *M. bovis* transmission is horizontal for mastitis (Simeka et al., 1992). There is evidence to suggest that severe infection may be acquired in young calves from ingestion of infected colostrum or milk (Gourlay, 1981). Brown and colleagues (1998) showed that in a Florida dairy herd all calves exposed to *M. bovis*-contaminated waste milk became infected in the upper respiratory tract with-in fourteen days. Most cases of *M. bovis*-associated mastitis are likely transmitted due to poor husbandry practices, improper milking technique, or teat trauma (Simeka et al., 1992; Pfutzner and Sachse, 1996). The lack of a cell-wall generally means that *Mycoplasma* is fragile in the environment and it is therefore likely to be restricted to direct contact and aerosol transmission (Nicholas, 2004). The possibility of wind-borne or indirect transmission may not be excluded (Ragalla, 1996). However, there are reports of *M. bovis* surviving in bedding sand, cooling ponds, and dry lots (Bray et al., 1997; Gonzalez and Wilson, 2003; Allen et al., 2010). Survival times for *M. bovis* in the

environment have been reported at 236 days in manure, 23 days in drinking water (~25°C), and 8 months in recycled bedding sand (Gonzalez and Wilson, 2003; Allen et al., 2010). Survival in stressful environmental conditions is likely aided by biofilm formation (McAuliffe et al., 2006). It is unclear what impact environmental sources of infection may have upon disease states in cattle (Allen et al., 2010). Based upon these reports fomites may serve as an important source of infection. Figure 2.2 shows the Maunsell and Donovan (2009) proposed model of transmission and infection dynamics for young calves.

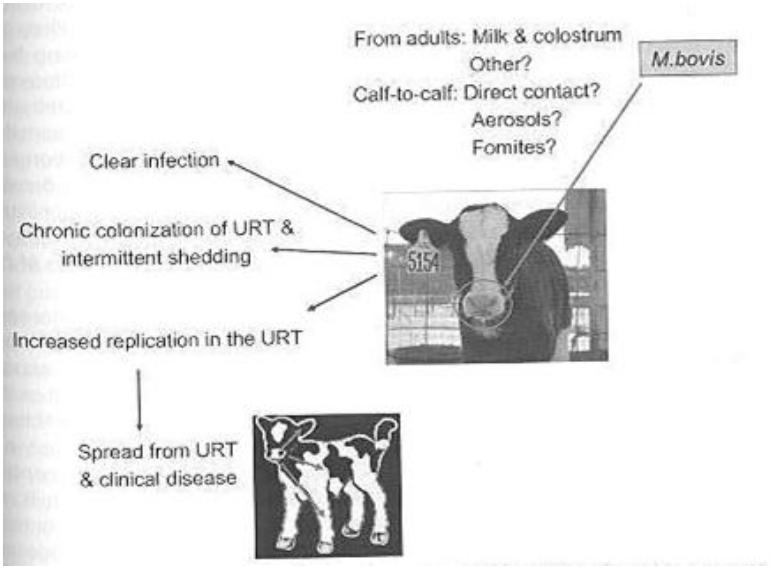

Figure 2.4 Proposed Transmission and infection dynamics in young calves. URT, upper respiratory tract (Maunsell and Donovan, 2009)

Mycoplasma sp. respiratory disease may be shed by infected animals, which may act as reservoirs of infection without symptoms of active disease, for many months for several months to years (Pfutzner et al., 1990; Pfutzner and Sachse, 1996; Nicholas, 2004). Transmission is likely direct contact, droplet, aerosol, or airborne in nature for most respiratory infections (Gonzalez and Sears, 1994; Nicholas, 2004). Viable fetuses and calves of cows with *M. bovis* mastitis have been shown to become infected either in-utero or during birth indicating the possibility of vertical

transmission too (Jasper, 1987). Lateral transmission between calves is well documented (Bennett and Jasper, 1977; Gonzalez and Sears, 1994). It has been suggested that spread of infection may occur from one organ site to another (Jasper, 1982). In these reports mastitis or arthritis develops following respiratory disease (Jasper, 1982). In multiple studies it has been demonstrated that when calves, heifers, and cows are housed together, mastitis in cows develops approximately one month after signs of respiratory disease or arthritis in calves or heifers (Gonzalez et al., 1993; Gonzalez and Sears, 1994; Gonzalez et al., 1995). Early detection of infection and culling of shedders and diseased animals has the potential to lessen the impact of *M. bovis* in a herd (Feenstra et al., 1991).

It has been reported that spread throughout the world occurred following the initial report of *M. bovis* infection in 1961 (Nicholas, 2004). Advancing research of transmission routes will be important for continuing control efforts of *M. bovis.* Reports of *M. bovis* infection in wildlife and non-bovine species means that potential reservoirs of disease exist not only in cattle and the environment, but perhaps also in other animals which may come into contact with cattle (Pfutzner and Sachse, 1996; Bray et al., 1997; Gonzalez and Wilson, 2003; Dyer, et al., 2004; Allen et al., 2010). Although there are suggestions that *Mycoplasma bovis* is recoverable directly from the air, the results are ambiguous in early studies due to identification only available by metabolic inhibition test and the collection process involved leaving uncovered plates in the barn during other sampling (Jasper et al., 1974). More recent studies have been able to use PCR methods to confirm identification of the swine pathogen, *Mycoplasma hyopneumoniae* obtained from air sampling using a membrane filter collection system (Stark et al., 1998). A consolidated list of the risk factors for *M. bovis* infection include mixing of different ages of calves and the presence of at least one infected animal in a group (Tschopp et al, 2001). These risks lead to lowered average daily gains and the prescription of two times the ordinarily prescribed antimicrobials (Tschopp et al, 2001). Although exposure to infected milk and direct exposure to infected cattle regardless of age are clear risk factors for developing disease, more research is necessary to elucidate other risk

factors, the impact of environmental exposures, and the exposure time and dose of bacteria required for clinical disease.

2.3.3 Clinical Disease and Pathology

Clinical disease-associated with *M. bovis* most frequently presents as respiratory disease, mastitis, otitis media, conjunctivitis, arthritis, or a combinations of these conditions (Hale et al., 1962; Pfutzner, 1990; Stipkovits et al., 1993; Kirby and Nicholas, 1996; Walz et al., 1997; Maeda et al., 2003; Lamm et al., 2004; Nicholas et al., 2008; Raedelli et al., 2008). Respiratory disease caused by *M. bovis* leads to fever (>40°C), depression weight loss, coughing, nasal discharge, tachypnea, and often dyspnea (Maunsell and Donovan, 2009; Caswell et al., 2010). The lungs will often present with focal bilateral chronic caseonecrotic bronchopneumonia in the cranioventral areas (Gagea et al., 2006a; Caswell et al., 2010). It is also possible to have lesions involving the whole lobe or the cranial portions of the cadual lobes (Maunsell and Donovan, 2009). It is more difficult to determine if *M. bovis* is an important finding in cases of bronchopneumonia without caseonecrotic foci (Caswell et al., 2010). *Mycoplasma bovis* has been confirmed as the only pathogen in numerous cases of bronchopneumonia, including cranioventral suppurative and catarrhal bronchopneumonia (Caswell et al., 2010). Feedlot and veal calves have been reported to have subacute and chronic cases of multifocal necrotizing lesions (Adegboye et al., 1996; Khodakaram-Tafti and Lopez, 2004; Gagea et al., 2006a; Maunsell and Donovan, 2009). The large number of differences between the reported microscopic and macroscopic lesions found throughout the respiratory tract may be due to differences in inoculation route, dosage, strain, health status of the host, and duration of infection (Maunsell and Donovan, 2009). Figure 2.5 represents some of the microscopic and macroscopic lung lesions associated with *M. bovis* (Madea et al., 2003; Gagea et al., 2006a; Radaelli et al, 2008).

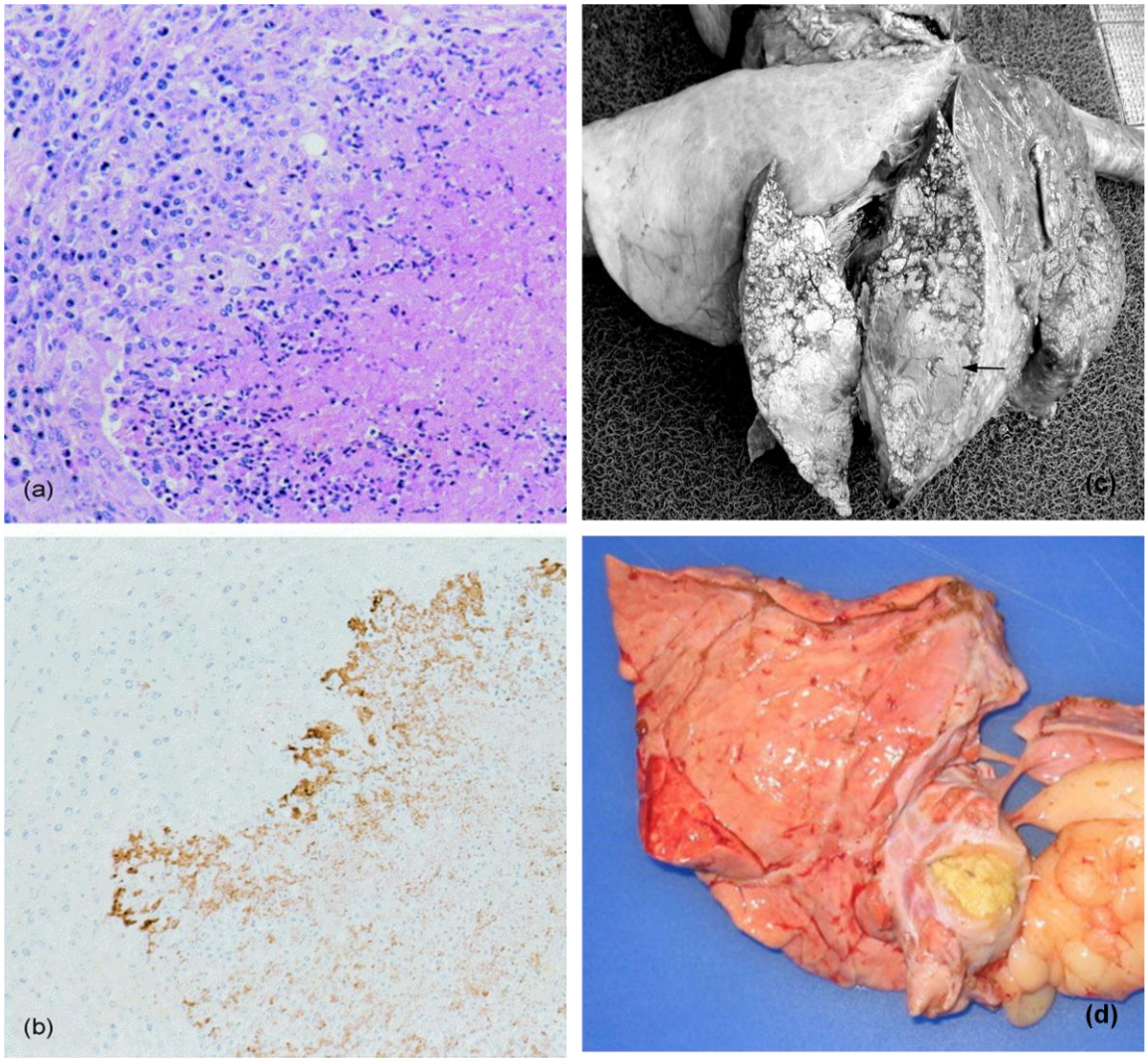

Figure 2.5 Microscopic and macroscopic *Mycoplasma bovis*-associated lung lesions. (a) Marked infiltration of neutrophils and macrophages in the bronchiolar lumina. HE. ×200. (b) *Mycoplasma bovis* antigen is detected in the necrotic exudates. SAB. ×200. (Maeda et al., 2003) (c) *Mycoplasma bovis* pneumonia. The right middle lung lobe contains numerous foci of caseous necrosis, with sequestration of the largest lesion (arrow). (Gagea et al., 2006a) (d) Veal calf, right middle lung lobe, necrosuppurative bronchopneumonia. On cut surface the nodular lesion consists of a thick fibrotic capsule surrounding necrosuppurative material. (Radaelli et al., 2008)

Similar to respiratory infections, *M. bovis* –associated mastitis is often difficult to differentiate from other causes. Frequently there are no clinical signs of mastitis, except for decreased production (Pourmarat et al., 1996). Common clinical mastitis symptoms for a single

quarter or multiple quarters infected with *M. bovis* include inflammation, increased leukocytes, swollen udders that are not painful, edema, and atrophy; however, fever may or may not be present in infected cows (Tanskanen, 1995; Pfutzner, 1996). Even low inoculations of *M. bovis* can lead to severe mastitis in experimentally infected cows (Bennett and Jasper, 1978b; Bennett and Jasper, 1978c). Figure 2.6 demonstrates some of the lesions seen in the mammary gland of cows experimentally infected with *M. bovis* (Bennett and Jasper, 1978b; Bennett and Jasper, 1978c).

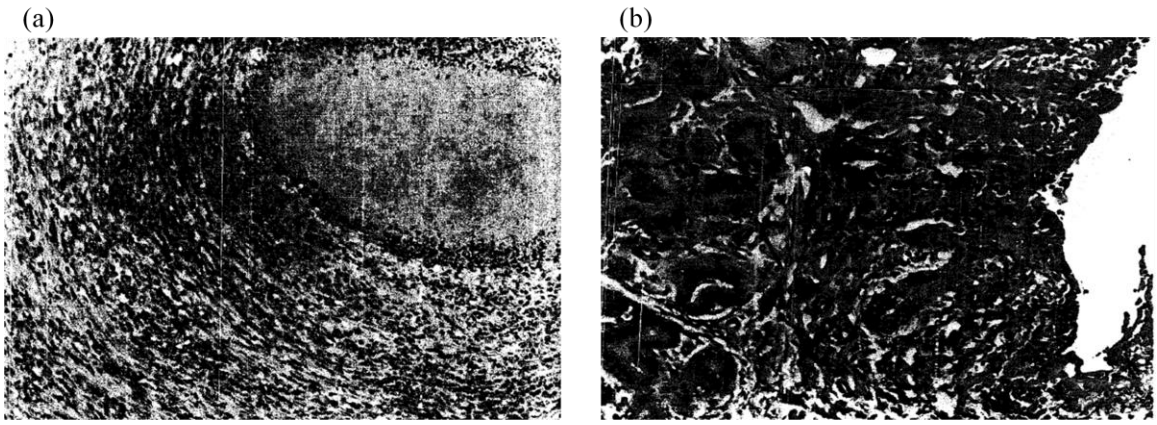

Figure 2.6 Microscopic *Mycoplasma bovis*-associated mammary gland lesions. (a) Abscess from the ventral portions of the right rear quarter of cow. The abscess wall is laden with macrophages and lymphocytes. Neutrophils ring the caseous material. H and E stain x130. (Bennett and Jasper, 1978c) (b) Epithelial hyperplasia of the milk duct with subepithelial fibrosis and mononuclear cell infiltrate. Adjacent alveoli (arrow) are totally involuted. H and E stain x240. (Bennett and Jasper, 1978b).

It has been reported that large numbers of neutrophils can be found in the alveoli, but there is the potential for varying levels of neighboring lobule involvement (Jasper, 1987). Eventually the interstitial tissue will be invaded by neutrophils, macrophages, lymphocytes, plasma cells, fibroblasts, and eosinophils with permanent changes to the normal architecture occurring (Kehoe et al., 1967; Jasper, 1987). Finally the severe cases will have persistent abscesses and fibrosis (Jasper, 1987).

Infections of the inner ear caused by *M. bovis* often begin in the respiratory tract before spreading upwards, likely through the Eustachian tube (Maunsell and Donovan, 2009). As demonstrated in Figure 2.7, unilateral or bilateral ear droop is a hallmark clinical sign of otitis media, but is not specific for *M. bovis* infection (Maeda et al., 2003; Nicholas et al., 2008; Maunsell and Donovan, 2009). Other commonly seen clinical signs include facial paralysis, ataxia, head tilt, and even nerve dysfunctions (Walz et al., 1997). As with other sites affected by *M. bovis*, a collection of neutrophils and macrophages, as well as tissue necrosis can be seen on histopathology sections (Maeda et al., 2003).

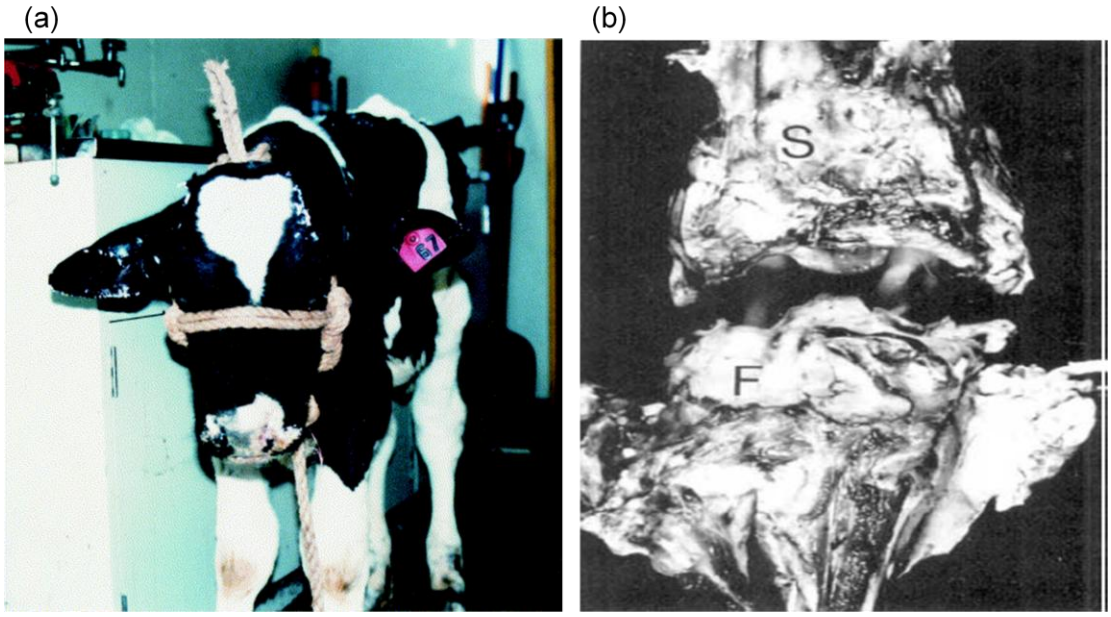

Figure 2.7 Otitis media and arthritis due to *Mycoplasma bovis* infection in calves. (a) Calf emaciated with bilateral ear droop and exudative otitis media. (Maeda et al., 2003) (b) Digital exterior tendons removed from anterior surface of right tarsal joint of calf, postinolcuation with live *M. bovis.* Fibrinous coagulum forms cast of joint capsule (F) and tendon sheath (S). Joint capsule thickened due to inflammatory edema. (Ryan et al., 1983)

Similarly, cases of *M. bovis*-associated arthritis may originate in the respiratory tract or from consumption of milk (Nicholas et al., 2008; Maunsell and Donovan, 2009). *Mycoplasma bovis*-associated arthritis is frequently considered a disease of calves, but it may also be observed

in adult cattle (Henderson and Ball, 1999). The carpal and tarsal joints are affected as well as the synovial becoming infiltrated by neutrophils, macrophages, and lymphocytes with an increase in immunoglobulin levels (Chima et al., 1981). Purulent exudates, erosion of cartilage, granulation tissue, ulceration of synovial membranes may be seen around or in the joint spaces (Chima et al., 1981; Ryan et al., 1983). An example of gross inflammation seen in the tarsal joint is shown in Figure 2.7. Conjunctivitis caused by *M. bovis* is often difficult to differentiate from *Moraxella bovis* with clinical symptoms including tearing, inflammation of the eyelid and eyeball, keratitis, and light sensitivity (Levisohn et al., 2004). Conjunctivitis with *M. bovis* is likely to be underreported due to the similarity to other causative agents and the difficulties of culturing *Mycoplasma* for many laboratories. The similarities of clinical symptoms of *M. bovis* infection and other bacterial pathogens increases the need to know the true distribution and determinants of the diseases of various regions around the country, including in the dairy, beef, and veal industries of Pennsylvania.

2.3.4 Epidemiology

Mycoplasma bovis has been isolated from North America, South America, Europe, Asia, and Australia. Estimates in Europe suggest that *M. bovis* is responsible for 25% to 35% of calf respiratory disease (Nicholas and Ayling, 2003). It is unclear what percentage of calf respiratory disease in the United States may be attributed to *M. bovis* since large scale epidemiological studies have not been performed. Studies from Ireland consist of 13% to 23% of pneumonic lungs being infected with *M. bovis* (Bride et al., 2000; Byrne et al., 2001). In France levels have been recorded at 30%, while England reported 20% - 25% (Le Grand et al., 2001; Nicholas et al., 2001). The Netherlands have reported 20% of pneumonic lungs infected with *M. bovis*, but only 0% to 7% of non-diseased lungs (ter Laak et al., 1992a; ter Laak et al., 1992b). Other studies have confirmed that *M. bovis* infection is more prevalent in the lungs of pneumonic calves rather than

healthy calves (Allen et al., 1991; Byrne et al., 2001; Gagea et al., 2006a; Gagea et al., 2006b Godinho et al, 2007). One pair of reports from Ontario, Canada noted that *M. bovis* was isolated from 98% of cattle in the study with chronic pneumonia and 46% of cattle with normal lungs (Gagea, 2006a; Gagea, 2006b). Feedlot field studies have also suggested reduction in weight gain in up to 50% of calves suffering respiratory disease (Tschopp et al., 2001).

It now appears to be widespread in the US dairy and beef cattle populations (Jasper et al., 1974; Feenstra et al., 1991; Gonzalez et al., 1992; Gonzalez and Wilson, 2003; NAHMS, 2003; Nicholas et al., 2008). In 2002, the USDA recoded that 7.9% of 871 dairies tested positive for *Mycoplasma* infection in bulk tank milk samples (NAHMS, 2002). States in different regions of the country demonstrated different prevalence indicating the likelihood that geographic region may play a role in *Mycoplasma* infections. It was shown that the Western and Southeast regions had the greatest percentage of positive operations at 9.4% and 6.6%, respectively (NAHMS, 2002). The Midwest (2.2%) and the Northeast (2.8%) regions had fewer operations with transmission dynamics of *M. bovis* specifically that it is intermittently shed in the milk from subclinically infected cows. The only report of *Mycoplasma* prevalence in Pennsylvania dairies is limited to a study of mastitis pathogens in central New York and parts of northern Pennsylvania (Wilson et al., 1997). In the study 85 cows of the 32, 978 sampled were found to have a *Mycoplasma* infection (Wilson et al., 1997). Importantly, mastitis associated with *Mycoplasma* infection led to some of the lowest 305-day mature equivalent milk production values (Wilson et al., 1997). This demonstrates the impact of *M. bovis*-associated mastitis for the Pennsylvania dairy industry. Unfortunately, the exact number of cases from Pennsylvania is not reported (Wilson et al., 1997).

Calves of different ages which are co-mingled, come in contact with a sero-positive animal, given prophylactic antibiotic treatments, or transported together appear to be at higher risk of developing infection (Boothby et al., 1983; ter Laak et al., 1992a; ter Laak et al., 1992b; Tschopp et al., 2001; Rifatbegovic et al., 2007; Caswell and Archambault, 2008). The precise

predisposing risk factors and mechanism of infection are unknown (Caswell and Archambault, 2008). The prevalence levels in older studies appear to be lower than recent studies (Caswell and Archambault, 2008). It is not clear if this increase is a real effect or if it is due to more robust detection methods or greater request for testing of *M. bovis* infection from clinicians and diagnosticians aware of the disease.

Although rare, *M. bovis* is capable of infecting non-bovine hosts. There are reports of white-tailed deer, buffalo, small ruminants, broiler chickens, and humans having *M. bovis* infections (Madoff et al., 1979; Pfutzner and Sachse, 1996; Nicholas and Ayling, 2003; Dyer et al., 2004; Dyer et al., 2008; Nicholas et al., 2008; Ongor et al., 2008; Janardhan et al., 2010). It is unclear what the prevalence of *M. bovis* in other hosts is actually due to the scarceness of reports. It is likely that infections are under-estimated in these other hosts since reports are generally of severe disease. Despite this, *M. bovis* is known to be wide-spread throughout the world. More prevalence studies in the United States are necessary to determine each impact of disease on each region of the country. Currently there is only the one article of Wilson et al. (1997) that reports any information regarding the prevalence of *M. bovis* in Pennsylvania.

2.3.5 Economic Impact

There are limited data available relating to the economic impact that *M.* bovis-associated disease has upon production and care of cattle. Additionally there are concerns regarding the accuracy of the economic reports due to the difficulty of attributing acute pneumonia cases to one particular pathogen (Caswell and Archambault, 2008). Despite this, the economic burden caused by BRD is one of the most profound in food animal production (Snowder et al., 2006). It is estimated that the cost of BRD from calf weaning through packing represents approximately 7% of total production costs when compared to animals with healthy respiratory tracts (Griffin, 1997). Based upon multiple reports, the estimated annual costs associated with BRD prevention

and treatment are reported to be over $3 billion for all beef animals (Griffin, 1997; Biss et al.,; 1994; Curtis et al., 1988; Houghton and Gourlay, 1983). Economic costs associated with *M. bovis* are likely due to reduced weight gain, pharmaceutical costs for treatment of ill animals, increased cost of labor, mortality losses, and cost of preventative measures (Caswell and Archambault, 2008).

Despite the costs associated with *M.* bovis-associated respiratory disease, mastitis likely represents a higher economic loss due to *M. bovis* infection (Rosengarten and Citti, 1999). Mastitis is the most expensive production disease of dairy herds in developed countries (Miller et al., 1993; Seegers et al., 2003). *Mycoplasma bovis* has been shown to be able to infect non-bovine hosts, such as broiler chickens, bison, and white-tailed deer, but the economic impact of these infections are unknown (Dyer et al., 2004; Dyer et al., 2008; Ongor et al., 2008; Janardhan et al., 2010). The combined direct and indirect costs associated with mastitis in the United States are estimated to be at least $2 billion (Smith and Hogan, 1990; Miller et al., 1993; Rosengarten and Citti, 1999; Nicholas et al., 2000). It is unknown what percentage of this economic burben is directly related to *Mycoplasma* mastitis.

Economic losses due specifically to *M.* bovis-associated mastitis have been estimated at $108 million per year in the U.S. dairy industry (Rosengarten and Citti, 1999). Costs associated with *M. bovis*-associated mastitis include: production loss from damaged quarters, failure to produce milk in infected quarters, prolonged milking times, increased treatment costs, increased costs of discarded milk following treatment, elevated somatic cell counts leading to lower milk quality, increased culling, and increased antibiotic residue risk for bulk tank milk (Boughton, 1979; Jasper, 1981). Cows at all points of the lactation cycle, dry and milking, are susceptible to infection by *M. bovis* (Gonzalez et al., 1993). Bulk tank milk sampling in the United States has estimated between 1 and 8% of herds have at least one cow suffering from *Mycoplasma* infection (NAHMS, 2002; Fox, 2005). In 2002, 7.9% of U.S. dairies tested positive for *Mycoplasma* in bulk tank milk sample (NAHMS, 2002). *M. bovis*, the most commonly isolated *Mycoplasma*, was

isolated in 86% of those *Mycoplasma* infected bulk tank milk samples (NAHMS, 2002). The profound impact of *M. bovis* upon the dairy industry indicates a need for optimized treatment options and prevention of infection. Mastitis caused by *M. bovis* is likely of particular concern for the state of Pennsylvania due to the large number of dairy operations and dairy cattle in the state. The last US census of Agriculture (2007) places Pennsylvania as the fourth largest dairy producing state in the country. The milking cows in the state are estimated at over 550,000 (US Census of Agriculture, 2007). The overall number of cattle at risk in Pennsylvania is over 1.6 million (US Census of Agriculture). A large outbreak of *M. bovis*- associated mastitis could have a severe economic impact upon the state.

Although the current economic loss estimates are likely largely underestimated, the importance of *M. bovis*-associated infections is clear. Most cost estimates are related to the direct and indirect *M. bovis*-disease associated costs, however, there are also concerns related to the impact upon international trade. International trade is directly linked to animal welfare, which may be influenced by *M. bovis* infections.

2.3.6 Animal Welfare Concerns

In addition to the economic impact that *M. bovis* has upon the dairy, beef, and veal industries; animal welfare is a concern. There are three major areas that are important to consider as benefits for improved animal welfare; protection of human health, efficiency of economic systems or alleviating trade concerns, and lastly, healthy and content animals (Blandford and Fulponi, 1999). The Council of the European Union directive on animal welfare issues of food production animals states:

> *"Any animal which appears to be ill or injured must be cared for appropriately without delay and, where an animal does not respond to such care, veterinary advice must be obtained as soon as possible. Where necessary sick or injured animals shall be isolated*

in suitable accommodation with, where appropriate, dry comfortable bedding." (Council of the European Union, 1998).

Although in the United States there are three federal statutes regarding animal welfare the most extensive was passed in 1966 for protection of research, zoo, and exhibition animals (U.S.C., The Animal Welfare Act of 1966). These laws are not as inclusive as the European Union equivalent and do not pertain to food animals while on the farm. However, US-based companies may choose to follow EU standards in order to protect international trade options at their discretion (Blandford and Fulponi, 1999). Trade concerns may be of particular interest to few countries left without confirmed cases of *M. bovis,* due to the belief that disease was originally spread from the United States to Europe through the export of infected cattle (Nicholas and Ayling, 2003). It is also likely that healthy cattle are safer for human consumption.

The impact of *M. bovis* infection is greatest for the animals. Calves are at especially high risk of morbidity and mortality due to *M. bovis*-associated disease (Nicholas and Ayling, 2003; NAHMS, 2007; Nicholas et al., 2008; Raedelli et al., 2008). In the United States, BRD is the second most important cause of morbidity and mortality in heifers and significant contributor for dairy calves (Virtala et al., 1996; Walz et al., 1997; Wells et al., 1997; Brown et al., 1998; Donovan et al., 1998; Arcangioli et al., 2008). Since current vaccines and antimicrobial therapy are of limited or poor efficacy the chronic nature of *M. bovis*-associated disease demonstrates even more importance in regards to long term calf, heifer, and cow well-being (Rosenbusch et al., 2005; Maunsell et al., 2009; Nicholas et al., 2008; Nicholas et al., 2009). These problems result in limited relief being available throughout long periods of illness.

Reduction of disease associated with *M. bovis* infection is an important animal welfare concern which highlights the need for efficacious vaccines selected and appropriately administered and efficacious antimicrobial therapies. Reduction of *M.* bovis-associated disease may allow for optimization of animal rearing systems which is vital to both the animal for health and wellness concerns and to the producers for the economic benefits it may provide.

2.4 Molecular Epidemiology of *Mycoplasma bovis*

Molecular epidemiological analysis allows for the tracing of outbreaks and the development of strategies for the control of disease through the implementation of genotyping methods. These analyses allow for assessment of the strain variability within populations which has the potential to provide important information to both researchers and clinicians. A large number of molecular typing methods may be employed including; random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), Vsp analysis, pulse-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, insertion sequence analysis (IS analysis), simple sequence repeats (SSRs), variable number tandem repeats (VNTR), multi-locus sequence typing (MLST) analysis, and gene sequencing apporaches (Kokotovic et al., 1999; Poumarat et al., 1999; Butler et al., 2001; Biddle et al., 2005; McAuliffe et al., 2004; Miles et al., 2005; Mrazek, 2006; Medini et al., 2008; Nicholas et al., 2008).

Often strains of pathogenic bacteria belongs to a specific clonal lineage, often due to virulence factor genes (or alleles), which aids in identification of disease severity, candidates for vaccine development, evolutionary importance, and population dynamics (Nicholas et al., 2008). The numerous typing methods available has reduced the costs associated with genotyping while increasing efficacy, high-throughput capabilities, reproducibility, turn-around time, and even the potential to apply methods directly to clinical specimens without need of culturing (Nicholas et al., 2008). It is the use of PCR that has increased the number and styles of typing available to laboratories. The testing choice for each laboratory depends upon available equipment, costs, level of discrimination and reproducibility required, training of personnel, and turn-around time considerations. Ideal genotyping methods will have a high discriminatory power; be reproducible, easy to interpret, quick to complete; and have inter-laboratory data transfer capabilities.

Unfortunately, all tests have limitations which require a researcher to select a method that best suits the needs of the specific research.

RAPD and AP-PCR which are techniques that involve the use of a single random primer or combination of variable length primers selected without *a priori* knowledge of the target amplicon sequence, are grouped under the heading of multiple arbitrary amplicon profiling (Welsh and McClelland, 1990). The commonly used primer(s) for *Mycoplasma bovis* are HUM1 or HUM4 for RAPD and the combination of REP1R-I and REP2-I for AP-PCR (Hotzelet al, 1998; Butler et al., 2001).These techniques are simple and fast to perform leading to very quick turn around times, although the coverage for such a procedure is <1% of the genome. The reproducibility of isolates analyzed using multiple arbitrary amplicon profiling is highly dependent upon the concentrations of Taq polymerase, $MgCl_2$, and the template DNA (Butler et al., 2001). Therefore, there are issues regarding the poor reproducibility of the results and more importantly what informational value there is for an isolate obtained using RAPD or AP-PCR profiles (Butler et al., 2001; McAuliffe et al., 2004; Stakenborg et al., 2006).

PFGE is a whole genome analysis method that has been considered the "gold standard" of typing systems for many bacteria. Patterns for PFGE profiles are highly reproducible and often highly discriminating since each gel image may be compared to all others using known standards to make corrections between runs. However, studies in the United Kingdom have shown that it had the lowest level of congruence compared to other methods tested for *M.* bovis (McAuliffe et al., 2004). The major issue related to the use of PFGE for *M. bovis* typing is that it is often unable to type numerous isolates based upon DNase activity and the presence of specific Vsps, especially Vsps A and C, which have chromosomal rearrangements that interfere with PFGE profiles, especially when using the *Sma*I restriction site (Citti et al., 2000; McAuliffe et al., 2004). The use of Southern blotting to determine the Vsp-antigen types present in isolates may improve the congruence of the test, although it not clear if Vsp profiles impact other typing methods (Poumarat et al., 1999; McAuliffe et al., 2004). PFGE is time consuming and can be expensive

when used for large numbers of isolates. Despite the issues associated with PFGE it has been successfully used for molecular epidemiological studies of *Mycoplasma* isolates by a few researchers (McAuliffe et al., 2004; Biddle et al., 2005).

AFLP is robust molecular technique which has been used for multiple *Mycoplasmas* that provides a nearly complete genomic coverage (Mueller and Wolfenbarger, 1999; Kokotovic et al., 1999). The fingerprinting pattern obtained is made more complex than that of PFGE by allowing for optimal separation and then sorting fragments by uniform sizes (Kokotovic et al., 1999; Kusiluka et al., 2000; McAuliffe et al., 2004; Nicholas et al., 2008). PFGE patterns normally have between 15-30 discernible bands, whereas AFLP can have upwards of 80 discernible bands (Kokotovic et al., 1999; Kusiluka et al., 2000; McAuliffe et al., 2004; Nicholas et al., 2008). The discriminatory power ahs been reported at >99% (Stakenborg et al., 2006). Reports of *M. bovis* typing via AFLP indicate that proper amplification selectivity uses the *Bgl*II and *Mfe*I restriction enzymes (Kokotovic et al., 1999). The technique can be tailored to account for differences in G+C content, size, and DNA modification may improve sensitivity (Kokotovic et al., 1999). The cost and extensive training required to perform AFLP makes it prohibitive for diagnostic laboratories, but results can be obtained for batches of isolates more readily than non-sequence based typing techniques with the ability to inter-laboratory data exchanges more feasible (Kokotovic et al., 1999). Interestingly, AFLP is one of the few techniques which may be used in long-term surveillance, short-term outbreak investigations, and in individual bacterial genomics studies (Kokotovic et al., 1999; Kusiluka et al., 2000; McAuliffe et al., 2004; Nicholas et al., 2008).

Insertion Sequence profiling, also called reverse dot blots, are a method that uses restriction enzymes and agarose gel separation with a transfer of the samples to a membrane which is probed to give different profiles (Miles et al., 2005; Lysnyansky et al., 2009). IS profiling may be considered comparable to RAPD or AFLP due to the presence of repetitive elements and neutral changes being capable of influencing the typing (Miles et al., 2005). The

method is to cleanly able to identify differences between insertion sequences elements (transposable elements) found within *M. bovis* isolates (Miles et al., 2005; Lysnansky et al., 2009). IS profiling is complicated, must have very high quality DNA samples, and takes a long time to run, making it a poor choice for high-throughput analysis. However, IS profiles could be particularly beneficial for studying how genes transfer between species or subtypes of a species (Miles et al., 2005; Lysnansky et al., 2009). IS probes are not frequently used in epidemiological analysis, but offer an attractive alternative to other typing techniques if the equipment is already available to a laboratory, especially since *Mycoplasma* species are believed to contain a high density of IS elements(Nicholas et al., 2008).

Variable number of tandem repeats (VNTRs) and short sequences repeats (SSR), originally referred to as mini-satellites and micro satellites, respectively, serve as targets useful in evolutionary and functional bacterial diversity studies (van Belkum et al., 1998). Several *Mollicutes* have been studied for the occurrence of VNTR and SSR (Mrazek, 2006; Nicholas et al., 2008). SSRs serve as important sources of clonal variance, which may come from the phase variation (Mrazek, 2006).SSRs appear to be relatively common in *Mycoplasma* genomes and offer a high potential for homologous recombination indicating the dynamic genome instability (Mrazek, 2006). The distribution of the SSRs patterns may offer future insight into the evolutionary and functional diversity of *M. bovis* (Mrazek, 2006). The functional diversity discovered through SSRs may in fact indicate the ability of specific strains to survive within a host, the level of virulence, and antigenic properties of the strain (van Belkum et al., 1998; Mrazek, 2006). SSRs may be used to deduce strain relatedness and even for strain identification, but the true functions and impact of corresponding DNA structure of SSRs is unknown (van Belkum et al., 1998).

VNTR analysis is well established for use in pedigree analysis and is becoming a more common technique for bacterial typing (van Belkum et al., 1998; Nicholas et al., 2008). VNTR analysis can generally be run on equipment already available for a laboratory and does not require

sequencing (Nicholas et al., 2008). Although VNTR has not be used for *M. bovis*, it has been successfully applied to *M. mycoides* subtyping (Nicholas et al., 2008). Despite the high discriminatory power, VNTR analysis is unlikely to overtake other typing methods, but does offer an early screening tool for isolates (Nicholas et al., 2008).

Multi-Locus Sequence Typing (MLST) and Multi-Virulence-Locus Sequence Typing (MVLST) are methods that use either housekeeping genes or virulence genes for characterization based upon unique allelic features (Mayor et al., 2008; Nicholas et al., 2008). The difference between these is that MLST uses 7-8 housekeeping genes while MVLST uses a collection of virulence genes. MLST and MVLST are relatively cheap and fast to run, although data analysis is longer and more challenging. Although this technique is considered a "gold-standard" for many bacterial studies of phylogenetic associations and is an excellent source of epidemiological data, it is difficult to perform for a species such as *M. bovis* because a complete genome sequence is not available and 7 housekeeping genes not known (Manso-Silvan et al., 2007; Mayor et al., 2008; Nicholas et al., 2008). However, other *Mycoplasma species* have shown that 3 housekeeping genes may be sufficient for data collection and that is a feasible number for *M. bovis* (Mayor et al., 2008). Additionally, it is possible that housekeeping genes in related species are likely to be present and of similar function in *M. bovis* meaning they could be used for early studies to determine the validity of such an idea.

One of the newest approaches to study molecular epidemiology is the use of sequencing methods (Enright and Spratt, 1999). Gene sequencing has been used for a wide range of *Mycoplasma* species, including *M. bovis* (Konigsson et al., 2002). Sequence differences in the 16S rRNA genes of homologous operons are of interest when attempting to elucidate the changes between and with-in species (Konigsson et al., 2002). The use of rRNA allows for high sensitivity and strong discriminatory power due to the high copy numbers and incredible accuracy of sequence data (van Kuppeveld et al., 1992; Johansson et al., 1998). Results obtained from 16S sequencing procedures have recently been applied in studies of an outbreak of *M. capricolum*

subsp. *capripneumoniae* in Turkey (Nicholas et al., 2008). Although expensive, sequencing can be performed directly without the creation of errors during the incorporation of deoxynucleotides as seen in other molecular techniques (Johansson et al., 1998). Until expenses present less of a challenge for many laboratories sequence techniques are unlikely to be used to their full potential.

There are many useful molecular methods that can be incorporated into molecular epidemiology studies regarding *M. bovis.* The proper selection of a method that gives the intended results and is not limited in the capacity for library typing verses comparative typing will give the best chance to draw inferences from the data.

2.5 Antimicrobial Susceptibility Testing

Mycoplasma species have been shown to harbor unique characteristics that allow for immune evasion, surprising stability in the environment, and importantly resistance to many antimicrobials (Gonzalez and Wilson, 2003; Rosenbusch et al., 2005; Nicholas et al., 2008; Allen et al., 2010). Due to the lack of a cell-wall, antimicrobials such as the penicillins and cephalosporins which target the cell-wall or more specifically, the cross-linking amino acids of the peptidogylcan, are ineffective. Although *M. bovis* is capable of forming biofilms, it is not clear if biofilm production actually reduces the susceptibility to antimicrobials or just allows for better survival within a host or the environment (McAuliffe et al., 2006). There are several mutations known to lead to resistance by mycoplasmas that affect humans including point mutations of the 23S DNA gene, and the DNA gyrase and topoisomerase gene (p*arC*) (Bebear et al., 2003; Pereyre et al., 2007). It has been shown that *M. bovis* also has the potential to contain a point mutation in the *parC* gene which leads to decreased susceptibility to fluoroquinolones (Lysnyansky et al., 2009). Interestingly, there are some antimicrobials that are effective against one species, but other species are refractory. An example of this phenomenon is demonstrated by

the susceptibility of *M. pneumoniae* to erythromycin, while *M. bovis* is not susceptible (Rosenbusch et al., 2005; Nicholas et al., 2008).

There are many considerations for selecting the most appropriate antimicrobial for treatment of a *Mycoplasma* infection, such as that many fluoroquinolones are known to concentrate at higher levels in the lung tissue than in the serum; macrolides have been shown to form complexes with other antimicrobials, and lastly that recommended use may be specific to certain species, weights, or ages of animals (Anadon and Reeve-Johnson, 1999; Wise and Honeybourne, 1999; Nicholas et al., 2008). *In-vitro* minimum inhibitory concentrations (MIC) levels are not necessarily identical to values that would be obtained with *in-vivo* testing, but are useful for predicting the antimicrobials most likely to be effective. Unfortunately, there are currently no Clinical Laboratory Standards Institute (CLSI) approved standardized testing methods for *Mollicutes* (Hannan, 2000). There are many methods that have been successfully employed in the testing of *M. bovis*, such as liquid broth microdilution, solid medium, and the E-test (Hannan, 2000; Francoz et al., 2005; Rosenbusch et al., 2005). A flow cytometry method has also been used for other *Mycoplasma* species (Assuncao et al., 2006).

All *in*-vitro susceptibility testing methods are recommended to follow a set of general technical considerations. Since there is no standard media available for the growth of all *Mycoplasma* ssp., one that offers optimal growth for the species being tested should be selected (Hannan, 2000). It should be noted that many types of mycoplasma media contain a β-lactam antibiotic, which may interact with the compounds being tested (Hannan, 2000). Although freshly thawed and cultures in logarithmic phase of growth give identical values for mycoplasmas, it is important to standardize the inoculum amount used in testing (Hannan, 2000). The generally accepted amount of inocula is $10^3 – 10^5$ colony forming units per plate or color changing units per mL (Hannan, 2000). If the inocula falls outside these ranges there may be too little or too much growth altering the MIC values (Hannan, 2000). The liquid broth dilution method and its several derivations are among the most commonly used animal mycoplasma methods (Hannan, 2000). A

method that incorporates alamarBlue, a redox reagent leading to a color change, was developed by Rosenbusch et al. (2005). Results from the Rosnebusch et al., (2005) study of 233 U.S. field isolates tested against chlortetracycline, enrofloxacin, erythromycin, florfenicol, oxytetracycline, spectinomycin, tilmicosin, ampicillin, and ceftiofur gave an MIC range (µg/mL) of 0.25 to >32, 0.03 to 4, 4 to >32, 0.06 to 8, 0.125 to >32, 1 to >>16, 0.5 to >128, >32, and 64 to >64, respectively. These data suggested that tilmicosin, erythromycin, ampicillin, and ceftiofur were ineffective and thereby not recommended for the treatment of *M. bovis* infections (Rosenbusch et al., 2005). Francoz et al. (2005) tested the susceptibility of a collection of *M. bovis* field isolates from Montreal, Canada, but used the E-test method. The MIC ranges (µg/mL) obtained in this study for azythromycin, tetracycline, spectinomycin, clindamycin, and enrofloxacin were 0.5 to >256, 0.094 to >256, 0.38 to >1021, 0.094 to >1021, and 0.047 to 0.5, respectively (Francoz et al., 2005). A comparison of five classes of antimicrobials tested against the *M. bovis* reference strain (Donetta, PG45) using both liquid medium and solid medium is shown in Table 2.2 (Hannan, 2000).

Table 2.2 Comparison MIC value of five classes of antimicrobial agents against *Mycoplasma bovis* reference strain using liquid and solid media (modified from Hannan, 2000)

Antimicrobial	**MIC (µg.mL^{-1})**		
	Liquid medium (Tanner and Wu, 1992)	Liquid medium (ter Laak et al., 1993)	Solid medium (Hannan et al., 1989)
Enrofloxacin	0.25	1-2	1*
Flumequine	10	-	≥ 10
Tiamulin	0.05	≤ 0.015	0.1
Tylosin	0.05	0.125	0.5
Oxytetracycline	0.1	4	0.5

* Results for ciprofloxacin, chemically similar to its analogue enrofloxacin

There is increasing attention to the possible mycoplasmastatic and mycoplasmacidal effects of novel natural and synthetic compounds. A few medicinal plants, such as *Artemesia herba-alba* and *Artemesia arborescens*, have previously been shown to have the potential to use in future treatments against *M. bovis* (Al-Momani et al., 2007). Most antimicrobials used for the treatment of a *Mycoplasma* ssp. infection only have a static effect upon the organisms, although some newer quinolones are reported to have cidal effects (Renaudin and Bebear, 1995; Taylor-Robinson and Bebear, 1997). Reports of differences based upon specific regions suggests a necessity to implement baseline testing programs in multiple regions of the United States in order to appropriately track emerging and new resistance patterns for *M. bovis* throughout the various afflicted cattle populations (Gerchman et al., 2009). Continued control of *M. bovis* infections will depend upon continuing good husbandry practices and improvements in the proper of selection effective antimicrobials whether from novel or traditional sources.

2.6 Vaccination

Due to the poor ability to control *M. bovis* using antimicrobials there has been increased attention on the possibility of control through vaccination. However, there are no licensed vaccines available in Europe and only a few in the United States and Canada (Nicholas et al., 2008). Unfortunately, even the vaccines found in North America have very little data published regarding the efficacy of each preparation (Nicholas et al., 2008; Maunsell et al., 2009). Despite this it is believed that due to data from immune response studies to *M. bovis* infection that vaccination should be able to contribute to the control and prevention of infection (Maunsell and Donovan, 2009).

The type of adjuvant delivery system selected with *M. bovis* may have a role in the effectiveness of a vaccine construct, but no research has been published to date specifically for *M. bovis.* Such advances in veterinary vaccine adjuvants include, emulsions, liposomes, and

microparticles which target the antigen presenting cells and lipopolysaccharide (LPS), monophosphoryl lipid (MPL), CpG DNA, saponins, and cytokines which are immunostimulatory adjuvants (Singh and O'Hagan, 2003). It is important to determine if the adjuvant is wholly responsible for the immune response of vaccinated animals or if the vaccine elicits an immune response. Some adjuvants appear to be more appropriate for use in development of *M. bovis* vaccines, such as saponins, which have documented success (Nicholas et al., 2002).

Some attempts at *M. bovis* vaccination have produced the unintended enhancement in the severity of pneumonia in calves treated with Triton X-114 membrane protein extracts and affinity-purified antigens (Bryson et al., 2002). Another batch of animals in an arthritis model were deemed unsuccessful (Poumarat et al., 1999). Experimental vaccine constructs aimed at mastitis have been largely unsuccessful and again show the potential to exacerbate the disease conditions (Boothby, 1986; Fox et al., 2005; Ross, 1993). Despite set-backs in the research for successful *M. bovis* vaccine constructs, there are has been some promising results reported. Most promising may be a saponized-inactivated vaccine developed by Nicholas et al. (2002) which has been shown to be safe, immunogenic, and protective against pneumonia in an experimental challenge with a virulent strain of *M. bovis*. The saponin vaccine was also successful in reducing loss of weight gain compared to the unvaccinated control calves (Nicholas et al., 2002). The use of an autogenous saponin vaccine for *M. bovis* infection in cases of mastitis has showed moderate improvements in culling rates and clinical signs, but it has been noted that it may be necessary to vaccinate calves before they are exposed to *M. bovis* in order to prevent disease manifestations (Nicholas et al., 2006). There are reports of formalin-inactivated bacterin vaccines given subcutaneously (sub-Q) that reduced levels of arthritis of *M. bovis*-associated arthritis to 13% of vaccinated calves compared to 100% of unvaccinated calves (Gagea et al., 2006a).

Despite limited data of field efficacy there are a few licensed *M. bovis* bacterin vaccines marketed in the United States. One vaccine (Mycomune, Biomune, Lenexa, KS, USA) is licensed for the reduction of severity and duration of *M. bovis*-associated mastitis in adult cattle (Maunsell

and Donovan, 2009). There are currently 3 other bacterin vaccines for prevention of respiratory disease associated with *M. bovis*-associated infections. There are no peer-reviewed reports of the efficacy of two of the bacterin vaccines, Pulmo-Guard (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) and Mycomune R (Biomune, Lenexa, KS, USA) which are marketed to the beef industry (cattle older than 45 days of age) and the use in calves 3 weeks of age or older, respectively. The efficacy of the third bacterin vaccine (Myco-Bac, Texas Vet Labs Inc., San Angelo, TX, USA) marketed for stocker and feedlot cattle has been reported by Maunsell et al. (2009). The study reported that the bacterin vaccine was ineffective at preventing nasal colonization and *M. bovis*-associated disease in pre-weaned endemically infected dairy herds from Florida (Maunsell et al., 2009).

Vaccination of older calves appears more successful than for young calves which indicates it will likely be more challenging to find appropriate vaccines for young calves. A stronger understanding of the calf immune system may help with the production and targeting of novel vaccine constructs that are produced. The lack of studies evaluating vaccines in specific age groups, using adequate power, blinding, and control groups is a gap in the understanding of the currently available vaccines which may be used for control of *M. bovis* infections, especially for young calves and those in group housing which are most likely to become colonized and subsequently infected (Nicholas et al., 2008; Maunsell and Donovan, 2009).

2.7 Summary

Although the literature reviewed here offers a glimpse into the extensive knowledge of *Mycoplasma bovis*, there are still many more holes in the data that need to be filled. There is very little data available regarding the prevalence of *M. bovis* in most states, including Pennsylvania. There is also little known regarding the efficacy of currently approved veterinary vaccines for use

in the US cattle industries, the impact of the current antimicrobial therapies in regards to developing resistance trends, and the genetic relationship of strains.

In order to determine the most appropriate course of action regarding selection of antimicrobials and the development of novel therapeutics and vaccines it is necessary to determine the impact of specific strains of *M. bovis* isolated in the US. As discussed in this review of the literature, *M. bovis* strains from some countries have strong diversity while others are heterogeneous. It has also been shown the antimicrobial susceptibility patterns varying between regions indicating the importance of testing isolates collected from the interested region. Once this data and epidemiological data are combined there is the ability to begin to determine the overall veterinary public health impact of *M. bovis* infections found in Pennsylvania. Vaccines and novel therapeutics may eventually be used for the control of infection throughout Pennsylvania after the gaps in the current knowledge of *M. bovis* begin to be filled.

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Chapter 3

Molecular epidemiological analysis of *Mycoplasma bovis* isolates from the Pennsylvania Animal Diagnostic Laboratory showing genetic diversity

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3.1 Abstract

We have examined the genetic variability of *Mycoplasma bovis* strains submitted to the Pennsylvania Animal Diagnostics Laboratory, University Park, PA (PA-ADL) between December 2007 and December 2008. Of 4,868 total samples submitted for *Mycoplasma* testing 302 were determined to be culture positive. *Mycoplasma bovis*, (63.6%), *M. californicum* (7.3%), *M. bovirhinis* (2.7%), *M. bovigenitalium* (0.7%), *M. alkalescens* (4.9%), *M. purtrefaciens* (0.3%), and *M. dispar (1.3%)* and unidentified *Mycoplasma* sp. (19.2%) were identified using polymerase chain reaction. *Mycoplasma bovis* represented the largest portion of isolates of the positive samples submitted. Each of the 192 *M. bovis* isolates were examined for variations in the *Bgl*II and *Mfe*I restriction sites of the DNA using amplified fragment length polymorphism (AFLP) fingerprinting and subsequently compared to the *M. bovis* type strain PG45 (ATCC 25523). Similarity between strains was calculated using the Dice similarity coefficient, ranging from approximately 0.7 to 1.0. When clustering the isolates at greater than 95% similarity, it was determined that 11 distinct clusters were present. The results are consistent with the existence of at least two clonally distinct groups. There was no clear geographical, month of isolation, or source origination relationship, indicating a currently unclassified characteristic is responsible for the strain heterogeneity. These data indicate strong heterogeneity of *M. bovis* isolates submitted to PA-ADL. Additionally, multiple sites throughout Pennsylvania had isolates of separate clonal lineages present concomitantly, indicating the ability of multiple overlapping outbreaks to occur at a single location. *Mycoplasma bovis* represents the largest portion of *Mycoplasma* sp. isolated from PA-ADL samples. We propose that AFLP may serve as a valuable tool for molecular characterization of *M. bovis* strains from the United States.

3.2 Introduction

Mycoplasma bovis is an important pathogen in the cattle industry. It is associated with multiple disease conditions, production losses, treatment expenses, increased case fatality rates, and high prevalence rates (Nicholas et al., 2000; Nicholas and Ayling, 2003). Diseases associated with *M. bovis* include mastitis, pneumonia, arthritis, otitis, and conjunctivitis, all resulting in significant economic losses in both the dairy and meat industries (Miller et al., 1993; Seegers et al, 2003). Although *M. bovis* itself is sufficient to cause bovine respiratory disease, the major role it plays in pneumonia is in co-infections with *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Arcanobacterium pyogenes*, leading to high morbidity and mortality. *Mycoplasma bovis* has been proposed to act as a predisposing factor, weakening the host immune system, and leading to invasion by other pathogenic bacteria or viruses (Rosengarten and Citti, 1999). It has been estimated that one quarter to one third of pneumonia related illnesses of growing cattle may be attributed at least in part to *M. bovis* infections (Nicholas et al., 2000). Cattle infected with *M. bovis* may shed the bacteria from the respiratory tract for many months serving as a reservoir of infection (Nicholas and Ayling, 2003).

When combining direct and indirect costs, the total economic burden of *M. bovis*-associated mastitis and losses due to diminished carcass values is estimated to be greater than $2 billion dollars in the United States annually (Smith and Hogan, 2001; Miller et al., 1993; Rosengarten and Citti, 1999; Nicholas et al., 2000). This makes it the most expensive production disease of dairy herds in developed countries (Smith and Hogan, 2001; Miller et al., 1993; Seegers et al., 2003).

The impact of *M. bovis* disease upon trade and cattle movement is of particular concern. There is a need for monitor the spread of *M. bovis* in cattle. Several DNA based typing methods have been employed for *M. bovis* intraspecies differentiation. They include pulsed-field gel

electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), insertion sequence profiling, restriction endonuclease analysis (REA), and amplified fragment length polymorphism (AFLP) (Kokotovic et al., 1999; Kusiluka et al., 2000; McAuliffe et al., 2004; Miles et al., 2005). Currently there is no "gold-standard" molecular typing method for *M. bovis*, but other studies have demonstrated the suitability of AFLP as a directly comparable inter-laboratory typing method that allows for a highly discriminatory, high-throughput, reproducible and robust analysis (Kusiluka et al., 2000; McAuliffe et al., 2004). AFLP analyses from the United Kingdom and Denmark has shown distinct *M. bovis* strain clusters and highly homogeneous clusters, respectively (Kusiluka et al., 2000; McAuliffe et al., 2004). This study was designed to assess the genetic relatedness of *M. bovis* isolates (n=192) submitted to the Pennsylvania Animal Diagnostic Laboratory (PA-ADL) over a one year period (2007- 2008) using AFLP analysis.

3.3 Materials and Methods

3.3.1 Isolation and identification of *Mycoplasma bovis*

A total of 4868 samples were submitted to PA-ADL over a one year time period (December 2007 to December 2008). All *Mycoplasma* positive samples, as determined by a positive culture, (n=302) submitted to the PA-ADL were grown on a pleuropneumonia-like organism (PPLO) agar plate (U.C. Davis Biological Media Services, Davis, CA) at 37° C under micro-aerophilic conditions, until growth was noted (Table 3.1). Under a light microscope a small section of the agar was identified that contained less than 5 *Mycoplasma* colonies and no gross contamination from other bacteria. The section was excised from the agar and placed in PPLO broth (U.C. Davis Biological Media Services, Davis, CA) for 48 hours at 37° C under micro-aerophilic condition. The resulting broth suspension was plated again and the process repeated. The final broth suspension was stored in with 10% glycerol at -80° C until further analysis.

Genomic DNA was extracted from 15-25 mL aliquots of stationary-phase cells using the phenol-chloroform procedure previously described (Sambrook and Russell, 2001; Pospiech and Neumann, 1995). Briefly, culture pellet was suspended in a Tris-EDTA solution (pH 7.5) with lysozyme and treated with proteinase K, before addition of phenol-chloroform. The aqueous phase was washed multiple times using ethanol and resuspended in sterile water. All DNA extracts were quantified spectrophotometrically. Isolates were confirmed as *M. bovis* using specific primers for the *uvrC* gene of *M. bovis* (Thomas et al., 2004). The isolates determined not to be *M. bovis* were confirmed as *M. dispar* (Marques et al., 2007; Miles et al., 2004), *M. bovirhinis* (Miles et al., 2004), *M. purtrefaciens* (Nicholas et al., 2008), *M. bovigenitalium* (Nicholas et al., 2008; Baird et al., 1999), and *M. californicum, M. canadense,* or *M. alkalescens* (Baird et al., 1999) based upon the PCR procedures and product sizes previously described. Based upon PCR speciation, 192 isolates were shown to be *M. bovis.* The *M. bovis* standard strain PG45 (accession number 25523), obtained from the American Type Culture Collection (ATCC) was used as a comparison strain. The strains *M. californicum* (ATCC 33461), *M. bovigenitalium* (ATCC 19852), and *M. dispar* (ATCC 27140), were also used in standardizing the PCR protocols.

Samples from Pennsylvania, Vermont, and Maryland were examined for *M. bovis.* A total of 18 counties were represented in Pennsylvania (n=186), 1 in Maryland (n=1), and 1 in Vermont (n=5). Multiple sites of infection including udder (n=151), lung (n=33), tympanic cavity (n=2), carpus joint (n=1), nasal swab (n=1), and bulk tank (n=4) were represented by the isolates (Table 3.2).

3.3.2 AFLP analysis

DNA digestion, ligation and amplification were carried out as described previously (Kokotovic et al., 1999). Briefly, the genomic DNA was simultaneously digested with 5 U *Bgl*II and 5 U *Mfe*I (New England Biolabs) at 37° C for 2 hours in digestion buffer containing 10 mM

Tris-acetate, 10 mM Mg acetate, 50 mM sp. K acetate, 5 mM dithioreitol and 50 ng bovine serum albumin (BSA). Total reaction volumes were 20 µl each. A 5 µl aliquot of the DNA digest was added to 15 µl of ligation mix containing 2 pmol of *Bgl*II adapter, 20 pmol of *Mfe*I adapter, and 1U of T4 DNA ligase (New England Biolabs), 2 µl of liagse buffer (New England Biolabs) and 8 µl of restriction buffer. Total ligation volume was 20 µl. Ligation reaction was carried out for 24 hours at room temperature. The modified genomic fragments were amplified with a Bgl-2F-0 primer labeled with a 5'- 6-carboxyfluorescein (FAM) and a Mfe-I-0 primer as previously described (Kokotovic et al., 1999). The PCR was performed in a 25 µl total reaction volume containing 4 µl of ligation product, 0.2 µM of each deoxynucleoside triphosphate, 2.5 mM of $MgCl_2$, standard polymerase buffer (New England Biolabs), 100ng of each primer, and 1.5 U of taq polymerase (New England Biolabs). The cycling conditions included an initial denaturation step of 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 54°C for 60 s, and extension at 72°C for 90 s. There was a final extension step of 72°C for 10 minutes. Amplification products were detected alongside a LIZ500 size standard at the Pennsylvania State University Genomics Core Facilities, University Park, PA under previously described conditions (Kokotovic et al., 1999). Eight isolates were selected and run in triplicate to verify the ability to accurately reproduce results. The *M. bovis* standard strain was run a total of 7 times, with at least one replicate per plate submitted for fragment analysis. Additionally an ATCC strain for *M. californicum* (33461), *M. bovigenitalium* (19852), and *M. dispar* (27140) was selected to compare outlying *M. bovis* samples against.

3.3.3 Data Analysis

Data collection, curve conversion, and pattern analysis were performed using GelCompar 6.0 (Applied Maths, Austin, TX). Level of similarity between fingerprints was calculated using the band-based Dice similarity coefficient (SD). Dendrograms were created using the unweighted pair group method with arithmetic means (UPGMA). Branch resampling support was

conducted using cophenetic correlation coefficient, which determines how accurately a dendrogram preserves the pairwise distances of the unmodelled data with values ranging from 60 to 100.

3.4 Results

A total of 4,868 samples were submitted to PA-ADL for *Mycoplasma* diagnosis between December 1, 2007 and November 30, 2008. Quarter milk and bulk tank milk samples represented 95.5% of samples submitted. The other 4.5% of samples were from various collection sites including tympanic, ear swab, nasal swab, nasal wash, lung, tracheal wash, carpus joint, ocular swab, pharyngeal swab, endotracheal wash, transtracheal wash, mammary tissue and urine. Samples submitted were from bovine, canine, caprine, cervine, feline, and ovine.

A total of 302 (6.2%) of all samples submitted for *Mycoplasma* testing were *Mycoplasma* positive. *Mycoplasma bovis* represented the largest percentage of species isolated from samples (n=192). *Mycoplasma bovis* isolates were from bovine (n=191) and cervine (n=1). January, 2008 and November, 2008 represented the months with the highest submission of samples (greater than 600). Other bovine *Mycoplasma* species represented included *M. californicum* (n=22), *M. bovirhinis* (n=8), *M. bovigenitalium* (n=2), *M. alkalescens* (n=15), *M. purtrefaciens* (n=1), *M. dispar* (n=4), and unknown (n=58) (Table 3.1). A total of 5 of the unknown samples were isolated from canine, feline, and caprine sources. *Mycoplasma bovis* was the species identified most frequently (64%) in the quarter and bulk tank milk samples (n=241). Of the quarter milk and bulk tank milk samples, *M. californicum, M. alkalescens*, *M. bovigenitalium*, *M. purtrefaciens* and *M. dispar* represented 8.7%, 5.4%, 0.4%, 0.4%, and 1.7%, respectively (Table 3.1). *Mycoplasma bovis* (63.5%) was also the most frequently isolated species from lung samples. *Mycoplasma*

bovirhinis, M. bovigenitalium, , M. californicum and *M. alkalescens* represented 15.3%, 1.9%, 1.9%, and 1.9% of lung isolates, respectively (Table 3.1).

The similarity between individual fingerprints, calculated by the Dice similarity coefficient, ranged from 0.70 to 1.0. A total of 192 *M. bovis* isolates and the PG45 standard strain were typed with AFLP, resulting in 39 different profiles at greater than or equal to 99% similarity. When comparing manually clustered AFLP fragments sized 100-490 bp at 95% similarity, the isolates could be separated into 11 clusters (A-K), clusters A and D, contained 136 and 37 isolates, respectively (Figure 3.1). The standard strain clustered within A. The similarity between the most distantly related clusters, A and K, was 70.5%. There was no temporal, geographical or source origination relationship to AFLP patterns found.

From 250-400 bp there is a difference of 10 (+/- 2 bands) bands between the isolates in clusters A and D with 40 bands and 50 bands, respectively. Strong genetic heterogeneity was observed for 3 strains, each of which had similarity values less than 80%. Clusters H to K exhibited the greatest genetic diversity (Figure 3.1). Each of these clusters contained fewer bands than the other clusters between 100-155bp. Only 9 strains, 3 from cluster F and one from clusters C, D, G, J, I, K were shown to be missing both bands at positions 118 and 122. Cluster A was shown to be missing a band at position 369 that was present in all samples of clusters C and D, while cluster B had one isolate with the band present and one without the band present.

3.5 Discussion

Since *M. bovis* represented the largest portion of *Mycoplasma* species identified, the focus of this study was to determine the clonal diversity of *Mycoplasma bovis* submitted to PA-ADL. The high percentage of samples submitted from milk sources (95.5%) indicates the perceived importance of *Mycoplasma* infection in the Pennsylvania dairy industry. A total of

6.2% of all samples submitted were positive for *Mycoplasma.* Of these milk samples, 64% were *Mycoplasma bovis*. The next most prevalent mycoplasmal mastitis agents were *M. californicum* and *M. alkalescens* representing 8.7% and 5.4% of milk isolates, respectively. Due to the similarity between *M. bovis* and *M. californicum* tetrazolium testing in aerobic conditions may sometimes be necessary (Nicholas, and Ayling, 2003). It has been suggested that *M. dispar* may be under-reported in milk samples due to the fastidious nature of the organism (Nicholas et al., 2008). Each of the *M. dispar* samples isolated was from quarter milk sources. Although *M. dispar* is present in Pennsylvania, *M. bovis*, *M. californicum*, and *M. alkalescens* are more prevalent in milk samples. Evidence suggests that *Mycoplasma* species are capable of infecting multiple organ sites (Table 3.1; Table 3.2). These results suggest that *M. bovis* is the most important *Mycoplasma* species for cattle in Pennsylvania. Each *Mycoplasma* species identified was found at multiple geographical locations indicating that it is unlikely that geography is responsible for the presence of specific species.

Although this is the first report of *M. bovis* isolated from wildlife in Pennsylvania, the identification of *M. bovis* from a cervine lung sample has previously been reported in the United States (Dyer et al., 2004). The unknown *Mycoplasma* samples are likely represented by one of the many known mycoplasmas without specific PCR primer sets available, such as *Mycoplasma verecundum* (Nicholas et al., 2008). There are few reports regarding the prevalence of the species without specific primer sets available for testing in diagnostic laboratories (Nicholas and Ayling, 2003; Fox et al., 2005; Nicholas et al., 2008).

AFLP analysis was standardized and validated in the laboratory prior to analysis of collected *M. bovis* isolates. The AFLP patterns produced in this study are highly reproducible, as demonstrated by selecting 8 isolates to run in triplicate and the standard strain run a total of 7 times, which returned patterns of >99% similarity, which is in agreement with the expected percentages of 0 to 2% average errors from repeated test samples (Mueller et al., 1999). Previous studies have demonstrated the comparability of the discriminatory power of AFLP to PFGE for

M. bovis isolates (Kusiluka et al., 2000; McAuliffe et al., 2004). This study represents the first use of AFLP to elucidate the genetic relatedness of strains of *M. bovis* isolated in the United States.

The Dice index was selected to use in conjunction with the UPGMA method since densitometric curves were converted into a band pattern for analysis. The phenogram type employed by this study is frequently used to infer epidemiological relationships (Riley, 2004). Genetic relatedness was selected at the level of 95% similarity to determine cluster relationships since it offers more flexibility than a cladistic approach while clearly defining highly homogeneous populations (Figure 3.1). The use of this index allowed for analysis to determine if geographic location, source of infection, or time of collection were responsible for the cluster patterns indicated. It was determined that geographic origin, source of infection, and time of collection were not responsible for the patterns identified. It is likely that extensive cattle transport throughout Pennsylvania and the rest of the United States, as well as the consolidation of herds and changes in management practices, influences the spread of a particular *M. bovis* pattern to farms that are separated by considerable distance. It would be informative to compare isolates collected over long time periods and distances in the U.S. in order to determine the likelihood of transmitting strains through long distance travel.

Use of an AFLP typing method may be useful for the tracing of outbreaks in the United States as well as to screen isolates from imported animals. Multiple individual sites throughout Pennsylvania had isolates from distinct clusters present at one time period indicating the ability of multiple overlapping outbreaks to occur at a single location. Tympanic samples collected from the same farm on the same day from two separate animals clustered in A and F (similarity ~90%) demonstrating this phenomenon.

The source of the isolates is weighted towards mastitis cases, at nearly 80% of isolates. *Mycoplasma bovis* infection is more frequently associated with mastitis in the United States when compared to European isolates (Kusiluka et al., 2000; McAuliffe et al., 2004). The strains submitted to PA-ADL in cluster A had a high degree of relationship to the type strain PG45

(Figure 3.1) and are therefore believed to be highly homogeneous with European strains that were previously reported as closely related to the standard strain (Kusiluka et al., 2000; McAuliffe et al., 2004). This suggests that these *M. bovis* strains that were isolated from mastitis cases and bulk tank milk samples are related to the isolates from pneumonic cattle in Europe. Expanding the comparison of various laboratory and pathologic specimens to obtain additional AFLP patterns would be very informative in our understanding of the most common genetic pattern (bands, sequences) and the distribution of the isolates.

It is hypothesized that clusters A, B, C, D are from one clonal line of decent while clusters E, F, G, H, I, J, and K represent another distinct lineage (Figure 3.1). It is also possible that clusters H, I, J, and K may represent novel clonal lineages since each is distinctly separated from the other clusters. As has been previously shown, *M. bovis* is occasionally isolated from sources other than cattle (Nicholas and Ayling, 2003). The cervine sample submitted to PA-ADL typed with group D suggesting that host species does not play a role in the clonal decent since this isolate typed with a large group of cattle isolates. It is unlikely that these isolates represent laboratory aberrations, as we feel that we have eliminated any potential source of laboratory variability. All media used for testing met quality control and assurance procedures of commercially available Mycoplasma media, there was an extensive enrichment and selection process to ensure a single genetic isolate, and the strains were each classified as being *M. bovis* by PCR classification of the *uvr*C gene. When compared to AFLP patterns for *M. bovis, M. dispar, M. bovigenitalium,* and *M. californicum,* the 3 isolates were shown to cluster in pattern similar to that shown in Figure 3.1 with the ATCC strains being more closely related than the 3 isolates (cluster I, J, K). This may be caused by the extremely small genome size (1080 kbp) and the ability to quickly genetically alter. Certainly, it would be interesting to use this type of methodology in comparison of pathologic conditions and disease outcomes in order to determine whether heterogeneity observed by AFLP analysis could account for differences in virulence, transmission, and evasion of the host's immune response. There is the possibility that J and K

separated at approximately the same time from the clonal line of descent giving rise to E-K, due to a distance of only 12 miles (19 km) between farms and 81% similarity between the two strains. It may be informative to compare these two strains acquired from quarter milk to others from other large dairy producing states in the United States. It is possible that the genomic plasticity of *M. bovis* may play a role in the heterogeneity due to recombination in repeat regions of the genome (Rocha and Blanchard, 2002). Such changes have been associated with antigenic variation and may be important for determining heterogeneity with AFLP (Kenri et al., 1999). Although previous work has not shown a connection between strain type and variable surface protein (Vsp) type, it is not known if US *M. bovis* strains have a relationship to Vsp type (McAuliffe et al., 2004). It may be useful to explore this relationship further.

3.6 Conclusions

In conclusion, this study has utilized a novel technology, AFLP, to examine the genetic relationships of *M. bovis* isolates submitted to the PA-ADL from the North Eastern United States, demonstrating the ability to separate isolates into clonally distinct groups. *Mycoplasma bovis* is the most important *Mycoplasma* species isolated from samples submitted to PA-ADL between December 1, 2007 and November, 30 2008. Isolates submitted to PA-ADL have shown strong genetic heterogeneity. We have shown AFLP to be a reliable and informative tool for these types of analyses: its ease and reproducibility should permit inter-laboratory comparison of isolates. This type of analysis has the potential to evolve into the standard tool for the characterization and tracing of various genetic isolates of pathogens in the US livestock industry.

3.7 References

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Table 3.1 *Mycoplasma* species identified from samples submitted to PA-ADL between Dec. 1, 2007-Nov. 30, 2008

Species	Collection Site			Totals
	Milk[1]	Lung	Other[2]	
M. bovis	155	33	4	**192 (63.6%)**
M. californicum	21	1	-	**22 (7.3%)**
M. alkalescens	13	1	1	**15 (4.9%)**
M. bovirhinis	-	8	-	**8 (2.7%)**
M. dispar	4	-	-	**4 (1.3%)**
M. bovigenitalium	1	1	-	**2 (0.7%)**
M. putrefaciens	1	-	-	**1 (0.3%)**
unknown	46	8	4	**58 (19.2%)**
Totals	**241**	**52**	**9**	**302**

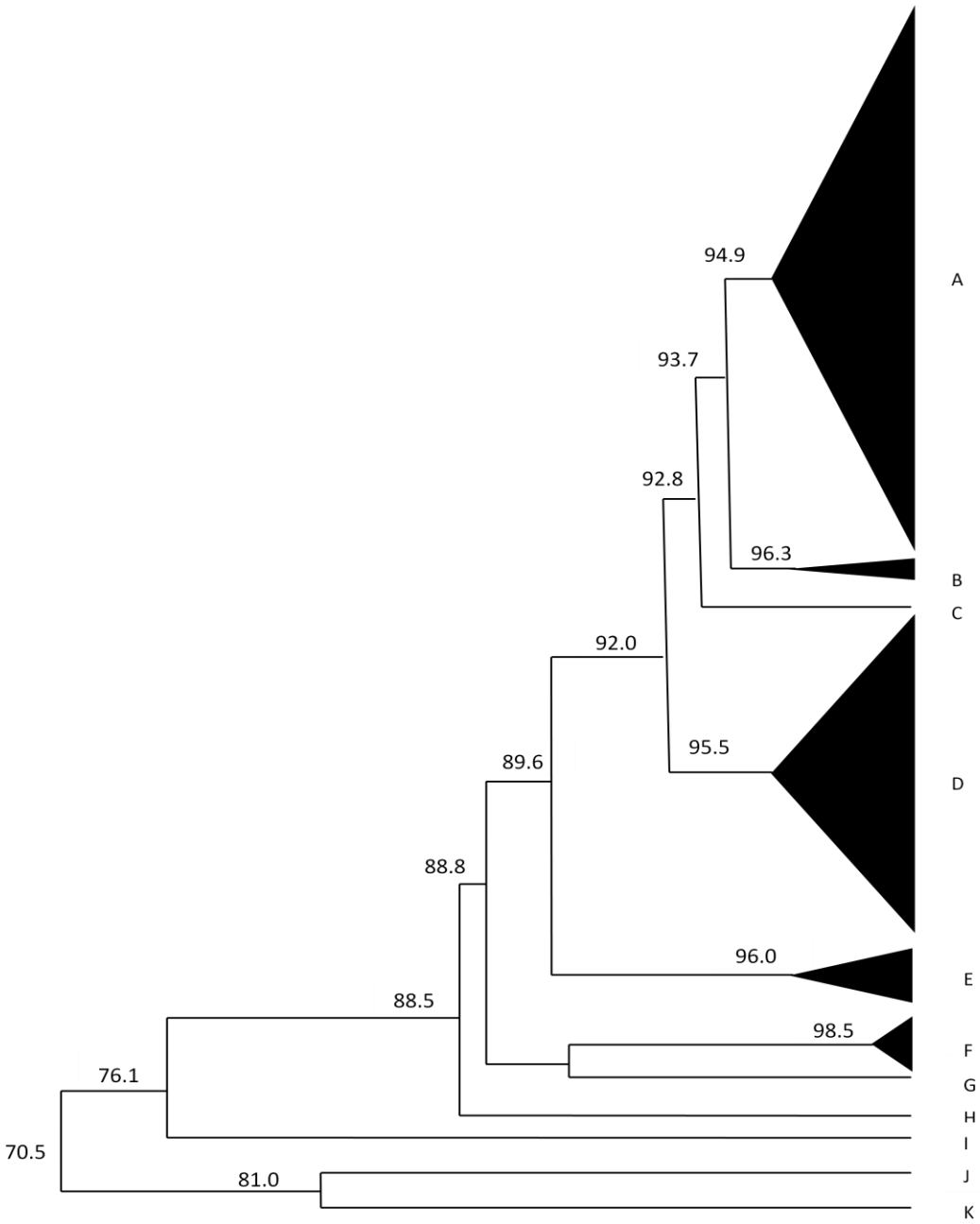
[1] Quarter milk and bulk tank
[2] typannic; carpus joint; nasal swab; respiratory swab; trachea

Table 3.2 Origins and characteristics of *M. bovis* isolates by AFLP pattern

AFLP Type at 95% Clustering	Geographical Origin[1]	Collection Source[2]	Month of Collection[3]
A	Addison, VT (n=5)	2	b, c, d, e, f
	Blair, PA (n=102)	1,2,4	a, b, c, d, e, f, g, h, i, j, k, l
	Bradford, PA (n=2)	2	b, c
	Centre, PA (n=1)	2	b
	Chester, PA (n=2)	1	h
	Clarion, PA (n=1)	2	b
	Franklin, PA (n=1)	2	c
	Fulton, PA (n=1)	2	e
	Garrett, MD (n=1)	1	h
	Huntingdon, PA (n=13)	1,2	b, c, h
	Indiana, PA (n=1)	1	b
	Lancaster, PA (n=1)	4	g
	Montgomery, PA (n=2)	1	a, c
	Montour, PA (n=1)	6	f
	Perry, PA (n=2)	1	d
	Somerset, PA (n=2)	2	b, c
	Sullivan, PA (n=1)	2	e
B	Adams, PA (n=1)	1	d
	Blair, PA (n=1)	1	k
C	Huntingdon, PA (n=1)	1	l
D	Blair, PA (n=30)	1,2	b, e, f, i, j, k, l
	Bradford, PA (n=1)	2	b
	Clarion, PA (n=1)	2	i
	Clinton, PA (n=1)	5	i
	Huntingdon, PA (n=3)	1,4	b, j, l
	Lancaster, PA (n=1)	1	l
E	Blair, PA (n=4)	1	c, i, j
	Huntingdon, PA (n=1)	1	b
F	Blair, PA (n=1)	3	f
	Crawford, PA (n=1)	2	h
	Sullivan, PA (n=1)	2	d
G	Fulton, PA (n=1)	2	f
H	Somerset, PA (n=1)	2	d
I	Crawford, PA (n=1)	2	i
J	Blair, PA (n=1)	1	j
K	Blair, PA (n=1)	1	l

[*] Geographical origin is listed by county.
[†] Collection source: 1 milk; 2 lung; 3 typannic; 4 bulk tank; 5 carpus joint; 6 nasal swab
‡ Month of Collection: a December, 2007; b January, 2008; c February, 2008; d March, 2008; e April, 2008; f May, 2008; g June, 2008; h July, 2008; i August, 2008; j September, 2008; k October, 2008; l November, 2008

Figure 3.1 Phenogram of *Mycoplasma bovis* isolates. Genetic relationship between *M. bovis* strains based upon AFLP profiles (100-490bp fragment size range) produced by amplification of *Bgl*II and *Mfe*I DNA templates with nonselective primers. The dendrogram was produced with the UPGMA method with the Dice similarity coefficient (S_D) and strains with greater than 95% similarity collapsed into single clusters.



Chapter 4

In vitro antimicrobial inhibition of *Mycoplasma bovis* isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and a broth microdilution method

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4.1 Abstract

Mycoplasma bovis is an important pathogen of cattle in the United States, causing mastitis, pneumonia, conjunctivitis, otitis, and arthritis. Currently there are only a few reports of sensitivity levels for *M. bovis* isolates from the United States. *Mycoplasma bovis* isolates submitted to the Pennsylvania Animal Diagnostic Laboratory between December 2007 and December 2008 (n=192) were tested for antimicrobial susceptibility to enrofloxacin, erythromycin, florfenicol, spectinomycin, ceftiofur, tetracycline, and oxytetracycline using a broth micodilution testing method. The most effective antimicrobials against *M. bovis* determined by using broth microdilution method were florfenicol, enrofloxacin, and tetracycline with a minimum inhibitory concentration (MIC) range of 2 - 32 µg/mL, 0.1 - 3.2 µg/mL, and 0.05 - >12.8 µg/mL, respectively. Spectinomycin, oxytetracycline, and tetracycline showed a wide ranging level of efficacy in isolate inhibition with broth microdilution MIC ranges of 4 - >256 µg/mL, 0.05 - >12.8 µg/mL, and 0.05 - >12.8 µg/mL, respectively. A significant difference in the susceptibility levels between milk and lung isolates was found for spectinomycin. When MIC values of a subset of the *M. bovis* isolates (n=12) were tested using a standardized flow cytometric technique, the MIC ranges of enrofloxacin, spectinomycin, ceftiofur, erythromycin, tetracycline, oxytetracycline, and florfenicol ranges were 0.1 – 0.4 µg/mL, 4 - >256 µg/mL, >125 µg/mL, >3.2 µg/mL, <0.025 - >6.4 µg/mL, 0.8 - >12.8 µg/mL, and <2 - 4 µg/mL, respectively. Flow cytometry offers potential in clinical applications due to high throughput-capability, quick turn-around time, and the objective nature of interpreting results.

4.2 Introduction

Mycoplasma bovis, a pathogenic *Mollicute*, is associated with pneumonia, mastitis, conjunctivitis, otitis, and arthritis in cattle. The first case of *M. bovis* was isolated in the United States during the summer of 1961 from cattle with mastitis (Hale, 1962). It has been estimated that the United States faces economic losses of greater than $32 million per year due to *M. bovis* alone (Nicholas and Ayling, 2002). Poor efficacy of *M. bovis* vaccines produces few clinical options. *Mycoplasma bovis* infections are either treated with antimicrobials which frequently do not clear the infection or infected animals are culled.

There is concern over the increasing resistance to frequently administered antimicrobials, including spectinomycin, tetracycline, and tilmicosin (Nicholas, 2004; Nicholas et al., 2009). The unique characteristics of *M. bovis*, such as the lack of a cell-wall, cause concern regarding treatment options due to the ineffectiveness of cell-wall targeting antimicrobial therapies. Beta-lactams, such as penicillin, which constitute the largest type and number of antimicrobials approved for therapeutic use in cattle, are ineffective against *M. bovis*. Clinical Laboratory Standards Institute (CLSI)-approved and standardized levels of minimum inhibitory concentrations (MIC) for antimicrobials have not been established regarding *Mycoplasma* species (Hannan, 2000; NCCLS, 2002; NCCLS, 2006).

Multiple methods have been employed in order to determine minimum inhibitory concentrations (MICs) in many species of *Mycoplasma.* These methods have included broth dilutions, solid agar testing, E-strip testing, and most recently, flow cytometry (Assuncao et al., 2006a; Francoz et al., 2005; Hannan, 2000; Hannan et al., 1989; Rosenbusch et al., 2005; Tanner and Wu, 1992; Waites et al., 1999). MIC values have been determined for *Mycoplasma agalactiae, Mycoplasma hyopneumoniae, Mycoplasma putreficans, Mycoplasma capricolum,* and *Mycoplasma mycoides* using a selection of antimicrobials with flow cytometry (Assuncao et al., 2006a; Assuncao et al., 2006b; Assuncao et al., 2006c; Assuncao et al., 2006d). It has been

reported that antimicrobial susceptibility profiles are influenced by geographical differences leading to a need for regional testing of isolates (Gerchman et al., 2009). The objectives of this study were to: 1) employ a microdilution method to determine the MIC of seven antimicrobials on *M. bovis* isolates (n=192) submitted to Pennsylvania Animal Diagnostic Laboratory (PA-ADL) between December 2007 and 2008, 2) develop a flow cytometry method for determination of antimicrobial sensitivity for *M. bovis* isolates that can be easily adopted in a diagnostic setting, 3) employ a flow cytometric method to determine the MIC of seven antimicrobials on twelve *M. bovis* field isolates from Pennsylvania, and 4) compare the microdilution and flow cytometry methods.

This is the first reported use of flow cytometry to determine MIC levels for *M. bovis.* The comparison of broth microdilution to flow cytometry allows for an opportunity to evaluate the use of a novel flow cytometry method. The inclusion of twelve field isolates during flow cytometric testing allows for a broader focus of antimicrobial effects than previously reported (Assuncao et al., 2006b; Assuncao et al., 2006c; Assuncao et al., 2006d). The impact and magnitude of resistance to frequently administered veterinary antimicrobials may be elucidated by testing the MIC values for all *M. bovis* isolates submitted to the PA-ADL over a one year period using the broth microdilution method.

4.3 Materials and Methods

4.3.1 Isolates and sample collection

All *Mycoplasma* positive samples sent to the PA-ADL were collected from December 2007 to December 2008 (n=192). Each sample was grown on a pleuropneumonia-like organism (PPLO) agar plate (U.C. Davis Biological Media Services, Davis, CA) at 37° C and 5% CO_2 until growth was noted. Under a light microscope a small section of the agar was identified that

contained less than 5 *Mycoplasma* colonies and no gross contamination from other bacteria. The section was excised from the agar and placed in PPLO broth (U.C. Davis Biological Media Services, Davis, CA) for 48 hours at 37° C and 5% CO_2. The resulting broth suspension was plated again and the process repeated. The final broth suspension was stored in with 10% glycerol at -80° C until ready to use in testing. All isolates submitted to the PA ADL were confirmed to be *M. bovis* (n=192) using specific primers for the *uvr*C gene of *M. bovis* before they were tested for antimicrobial activity using 96-well round bottom plates (Thomas et al., 2004).

The *M. bovis* isolates sent to PA-ADL from Vermont, Maryland, and Pennsylvania represented 27 U.S. zip-codes from 34 individual farms. A total of 18 counties were represented in Pennsylvania (n=186), 1 in Maryland (n=1), and 1 in Vermont (n=5). Multiple sites of infection including udder (n=151), lung (n=33), tympanic cavity (n=2), carpus joint (n=1), nasal swab (n=1), and bulk tank (n=4) were represented by the samples.

4.3.2 Colorimetric broth microdilution assay

Antimicrobial susceptibility testing was performed using the redox reagent alamarBlue (AbD Serotec, Raleigh, NC) (resazurin) following to a modification of a previously described broth microdilution procedure (Rosenbusch et al., 2005). Briefly, early log phase suspensions of *M. bovis* (3×10^3 - 3×10^5 cfu/ml) grown in Mycoplasma Enrichment Broth without supplements (U.C. Davis Biological Media Services, Davis, CA) at 37° C and 5% CO_2 were exposed to doubling concentrations of enrofloxacin (MP Biomedicals Inc., Solon, OH) starting at 0.0125 µg/mL, spectinomycin (MP Biomedicals Inc., Solon, OH) at 1 µg/mL, erythromycin (MP Biomedicals Inc., Solon, OH) at 0.0125 µg/mL, tetracycline (MP Biomedicals Inc., Solon, OH) at 0.025 µg/mL, oxytetracycline (MP Biomedicals Inc., Solon, OH) at 0.05 µg/mL, ceftiofur (SmithKline Beecham Corp., Philadelphia, PA) at 0.49 µg/mL, and florfenicol (Sigma-Aldrich, St. Louis, MO) at 2 µg/mL in the presence of 5% alamarBlue. These levels were selected as starting concentrations based upon previous research (Assuncao et al., 2006a; Francoz et al.,

2005; Hannan, 2000; Hannan et al., 1989; Rosenbusch et al., 2005; Tanner and Wu, 1992; Waites et al., 1999). All plates were sealed to prevent gas exchange between wells. Each isolate was run in duplicate on a sterile 96-well round bottom plate composed of nine dilutions of the seven antimicrobials, one negative control (no antimicrobial and no culture) and two positive controls (no antimicrobial present with culture). Additional plates containing each concentration of the seven antimicrobials in the presence of 5% alamarBlue infused broth without culture were sealed and incubated for 10 days. There was no indication of color change in any well, indicating that antimicrobials alone were unable to induce the redox reaction. MIC was determined as the lowest concentration of antimicrobial suppressing growth, as expressed by a blue to red shift. The MIC for each isolate and antimicrobial was recorded. If a color shift occurred in all wells before day 10 of incubation then it was determined that antimicrobials were mycoplasmastatic in nature. The highest MIC of the duplicate runs was used for further analysis. All sample duplicates were within at least 2 dilution levels of each other.

4.3.3 Flow cytometry

Isolates selected for flow cytometry included *M. bovis* isolates from milk sources (n=6) and lung sources (n=6) from the collection of 192 *M. bovis* isolates received at the PA-ADL between December 2007 and December 2008. Two of the isolates selected were obtained from the same farm location; the other samples were unrelated by farm location. Mycoplasma Enrichment Broth without supplements (U.C. Davis Biological Media Services, Davis, CA) was filtered using a 0.2 µm pore size (VWR, West Chester, PA). Mycoplasma absolute cell counts were gathered using 6 µM Fluoresbrite™ Polychromatic Red Microspheres (PolyScience Inc., Warrington, PA) and regions of analysis were determined using heat injured *M. bovis* cells according to the procedures previously described (Assuncao et al., 2006a). Early log phase suspensions of *M. bovis (*~$4.5x10^5$ cfu/ml) grown at 37° C and 5% CO_2 were exposed to doubling concentrations of enrofloxacin (MP Biomedicals Inc., Solon, OH) starting at 0.0125 µg/mL,

spectinomycin (MP Biomedicals Inc., Solon, OH) 1 µg/mL, erythromycin (MP Biomedicals Inc., Solon, OH) 0.0125 µg/mL, tetracycline (MP Biomedicals Inc., Solon, OH) 0.025 µg/mL, oxytetracycline (MP Biomedicals Inc., Solon, OH) 0.05 µg/mL, ceftiofur (SmithKline Beecham Corp., Philadelphia, PA) 0.49 µg/mL and florfenicol (Sigma-Aldrich, St. Louis, MO) 2 µg/mL. Each antimicrobial stock solution was prepared on the day of analysis. Cell populations were examined for living and injured or dead *M. bovis* at 3, 6, 12, and 24 hours. A final MIC_{90} value was assigned at the 24 hour reading. Propidium iodide (Invitrogen, Eugene, OR) at a concentration of 4 mg/mL was added to each sample to determine the amount of cell membrane damage. SYBR Green I (Invitrogen, Eugene, OR) at a 1:10,000 vol/vol concentration was used to identify nucleic acids corresponding to a viable cell population in samples. SYTO (Invitrogen, Eugene, OR) stain was tested, but SYBR Green I was shown to give a stronger signal. Each sample was incubated for 15 minutes in the dark before the analysis on the flow cytometer. Analyses were performed on an XL-MCL flow cytometer (Beckman-Coulter, Miami, Lakes, FL) with System II Software Version 3.0 using forward scatter (FS) and side scatter (SSC) dot plots to count 20,000 cells at low flow rate providing the cellular physical properties of size and granularity. After 488 nm laser excitation, a 550 nm dichoric long pass filter split the emission, sending the green light to PMT1 which has an additional 525 nm band-pass filter and the red light to PMT3 which has an additional 610 nm band-pass filter. Propidium iodide positive cells were eliminated from the green distribution. Data, collected in a four-decade logarithmic scale, were analyzed using WinMDI (v. 2.8) (Joseph Trotter, Scripps Research Institute, La Jolla, CA) and FCS Express Research Edition (v. 3) (De Novo, Los Angeles, CA) software packages. All data for this study were found in the F2 quadrant. Additional quadrants were created and color schemes assigned for cells positive for each fluorochrome. All experiments were duplicated on a separate day.

4.3.4 Statistical analysis

Only the antibiotics that demonstrated activity against at least one sample were considered for statistical analysis. The MIC_{90} values were analyzed separately using G-test of independence (r x c contingency tables), testing the independence of the active antimicrobials and grouping of isolates by geographic region and sample origin. Only variables with greater than 5 samples were tested, therefore only quarter milk and lung were used. Results were considered significant at a p-value of less than 0.05, confidence interval 95%.

4.4 Results

4.4.1 Broth microdilution

Samples submitted from Pennsylvania, Maryland, and Vermont collected from lung tissue, nasal swab, carpus joint, tympanic cavity, quarter milk and bulk tank milk showed similar antimicrobial activity profiles between farm location and antimicrobial susceptibility level (no significant interactions between location and antimicrobial action was found at $p<0.05$). However, 67.5% of quarter milk samples versus 24.2% of lung samples had a MIC level greater than or equal to 128 µg/mL, indicating resistance if using CLSI interpretative criteria for bovine respiratory disease pathogens (*Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni)* (NCCLS, 2002; NCCLS, 2006). The difference between spectinomycin resistance lung and quarter milk samples is significant ($p<0.001$) using a G-test of independence. There was no significance difference between tetracycline, oxytetracycline, enrofloxacin, or florfenicol activity and sample origin. Erythromycin and ceftiofur did not demonstrate growth inhibition of *M. bovis* at the concentrations tested and were therefore not included in any statistical comparisons. The MIC of oxytetracycline was most commonly one or two dilutions higher than that demonstrated

for tetracycline. The broth microdilution antimicrobial susceptibility ranges for enrofloxacin, spectinomycin, ceftiofur, erythromycin, tetracycline, oxytetracycline, and florfenicol were 0.025 to 3.2 µg/mL, 2 to >256 µg/mL, >125 µg/mL, >3.2 µg/mL, 0.05 to >12.8 µg/mL, 0.05 to >12.8 µg/mL, and <1 to 32 µg/mL, respectively (Table 4.1).

4.4.2 Flow cytometry

The MIC_{90} values for seven veterinary approved antimicrobials were determined for the twelve isolates using flow cytometry. Figure 4.1 shows a representative sample of an enrofloxacin tested sample with appropriate controls. Each histogram represents the F2 region split into quartiles with the living cell population highlighted by blue dots (left side) and the dead or injured cell population highlighted by green dots (right side). All samples were compared to a control with no antimicrobial at each reading. These controls were used to determine the appropriate living percentages before calculating the MIC values. Using flow cytometry, antimicrobial susceptibility ranges for enrofloxacin, spectinomycin, ceftiofur, erythromycin, tetracycline, oxytetracycline, and florfenicol were 0.1 to 0.4 µg/mL, 4 to >256 µg/mL, >125 µg/mL, >3.2µg/mL, <0.025 to >6.4 µg/mL, 0.05 to >12.8 µg/mL, and <2 to 4 µg/mL, respectively (Table 4.2).

Ceftiofur and erythromycin showed no growth inhibition at the concentrations tested throughout any of the time points. Enrofloxacin, spectinomycin, florfenicol, tetracycline and oxytetracycline first began to show low level changes in living and dead/injured cell population proportions between the 6 hour and 12 hour time points. At 24 hours, 6 samples were shown to have MIC_{90} values equivalent to the broth microdilution test. Four samples had higher MIC_{90} values reported for tetracycline and oxytetracycline (M3I9, M4B2, M1H9, and M2A8), while 2 samples had higher MIC_{90} values for spectinomycin (M2A7 and M2H9) when compared to the broth microdilution method. The MIC_{50} value for the spectinomycin samples was equivalent to the MIC_{90} values of the broth microdilution method. The 24 hour flow cytometry results of M3I9,

M4B2, M1H9, M2A8, M2A7, and M2H9 matched the MIC_{90} values of the broth microdilution method by 48 hours.

4.5 Discussion

The current lack of CLSI-approved MIC breakpoints or standardized methods for testing of *Mycoplasma* species of veterinary importance leads to difficulty with interpreting the impact of antimicrobial activity *in-vitro*. The use of CLSI-approved interpretative criteria for other bovine pathogens is frequently used to understand the implication of *in-vitro Mycoplasma* sensitivity testing (Ayling et al., 2000; Francoz et al., 2005; Gerchman et al., 2009; Hannan, 2000; Rosenbusch et al., 2005). It has been suggested that if the actual MIC values are significantly higher than those established by CLSI for other important pathogens of veterinary medicine, it is unlikely that the antimicrobial will be capable of being efficacious (Ayling et al., 2000; Francoz et al., 2005; Gerchman et al., 2009; Hannan, 2000; Hannan et al., 1989; Rosenbusch et al., 2005). This indicates that *M. bovis* may be categorized as susceptible to an antimicrobial if it falls in the susceptible category of the CLSI-approved criteria for the other veterinary pathogens. Therefore, enrofloxacin, florfenicol, spectinomycin, tetracycline, oxytetracycline, ceftiofur, and erythromycin meet the criteria for the susceptible category for respiratory pathogens at less than or equal to 0.25 µg/mL, 2 µg/mL, 32 µg/mL, 4 µg/mL, 4 µg/mL, 0.5 µg/mL, and 2 µg/mL, respectively (NCCLS, 2002; NCCLS, 2006). However, it has been reported that beta lactams and similar cell-wall targeting therapeutics have minimal or no effect on *M. bovis*. In our study, ceftiofur, a cephalosporin that shares a ring structure with beta-lactams, had no effect upon *M. bovis* in either the broth microdilution or flow cytometry testing. These observations are in agreement with those that previously reported of no effect on *Mycoplasmas* by antimicrobials

which target the cell wall (Nicholas, 2004). Similar to previous work, erythromycin was unable to show an effect upon *M. bovis* using either method (Rosenbusch et al., 2005).

Although the *in-vitro* susceptibility testing is not intended to be a definitive statement on the impact an antimicrobial may have *in-vivo,* it may provide guidance for a veterinarian seeking an appropriate antimicrobial selection. It is also important to note that there was no treatment data available for the samples in this study. It is unknown whether acute, chronic, treated, and untreated samples may have significantly different antimicrobial sensitivity profiles. For this reason, the higher of the two broth microdilution runs was selected for this study.

Varying levels of susceptibility and increased resistance to spectinomycin by *M. bovis* has been observed (Ayling et al., 2000; Francoz et al., 2005; Gerchman et al., 2009; Rosenbusch et al., 2005). The results of this study also suggest this phenomenon, as that 58% of the isolates had MIC's at or above 128 µg/mL, which is considered resistant using the CLSI standards for other veterinary pathogens. Tetracycline and oxytetracycline have a wide distribution of antimicrobial activity with many of the duplicate samples being +/- 2 dilutions from the first run. This suggests a wider range of antimicrobial MIC values for these antimicrobials which may lead to difficulty accurately interpreting the *in-vitro* susceptibility testing results.

During the development of the flow cytometry assay, it was determined that SYTO staining did not result in a fluorescent output high enough for clear identification of *M. bovis* populations. The strength of the SYBR fluorescence leads to mild levels of bleed through into the same range as PI and it is necessary to adjust the distribution on the flow cytometer. Dying cells may release nucleic acids which can bind SYBR leading to overlap.

The findings of the study suggest that flow cytometry may be employed successfully in the real time collection of MIC values for *M. bovis* field isolates. Similar versions of the flow cytometry results recording a different MIC value than other methods has been previously reported for other *Mycoplasma* species (Assuncao et al., 2006a; Assuncao et al., 2006c; Assuncao et al., 2006d). The antimicrobials which were most effective for *M. bovis* using flow cytometry in

this study were florfenicol and enrofloxacin. Using flow cytometry spectinomycin, oxytetracycline, and tetracycline showed a wide ranging level of efficacy for Pennsylvania *M. bovis* field isolates. A similar pattern of diverse MIC levels for these antibiotics was also seen with the broth microdilution method. The shift from a living cell to a dead cell occurs between 6 and 12 hours. The 24-hour time point may be too long in some instances, because the MIC has moved to a higher dilution of antimicrobial in order to inhibit growth. Therefore the results suggest the most appropriate time point for collecting data with flow cytometry is between 12 and 24 hours. This indicates that flow cytometry may be capable of producing results in less time than the broth microdilution method.

4.6 Conclusions

In conclusion, both flow cytometry and broth microdilution with 5% alamarBlue demonstrated that florfenicol and enrofloxacin are most efficacious in inhibiting *M. bovis* isolates of bovine origin from Pennsylvania in this study. *In-vitro* susceptibility breakpoints do not necessarily correspond to *in-vivo* results, but they provide a method of determining the agents which are most likely to be effective treatment options. The use of 5% alamarBlue with the broth microdilution method allows for clear differentiation of breakpoints and ease of use by technical staff. This study is the first report of the use of flow cytometry for susceptibility testing of *M. bovis*. Through the use of flow cytometry, better turn-around time for sensitivity data can be achieved. In certain regions there is potential for real time diagnostic data to be available within 24 hours.

4.7 References

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Table 4.1 MIC values (24 h) of *Mycoplasma bovis* isolates with broth microdilution (n=192)*

	Range (n=192)	Mode (n=192)	Median (n=192)	Median of Milk Isolates (n= 151)	Median of Lung Isolates (n= 33)
Ceftiofur	>125	>125	>125	>125	>125
Enrofloxacin	0.025 to 3.2	0.2	0.2	0.2	0.2
Erythromycin	>3.2	>3.2	>3.2	>3.2	>3.2
Florfenicol	<1 to 32	4	4	4	4
Oxytetracycline	0.05 to >12.8	6.4	6.4	6.4	6.4
Spectinomycin	2 to >256	>256	>256	>256	8
Tetracycline	0.05 to >12.8	1.6	3.2	3.2	3.2

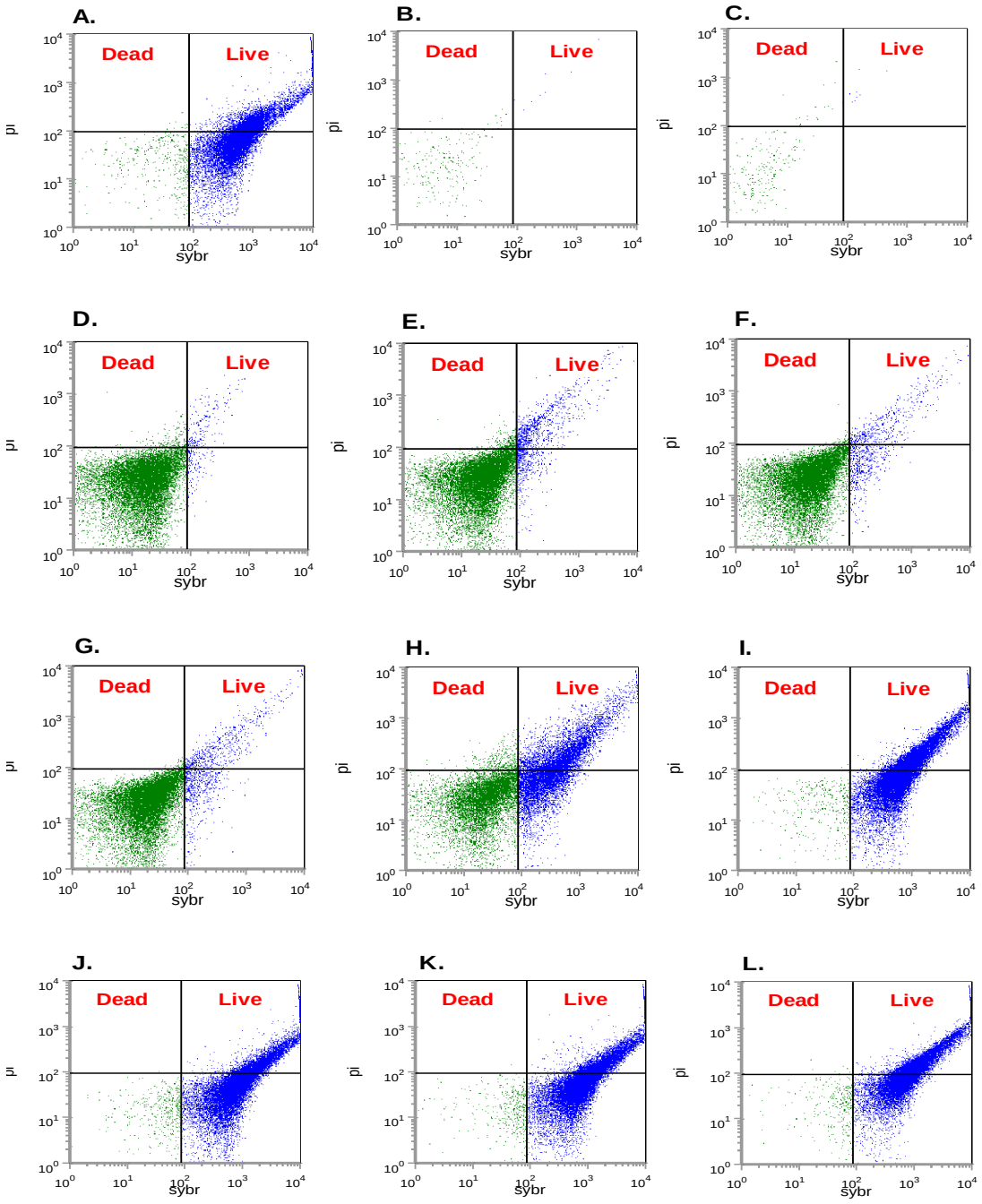
*All antimicrobial concentrations are presented in µg/mL.

Table 4.2 MIC values (24 h) as determined by Flow Cytometry using SYBR green I and Propidium Iodide stains*

Milk Isolate	Spectinomycin	Ceftiofur	Florfenicol	Oxytetracycline	Erythromycin	Enrofloxacin	Tetracycline
M2B4	8	>125	<2	1.6	>3.2	0.2	0.8
M3I9	>256	>125	<2	>12.8	>3.2	0.1	6.4
M2H4	>256	>125	<2	>12.8	>3.2	0.1	>6.4
M2A4	>256	>125	<2	0.8	>3.2	0.4	0.4
M4B2	8	>125	<2	0.05	>3.2	0.4	<0.025
M2C3	>256	>125	<2	1.6	>3.2	0.4	1.6
Lung Isolate	Spectinomycin	Ceftiofur	Florfenicol	Oxytetracycline	Erythromycin	Enrofloxacin	Tetracycline
M2A7	32	>125	4	>12.8	>3.2	0.4	>6.4
M2B8	>256	>125	<2	>12.8	>3.2	0.4	>6.4
M1H4	>256	>125	4	>12.8	>3.2	0.4	>6.4
M2H9	>256	>125	4	>12.8	>3.2	0.4	>6.4
M1H9	>256	>125	<2	>12.8	>3.2	0.4	>6.4
M2A8	4	>125	<2	3.2	>3.2	0.4	>6.4
Range	**4 to >256**	**>125**	**<2 to 4**	**0.05 to >12.8**	**>3.2**	**0.1 to 0.4**	**<0.025 to >6.4**

*All antimicrobial concentrations are presented in µg/mL

Figure 4.1 Representative Histograms of Flow Cytometry Results at 24 Hours



Dot plot histograms (SYBR verses PI on a logarithmic based scale) show a representative sample (M2A7) at each dilution of enrofloxacin at 24 hours. Green dots highlight the PI positive cell population (log scale Y-axis), and blue dots show the SYBR-positive population (log scale X-axis). Plate A represents sample M2A7 at 24 hours of growth with no antibiotic added. Plate B is enrofloxacin in filtered broth at 24 hours. Plate C is filtered sterile water at 24 hours. The µg/mL concentration for enrofloxacin is 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 for plates D through L, respectively.

Chapter 5

Identification of novel small molecule antimicrobials targeting *Mycoplasma bovis*

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5.1 Abstract

Mycoplasma bovis, a major cause of pneumonia and mastitis in cattle, is naturally resistant to all cell-wall targeting antimicrobials. Beta-lactams, a commonly used class of drugs in cattle, are among the antimicrobials that are ineffective against *M. bovis*. Using a tetrazolium salt cytotoxicity assay, 483 natural compounds were screened to determine which of the small molecules serve the potential to become therapeutic options for *M. bovis* prevention and treatment. Data suggest that 32 of the 483 compounds tested were able to inhibit growth of *M. bovis* using a tetrazolium salt assay. Methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate were selected for further concentration dependent studies and testing in fresh quarter milk. Small molecule natural compounds are capable of inhibiting growth of *M. bovis* in both a pleuropneumonia-like organism (PPLO) media and in fresh quarter milk. Results suggest that the compounds are mycoplasmastatic in a dose dependent manner. By inhibiting *M. bovis*, small molecule natural compounds offer the potential for prophylactic or therapeutic use on organic and natural farms as a viable alternative to traditional antimicrobial agents.

5.2 Introduction

Various species of *Mycoplasmas* have been shown to be pathogens of humans, cattle, sheep, goats, swine, cats, dogs, rodents, horses, and poultry. *Mycoplasma bovis*, a pathogenic mycoplasma that affects cattle, is associated with pneumonia, arthritis, mastitis, otitis, and conjunctivitis, which leads to significant economic losses in the dairy and meat industries. *Mycoplasma* species are extracellular parasites with membrane-membrane interactions leading to pathogenesis in host cells, especially epithelial cells (Simecka et al., 1992).

Bovine mastitis and pneumonia caused by *M. bovis* are complex multifactorial diseases that are some of the most difficult bacterial infections to treat and control. The lack of a cell-wall in *Mycoplasma* species raises concerns regarding treatment. Cell-wall targeting antimicrobials, such as beta-lactams, are ineffective against *M. bovis*. In addition to natural resistance against cell-wall targeting drugs, increased resistance in frequently administered veterinary antimicrobials, such as spectinomycin, tetracycline, and tilmicosin have been recorded for *M. bovis* (Nicholas, 2004; Nicholas et al., 2009). It may be necessary to consider alternatives to the frequently used antimicrobials in order to continue combating *M. bovis* infections in the future.

One suggested method is through the use of novel natural compounds. Toxicity assays allow for the fast and efficient screening of the compounds that may possess antimicrobial activity. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) assays, a colorimetric test based upon reduction of tetrazolium, have been successfully used on multiple mycoplasma species allowing for quantitative evaluation of growth, inhibition, or chemosensitivity of mycoplasmas (Bredt, 1976; Kirchhoff et al., 1992). Formazan, created by the reduction of tetrazolium salt, has been shown to be cleaved by living cells while dead cells are almost completely negative for this action due to the requirement of active mitochondria

(Mosmann, 1983). In order to select the most appropriate therapeutic and prophylactic options, it is necessary to consider how to target the compounds. The objectives of this study were to 1) screen novel small molecule compounds for inhibition of *M. bovis* growth, 2) evaluate the potential of a dose-dependent response; 3) determine whether inhibition of *M. bovis* was possible in a milk environment using novel small molecule natural compounds.

5.3 Materials and Methods

5.3.1 Natural compound library cytotoxicity screening

Mycoplasma cytotoxicity assays were performed using Cell Counting Kit-8 (CCK-8) which utilizes a water soluble tetrazolium salt (Dojindo Technologies, Rockville, MD). The production of a yellow color is the result of dehydrogenase reduction, which is directly proportional to the number of living cells in the assay. The TimTec (Newark, DE) Natural Compound library of 480 compounds was screened. Three additional compounds, monocaprylin, caprylic acid, and epigallocatechin gallate (EGCg) were screened (Sigma-Aldrich, St. Louis, MO) due to previous reports of antibacterial activity for each (Nagle, 2006; Nair, 2005; Taylor, 2005). *Mycoplasma bovis* PG45 cultures (ATCC 25523) were grown to log phase growth (~$5x10^3$ cells/mL). A final compound concentration of 10µM was added to each inoculum broth well in a sterile 96-well round bottom plate. Enrofloxacin (Sigma-Aldrich, St. Louis, MO) and florfenicol (Sigma-Aldrich, St. Louis, MO) were selected as a kill level control due to the common use of each antimicrobial as a treatment for *M. bovis* associated disease in cattle. Florfenicol final concentration levels of 512 µg/mL, 256 µg/mL, and 128 µg/mL and enrofloxacin levels of 3.2 µg/mL, 1.6 µg/mL, and 0.8 µg/mL were run in duplicate on each plate allowing for comparisons to the small molecule natural compound results. Additionally, media alone, media with 10%

dimethyl sulfoxide (DMSO), culture alone, and culture alone with 10% DMSO were run with every plate (Sigma Aldrich, St. Louis, MO). Samples were then incubated at 37° C in a 5% CO2 environment for 4 hours when 10% vol/vol of CCK-8 was added. The absorbance was read at 450nm using a microplate reader (FlexStation3, Molecular Devices, Sunnyvale, CA) at 2 hours and 20 hours after addition of CCK-8. The media alone and media with 10% DMSO wells showed no notable difference in absorbance reading. The absorbance level in the media wells was used to determine the background levels, with a range of 0.2 to 0.5.

Necessitated by a report that some compounds with a thiol group could interfere with MTT assays (Natarajan et al., 2000), the impact of a small molecule natural compounds ability to interfere with the cytotoxicity assay was determined. Each of the 32 compounds identified as able to inhibit the growth of *M. bovis* were tested in the presence of CCK-8 at the testing conditions without the presence of *M. bovis.* Additionally a 40µM concentration of methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, (+)-alpha-tocopherol acid succinate or 99.9% pure DMSO was added to a plastic 5 mL culture tube containing 5% alamarBlue (AbD Serotec, Raleigh, NC) which was sealed with parafilm (Peachiney Plastic Packaging, Menasha, WI) at 37° C in a 5% CO2 environment.

5.3.2 Dose response testing of selected small molecule compounds

Methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate (TimTec, Newark, DE) were selected for dose response cytotoxicity assays. Cultures of log-phase *M. bovis*, PG45 (~$5x10^3$ cells/mL) were tested with final compound concentrations of 20µM, 10µM, and 1µM of

each compound in a sterile 96-well round bottom plate. Media alone, media with 10% DMSO, culture alone, and culture alone with 10% DMSO were run with every plate. Florfenicol (512 µg/mL) and enrofloxacin (3.2 µg/mL) were run in duplicate on each plate as a kill control/comparison set. Samples were then incubated at 37° C in a 5% CO2 environment for 4 hours, 10% vol/vol of CCK-8 was added, 20 hours later the absorbance was read at 450nm using a microplate reader.

5.3.3 High throughput screening power and validation

The Z-factor statistic was calculated to provide evaluation and comparison of the quality of the high throughput assay used in this study. The Z-factor was determined using the ratio of separation band to signal dynamic range as previously described (Zhang et al., 1999). The power (Z-factor) of the high throughput screening was calculated to be 0.6749, representing an "excellent assay" as described by Zhang et al. (1999) due to a large separation band indicated by a value less than 1 yet greater than 0.5. Determination of a "hit" was determined by comparing the sample tested to the absorbance levels of untreated growth wells and wells treated with known antimicrobial (florfenicol and enrofloxacin). Samples that were shown to have absorbance of untreated control wells or were nearly equivalent to the absorbance levels recorded for the known antimicrobials were determined to be a "hit".

5.3.4 Cytotoxicity in fresh quarter milk

Methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate were tested for their ability to inhibit growth of *M. bovis* in a milk environment. Fresh, non-mastitic, mycoplasma-free

quarter milk samples were obtained on the day of testing from the Pennsylvania State University Department of Dairy and Animal Sciences Dairy barns (average somatic cell count 3/µL). Each sample of milk was spiked with the *M. bovis* standard strain, PG45 (~$2x10^4$ cells/mL). Each of the compounds was added to the fresh quarter milk at 1µM, 10µM, 20µM, and 40µM toxicant concentrations. Samples were incubated at 37° C in a 5% CO2 environment. At hours 0, 3, and 24 a 10µL aliquot was spread plated on pleuropneumonia-like organism (PPLO) agar plates (U.C. Davis Veterinary Biological Media Services, Davis, CA). Plates were incubated for 48 hours. Colony counts were determined using a light microscope. All samples were run in duplicate and the average colony count was recorded. All negative control plates of quarter milk alone had no colonies of *Mycoplasma* present. The proportion of growth in the presence of each compound was compared to the growth of untreated controls, which were set at 1.

5.4 Results

The two hour absorbance readings did not indicate mycoplasmastatic action for any of the natural compounds. Monocaprylin, caprylic acid, and EGCg were unable to inhibit growth of *M. bovis*. It was determined that 32 of the 480 TimTec natural library compounds inhibited the growth of *M. bovis* at 10µM (Table 5.1). Following the first screening, each of the 32 compounds with inhibitory effects was tested alone in order to determine the effect of each compound on absorbance readings in the presence of CCK-8. It was shown that each of the 32 compounds did not increase absorbance above the levels of enrofloxacin or florfenicol in the absence of cells and were equivalent of media with 10% DMSO alone controls. It was shown that neither a 40µM concentration of natural compound or DMSO led to color change in the presence of 5% alamarBlue (resazurin), a redox reagent.

Based upon available pharmacology data, 7 of the 32 compounds (Table 5.1) were determined to be the best candidates for further testing. Compounds that were not tested further despite showing reduction of Mycoplasma growth included hormones and current compounds used in human cardiac and cancer therapies (Table 5.1). The seven compounds of interest were further tested against *M. bovis* at three concentrations of toxicant, 20µM, 10µM, and 1µM. Growth of *M. bovis* treated with the compounds of interest is shown to be dose-dependent (Figure 5.1). Testing indicated the absorbance levels of (+)-alpha-tocopherol acid succinate at 0.5918, 0.6803, 0.5352; eriodictyol at 0.5435, 0.5161, 0.4160; dihydrotachysterol at 0.6532, 0.6048, 0.1692; L-aspartic acid at 0.719, 0.583, 0.3006; S-carboxymethyl-L-cysteine at 0.7061, 0.3901, 0.3294; 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid at 0.7160, 0.4123, 0.2223; and methanesulfonic acid at 0.6010, 0.3826, 0.1769 for 1µM, 10µM, and 20µM, respectively. The average untreated cells absorbance level was 0.8207. Florfenicol (512µg/mL) and enrofloxacin (3.2µg/mL) showed average absorbance readings of 0.132 and 0.1052, respectively.

In order to determine the effectiveness of the seven natural compounds of interest in a complex environment against *M. bovis (*~$2x10^4$ cfu/mL), each was tested in fresh, non-mastitic, quarter milk samples (somatic cell count 3/µL) at 1µM, 10µM, 20µM, and 40µM toxicant concentrations. At time 0 treated samples had the same level of growth as the untreated control samples. At 3 hours the seven natural compounds at 20µM, and 40µM concentrations showed proportions of growth similar to florfenicol (512µg/mL) (Figure 5.2). The 24 hour time point indicates that all 4 concentrations of natural compound have a similar level of impact for L-aspartic acid and S-carboxymethyl-L-cysteine (Figure 5.3). The 24 hour time point indicates that all 4 concentrations of L-aspartic acid and S-carboxymethyl-L-cysteine caused a similar level of inhibition whereas (+)-alpha-tocopherol acid succinate, eriodictyol, and 3-[(2E)-3-(3,4-

dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid showed poor inhibition at a 1μM concentration at 24 hours (Figure 5.3). Based upon the results, there is a dose-dependent effect of each compound relating to inhibiting growth of *M. bovis* in a fresh quarter milk environment.

5.5 Discussion

The purpose of this study was to determine whether novel small molecule compounds have the potential to inhibit growth of *Mycoplasma bovis*. Recent developments in the understanding of the effects of natural compounds has lead to increased interest in using natural compounds to combat microbial and viral infections, increase airway space, to reduce production of mucus in the airways or reduce production of reactive radicals (Ahmed et al., 2009; Gillissen et al., 1991; Johnson et al., 2009; Lee et al., 2007; Matsuo et al., 2005; Rahman, 2008; Richmond, 1962; Takeda et al., 2005; Yasuda et al., 2006). A tetrazolium assay was selected to conduct these experiments due to previous success in *Mycoplasma* studies and suitability as a drug screening method (Bredt, 1976; Kirchhoff et al., 1992; Mosamnn, 1983).

However, natural compound antioxidants, such as thiol compounds, inducers of glutathione biosynthesis, antioxidant vitamins, and polyphenols are all capable of effecting the reactive oxygen species (ROS) and reactive nitrogen species (RNS) actions which may interfere with testing conditions (Aghdassi et al., 1999; Allard et al., 1994; Cross et al., 1994; Habib et al., 1999; Huang et al., 2009; Lykkesfeldt et al., 2000; Rahman, 2008). Previous reports demonstrated the potential of compounds with thiol group to interfere with MTT assays (Natarajan et al., 2000). The data demonstrates that CCK-8, which is similar to MTT, is not impacted by the selected compounds that contain thiol groups, such as (+)-alpha-tocopherol acid succinate. In addition to

testing the 32 compounds shown to have ability to inhibit growth of *M. bovis* by testing the impact of the compounds on the assay alone, the seven natural compounds selected for testing in milk were tested in the presence of a redox reagent under the assay conditions. Since compounds did not produce a color change, which would falsely indicate bacterial growth had occurred, it was demonstrated that compounds are unlikely to interfere with the cytotoxicity assay.

The ability to appropriately screen small natural compounds for ability to inhibit growth of *Mycoplasma bovis* with a high quality, high throughput assay is necessary to identify compounds with the potential for further testing. The Z-factor is sensitive to changes in data variability, which is capable of being tightly controlled (i.e. low standard deviation or a narrow dynamic range) by the procedure, instrumentation and compound concentration selection (Zhang et al, 1999). Based upon the acquired Z-factor value of 0.6749 the assay used for this study should be considered appropriate and capable of identifying "hits" which are compounds capable of inhibiting growth of *M. bovis.*

The results of the initial small molecule library screen indicated that 32 compounds had the ability to inhibit growth of *M. bovis*. Many of the compounds, such as hormones or contraceptives, would be inappropriate for use in cattle so they were not tested further. The seven compounds selected for testing across a selection of concentrations to see the potential for a dose dependent response. A dose dependent response was noted for all 7 of the compounds. Although none of the compounds at 20µM were able to inhibit growth to the degree of florfenicol and enrofloxacin. It is anticipated that higher concentrations of the small molecule natural compounds may inhibit growth further. The concentrations of florfenicol (512µg/mL) and enrofloxacin (3.2µg/mL) used in this study were higher than the *M. bovis* PG45 minimum inhibitory concentration (MIC) level of 4µg/mL and 0.4µg/mL, respectively. The testing of multiple

concentrations of each antimicrobial in the screening panels indicated no change in the level of inhibition, therefore the highest concentration of each was selected for use in further testing.

The compounds which were most effective at a the20μM concentration were methanesulfonic acid and dihydrotachyesterol in the dose-response testing. Dihydrotachyesterol at a 40μM was also the most effective at both 3 hours and 24 hours in the fresh quarter milk experiments. This may be due to interactions with the somatic cells and lipids present in the milk. These results also indicate that the compounds begin to inhibit growth by 3 hours as indicated by no inhibition of *M. bovis* at time zero and growth inhibition by hour 3. Colony counts increased on each plate at time 3 and 24, but the overall proportion of growth as compared to the untreated control is reduced at both time points. This indicates that compounds act in a mycoplasmastatic, not mycoplasmacidal manner.

Although many natural compounds have been shown to have antibacterial or antiviral abilities some natural compounds, such as S-carboxymethyl-L-cysteine, are also capable of acting as an anti-mucolytic and reducer of ROS and RNS (Nogawa et al., 2009; Panagopoulos et al., 2010; Yasuda et al., 2006). It has been used for normalization of airway responsiveness and reduction of ROS *in vitro* in human cell lines and *in vivo* in mice, dogs, sheep, and calves (Nogawa et al., 2009; Panagopoulos et al., 2010; Takeda et al., 2005; Yasuda et al., 2006). Compounds with antibacterial and antiviral capabilities that also possess the ability to normalize airway responsiveness offer intriguing opportunities to develop into therapeutics as well as serve as a platform to elucidate the cellular-compound interactions leading to antimicrobial ability. These compounds offer a viable alternative to traditional antimicrobial agents allowing farms to wean away from the use of less effective antimicrobials. Reports of increasing antimicrobial resistance in Europe and North America and the controversial use of certain antimicrobials, such as fluoroquinolones, has increased the need for alternative strategies (Nicholas et al., 2008).

Although the prophylactic use of antimicrobials is generally undesirable, the use of small molecule natural compounds may alleviate the need for traditional antimicrobial agent prophylactic uses. The potential applications in organic and natural farming offer a new method for treating or preventing difficult to control diseases, such as *M. bovis*-associated mastitis. This is especially important since the United States Food and Drug Administration does not support the use of unapproved products in food animals. The use of an antimicrobial in a dairy cow results in a loss of the "organic" status (Reugg, 2009). However, there is no currently approved therapeutic capable of treating mycoplasma mastitis for organic farmers (Reugg, 2009). Small molecule natural compounds may have the potential to serve the organic and naturally farming communities in an effective and safe manner, although there are not automatically approved for use.

5.6 Conclusions

The data suggest that CCK-8 may be successfully used for high-throughput screening of small molecule compounds for ability to inhibit growth of *M.* bovis. The overall results of the study suggest that the small molecule natural compounds methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate are capable of inhibiting the growth of *M. bovis* in a dose-dependent manner suiting the compounds for potential therapeutic and prophylactic uses. Further, the ability to inhibit growth of *M. bovis* in milk suggests that each of the compounds is likely mycoplasmastatic due to the increased colonies numbers at 24 hours despite the reduced proportion of growth as compared to the controls. Since most antimicrobials used to treat *M.*

bovis infections are static, it is possible that natural compounds may be further developed for use on organic farms. Based upon this information, these natural compounds have the potential to serve as therapeutics and prophylactics lessening the dependency on current antimicrobials to which bacteria show increasing resistance, allowing for a promotion of animal and human health and well-being.

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Table 5.1 List of compounds shown to inhibit growth of *Mycoplasma bovis* using an *in-vitro* tetrazolium salt reduction assay. Bolded compounds were selected for further study.

Name of Compound	Chemical Formula
(+)-(4,6-O-Benzylidene)methyl-alpha-D-glucopyranoside	$C_{14}H_{18}O_6$
9-methoxy-2,2-dimethyl-2H,5H,6H-pyrano[3,2-c]quinolin-5-one	$C_{15}H_{15}NO_3$
2'-Deoxycytidine 5'-monophosphate	$C_9H_{14}N_3O_7P$
9-[3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]hydropurin-6-one	$C_{10}H_{12}N_4O_5$
(1S,7aS)-1-hydroxy-2,3,5,7a-tetrahydro-1H-pyrrolizin-7-yl 2-hydroxy-3-methoxy-2-(propan-2-yl)butanoate	$C_{15}H_{25}NO_5$
(5aS)-3,5a,9-trimethyl-2H,3H,3aH,4H,5H,5aH,8H,9bH-naphtho[1,2-b]furan-2,8-dione	$C_{15}H_{18}O_3$
disodium[(2R,3S,4R,5R)-5-(2-amino-6-oxo-6,9-dihydro-1H-purin-9-yl)-3,4- dihydroxyoxolan-2-yl]methyl phosphate	$C_{10}H_{14}N_5Na_2O_8P$
Brucine	$C_{23}H_{26}N_2O_4$
(3R)-3-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5 trihydroxycyclohexane-1-carboxylic acid	**$C_{16}H_{18}O_9$**
Methanesulfonic acid	**$C_{34}H_{41}N_5O_8S$**
S-Carboxymethyl-L-cysteine	**$C_5H_9NO_4S$**
Isosorbide, 98%	$C_6H_{10}O_4$
Deoxycorticosterone acetate	$C_{23}H_{32}O_4$
L-Aspartic Acid	**$C_4H_7NO_4$**
(-)-6beta-Hydroxymethyl-7alpha-hydroxy-cis-2-oxabicyclo[3.3.0]octan-3-one	$C_8H_{12}O_4$
Dihydrotachysterol	**$C_{28}H_{46}O$**
17a-Hydroxyprogesterone hexanoate	$C_{27}H_{40}O_4$
Himbacine	$C_{22}H_{35}NO_2$
Estradiol valerate	$C_{23}H_{32}O_3$
Lanatoside C	$C_{49}H_{76}O_{20}$
Adenosine 5'-triphosphate disodium salt	$C_{10}H_{14}N_5O_{13}P_3$
Beta-estradiol 17-cypionate	$C_{26}H_{36}O_3$
Lividomycin A sulfate salt	$C_{29}H_{55}N_5O_{18}$
(+)-alpha-Tocopherol acid succinate	**$C_{33}H_{54}O_5$**
Thyroliberin	$C_{16}H_{22}N_6O_4$
Eriodictyol [552-58-9] (3',4',5,7-tetrahydroxyflavanone)	**$C_{15}H_{12}O_6$**
Chlormadinone acetate	$C_{23}H_{29}ClO_4$
Proscillaridin A	$C_{30}H_{42}O_8$
L-Prolinamide	$C_5H_{10}N_2O$
Clindamycin 2-phosphate	$C_{18}H_{34}ClN_2O_8PS$
Khellin	$C_{14}H_{12}O_5$
Rolitetracycline	$C_{27}H_{33}N_3O_8$

Figure 5.1 Growth inhibition of *M. bovis* using selected natural compounds

Comparison of the ability of small molecule natural compounds at multiple concentrations to inhibit the growth of *M. bovis*. Florfenicol and enrofloxacin are negative ("kill level") controls. Growth of *M. bovis* is represented by increased levels of absorbance using a microplate reader (450nm) in the presence of CCK-8, a tetrazolium salt.

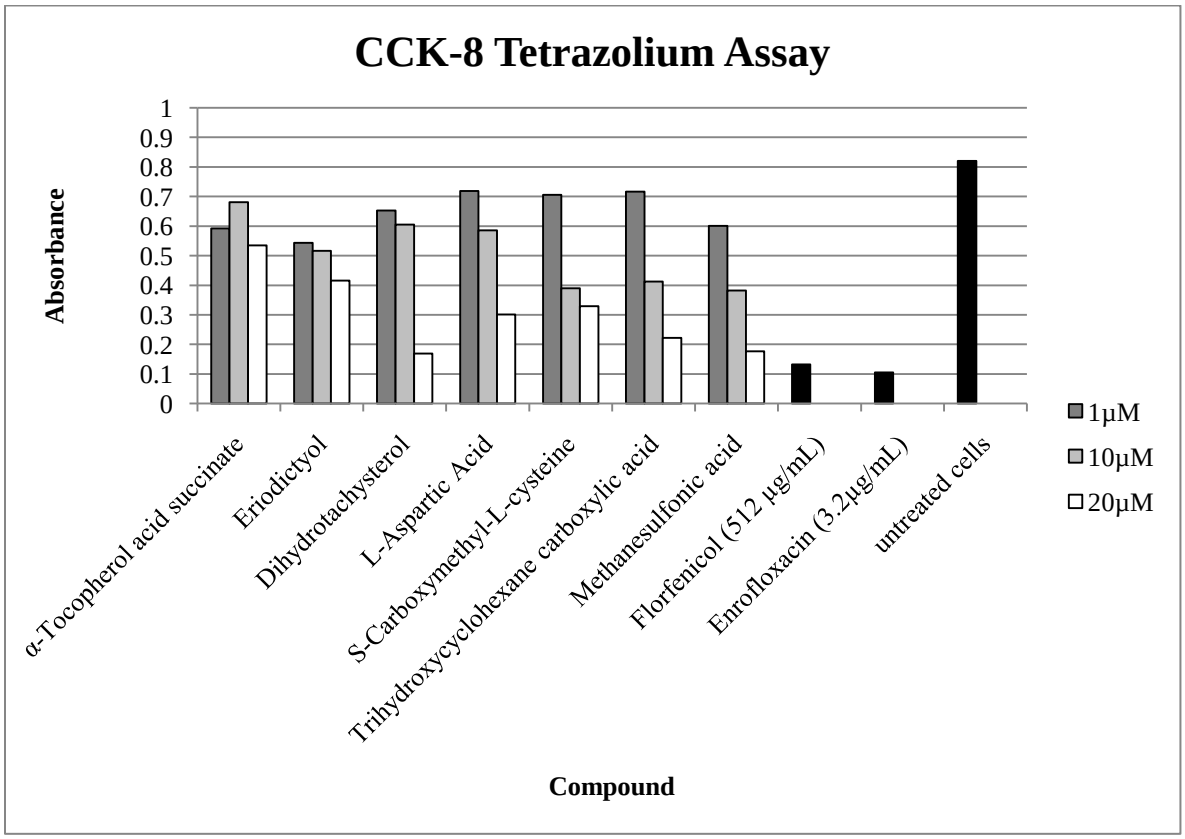


Figure 5.2 Relative growth of *M. bovis* in the presence of selected natural compounds in fresh quarter milk at 3 hours

The relative growth of *M. bovis* in fresh quarter milk in the presence of increasing levels of small molecule natural compounds as compared to untreated cells (set at 1). Samples were plated on pleuropneumonia-like organism agar (PPLO) 3 hours after the addition of natural compound and incubated for 48 hours at 37°C, 5% CO_2.

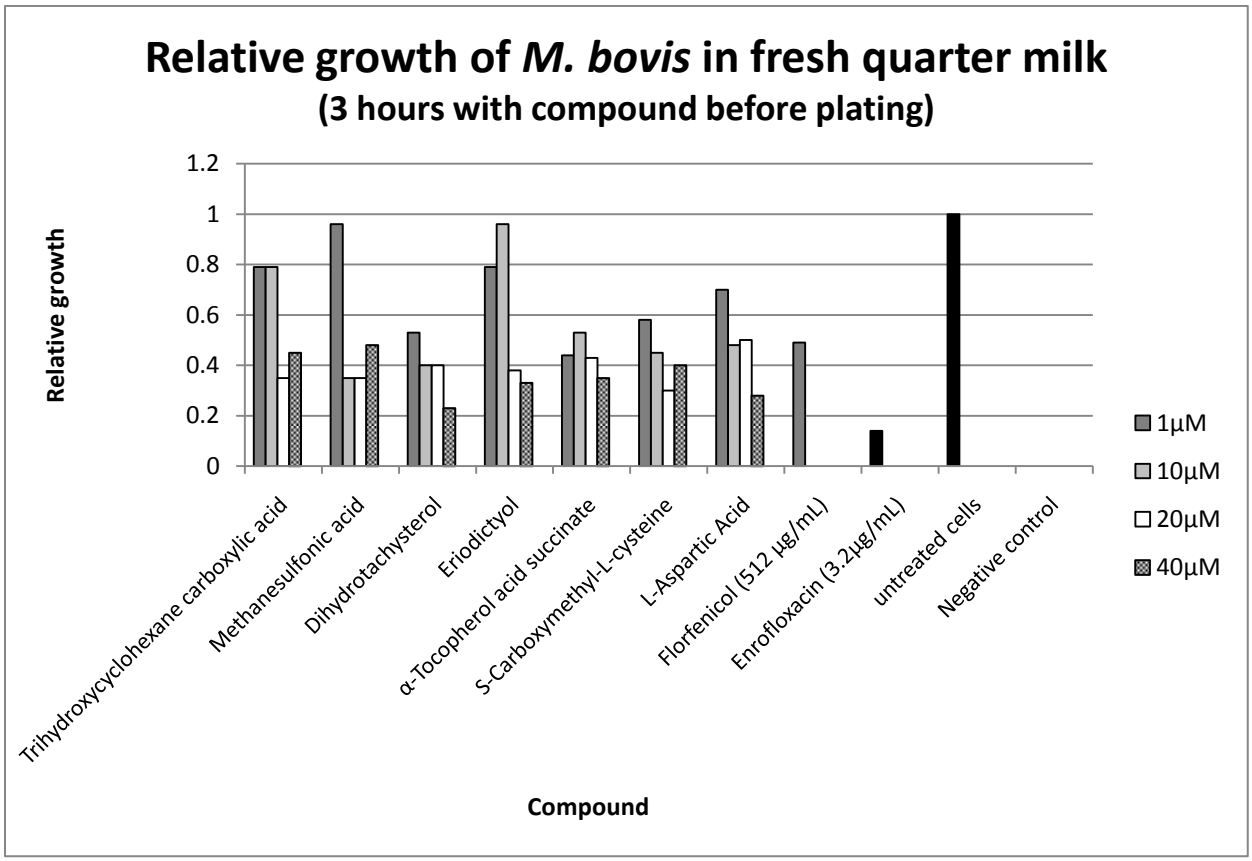
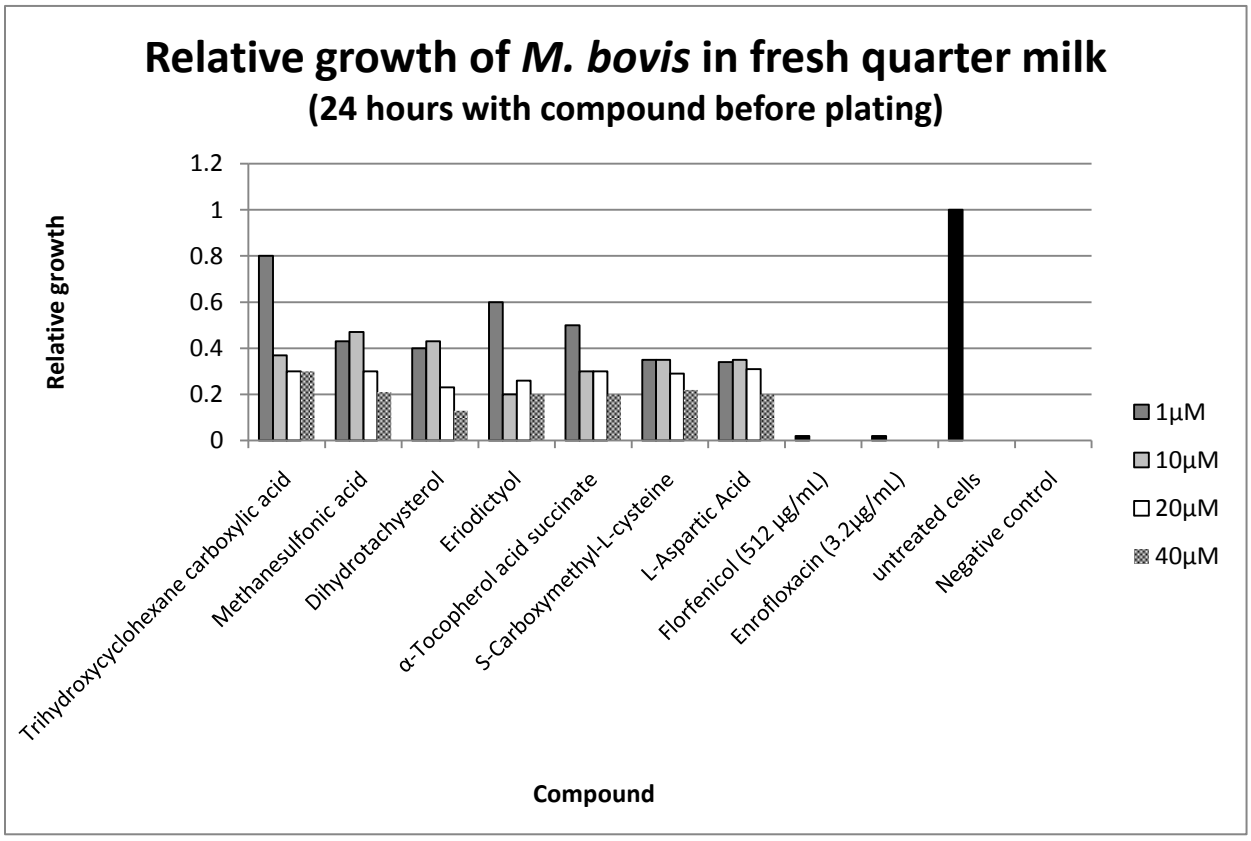


Figure 5.3 Relative growth for *M. bovis* in the presence of selected natural compounds in fresh quarter milk at 24 hours

The relative growth of *M. bovis* in a fresh quarter milk in the presence of increasing levels of small molecule natural compounds as compared to untreated cells (set at 1). Samples were plated on pleuropneumonia-like organism agar (PPLO) 24 hours after the addition of natural compound and incubated for 48 hours at 37°C, 5% CO_2.



Chapter 6

Epidemiology of *Mycoplasma bovis* in Pennsylvania veal calves

6.1 Abstract

Pennsylvania is one of the major special-fed veal raising areas in the United States. The objective of the study was to estimate the prevalence and incidence of *Mycoplasma bovis*, a common cause of pneumonia, in veal calves. Using simple random sampling, nasal swabs were collected from 252 calves from 4 veal herds located in central Pennsylvania. Nasal swabs were collected on a monthly basis. Calves were followed from veal farms to a USDA approved processing facility where lung lesions and swabs collected from the bronchial bifurcation were collected post-slaughter. Swabs were cultured for *M. bovis* and identification of isolates confirmed through polymerase chain reaction (PCR) using the *M. bovis* specific primers targeting the *uvr*C gene. The period prevalence of *M. bovis* colonization was 90.5% for the nasal swabs and 38.0% for the bronchial swabs. A total of 90 lung lesions were identified of which 42.2% were culture positive for *M. bovis*. Co-infections of *M. bovis* and *Pasteurella multocida* or *Mannheimia haemolytica* were present in 8 (8.9%) of the 90 lung lesions collected. In this study, *M. bovis*, in concert with other respiratory pathogens, is responsible for pneumonic lesions in veal calves. Our data suggest that nasal swab testing on special-fed veal farms can indicate which calves are at increased risk, but are unable to identify differences in the weight gain of colonized calves.

6.2 Introduction

Mycoplasma bovis, an important etiologic agent of calf pneumonia, is also commonly associated with arthritis, mastitis, conjunctivitis, and otitis (Nicholas and Ayling, 2004). An estimated one-quarter to one-third of pneumonia-related illnesses in growing cattle may be attributed to *M. bovis* infections (Nicholas et al., 2000). Additionally, cattle infected with *M. bovis* may serve as a reservoir of infection by shedding the bacteria from the respiratory tract for multiple months following initial colonization (Pfutzner et al., 1990). Calves less than 4 months of age are at a highest risk of respiratory disease due to *M. bovis* (Stipkovits et al., 2000; Nicholas and Ayling, 2004). *Mycoplasma bovis* may act as a predisposing factor that weakens the host immune system leading to invasion by other pathogenic bacteria or viruses, which may explain the chronic and polymicrobial nature of *M. bovis* infections (Rosengarten and Citti, 1999; Snowder et al., 2006). The severity of calf pneumonia is further compounded by animal husbandry, the environment, low efficacy of many antimicrobials, and unknown efficacy of vaccines (Nicholas et al., 2000, Nicholas et al., 2009).

Little is known about the age of onset and duration of nasal shedding of *M. bovis* in young calves. It has been suggested that *M. bovis* prevalence peaks between 1 and 4 months of age, but it is not known which point in those months is highest regardless of differences between local conditions (Bennett and Jasper, 1977). Special-fed veal calves are reared in temperature-controlled housing in which they can interact with neighboring calves, potentially leading to the transmission of *M. bovis*. Calves may become infected with *M. bovis* within the first days of life through ingestion of infected colostrum from the dam (Pfutzner and Sachse, 1996). Airborne exposure is likely a major route of transmission in barns (Jasper et al., 1974).

Estimates of the prevalence of *M. bovis* infection on farms are wide ranging with reports around the world between 26% and 100% (Langford, 1977; Pignatelli, 1978; Muenster et al.,

1979; Adegboye et al., 1995; Poumarat et al., 2001; Arcangioli et al., 2008; Gerchman et al., 2009). One pair of reports from Ontario, Canada noted that *M. bovis* was isolated from 98% of cattle with chronic pneumonia (Gagea et al., 2006a). Another report from Ontario, Canada indicated 46% of healthy calves had *M. bovis* infection (Gagea et al., 2006b). Since there are few reports of *M.* bovis-associated pneumonia in young calves it is difficult to estimate the impact of the individual components of multifactorial diseases, such as pneumonia. Currently there are no reports regarding the incidence, prevalence, or attack rates of veal calf pneumonia associated with *M. bovis* in the United States.

The similarity of age, sex, barn temperature, and diet of special-fed veal calf populations from this study are especially advantageous to research of infectious diseases that affect young calves. The objectives of this study were to: 1) determine the incidence, prevalence, and attack rates of *M. bovis* in Pennsylvania special-fed veal calves, 2) examine whether *M. bovis* found through nasal swab screening is associated with disease of the lower respiratory tract and, 3) assess the impact of the veal housing environment upon disease.

6.3 Materials and Methods

6.3.1 Study Populations and Sample Collection

The largest veal production company in the United States was recruited for participation in this study. According to company policy, healthy unaltered 3-7 day old male dairy calves with an average weight of 105 lbs (~48kg) were purchased from livestock auctions in Pennsylvania and lower New York and transported to one of four cooperating veal growers. Calves were cared for according to the standard operating procedures of the veal production company. Individual sick calves were treated per normal farm protocols under the supervision of their veterinary

practitioner. Diet consisted of an all-milk milk replacer containing iron and ~40 other essential nutrients and routine veterinary care, including vaccination against BVDV (types 1 and 2), BHV-1, BRSV, and PI_3.

Calves in three (herds 2, 3, 4) of the four herds were housed in separate stalls. Calves in herd 1 were housed 2 per stall; dividers in each stall separated the calves until approximately 8 weeks of age when dividers were removed. There were a total of three different managers for the four selected herds. The distance between herds ranged between 10 miles (16km) and 40 miles (64km). There were different managers/workers for herds 1 and 4, while herds 2 and 3 shared the same manager/workers. Herds 2 and 3 were raised during the time period of October, 2008 to March, 2009 and April, 2009 to August, 2009, respectively. Herd 1 was raised during September, 2008 to January, 2009. Herd 4 was raised during April, 2009 to July, 2009. When the calves reached a sale weight of ~450 lbs (~145 days of age), they were shipped to a common facility for slaughter and processing.

A total of 252 calves from 4 herds (identified by ear tag) were selected based upon simple random sampling following random assignment to a stall upon entry into each barn and subsequently sampled for bacterial respiratory pathogens. A sterile rayon-tipped swab with polyurethane plastic shaft (BBL™ Aimes media CultureSwab™) was inserted in the nostril to a depth of ~4 inches. Sampling of calves began 3 days after arrival in the barn and at 30-40 day intervals thereafter; ending with a final swab collection 7 days prior to slaughter, for a total of 4-5 samplings. Swabs were transported on ice and cultured within 7 hours of collection.

At slaughter, an incision was made into the trachea at the bronchial bifurcation and a swab was inserted into the insertion for sampling the bifurcation. Lung lesions were identified by visual inspection for lesions characteristic for pleural pneumonia, pleurtitis, or gross pathology consistent with pneumonia and excised by a veterinarian (DRW, JWB, and BMJ). All bronchial

swabs and lung lesions were transported on ice and cultured within 24 hours of collection. A representative sample of sections of tissue (n=9) were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) stain at the Pennsylvania State University Animal Diagnostic Laboratory, University Park, PA. Histopathology was performed by a veterinary pathologist (ALH).

Lung lesion morbidity due to *M. bovis*-associated infection and upper-respiratory tract colonization associated with *M. bovis* were the major outcomes of interest. The impact of *M. bovis* upon weight gain was a secondary outcome of interest.

6.3.2 Environmental Air Sampling

Environmental air samples were collected using the all glass impinger bio-aerosol system, Vac-U-Go (SKC, Inc., Eighty-Four, PA) with a 12.5 L/minute air intake. The sampler was run from a height of 0.4 meters for 30 minutes in the center of the barn. Sampling was performed prior to entry of calves and at approximately one-month intervals after calves arrived for a total of 22 sample collections. The sampling medium placed in the collection vial of the impinger was a PPLO broth with Mycoplasma enrichment without inhibitors (Becton Dickinson, Sparks, MD). No antifoam A was added as no issues of foaming occurred during sampling during the preliminary testing. Preliminary testing with an aerosolized *M. bovis* sample (ATCC 25523) under a closed laminar hood indicated the ability of the media and unit to adequately collect sample. The sample was stored on ice in a cooler for transportation to the lab. Samples were cultured within 7 hours of collection.

6.3.3 Culturing

6.3.3.1 Calves

In the laboratory, the surface of the lung lesion tissue was seared. An incision was then made with a sterile scalpel and a sterile cotton tipped swab (Puritan Medical Products, Guilford, ME) was inserted into the core of the tissue sample. Nasal swabs, bronchialal bifurcation swabs, and lung lesion swabs were cultured on blood agar (BA) (Becton, Dickinson and Co., Sparks, MD), MacConkey (MAC) agar (Becton, Dickinson and Co., Sparks, MD) at 37°C in an aerophilic environment, and chocolate agar (CA) (Becton, Dickinson and Co., Sparks, MD) at 37°C in a microaerophilic environment. Standard biochemical identification procedures and API (BioMerieux, France) for identification of common bovine respiratory pathogens was used. All swabs were then enriched in Mycoplasma enrichment broth supplemented with penicillin (UC Davis Biological Media Services, Davis, CA) for 48 hours before being plated on pleuropneumonia-like organism (PPLO) agar plates (UC Davis Biological Media Services, Davis, CA). All mycoplasma cultures were grown at 37°C in a microaerophilic environment. PPLO agar plates were considered negative if no growth was noted by day 10.

Confirmation of mycoplasma growth was performed using PCR of the *uvr*C *M. bovis* housekeeping gene as described by Thomas et al. (2004) and general mycoplasma primers. DNA was extracted from 3 mL aliquots of stationary-phase growth using the phenol-chloroform procedure previously described (Sambrook and Russell, 2001; Pospiech and Newmann, 1995). Briefly, culture pellet was suspended in a Tris-EDTA solution (pH 7.5) with lysozyme and treated with proteinase K, before addition of phenol-chloroform. The aqueous phase was washed multiple times using ethanol and resuspended in sterile water. The American Type Culture Collection (ATCC) *M. bovis* standard strain PG45, accession number 25523, was used as a positive control.

6.3.3.1 Air Samples

Aliquots of inoculum (100µL each) were spread-plated on BA, MAC, CA, Sabouraud Dextrose agar (SDA) (Becton, Dickinson and Co., Sparks, MD), and PPLO agar. BA and MAC were incubated at 37°C under aerophilic conditions. One plate of SDA was incubated at 32°C and another at 37°C under aerophilic conditions. The CA and PPLO were incubated at 37°C in a microaerophilic environment. Standard biochemical identification procedures and API (BioMerieux, France) for identification of bacteria was used.

6.3.4 Statistical Methods

Risk factors for the categorical outcome variables were compared using chi-square tests, while risk factors for quantitative outcome variables were compared using independent sample *t*-tests using GraphPad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA). 95% confidence intervals (CI) were calculated using the modified Wald method. For the cumulative incidence the goodness of fit for the curve linearity was determined using R-squared analysis (Microsoft Office Excel 2007). The Odds Ratio (OR) was calculated using 2x2 contingency tables using EpiInfo2002 v 3.5.1 (Atlanta, GA). The p-value of OR analysis was determined using Fisher's exact test. One-way ANOVA and z-test (independent groups) was performed using SAS (Cary, NC). For two-sided tests, a p-value of <0.05 was considered significant.

6.4 Results

6.4.1 Upper-Respiratory Tract Colonization and Lung Infection

Samples were collected from a total of 252 calves (3 additional calves died before completion of sampling and were considered lost to follow-up and not included in any statistical

analysis) which represented 25% of the total population of the 4 herds. The period prevalence of *M. bovis* colonization across all observation points and herds sampled was 90.5% (95% CI, 86.2%-93.6%) for the nasal swabs and 40.0% (95% CI, 32.3%-44.2%) for the bronchial swabs. The highest point prevalence of *M. bovis* colonization from nasal swabs in herds 1, 2, 3, and 4 was 76.0%, 64.0%, 90.4%, and 70.0% was acquired after day 50 for each herd, respectively. For bronchial swabs the prevalence at slaughter was, 71.0%, 40.0%, 23.1%, and 31.1%, respectively. The overall percentage of calves with at least one positive nasal swab were 77.8%, 90.0%, 92.3%, and 93.0% for herds, 1, 2, 3, and 4, respectively (Table 6.1). The attack rate for *M. bovis* upper respiratory tract colonization was shown to be highest after 50 days of age for all the herds sampled (Table 6.1). Herds 1, 2, and 4 had the highest attack rate between 81-140 days of age, while herd 3 had the highest between 51-80 days of age. The cumulative incidence rate for each herd is similar to the attack rate; however herds 2 and 3 indicate approximately level attack rates between 51 and 140 days of age while the cumulative incidence continues to increase. Herds 1 and 4 indicate the largest increase in attack rates between 81-140 days of age. The cumulative incidence of *M. bovis* URT colonization ($R^2 = 0.9655$) for each herd is shown in Figure 6.1.

Ten calves from Herd 4 were unable to be identified by ear tag at the abattoir and were considered lost to follow-up for all calculations involving bronchial swabs and lung lesions. A total of 90 (37.2%; 95% CI 31-43%) lung lesions were identified from the 242 individual calves sampled at slaughter for the 4 herds, of which 42.2% contained *M. bovis* (Table 6.2). Herd 1, herd 2, herd 3, and herd 4 represented 27, 4, 37, and 22 of the total lung lesions collected, respectively. *Mycoplasma bovis* was successfully cultured from 15, 3, 7, and 12 lung lesions collected from herds 1, 2, 3, and 4, respectively. Ninety-four percent of calves that were culture positive at slaughter also had at least one culture positive nasal swab during the study. Lung lesions were 2 times more likely to be observed in calves that had at least one *M. bovis* positive nasal swab

(OR=1.99; 95% CI 0.45-8.87). A single *M. bovis* positive nasal swab was not shown to be linked to positive bronchial swab (OR=0.17; 95% CI 0.06-0.45). Histopathology of a representative sample of collected tissues (n=9) were each reported to have minimal, mild or moderate multifocal interstitial pneumonia.

Co-infections of *M. bovis* and either *P. multocida* or *Mannheimia haemolytica* were present in 11 (12.2%) of the 90 lung lesions (Table 6.2). A total of 1 (3.7%), 3 (75%), 3(8.1%), and 4 (18.2%) lung lesions were associated with more than one respiratory pathogen for herd 1, herd 2, herd 3, and herd 4, respectively. *Pasteurella multocida* was found concurrently with *M. bovis* most often. Calves with a member of the Pasteurellaceae family found in lung lesions were 10.8 (OR=10.79; 95% CI 2.23-52.32) times more likely to also have *M. bovis* present in the lesion than *Pasteurellaceae* alone. Forty-two percent of bronchial swabs had at least one respiratory bacterial pathogen cultured. The most frequently cultured respiratory pathogen from lung lesions (42.2%) and bronchial swabs (37.0%) was *M. bovis* (Table 6.2). It was determined that calves with a member of the *Pasteurellaceae* family found in bronchial swabs were not significantly more likely to also have *M. bovis* present at the bronchial bifurcation than *Pasteurellaceae* alone (OR=1.26; 95% CI 0.37-4.32).

6.4.2 Effects of M. bovis on Weight Gain

Individual calf weights at time of entry into the barn and the dress weights of the calves at the slaughterhouse were normally distributed for each individual herd and across herds. The average dress weight (carcass weight) for calves from herd A, herd B, herd C, and herd D was 112.45 ±16.90, 126.05 ± 13.21, 126.05 ± 11.02, and 125.30 ± 13.19 kg, respectively. There was a significant difference between the dress weight of calves from herd A and the other herds (p=0.0001). Difference in the dress weight for calves with *M. bovis* culture positive lung lesions

compared to calves without lung lesions was not significant for any herd. The difference in dress weight for calves with *Pasteurellaceae* culture positive lung lesions compare to calves without lung lesions was not significant. The overall mortality rate for calves in the 140 day study was 11.88 per 1,000 calves.

6.4.3 Environmental Air Sampling

Neither *Mycoplasma* nor *Pasteurella* species were isolated the air samples (n=22). The genera of bacteria that were identified from the air samples included *Staphylococcus, Aerococcus, Micrococcus, Kocuria, Enterococcus, Lactococcus, Pseudomonas,* and *Bacillus (*Table 6.3).

6.5 Discussion

Bovine respiratory disease (BRD), an infectious bronchopneumonia, is a disease complex associated with a variety of bacterial and viral pathogens and environmental/host factors. BRD is the most common and costly disease of U.S. feedlots (Snowder et al., 2006; Griffin, 1997) and one of the most common diseases of veal operations (Catry et al., 2008). The major bacterial pathogens of BRD include *Mycoplasma bovis, Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Arcanobacterium pyogenes* (Bryson, 1985; Sivula et al., 1996; Mosier, 1997). The viral pathogens associated with BRD include, bovine herpes virus (BHV-1), bovine viral diarrhea virus (BVDV), bovine respiratory synctial virus (BRSV), and parainfluenze type 3 virus (PI_3) (Snowder et al., 1999). It is suggested that *M. bovis* and *P. multocida* are frequently secondary pathogens, but they have been associated with the chronic versions of BRD while having poor treatment success (Smith, 2009; St. Jean, 1997). *Mycoplasma bovis* has been increasingly implicated in veal calf populations of Europe and Canada, with some reported

seroconversion rates of 65-100% occurring in feedlots (Ter Laak et al., 1992; Gagea et al., 2006b; Arcangioloi et al., 2008; Radaelli et al., 2008). The objectives of this study were to gain an understanding of the role *M. bovis* and other bacterial respiratory pathogens in the special-fed veal industry of Pennsylvania. Four herds were selected to survey for the impact of *M. bovis.* According to standard operating procedures, all calves in the study herds were vaccinated against BVD (types 1 and 2), IBR, BRSV, and PI_3 infection. Viral and bacterial co-infections are likely to be decreased in the study populations due to vaccinations against common BRD viruses, but viral titers were not performed in this study.

Mycoplasma bovis was not isolated in environmental air samples collected monthly from the veal raisers facilities during this study. There is one previous report of *M. bovis* being successfully cultivated from the air by leaving an agar plate open in the barn (Jasper et al., 1974). Preliminary testing of the sampling media and all-glass impinger collection vessel in a closed biological hood with aerosolized *M. bovis* (ATCC 25523) indicated the ability of the system in our laboratory to be used to successfully culture *M. bovis* from the air. This method should not be directly used to imply the absence of *M. bovis* from the air of the facilities in this study. It is possible that sample collection times, transport, the number colony forming units of *M. bovis* present in the air, or the presence of other air-borne bacteria, viruses, and fungi may impact the ability to cultivate *M. bovis* in this study. Other bacteria were able to be successfully isolated from the environmental air samples. The largest percentage of bacteria isolated in this study was from the genus *Staphylococcus.*

The shedding of *M. bovis* by dairy calves with no clinical signs of disease has been reported to range from 0 - 30% in feedlots (Bennett and Jasper, 1977; Springer et al., 1982; Boothby et al., 1983; Wiggins et al., 2007). Therefore, since 30% is the higher range it was selected to determine the normal colonization or infection percentage for veal calves used to

calculate the significance. The results of this study indicate that when a lung lesion with *M. bovis* is identified a nasal swab will likely have been positive (94%). However, a *M. bovis* positive nasal swab or bronchial swab was not shown to significantly influence the future presence of a lung lesion associated with *M. bovis* infection. This suggests that nasal swabs should only be used as a screening tool of *M. bovis* colonization in a special-fed veal herd rather than an indicator of risk of developing a lung lesion due to *M. bovis* infection.

The results of this study suggest that *M. bovis*, in concert with other bacterial respiratory pathogens, is likely responsible for pneumonic lesions in veal calves. Of collected lung lesions, it was shown that 41.1% contained *M. bovis*, indicating that *M. bovis* serves an important role in bacterial respiratory disease of Pennsylvania veal calves. The importance of polymicrobial bacterial infections in the formation of pneumonic lesions in the lung is supported by an odds of 10.8 that calves with a lung lesion with a member of the Pasteurellaceae family also has *M. bovis* present in the lesion. As demonstrated in this study, *M. bovis* occurred early in the disease process. The lowest incidence and attack rates were seen in calves prior to 50 days of age. The minor differences between the pattern of attack rates for herds A and D compared to herds B and C may be due to the ventilation systems of the barn. Herd B and C were in fan-ventilated systems while herd A and D were natural ventilation. In agreement with previously published reports, *M. bovis* may therefore be a primary pathogen or initiating factor for polymicrobial bacterial infections (Gourlay and Houghton, 1985). The trends of colonization for *M. bovis* across all animals sampled indicate that vaccines and other prophylactic strategies targeted against *M. bovis* colonization before 50 days old would be likely to reduce the impact of *M. bovis* infection.

Mycoplasma bovis infection in the lung was not shown to have a significant impact on the final dress weight for the 4 special-fed veal calf herds in Pennsylvania. The mortality rate of calves in the study was low, but since field necropsy was not performed was not implicate *M.*

bovis in the mortality. Based upon these results, it is difficult to assess the impact of *M. bovis* on herd health in the United States. Despite this, previous studies have reported that *M. bovis* is often found early in the BRD disease process of veal calves (Gourlay and Houghton, 1985; Arcangioloi et al., 2008). The results of this study indicate that pneumonia associated with *M. bovis* occurred in 15% of the calves sampled at the abattoir. This percentage is lower than the anticipated percentage of calf pneumonia cases in Europe attributed at least in part to *M. bovis* (Nicholas et al., 2002). This may be due to the strict nutritional care and moderated temperature and ventilation afforded to veal calves versus calves on stocker, feeder, or heifer raiser operations, the younger age of calves which were sampled in this study, or the number of infected calves purchased.

6.6 Conclusions

In conclusion, *M. bovis* was able to be isolated from URT via nasal swabs frequently, including within three days of arrival in the barn for one herd. The cumulative incidence, attack rates, and the frequency of multiple bacterial respiratory pathogens being cultured from nasal swabs, bronchial swabs, and lung lesions suggests that *M. bovis* is an initiating factor of respiratory disease in special-fed veal calves from Pennsylvania. For future studies, attack rates and cumulative incidence rates may be used in future studies for modeling of *M. bovis* transmission differences in fan ventilation and natural ventilation barns. There is a high prevalence of *M. bovis* colonization which begins in the first few months of life for calves. This indicates a need for successful vaccines that may induce the immune response of young calves, preferably prior to 50 days of age. Further investigation of the impact of *M. bovis* in special-fed

veal from other regions of the United States, as well as vaccine trials targeting young calves would be beneficial.

6.7 References

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Table 6.1 Summary of incidence rates, attack rates, and mortality for calves in study. Attack rates are reported for *M. bovis* in the upper –respiratory tract (URT) across the common age ranges of each herd.

	Herd 1	**Herd 2**	**Herd 3**	**Herd 4**	**Combined**
Sample size	50	50	52	100	252
Percentage of calves with at least one positive *M. bovis* sample from the URT by 140 days of age (95% CI)[1] (p-value)[2]	77.8% (64.6- 87.4%) <0.001	90.0% (78.2- 96.1%) <0.001	92.3% (81.3- 97.5%) <0.001	93.0% (86.0- 96.8%) <0.001	90.5% (86.2-93.6%) <0.001
Attack rates for *M. bovis* URT colonization (95% CI)					
Age (days old) 0-15	0.0% (0.0-0.09%)	0.0% (0.00-0.09%)	0.0% (0.0-0.06%)	14.0% (0.08-0.23%)	5.6% (3.3-9.2%)
16-50	6.0% (1.4-16.8%)	16.0% (8.1-28.8%)	17.0% (9.2-30.0%)	34.8% (25.6-45.4%)	21.0% (16.3-26.6%)
51-80	10.6% (4.2-23.0%)	66.7% (51.5-79.1%	76.7% (62.1-87.0)	23.2% (14.0-36.0%)	42.0% (35.2-49.2%)
81-140	76.2% (61.3-86.7%)	71.4% (45.0-88.7%)	60.6% (43.7-73.4%)	85.7% (74.0-92.8%)	78.0% (69.3-84.8%)
Mortality					
M. bovis-associated	0	0	1	0	1
All causes	1	0	2	0	3

[1] 95% confidence intervals by the modified Wald method.

[2] P-value determined by Z-test.

Table 6.2 Percentage of Bacterial Respiratory Pathogens in Veal Calves. The percentage of observed respiratory disease agents in lung lesions collected at abattoir and from the bronchial bifurcation for 4 herds of special-fed veal calves from Pennsylvania.

	Percentage 95% CI	
Condition	Lung Lesion + (n=90)	Bronchial Bifurcation + (n=242)
Mycoplasma bovis only	28.9% 20.5-39.0%	36.8% 30.9-43.0%
Pasteurellaceae only	2.2% 0.13-8.2%	1.7% 0.5-4.3%
Co-infection of *M. bovis* and *Pasteurellaceae*	12.2% 6.8-20.7%	3.3% 1.6-6.5%

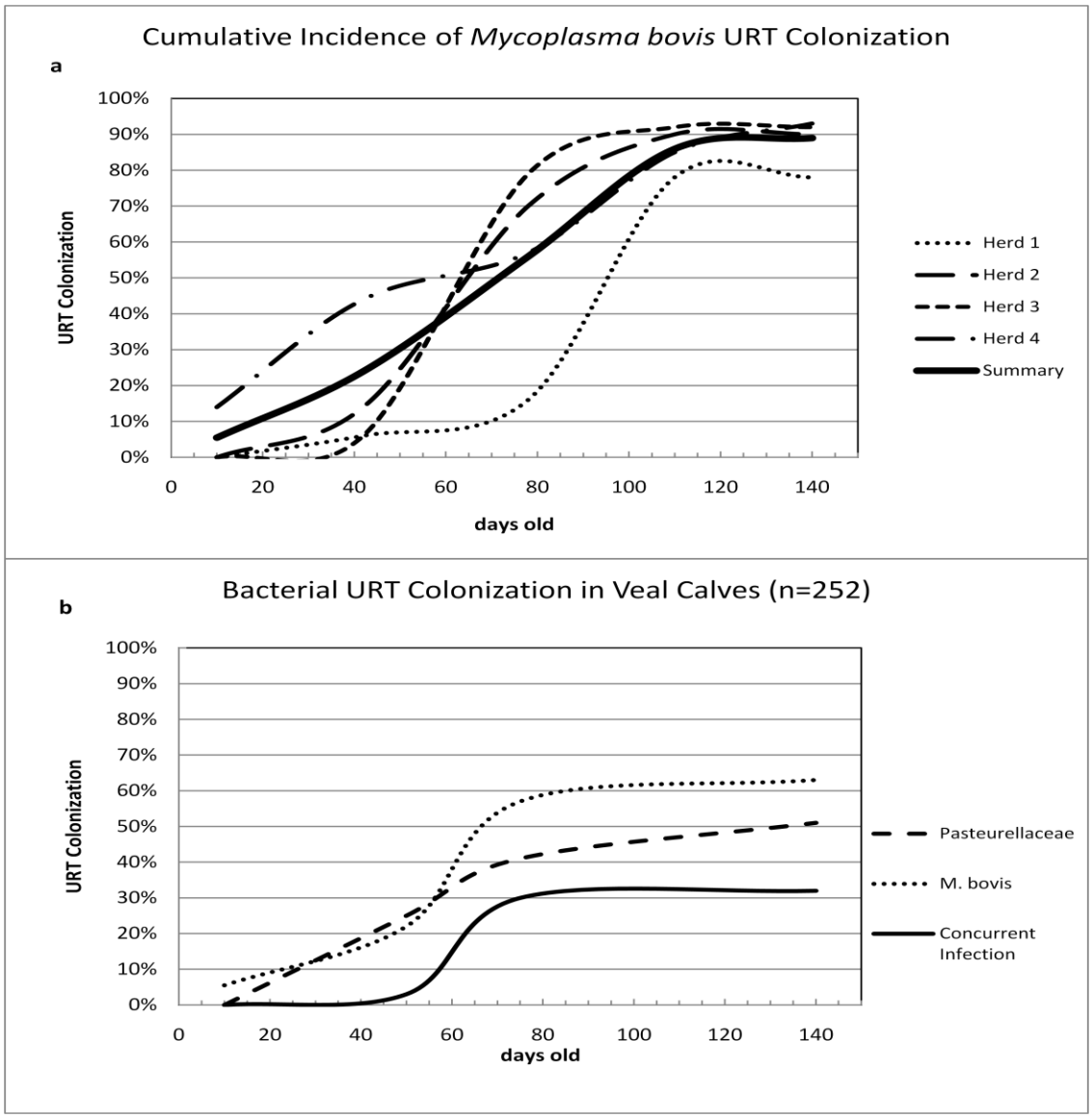
[1] 95% confidence intervals by the modified Wald method.

Tble 6.3 Cumulative list of bacteria isolated from environmental air samples at veal farms

Herd 1	Herd 2	Herd 3	Herd 4
Bacillus brevis	*Aerococcus viridans*	*Bacillus ssp.*	*Staphylococcus haemolyticus*
Staphylococcus saprophiticus	*Staphylococcus xylosus*	*Micrococcus ssp.*	*Micrococcus ssp.*
Staphylococcus cohnii	*Staphylococcus capitis*	*Staphylococcus xylosus*	*Kocuria varians*
Staphylococcus lentus	*Staphylococcus sciuri*	*Aerococcus viridans*	*Bacillus megaterium*
Micrococcus ssp.	*Staphylococcus aureus*	*Staphylococcus capitis*	*Bacillus stearothermophilus*
Staphylococcus cohnii	*Staphylococcus cohnii*	*Staphylococcus cohnii*	*Staphylococcus cohnii*
Staphylococcus capitis	*Bacillus ssp.*	*Staphylococcus carnosus*	*Aerococcus viridans*
Aerococcus viridans		*Staphylococcus lentus*	*Staphylococcus capitis*
Pseudomonas aeroginosa		*Staphylococcus haemolyticus*	*Staphylococcus hominis*
Pseudomonas putida			*Staphylococcus auricularis*
Kocuria varians			*Bacillus brevis*
Staphylococcus epidemidis			*Staphylococcus carnosus*
Staphylococcus xylosus			*Staphylococcus sciuri*
CDC EF4 RG-			*Staphylococcus epidemidis*
Staphylococcus sciuri			*Staphylococcus auricularis*
Staphylococcus caprae			*Staphylococcus xylosus*
Lactococcus lactis			
Staphylococcus epidermidis			
Enterococcus faecium			
Staphylococcus haemolyticus			

Figure 6.1 *Mycoplasma bovis* colonization trends in veal calves from Pennsylvania.

a) Cumulative incidence of *M. bovis* upper-respiratory tract (URT) for herd 1 (dots) (n=50), herd 2 (long dashes) (n=50), herd 3 (short dashes) (n=52), herd 4 (line with dot) (n=100), and summary of all veal calves studied (solid line) (R^2 of summary line=0.9655). b) Combined veal farm colonization trends of *M. bovis* (dotted line), Pasteurellaceae (dashes), and concurrent *M. bovis* and Pasteurellaceae cultured from the URT for all calves sampled (n=252).



Chapter 7

Blinded, controlled field trial of two commercially available *Mycoplasma bovis* bacterin vaccines in Pennsylvania veal calves

7.1 Abstract

Mycoplasma bovis is an etiologic agent of pneumonia, arthritis, and otitis in young calves, such as those found in the special-fed veal industry. We conducted a blinded, controlled trial of two commercially available *M. bovis* bacterin vaccines for the prevention of respiratory disease in calves associated with *M. bovis* infection. Calves were randomly assigned to a subcutaneous treatment of vaccine A (n=50), placebo A (n=50), vaccine B (n=50), or 0.9% sterile saline solution (n=50) beginning at 27 days of age. The on-label recommendation for vaccine A and vaccine B was 3 weeks of age and older and 45 days of age and older, respectively. Upper-respiratory tract colonization was not impacted by vaccination status. The presence of lung lesions was significantly reduced by treatment of vaccine A (p=0.0325), however lung lesions from tissues positive for *M. bovis* was not significantly reduced. Vaccine B was not shown to significantly reduce total lung lesions or *M. bovis*-specific lung lesions. There was no association between specific antibody concentrations and *M. bovis*-associated morbidity in the veal calves. Detectable differences in the IL-1β and TNF-α level for vaccine A and vaccine B were seen as compared to placebo A and saline. Under the field conditions of this study, vaccine efficacy was determined to be 44% and less than 1% for vaccine A and vaccine B, respectively.

7.2 Introduction

Mycoplasma bovis infection is an important disease of cattle causing pneumonia, mastitis, otitis, conjunctivitis, and arthritis which is naturally refractory to numerous antimicrobials (Nicholas and Ayling, 2003). Young calves less than 4 months of age are at high risk for respiratory disease due to *M. bovis* (Stipkovits et al., 2000; Nicholas and Ayling, 2003). *Mycoplasma bovis* may also act as a predisposing factor that could weaken the host immune system leading to invasion by other pathogenic bacteria or viruses (Rosengarten and Citti, 1999). Between one-quarter and one-third of pneumonia-related illnesses in growing cattle may be attributed at least in part to *M. bovis* infections (Nicholas et al., 2000). Additionally, cattle infected with *M. bovis* may serve as a reservoir of infection by shedding bacteria from the respiratory tract for many months (Pfutzner, 1990). The largest economic impact of *M. bovis* infections is due to its chronicity and polymicrobial nature of the infections (Snowder et al., 2006).

Special-fed veal represents nearly a 1 billion dollar industry in the United States, of which Pennsylvania is one of the five largest veal producing states. In the Pennsylvania special-fed veal industry, unaltered male calves are purchased from the dairy industry and raised to ~450 lbs (~205 kg). The special-fed veal industry in the US follows “all in/ all out” agricultural bio-secure management protocols to reduce exposure to outside diseases. The age of the animal, as well as the chronic nature of the disease, and poor response to most antimicrobial therapies makes veal calves vulnerable to *M. bovis*-associated respiratory disease.

One potential method of bacterial disease control in veal calves is the use of vaccines. Some European and Asian field trials have indicated protection from respiratory disease due to vaccination (Stott et al., 1987; Urbaneck et al., 2000; Cho et al., 2008; Nicholas et al., 2009).

Currently there are only a few commercially available *M. bovis* vaccines approved in the United States including bacterin and autogenous vaccines. However, there is little evidence of efficacy as there are few published reports on *M. bovis* vaccines in the United States (Nicholas et al., 2009; Maunsell et al, 2009). In an animal host, *M. bovis* has the potential to serve both immune reactive and immunosuppressive functions. This is shown by the activation of TNF-α and nitric oxide in alveolar macrophages as well as inhibition of polymorphonuclear neutrophil (PMN) degranulation, oxidative bursts, and induction of lymphocyte apoptosis (Jungi et al., 1996; Finch and Howard, 1990; Razin and Hermann, 2002; Vanden Bush and Rosenbusch, 2002; Vanden Bush and Rosenbusch, 2003). There is a measurable humoral and cellular immune response to *M. bovis;* in cattle, the immune response has been reported to be characterized by Th2-skewed cytokine production (Vanden Bush and Rosenbusch, 2003). However, as seen in murine models, the immune response of the host may be responsible for some of the lung damage associated with *M. bovis* (Vanden Bush and Rosenbusch, 2003; Cartner et al., 1998). To appreciate the efficacy of vaccination of individual animals or of entire herds a better understanding of the immune response and distribution of *M. bovis* in affected animals is necessary.

The lack of published reports regarding the efficacy of currently available *M. bovis* vaccines impedes the ability to determine if vaccination is a viable strategy for the reduction of *M. bovis*-associated disease in calves. The objectives of the study were to determine the efficacy of a *M. bovis* bacterin vaccine in veal calves in a natural exposure setting. The outcomes of interest for this trial were: 1) to determine the ability of *M. bovis* bacterin vaccines to reduce incidence of lung lesions 2) to determine the ability of *M. bovis* bacterin vaccines to reduce *M. bovis* nasal colonization and 3) to determine the immunoglobulin and cytokine response of calves to *M. bovis* bacterin vaccination in a natural setting.

7.3 Materials and Methods

7.3.1 Population and Sample Size

Using a blinded, systematically randomized field trial, we studied 200 unaltered male Holstein calves housed at a single location in northwestern Pennsylvania. The herd was selected because the herd producer reported respiratory problems in previous herds and a willingness to participate. Calves were followed through the entire growing period from November 2009 through April 2010. Each calf received a diet of non-soy milk replacer containing iron and 40 other essential nutrients and routine veterinary care, including vaccination against BVD (types 1 and 2), IBR, BRSV, and PI_3. When the calves reached sale weight of ~450 lbs (approximately 145 days of age), they were shipped to a common facility for processing. Any individual calves that became ill during the study were treated under the supervision of a veterinary practitioner.

Lung lesion morbidity due to *M. bovis*-associated infection was the major outcome of interest and its anticipated incidence was used to calculate the required sample size. We hypothesized that a 20% reduction in lung lesions would be biologically impactful. The sample size was determined using an alpha value of 95% and a power of 80%. It was determined the ideal sample size per group would range between 43 calves based upon the prevalence of *M. bovis* (90.5%) in veal calf herds from Pennsylvania. In order to account for attrition a total of 50 calves each were assigned to the following groups; vaccine A, placebo A (all vaccine components except antigen; control group), vaccine B, and 0.9% sterile saline (control group).

7.3.2 Vaccines, Group Assignment, and Blinding

Vaccine A and placebo A were provided blinded by the manufacturer. Vaccine B was purchased from PBS Animal Health Inc. (Massillon, Ohio). To ensure differences in air-flow

patterns in the barn were not likely to result in an increased risk of infection; groups of 4-5 calves per section were systematically assigned to vaccine A, vaccine B, placebo A or sterile saline. The order for the systematic arrangement was randomly assigned using Excel (Microsoft Office Excel 2007). The systematic assignment pattern was followed throughout the barn of 200 calves, resulting in equal groups of 50 calves.

The calf owners, caretakers, and processing personnel were blinded during the trial. The researchers involved with data analysis were back-end blinded to the treatment-type administered to each calf.

7.3.3 Interventions and Outcomes of Interest

Following Pennsylvania Beef Quality Assurance guidelines (BQA, 2010) and manufacturer's recommendations, all injections were 2 mL doses given subcutaneously in the neck beginning at approximately 4 weeks of age. According to the manufacturer's instructions vaccine A (Mycomune® R, BIOMUNE Co., Lenexa, KS) and placebo A (BIOMUNE Co., Lenexa, KS), were administered as three doses to the calves at ~27, 38, and 56 days old. According to manufacturer's instructions, the doses of vaccine B (Pulmo-Guard™ MpB, American Animal Health, Inc., Grand Prairie, TX), and 0.9% sterile saline solution (Agri Labs, Ltd., St. Joseph, MO) were given to the calves as two doses at ~27, and 38 days old. Vaccination of calves was administered by a veterinarian (DRW). The focus of this study was to determine the efficacy of two commercially produced *M. bovis* bacterin vaccines for the prevention of morbidity associated with *M. bovis* colonization and infection leading to the development of lung lesions. Additional objectives were to compare the vaccinated and control group calves (either placebo or sterile saline) with respect to the percentage of nasal colonization and otitis morbidity

from *M. bovis*, immunoglobulin sub-type and cytokine concentrations, *M. bovis*-specific serum immunoglobulin concentrations, and potential of adverse events associated with vaccination.

7.3.4 Outcomes

On farm, nasal swabs were collected at 7, 38, 88, and 136 days of age. A sterile rayon-tipped swab with polyurethane plastic shaft (BBL™ Aimes media CultureSwab™, BD, Franklin Lakes, NJ) was inserted in the nostril to a depth of ~4 inches. Swabs were transported on ice and cultured with-in 6 hours of collection. Due to the effect that colostral immunoglobulins may have upon infection and response to vaccination, blood was collected upon acclimation to barn (3 days post arrival).

A total of 4 blood samples were collected from each veal calf at 7, 38, 88 and 136 days of age. At each sampling, two 3 mL tubes of blood (BD vacutainer®, BD, Franklin Lakes, NJ) were collected through venipuncture to gather plasma and serum. Samples were centrifuged at 3000 rpm at room temperature for 15 min.; collected serum was stored at -20°C until further testing.

At a USDA approved processing facility where lungs were excised post slaughter. A swab was then inserted into an incision made into the trachea to sample both sides of the bronchial bifurcation. Lung lesions were identified and excised for examination by culture analysis. A random selection of tissue samples from normal trachea and lung without lesions (n= 4) were collected for later histological comparison to lung lesions tissue. All bronchial swabs and lung lesions were transported on ice. Bronchial swabs were cultured within 24 hours of collection. In the laboratory, the surface of the lung lesion tissue collected was seared. An incision was then made with a sterile scalpel and a sterile cotton tipped swab (Puritan Medical Products, Guilford, ME) was inserted into the core of the tissue sample and immediately cultured.

7.3.5 Culture Methods

Nasal, bronchial bifurcation, and lung lesion swabs were cultured on blood agar (BA) and MacConkey (MAC) agar at 37°C in an aerophilic environment, and chocolate agar (CA) at 37°C in a microaerophilic environment. Standard biochemical identification procedures and API (BioMerieux, France) for identification of common bovine respiratory pathogens was used. All swabs were then enriched in Mycoplasma enrichment broth supplemented with penicillin (UC Davis Biological Media Services, Davis, CA) for 48 hours before being plated on pleuropneumonia-like organism (PPLO) agar plates. All mycoplasma cultures were grown at 37°C in a microaerophilic environment. PPLO agar plates were considered negative if no growth was noted by day 10. Confirmation of mycoplasma growth was performed using PCR of the *uvr*C *M. bovis* housekeeping gene as described by Thomas et al. (2004).

7.3.6 Total Protein and Hematocrit

In order to determine the health of calves prior to vaccination, total protein was determined for all samples using a refractometer (Atago Inc., Tokyo, Japan). Hematocrit readings were also performed on a random selection of calves (n=31). All calves determined to have abnormal total protein readings were tested again one and two months later.

7.3.7 Nutrient Profiling

Nutrient profiles for two calves culled from the herd by the veal raiser due to poor weight gain (in the study until ~90 days old) were submitted to the Michigan State University, Diagnostic Center for Population and Animal Health, Lansing MI in order to determine the overall health of the calves. Freshly frozen liver tissue and serum were tested for selenium, cobalt, copper, iron, manganese, molybdenum, and zinc.

7.3.8 Histology

Sections of normal and abnormal tissue were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) stain at the Pennsylvania State University Animal Diagnostic Laboratory, University Park, PA.

7.3.9 Serology

Serum, harvested after clotting, was stored at -20°C. Serum of all calves at each time point was analyzed for IgM, IgA, IgG1, and IgG2 by ELISA (Bethyl Laboratories, Montgomery, TX) per manufacturer's instructions and suggested optimization procedures. The optical density of each well was measured at 450nm using an automated plate reader (ELx800 Microplate Reader, BioTek Instruments, Inc., Winooski, VT). Data was gathered using KC junior (BioTek Instruments, Inc., Winooski, VT).

For each plate duplicate sets of bovine reference standards (Bethyl Laboratories, Montgomery, TX) were analyzed under the same conditions as samples. Reference serum concentrations of 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL, 31.25ng/mL, 15.625ng/mL, and 0ng/mL were prepared and used to calculate the standard curve using linear regression analysis. Duplicate run OD values were averaged. Sample values were converted to a serum concentration.

Serum of all calves at each collection point was analyzed for *M. bovis* antibody via semi-quantitative ELISA (Biovet Inc., St. Hyacinthe, Canada) per manufacturer's instructions. The optical density of each well was measured at 450nm using an automated plate reader according to the same conditions as reported above for sub-type antibody ELISA. Positive and negative controls, provided by the manufacturer were run with each plate.

A subset of calves was selected for further testing. A total of 5 calves (10%) from each treatment subgroup were randomly selected for cytokine screening. Sera from each collection date were sent to Aushon Biosystems, Inc. (Billerica, MA) for the bovine chemiluminscent multiplex enzyme-linked immunosorbent assay (ELISA) panels for IL-1β, IL-2, IL4, IL-6, IFN-γ, and TNF-α.

7.3.10 Statistical Methods

The statistical significance of *M. bovis* morbidity was determined using Fisher's exact test and 2*x*2 contingency tables. Categorical outcome variables were compared using chi-square tests. Quantitative outcome variables were compared using independent sample *t*-tests. Vaccine efficacy was determined using the relative risk, a ratio of the risk among the vaccinated and the risk among the unvaccinated (2). Vaccine A and placebo A were compared and vaccine B and sterile saline were compared. ELISA data were compared using two-factor ANOVA with repeated measures. A p-value of <0.05 with two-sided tests was considered significant for all analyses. Statistical analyses were performed using GraphPad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA).

7.4 Results

7.4.1 Baseline Data and Vaccine-Associated Adverse Events

After attrition (unidentifiable eartags at slaughterhouse (n=13) the vaccine A, placebo A, vaccine B, and sterile saline groups included 45, 45, 48, and 49 calves, respectively. The average readings for total protein (TP) and hematocrit upon entry to the barn were 5.9g/dL and 24%, respectively. Nineteen calves were shown to have TP levels less than 5.0g/dL upon entry into the

barn, likely due to poor transfer of colostrum from the dam. All calves were found to be of acceptable health for vaccine regiment. A total of 7 subcutaneous granuloma vaccine associated adverse events were noted at injection sites after vaccination with vaccine A (n=2) and placebo A (n=5). Upon examination of the carcasses, no granulomas were found. No adverse reactions at injection sites were noted for vaccine B or 0.9% sterile saline solution. The average weight gain over the course of the study for calves receiving vaccine A, placebo A, vaccine B, and saline was 167, 168, 171, and 170 pounds, respectively.

7.4.2 Lung Infection

A total of 81 lung lesions were identified at the abattoir. At slaughter, lung lesions were identified in a total of 14, 25, 24, and 18 calves treated with vaccine A (n=43), placebo A (n=45), vaccine B (n=48), and sterile saline (n=49), respectively. Of these lung lesions from calves receiving vaccine A, placebo A, vaccine B, and sterile saline, 25.0%, 40.0%, 29.0%, and 45.0% of the lesions were culture positive for *M. bovis,* respectively. Other bacterial respiratory pathogens cultured from the lung lesions of calves treated with vaccine A were *Pasteurella multocida* (n=1); placebo A, *P. multocida* (n=1) and *Mannheimia haemolytica* (n=1); vaccine B, *P. multocida* (n=1), *Klebsiella pneumoniae* (n=1), and *Arcanobacterium pyogenes* (n=2); and for sterile saline, *P. multocida* (n=1), *K. pneumoniae* (n=1), and *Arcanobacterium pyogenes* (n=1).

7.4.3Upper-Respiratory Tract Colonization

Upper-respiratory tract (URT) colonization was neither significantly different for vaccine A compared to placebo A nor vaccine B compared to sterile saline (Figure 7.1). The cumulative incidence at day 136 of *M. bovis*-associated URT colonization for vaccine A, placebo A, vaccine B, and saline were 94.0% (95% CI, 83.1-97.9%), 92.0% (95% CI, 80.7-97.4%), 96.0% (95% CI,

85.8-99.7%), and 90.0% (95% CI, 78.2-0.96.1%), respectively. *Mycoplasma bovis* was recovered from a total of 62.0% (95% CI, 47.6-74.9%), 49.0% (95% CI, 37.1-60.9%), 56.0% (95% CI, 42.3-0.69.3%), and 59.0% (95% CI, 45.2-71.8%) of swabs collected at the bronchial bifurcation of calves treated with vaccine A, placebo A, vaccine B, and sterile saline, respectively.

7.4.4 Efficacy of Vaccination on *M. bovis*-Associated Morbidity

It was determined that vaccine A significantly reduced the number of lung lesions identified in veal calves compared to placebo A ($p=0.0325$), but did not significantly reduce *M.* bovis-specific lung lesions ($p=0.0690$). It was determined that vaccine B neither significantly reduced the total number of lung lesions identified in veal calves as compared to 0.9% sterile saline ($p=0.2218$), nor significantly reduced the number of veal calves with *M. bovis* lung lesions ($p=1.000$). It was determined that calves in the center of the systematically randomized groups of five for the vaccinated were not significantly ($p>0.05$) less likely to develop lung lesions than the assigned control group.

The relative risk of developing a lung lesion for vaccine A compared to placebo A and vaccine B compared to sterile saline was 0.56 and 1.36, respectively. The vaccine efficacy related to overall reduction of lung lesions in veal calves of vaccine A compared to placebo A was 44%. Vaccine B compared to sterile saline showed no vaccine efficacy (defined as less than 1%).

7.4.5 Antibody Response

The serum antibody subclass response (IgM, IgA, IgG1 and IgG2) was assessed via ELISA at 7, 38, 88, and 136 days of age. The trends across the sampling times for the averaged IgM, IgA, IgG1, and IgG2 values for vaccine A, placebo A, vaccine B, and sterile saline are shown in Figure 7.2. The differences across sampling time points for each immunoglobulin tested

for all treatment groups was significant ($p<0.0001$). No significant differences between vaccinated or control calves was detected for IgM, IgA, IgG1, or IgG2. The largest differences between vaccine A and placebo A and between vaccine B and saline occur at 88 days for each immunoglobulin tested (56 days post final dose of vaccine A and placebo A; 38 days post final dose of vaccine B and saline). There was a significant difference in the IgG1 antibody subclass response of vaccine A calves which had URT colonization of *M. bovis* and those without URT colonization at day 7 ($p=0.01$) and day 88 ($p=0.05$), while a significant difference between vaccine B calves was found for IgG2 subclass response at day 38 ($p=0.03$) and for IgA at day 88 ($p=0.006$). There were no significant differences for the IgM antibody subclass regardless of URT status.

At three days post-arrival to the barn, *M. bovis* seropositivity was noted for of 38.0% (95% CI 25.8-51.9%), 20.0%, (95% CI 11.1-33.2%), 28.0% (95% CI 17.4-41.8%), and 36.0% (95% CI 24.1-49.9%) of calves in vaccine A, placebo A, vaccine B, and saline, respectively. At 138 days of age 100% of calves in each intervention group were *M. bovis* seropositive.

7.4.6 Cytokine Response

A subset of 5 calves of was randomly selected from each treatment group for cytokine assays. The average cytokine concentrations (pg/mL) for days 7, 38, 88, and 138 for IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α are shown in Figure 7.3. Most notably, calves receiving either vaccine A or vaccine B showed a peak in Il-1β and TNF-α concentration at day 88. Calves receiving either placebo A or saline remained stable across sampling for Il-1β, and TNF-α (Figure 7.3). Additionally, a peak in IL-2 concentrations was seen for vaccine A and saline at 88 days. Differences between vaccinates and the respective controls were no significant for IL-1β, IL-2, IL-4, IL-6, IFN-γ, nor TNF-α.

7.4.6 Additional Results

Otitis, identified by characteristic unilateral or bilateral ear droop, was noted in 16 calves throughout the study period. Otitis was present in calves vaccinated with vaccine A (n=4), placebo A (n=6), vaccine B (n=3), and saline (n=3) groups. Fifteen of the 16 calves with otitis also had at least one *M. bovis* positive nasal swab during the study period. Vaccination with either vaccine A or vaccine B did not result in a significant reduction of otitis morbidity. No cases of arthritis were noted in this study.

Two calves (both from vaccine A group) were culled from the herd by the veal-grower at ~90 days of age due to poor weight gain. Field necropsy was performed on both calves at the farm. Both calves had lung lesions. Based upon histopathology, calf A was diagnosed with moderate to moderately severe broncho-interstitial pneumonia and calf B was diagnosed with mild multifocal interstitial pneumonia. *Mycoplasma bovis* was not cultured from either lung lesion. No significant lesions were noted in the kidney, heart, spleen, or liver collected from each calf. Calves A and B were determined to have serum micronutrient levels of 82 and 81ng/mL (selenium), 5.43 and 4.76ng/mL (cobalt), 1.01 and 1.41µg/mL (copper), 142 and 122µg/dL (iron), 5.5 and 10.1ng/mL (manganese), 9.4 and 17.5ng/mL (molybdenum), and 1.44 and 1.60µg/mL (zinc), respectively. The liver tissue micronutrient profiles were 3.5 and 2.1µg/g (selenium), 0.71 and 0.86µg/g (cobalt), 1308 and 856µg/g (copper), 380 and 294µg/g (iron), 10.1 and 8.3µg/g (manganese), 2.2 and 2.7µg/g (molybdenum), and 587 and 730µg/g (zinc) for calves A and B, respectively. The micro-nutrient profiles performed indicated that neither calf was deficient of selenium, cobalt, copper, iron, manganese, molybdenum, or zinc in the serum and liver. Lung lesions from both calves were negative for *M. bovis.* Due to the intent to treat, the lung lesions identified were included in the statistical analyses of the vaccine trial.

7.5 Discussion

Protection from *M. bovis*-associated respiratory disease has been reported for killed whole cell bacterin vaccines in older calves (Howard et al., 1987; Stott et al., 1987; Uranbaneck et al., 2000; Cho et al., 2008; Nicholas et al., 2009), but there have been reports of adverse events associated with vaccination against *M. bovis* (Boothby et al., 1987; Bryson et al., 2002). Efficacy has also been reported for a saponin bacterin vaccine in 3 week old dairy calves in Europe (Nicholas et al., 2009). The efficacy of the commercially available bacterin vaccine in the United States, marketed for stocker and feedlot cattle, has been reported by Maunsell et al. (2009) in young dairy calves from Florida. The study reported that the bacterin vaccine was ineffective at preventing nasal colonization and *M. bovis*-associated disease in pre-weaned endemically infected dairy herds from Florida (Maunsell et al., 2009).

The purpose of this study was to determine the efficacy of two commercially available *M. bovis* bacterin vaccines for use in veal calves. The vaccine efficacy related to reduction of lung lesions in veal calves was 44% and <1% for vaccine A and vaccine B, respectively. Vaccine efficacy in this study may be different from previous studies due to the strain of bacteria used in the vaccines, the adjuvant used (the adjuvant of both vaccines used in this study are proprietary; only the adjuvant of vaccine A was acquired for this study), the method of inactivation, the high URT colonization (44%) of the total study calves prior to vaccination for this endemically infected herd, the impact of herd immunity, and the housing differences of veal calves compared to other calves. The vaccines used in this study were ineffective at preventing URT colonization with *M. bovis* in calves. Vaccine A and vaccine B were also ineffective at preventing *M. bovis* infection of the bronchus. This indicates that *M. bovis* infection at the intersection of the upper-respiratory and lower-respiratory tract is not affected by vaccination. Although serum IgG1

response was shown to increase throughout the duration of the study there was not a significant difference between calves vaccinated with vaccine A and placebo A or vaccine B and sterile saline.

Previous research has shown no relationship between indirect hemagglutination titers and IgM or IgG concentrations in calves (Carroll et al., 1977). Local immune responses are likely better indicators of *M. bovis* protection than serum antibody responses (Howard et al., 1980). The role of specific antibody levels in susceptible and immunized animals is poorly understood, but it has been suggested that IgG responses of vaccinated calves and naturally infected calves are similar (Boothby et al., 1987). The lack of a significant difference between IgA concentrations, in vaccinated and control calves is likely due to the similarity of infection status across the herd and that vaccines may not be designed to stimulate the mucosal system. It has been suggested that IgA antibodies are unlikely to be produced following subcutaneous vaccination with killed *Mycoplasma* species (Howard and Gourlay, 1983). The results of this study are in agreement with previously reported immune responses to a *M. bovis* vaccine subcutaneously administered to heifer calves (Maunsell et al., 2009). The lack of notable differences in the tested antibody sub-type responses for vaccinated and control calves may be due to the constant stimulation of the immune response in all calves due to the endemic nature of *M. bovis* infections in the study herd.

It is unclear how vaccination would impact naïve calves instead of calves which had already begun to show *M. bovis* seropositivity prior to vaccination. A total of 30.5% of all calves in the barn, regardless of intervention group were seropositive at three days post arrival to the barn indicating that it is likely at least a portion of these calves had become *M. bovis* seropositive at their origination farms. Presumably, *M. bovis* antibodies which are transferred to new born calves via maternal colostrum may indicate the prevalence in the maternal population of a farm if one single batch of colostrum is not used to feed all new-born calves. Previous studies throughout

Europe have found the seropositivity to range from 2.2% to 14% (Le Grand et al., 2002; Arcangioli et al., 2008). The high seroprevalence of *M. bovis* antibody results in this study indicate a need for further studies.

It has been reported that in cattle an immune response to *M. bovis* is characterized by a Th2-skewed cytokine response (Vanden Bush and Rosenbusch, 2003). The increased levels of IL-1β and TNF-α in vaccine A and B as compared to placebo A and saline suggest that a pro-inflammatory effect in vaccinated calves, which is likely in epithelial cells. In murine models it has been shown that IL-1β augments the TNF-α responses in lung epithelial cells (Saperstein et al., 2009). The increase in IL-2 found in the vaccine A and saline groups may be the result of adaptive immune responses in those calves. It is likely that the early (~7 days age) sampling impacted the cytokine and IgG specific antibody titers reported, which may represent the maternal transfer of antibody to the calf. The changes noted in IL- 4, IL-6, and IFN-γ level for the subset of calves selected may be impacted by the colostral status of calves upon entry into the barn which are shown to stabilize by the second and third samplings. The detectable differences between intervention groups for IL-1β, IL-2, and TNF-α are not significant and therefore it is recommended that larger sampling groups are tested in future studies to determine the nature of the differences.

Importantly, it was shown that *M. bovis* colonization was established prior to vaccination. If the adaptive immune responses were unable to develop there may have been chronic inflammation, which may limit vaccine efficacy. It should be noted that vaccine A is labeled for use in calves 3 weeks of age or older while vaccine B is labeled for use in calves 45 days of age or older. Calves in this study were vaccinated at 27, 38, and 56 days of age or 27 and 38 days of age for vaccine A and vaccine B, respectively. It is possible that vaccination at 27 and 38 days of age does not elicit the same immune response as in calves 45 days of age or older, as

recommended for vaccine B. Calves should not be vaccinated within 21 days or 60 days prior to slaughter for vaccine A or vaccine B, respectively. Vaccination at a later age, as recommended by vaccine B, plus the need for 60 days between vaccination and slaughter will likely prove to be challenging for veal producers due to the total length of time that calves are fed prior to processing (~145 days). The vaccination protocol was selected based upon the early age of URT colonization and lung infection observed in endemically exposed veal calves from Pennsylvania in previous work.

The systematic assignment of calves in groups of five was selected in order to control for the possibility of air flow differences in the barn. Calves were randomly assigned a specific stall upon entry to the barn. The random assignment should minimize the influence of herd immunity. It was determined that the calves in the center of the group of five were not significantly less likely to have lung lesions. Therefore, it is unlikely that herd immunity is acquired by vaccination with vaccine A or vaccine B for *M. bovis*-associated lung lesions. It is possible that clusters greater than five are necessary to identify herd immunity effects for *M. bovis*-associated lung lesions. Based on this data, it is unlikely that a selection bias from the randomization method resulted in a significant impact upon the measured outcomes in this study.

It has been suggested that calves with TP values less than 5.0g/dL have failure of passive transfer of antibody and are 3 to 6 times more likely to die before 6 months of age, although mortality in special-fed veal herds is generally less than in other calf raising operations (Wilson et al., 1994; Donovan et al., 1998). In order to assess the overall health of calves upon entry into the barn, total protein and hematocrit were performed. A total of 19 calves were found to have TP values below 5.0g/dL upon entry into the veal raiser barn. Six of the 19 calves (0.3158; 95% CI, 0.1516-0.5420) were found to have lung lesions at slaughter. Forty-three percent of calves with a TP value greater than or equal to 5.0g/dL were found to have lung lesions at slaughter (0.4345;

95% CI, 0.3618-0.5101). These results are similar to previously reported results of another *M. bovis* bacterin vaccine, indicating that TP concentrations do not play a role in the incidence of respiratory disease (Maunsell et al., 2009). The micro-nutrient profiles performed on two calves culled from the herd in order to asses the health of the calf at time of post-mortem inspection indicated that neither calf was deficient of selenium, cobalt, copper, iron, manganese, molybdenum, or zinc in the serum or liver. These results in conjunction with the negative lung lesion bacterial cultures suggest that other factors were responsible for the poor weight gain of these calves. The average weight gain of calves was not significantly impacted by vaccination against *M.* bovis. These results agree with previous reports that antimicrobial treatment rates for respiratory disease and weight gain do not differ between vaccinated and unvaccinated groups (Martin, 1983). In this study, treatment group did not impact the presence of otitis cases in the veal calves.

7.6 Conclusions

To the best of the authors' knowledge this is the first report of a blinded, controlled field trial of these two *M. bovis* vaccines available in the United States in young calves. Vaccination with either vaccine A or vaccine B was not efficacious in preventing upper-respiratory tract colonization of *M. bovis*, nor effective in preventing *M. bovis*-specific lung lesions in special-fed veal calves. Vaccine A was shown to have a vaccine efficacy of 44% in preventing the presence of lung lesions. Vaccination resulted in a detectable difference in IL-1β and TNF-α following the final dose of vaccine. The results of this study suggest that vaccine A is more likely to be efficacious for use in young veal calves (starting at 3 weeks of age). The results of this study should not be used to infer the ability of either vaccine to effectively prevent *M. bovis*

colonization or disease in animals of other ages. This research demonstrates the difficulty of current vaccination strategies for use with special-fed veal calves which are much younger than most dairy or beef calves are when vaccinated. As well as the inherent higher risk for sharing respiratory pathogens due to the close proximity of uninfected to infected calves.

7.7 References

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Figure 7.1 Cumulative incidence of *M. bovis* upper-respiratory tract (URT) colonization of vaccine A (solid line), placebo A (solid with a dot line), vaccine B (dashed line), and 0.9% sterile saline solution (dotted line).

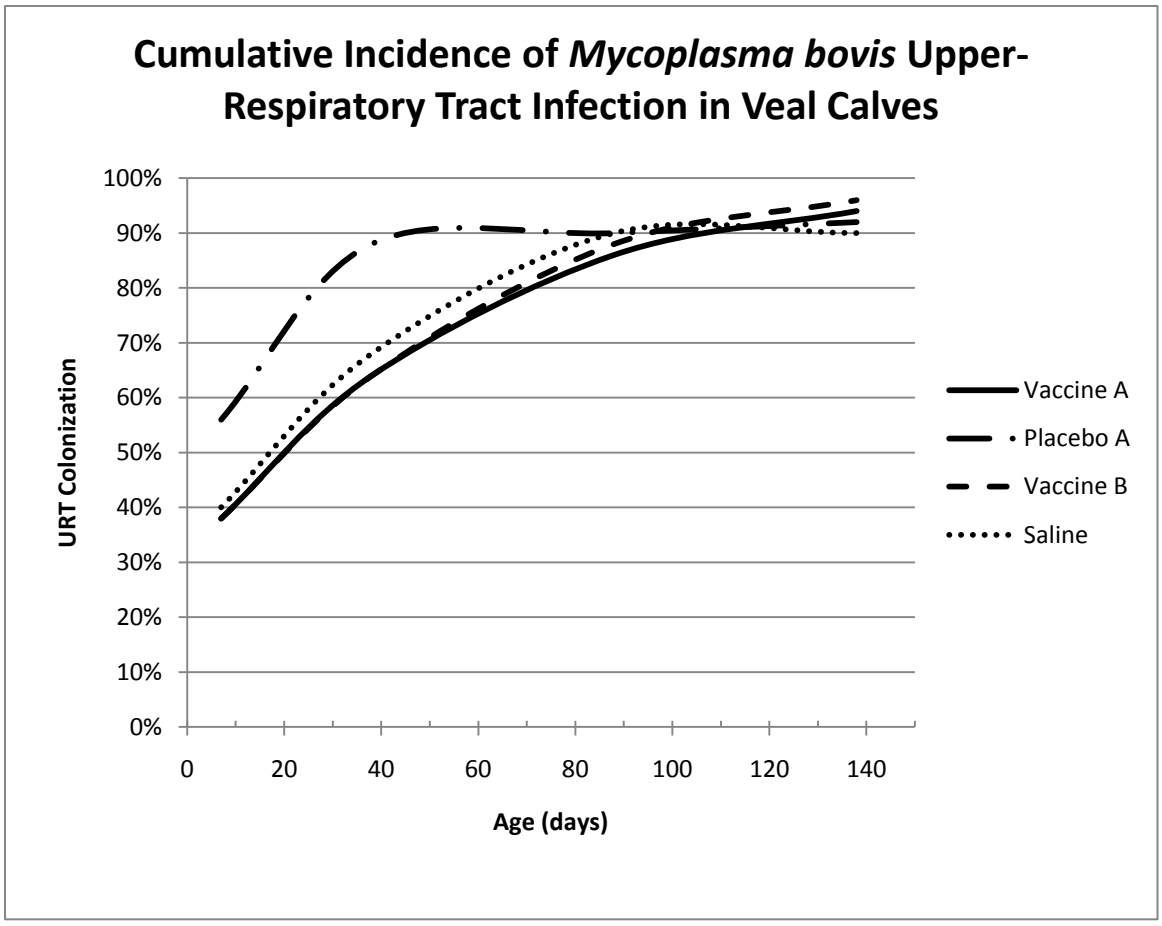


Figure 7.2 Trends of averaged IgM (a), IgA (b) IgG1 (c), and IgG2 (d), by vaccine A (solid line), placebo A (solid with a dot line), vaccine B (dashed line), and 0.9% sterile saline solution (dotted line) represented in ng/dL concentrations.

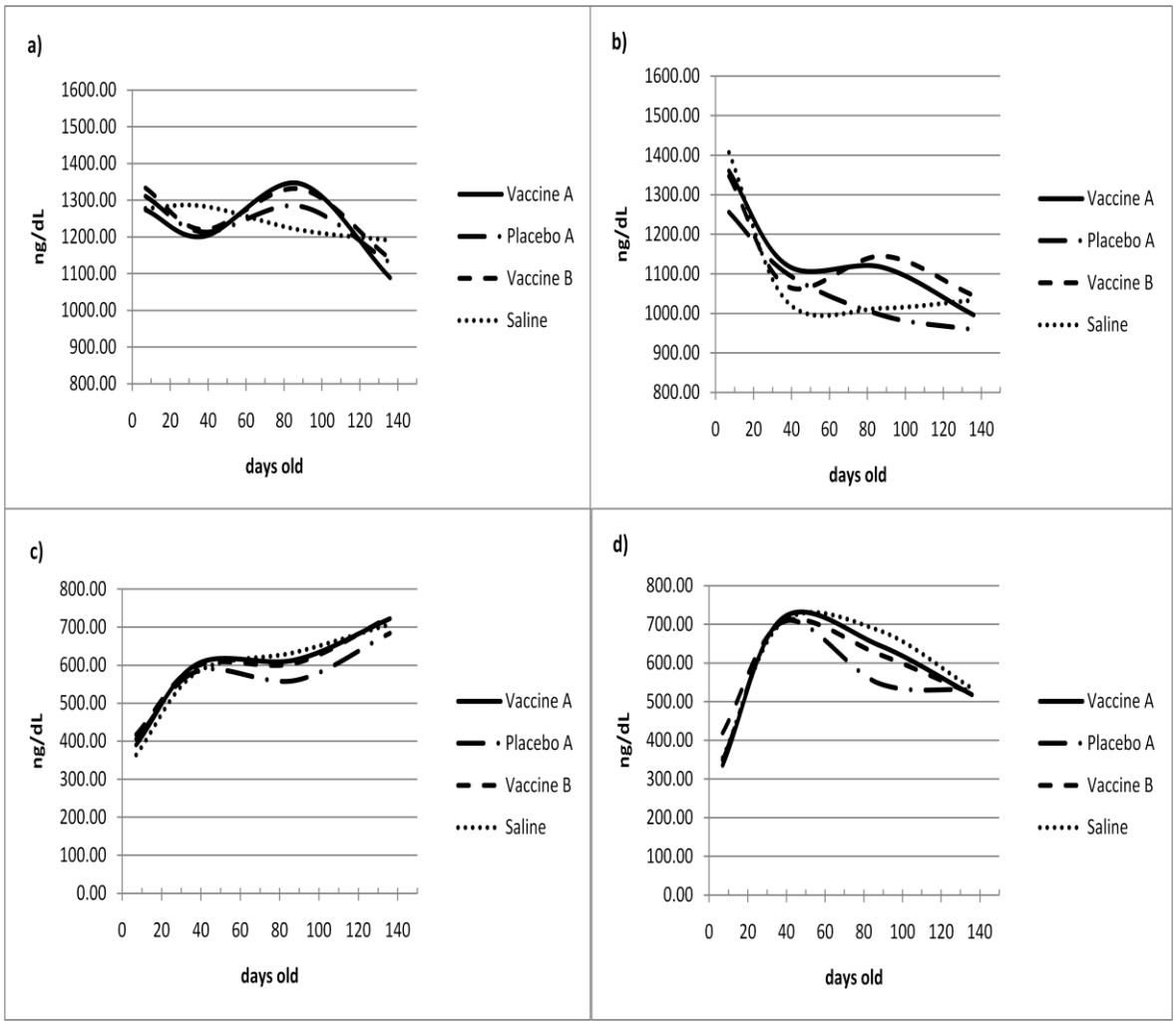
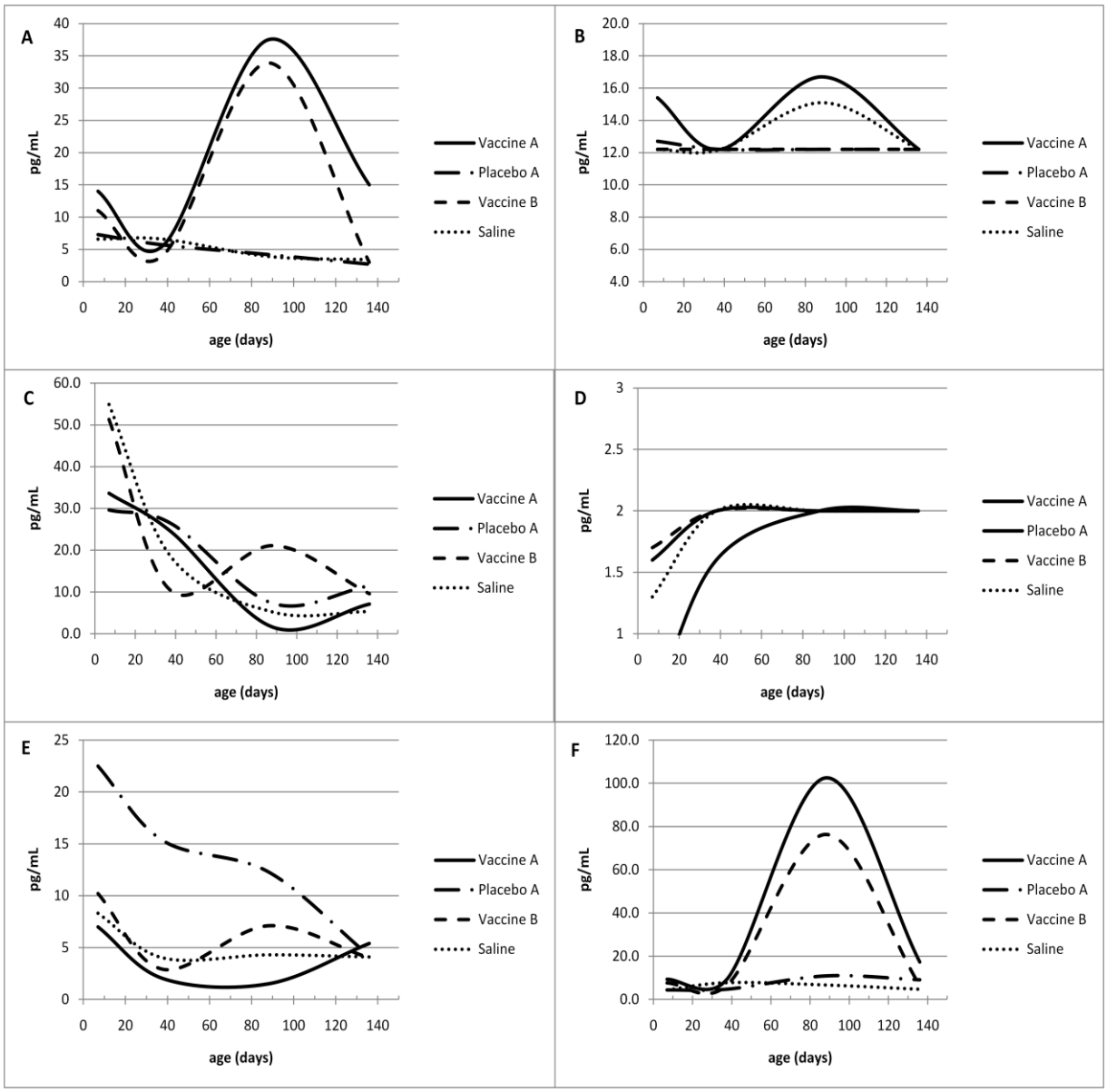


Figure 7.3 Average cytokine responses of a subset of calves receiving vaccine A (solid line), placebo A (solid with a dot line), vaccine B (dash line) or sterile saline solution (dotted line). Bovine cytokines represented in pg/mL are A. IL-1β, B. IL-2, C. IL-4, D. IL-6, E. IFN-γ, and F.TNF-α.



Chapter 8

Summary and Conclusions

8.1 Summary and Conclusions

Since *M. bovis* represented the largest portion of *Mycoplasma* species submitted to the Pennsylvania Animal Diagnostic Laboratory between December 1, 2007 and November 30, 2008, the focus of a laboratory standardized and validated AFLP analysis was to determine the clonal diversity of *Mycoplasma bovis.* AFLP analysis indicated strong genetic heterogeneity of isolates indicating the importance and genome plasticity of *M. bovis* isolated from the North-Eastern United States. AFLP analysis offers a typing method may be useful for the tracing of outbreaks in the United States as well as to screen isolates from imported animals with inter-laboratory capabilities. This research has demonstrated that Pennsylvania has the possibility for multiple overlapping outbreaks to occur at a single location.

Although *in-vitro* susceptibility breakpoints do not necessarily correspond to *in-vivo* results, they can provide a method of determining the agents which are most likely to be effective treatment options. Two methods, broth microdilution, and flow cytometry, were considered and tested for the ability to identify MIC for *M. bovis.* Both antimicrobial susceptibility testing methods demonstrated that florfenicol, enrofloxacin, and tetracycline are most efficacious in inhibiting *M. bovis* isolates of bovine origin from Pennsylvania. The wide sensitivity range for several commonly administered antimicrobials suggests that *in-vitro* susceptibility testing prior to application may help optimize *in-vivo* applications.

The use of a broth microdilution assay with a color changing redox reagent offers an assay which is effective and highly reproducible which can be easily adapted to a diagnostic setting without requiring additional training or specialized equipment. However, the use of flow cytometry offers better turn-around time, high-throughput testing capabilities, inter-laboratory comparisons capability, and the objective collection of results for sensitivity data. There is

potential for real time diagnostic data to be available in less than 24 hours. Flow cytometry offers great potential as a valuable tool in diagnostic laboratories, but may currently be cost prohibitive to some laboratories.

Results presented here-in suggest that the small molecule natural compounds methanesulfonic acid, 3-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyloxy](1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane carboxylic acid, S-carboxymethyl-L-cysteine, L-aspartic acid, dihydrotachysterol, eriodictyol, and (+)-alpha-tocopherol acid succinate are capable of inhibiting the growth of *M. bovis* in a dose-dependent manner. These natural compounds have the potential to become future therapeutics and prophylactics capable of lessening the dependency on the current antimicrobials with increasing resistance. The introduction of novel compounds with inhibition properties allows for the promotion of animal and human health and well-being.

In the epidemiological study of *M. bovis* in special-fed veal calves it was shown that *M. bovis* can be isolated from upper-respiratory tract of calves beginning at an early age. *Mycoplasma bovis* is an initiating factor of respiratory disease in special-fed veal calves from Pennsylvania as supported by the cumulative incidence, attack rates, and the frequency of multiple bacterial respiratory pathogens being cultured from nasal swabs, bronchi swabs, and lung lesions. The high prevalence of *M. bovis* colonization beginning in the first few months of the calf's life indicates a need for successful vaccines that may induce the immune response of very young calves, preferably prior to 50 days of age.

The use of a blinded, controlled trial of commercially available *M. bovis* bacterin products demonstrates the difficulty of current vaccination strategies for use with special-fed veal calves which are younger than most dairy or beef calves would be at the recommended time for vaccination. There is an inherent higher risk for sharing respiratory pathogens due to the close proximity of uninfected to infected calves in veal raiser operations. Analysis of the immune

responses to vaccination indicates a detectable difference in TNF-α and IL-1β levels for vaccinated and unvaccinated animals, but the sub-type antibody responses are not significant.

Vaccination with either vaccine A or vaccine B was not shown to be efficacious in preventing upper-respiratory tract colonization nor effective in preventing *M. bovis*-specific lung lesions in special-fed veal calves. However, vaccine A demonstrated a vaccine efficacy of 44% in preventing the presence of lung lesions. Vaccination resulted in a detectable difference in IL-1β and TNF-α following the final dose of vaccine. Although the results suggest that vaccine A is most likely to be efficacious in young veal calves, inference of the ability of either vaccine to effectively prevent *M. bovis* colonization or disease in animals of other ages is not recommended.

A more complete understanding of the molecular characterization and epidemiology of *M. bovis* allows for more effective prevention and treatment strategies which may reduce the veterinary public health burden of *M. bovis*-associated disease and the polymicrobial infections involving *M. bovis*. Further characterization of *M. bovis*, especially of virulence factors, would contribute to the knowledge regarding the mechanism of *M. bovis*-associated diseases in cattle.

Appendix A

IACUC Approval #28296

Date: May 1, 2008

From: William G. Greer, IACUC Administrator

To: David R. Wolfgang

Subject: Results of IACUC Protocol Review – New Protocol **(IACUC# 28296)**

Approval Expiration Date: April 27, 2009

"Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate"

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your protocol for the use of animals in your research. **This approval has been granted for a one-year period.**

Approval for the use of animals in this research project is given for a period covering one year from the date of this memo. **If your study extends beyond this approval period, you must contact this office to request an annual review of this research.**

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. As of February 13, 2001, The Pennsylvania State University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/alb

Attachment

cc: Bhushan M. Jayarao

To the Investigator:

Please forward the enclosed original approval letter to your funding agency, if applicable. This approval is effective for one year. During this time, you should notify this office of any changes in the protocol that will affect the care and use of the approved animals or that will result in the use of additional animals.

In a continuing effort to comply with federal regulations, this office reviews IACUC approvals on an annual basis. On the anniversary of this approval, you should expect to receive a letter soliciting your request for an "annual review" by the IACUC. It is my hope that this process aids researchers in maintaining active IACUC approvals and avoids the use of animals without the proper approval.

Also, in order for records of your animal usage at ARP and ORP to remain current, please review the information below. If you feel there is any discrepancy between this information and your request, please contact our office (ORP) immediately at 865-1775. Thank you.

IACUC#: 28296 **Approved:** May 1, 2008

PI: David R. Wolfgang

Title: "Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate"

Species	Total # Approved	# Used to Date	# Not Yet Used
Cattle: Beef	1000	0	1000

Appendix B

Final IACUC Renewal Approval #28296

Date: March 29, 2010

From: William G. Greer, Assistant Director, Animal Care, Biosafety and Radiation Programs

To: David R. Wolfgang

Subject: Results of IACUC Protocol Review – Annual Review **(IACUC# 28296)**

Approval Expiration Date: March 28, 2011

"Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate"

The Annual Project Review form for your protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) for the continued use of animals in your research. **This approval has been granted for a one-year period.**

Comment: Removal of Ginger Fenton as personnel.

Approval for the use of animals in this research project is given for a period covering one year from the date of this memo. **If your study extends beyond this approval period, you must contact this office to request an annual review of this research.**

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. The Pennsylvania State University is also registered with the US Department of Agriculture (Certificate No. 23-R-0021). As of February 13, 2001, The Pennsylvania State University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

The IACUC does not require the principal investigator to provide copies of permits (e.g., PA Game Commission, Bird Banding, US Fish and Wildlife Service) prior to approval. However, if your research mandates a permit requirement, it is your responsibility to acquire such permits prior to conducting the research described in your IACUC protocol.

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/ci

Attachment

cc: Bhushan M. Jayarao

Marty K. Soehnlen

To the Investigator:

Please forward the enclosed original approval letter to your funding agency, if applicable. This approval is effective for one year. During this time, you should notify this office of any changes in the protocol that will affect the care and use of the approved animals or that will result in the use of additional animals.

In a continuing effort to comply with federal regulations, this office reviews IACUC approvals on an annual basis. On the anniversary of this approval, you should expect to receive a letter soliciting your request for an "annual review" by the IACUC. It is my hope that this process aids researchers in maintaining active IACUC approvals and avoids the use of animals without the proper approval.

Also, in order for records of your animal usage at ARP and ORP to remain current, please review the information below. If you feel there is any discrepancy between this information and your request, please contact our office (ORP) immediately at 865-1775. Thank you.

IACUC#: 28296 **Approved:** March 29, 2010

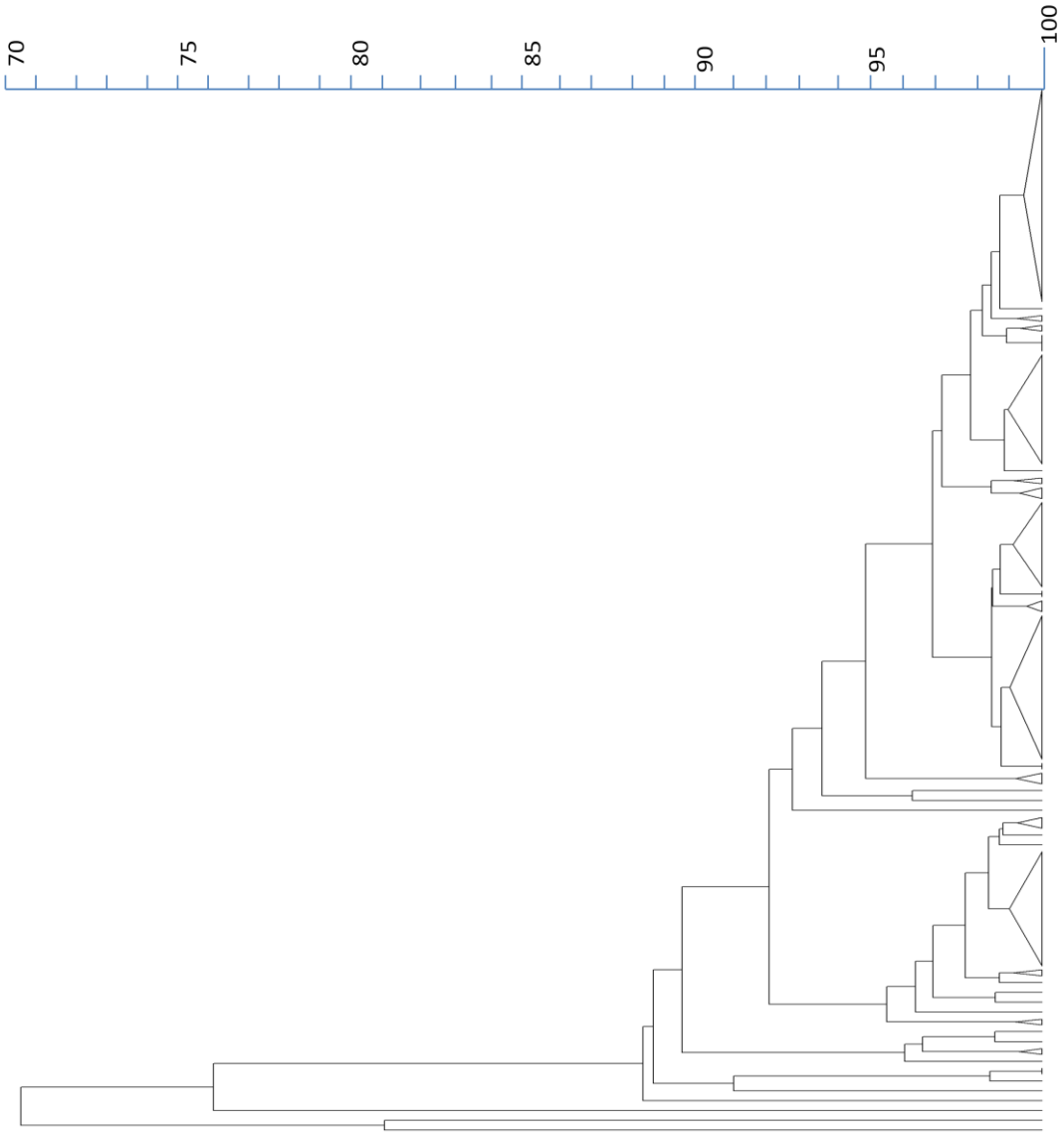
PI: David R. Wolfgang

Title: "Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate"

Species	Total # Approved	# Used to Date	# Not Yet Used
Cattle: Beef	1000	456	544

Appendix C

Genetic relationship between *Mycoplasma bovis* strains based upon AFLP profiles (100-490bp fragment size range) produced by amplification of *Bgl*II and *Mfe*I DNA templates with nonselective primers. The dendrogram was produced with the UPGMA method with the Dice similarity coefficient (S_D) and strains with greater than 99% similarity collapsed into single clusters. Each main tick mark on the similarity scale represents a change of 1%.



Appendix D

Minimum Inhibitory Concentration values for *M. bovis* (n=192) isolates

Antibiotic	MIC (µg/mL)[1]										
	<[2]	1	2	4	8	16	32	64	128	256	>[3]
Florfenicol	13	-	64	93	19	2	1	-	-	-	-
Spectinomycin	-	-	7	32	30	5	6	1	1	1	109
	<[2]	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	>[3]
Oxytetracycline	-	1	1	2	-	6	22	39	50	28	43
Tetracycline	-	1	3	1	-	13	44	37	37	32	24
	<[2]	0.0125	0.025	0.5	0.1	0.2	0.4	0.8	1.6	3.2	>[3]
Enrofloxacin	-	-	2	4	25	106	40	9	2	4	-
Erythromycin	-	-	-	-	-	-	-	-	-	-	192
	<[2]	0.49	0.98	1.95	3.9	7.8	15.63	31.3	62.5	125	>[3]
Ceftiofur	-	-	-	-	-	-	-	-	-	-	192

[1]values listed in table represent the number of isolates with MIC values respective to each antimicrobial concentration

[2]MIC less than the lowest concentration tested, no growth observed at all concentrations tested

[3]MIC greater than the highest concentration tested, growth observed at all concentrations tested

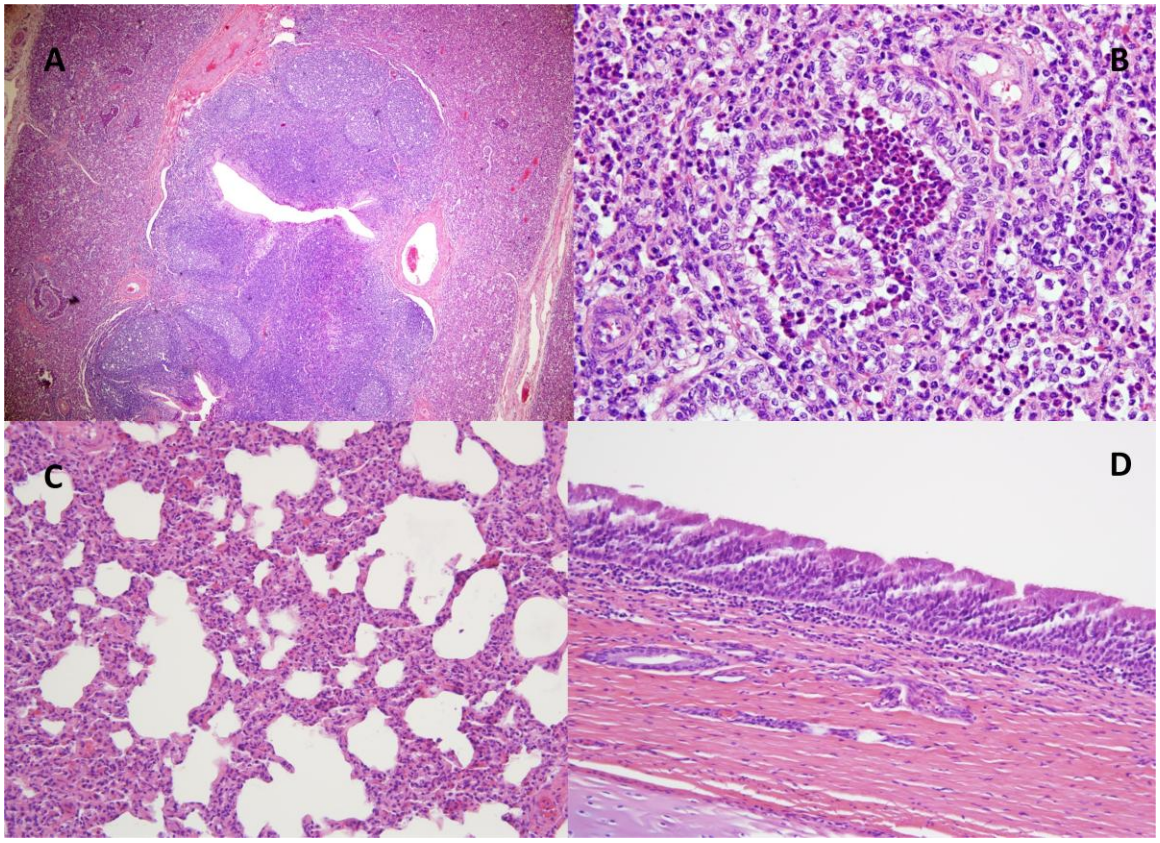
Appendix E

Systematically randomized set-up for vaccine efficacy trial[1]

[1] Red - Vaccine A Black – Placebo A Blue - Vaccine B Green - Saline

Appendix F

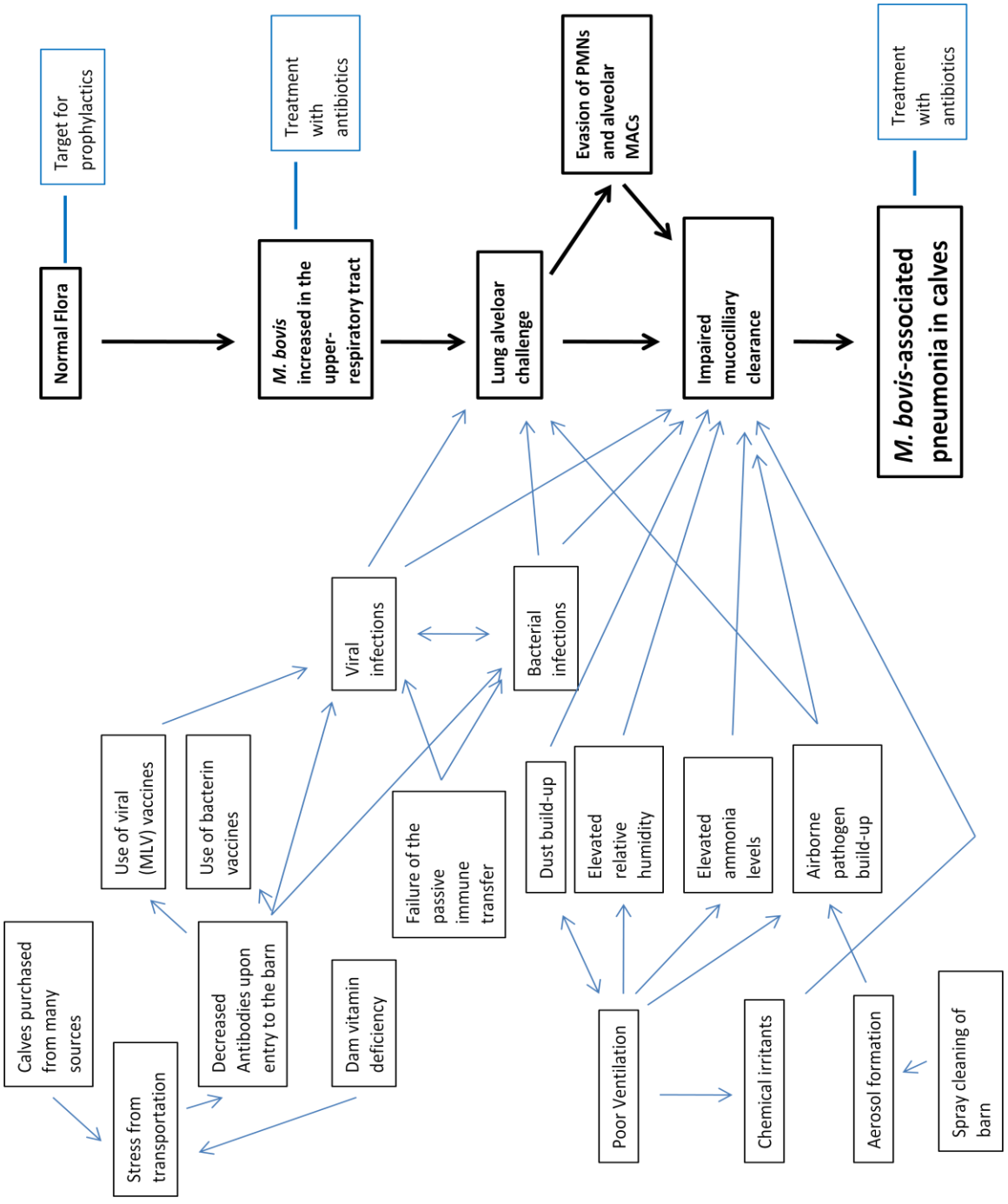
Histopathology of *Mycoplasma bovis*-associated pneumonia



A and B) Severe broncho-interstitial pneumonia with intrabronchiolar accumulations of neutrophils and cellular debris.Marked peribronchial lymphoid hyperplasia is also evident, characteristic of Mycoplasma sp. infection. **C)** Mild to moderate interstitial pneumonia characterized by interstitial thickening. **D)** Normal calf trachea

Appendix G

Proposed path model of *Mycoplasma bovis*-associated pneumonia in veal



Proposed path model (causal web) of *Mycoplasma bovis*-associated pneumonia in special-fed calves.

VITA

Marty K. Soehnlen

EDUCATION

Degree	Date	Institution	Major
Ph.D.	2011	The Pennsylvania State University	Pathobiology
M.P.H.	2006	The University of Michigan	Hospital and Molecular Epidemiology
B.S.	2004	The Ohio State University	Medical Technology

FELLOWSHIPS

2006-2007 APHL/CDC Emerging Infectious Diseases Advanced Laboratory Training Fellowship Class XII

CERTIFIED TRAINING

2010 Graduate Teaching Certificate, The Pennsylvania State University
2010 Large Animal Rescue Training, Pennsylvania Agriculture Rescue Program
2010 Beef Quality Assurance Training, Pennsylvania Beef Council
2007 Basics of Laboratory Animal Handling, American Association of Laboratory Animal Science (AALAS)
2007 Laboratory Animal Care Standards Training, AALAS

HONORS AND AWARDS

2010 Richard L. Walker Bacteriology Award
American Association of Veterinary Laboratory Diagnosticians

2010 Second Place- Biological Sciences
College of Agricultural Sciences/Gamma Sigma Delta Research Expo

2007 Dean's Scholarship
The Pennsylvania State University, Dept. of Veterinary and Biomedical Sciences

PUBLICATIONS

Soehnlen, Kunze, Karunathilake, Henwood, Kariyawasam, Wolfgang, and Jayarao. 2011. *In vitro* antimicrobial inhibition of *Mycoplasma bovis* isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and a broth microdilution method. Journal of Veterinary Diagnostic Investigation. In press.

Soehnlen, Tran, Lysczek, Wolfgang, and Jayarao. 2011. Identification of novel small molecule antimicrobials targeting *Mycoplasma bovis.* Journal of Antimicrobial Chemotherapy. In press.

Soehnlen, Kariyawasam, Lumadue, Pierre, Wolfgang, Jayarao. 2011. Molecular epidemiological analysis of *Mycoplasma bovis* isolates from the Pennsylvania Animal Diagnostic Laboratory showing genetic diversity. Journal of Dairy Science. In press.

Soehnlen, Aydin, Murthy, Hattel, Houser, Fenton, Lysczek, Burns, Townsend, Wolfgang, and Jayarao. 2011. Epidemiology of *Mycoplasma bovis* in Pennsylvania veal calves. In preparation.

Soehnlen, Aydin, Houser, Fenton, Lysczek, Burns, Byler, Hattel, Wolfgang, and Jayarao. 2011. Blinded, controlled field trial of two commercially available *Mycoplasma bovis* bacterin vaccines in Pennsylvania veal calves. In preparation.