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SURVIVAL OF *MYCOPLASMA* SPECIES IN RECYCLED BEDDING SAND AND POSSIBLE IMPLICATIONS FOR DISEASE TRANSMISSION TO RUMINANTS

by

Anne E. Justice-Allen

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Bioveterinary Science

Approved:

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UTAH STATE UNIVERSITY
Logan, Utah

2010

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ABSTRACT

Survival of *Mycoplasma* Species in Recycled Bedding Sand and Possible Implications for Disease Transmission to Ruminants

by

Anne E. Justice-Allen, Master of Science

Utah State University, 2010

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Department: Animal, Dairy and Veterinary Sciences

Mycoplasmas are a group of bacteria which are small in size, lack a cell wall, and have small genomes in comparison to other bacteria. The members of the group that are pathogenic utilize several mechanisms to evade the host's immune system. These processes affect surveillance and control mechanisms such as serologic testing and vaccination. Many of these organisms cause diseases of livestock, which heavily impact production parameters such as weight gain, milk yield, and egg production. Mycoplasmas also cause disease in people.

Mycoplasma spp. can cause mastitis, metritis, pneumonia, and arthritis. The currently documented routes of transmission of *Mycoplasma* spp. are through fomites and by direct animal contact. The existence of environmental sources for *Mycoplasma* spp. and their role in transmission are poorly characterized. *Mycoplasma* spp. (confirmed as *M. bovis* using PCR) was found in recycled bedding sand from a dairy experiencing an

outbreak of mycoplasma mastitis. The possibility of a persistent environmental source of *Mycoplasma* spp. in recycled bedding sand was further investigated using recycled sand from the dairy. Study objectives included determining factors associated with the persistence of *Mycoplasma* spp. in recycled bedding sand and the duration of survival of mycoplasmas in the sand. We also evaluated 2 disinfectants at 2 different concentrations each for the elimination of *Mycoplasma* spp. from contaminated sand.

Mycoplasma spp. survived in the sand pile intermittently over a period of 8 months. The concentration of *Mycoplasma* spp. within the sand pile was directly related to temperature and precipitation. The survival of *Mycoplasma* spp. at a greater than expected range of temperatures suggests the formation of a biofilm. Ideal temperatures for replication of *Mycoplasma* spp. occurred between 15 °C and 20 °C. Moisture in the sand and movement of the sand pile also appeared to play a role in replication of mycoplasmas. Sodium hypochlorite (0.5%) and chlorhexidine (2%) were efficacious in eliminating *Mycoplasma* spp. from contaminated bedding sand. Recycled bedding sand could be an environmental source of *Mycoplasma* spp. infections, including *M. bovis*, in dairy cows. Future studies should investigate the contribution of this environmental source to the epidemiology of mycoplasma infections in dairy cattle and other ruminants.

(124 pages)

ACKNOWLEDGMENTS

I would like to thank Dr. Tom Baldwin and the personnel at the Utah Veterinary Diagnostic Laboratory for providing me space to work on my research and technical support. I would especially like to thank my major professor, Dr. David Wilson, whose guidance and support were invaluable. I also appreciate the efforts of my committee members, Drs. Kerry Rood and Brian Gowen, in assisting in the development of my research program and the review of my thesis.

My husband, Kelly, deserves special recognition for his support, patience, encouragement, and occasional calf care duties.

Thank you all.

Anne E. Justice-Allen

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CHAPTER 1

INTRODUCTION

Disease is a state where normal physiological processes are disrupted in a part, organ, or system of an organism resulting in signs or symptoms. Disease can be caused by many different mechanisms and agents, such as infection, genetic defect or environmental stress. Bacteria can be both disease agents as well as participants in a host's normal physiological processes. Microbiologists and epidemiologists have become increasingly aware that just as with macroorganisms, microorganisms fit into ecological niches both within and outside of the host. One current model describes disease as an interaction between the host, the environment, and the agent.

This thesis describes the characteristics of one set of organisms, the mycoplasmas, and the mechanisms by which a number of them cause disease in man and animals. Characteristics of mycoplasmas that are important to consider in the investigation and management of disease outbreaks are the requirements for growth and laboratory isolation, the routes of transmission between animals, the methods available for identification of infected animals, and the value of vaccines, antibiotics, or management and husbandry practices for control. The diseases caused by mycoplasmas are both acute and chronic, and many are important because of the impact of the infections on the productivity of livestock animals and birds.

Because this group of organisms lacks a cell wall, environmental survival has been considered to be short and the primary routes of transmission have been described

as by direct contact between animals or by contaminated wet surfaces such as milking equipment (Jasper, 1977; Nagatomo et al., 2001; González and Wilson, 2003). During an investigation into an outbreak of mastitis, *Mycoplasma bovis* was isolated from the infected dairy cows and from the sand which was used to bed their freestall housing. Evaluation of the bedding materials at several other mycoplasma-positive dairy farms found 2 additional farms also had *M. bovis* in the bedding. This finding led to the development of the observational study of the survival of a mycoplasma species, confirmed as *M. bovis* by PCR, described in this thesis. The results of this research could affect how mycoplasmal diseases, specifically mycoplasmal mastitis, are managed.

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CHAPTER 2

LITERATURE REVIEW OF MYCOPLASMAS—PHYLOGENY, GROWTH REQUIREMENTS, DISEASES, AND PATHOGENESIS

History and Phylogeny

The term mycoplasma has been used to designate both members of a class of organisms Mollicutes as well as members of the genus *Mycoplasma* (Razin, 1978). In this thesis, the term mycoplasma will refer only to those members of the genus. The Mollicutes have been grouped together because of the common characteristics of a small genome (0.58 - 2.2 Mbp) with low guanine-cytosine content (a measure of DNA variability used to characterize prokaryotes) and an absent cell wall (Razin and Hermann, 2002). The first mycoplasma organism was identified in 1896 by Nocard and Roux as the agent of bovine pleuropneumonia (Nocard et al., 1896). Originally the organism was classified as a virus because it was filterable (passed through a Chamberland filter), could not be cultured using the solid agar media available, and could just be detected with the light microscopes in use at the time (Nocard et al., 1896; Eaton et al., 1945; Goodburn and Marmion, 1962; Harwick et al., 1972). Because the organism was not readily visible, Koch's postulates were not technically satisfied but the researchers were able to remove the organism from infected calves, grow it in semi-permeable, membranous pouches in the peritoneal cavities of rabbits, (without the rabbits becoming infected), inoculate a naïve calf with the culture, and cause the classic form of the disease (Nocard et al., 1896). The second mycoplasma to be identified and isolated by inoculation of chick embryos with filtered (600 mμ collodion

membrane) sputum from human patients was *M. pneumoniae* (Eaton et al., 1945; Goodburn and Marmion, 1962; Harwick et al., 1972). There are now approximately 200 validly described species in 8 genera (Table 2.1, Johansson and Peterson, 2002; International Committee on Systematics of Prokaryotics, 2003). These organisms occur in insects, plants, plant surfaces, animals, bovine and ovine rumens, and people. The Mollicutes have been placed in the phylum Firmicutes with the classes Bacilli and Clostridia based on rRNA sequence analysis (Maniloff, 2002). This also reflects their evolutionary origin in the Late Proterozoic when the AAP (Acholeplasmataceae, Anaeroplasmataceae, and

Table 2.1: Classification of Mollicutes (adapted from Razin and International Committee on Systematics of Prokaryotics)

Order	Family	Genus	No. of species[1]	Habitat
Mycoplasmatales	Mycoplasmataceae	Mycoplasma	111(5)	man, animals
		Ureaplasma	7	man, animals
Entomoplasmatales	Entomoplasmataceae	Entomoplasma	6	insects, plants
		Mesoplasma	12	insects, plants
	Spiroplasmataceae	Spiroplasma	45[2]	insects, plants
Acholeplasmatales	Acholeplasmataceae	Acholeplasma	14	animals, plant surfaces
		Phytoplasma[3]	(8)	insects, plants
Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	4	rumen
		Asteroplasma	1	rumen

[1]Candidate species in parentheses; from ICSP
[2]Includes 11 unnamed species
[3]Razin tentative classification; not in ICSP

Phytoplasma) branch diverged from the Streptococcus branch (Maniloff, 2002). This divergence coincided with genome reduction and loss of the ability to produce a cell wall (Maniloff, 2002). Two more divergences occurred in the Middle Ordovician and at the Silurian-Devonian boundary which resulted in the separation of the Mycoplasmataceae branch containing the genera *Mycoplasma* and *Ureaplasma* (Maniloff, 2002). Mycoplasmas evolved dramatically with the appearance of mammals and other vertebrates approximately 191 million years ago (Maniloff, 2002).

With the advent of DNA sequencing methods, microorganisms are being reclassified based on similarities in the nucleotide sequence of all or part of the genome. Recently, 2 groups of previously identified red blood cell (RBC) parasites, formerly called *Eperythrozoon* spp. and *Haemobartonella* spp., have been moved from the Rickettsiales to Mycoplasmatales class Mollicutes. Most microbiologists agree with this change because the similarity between the sequences of 2 gene segments, the 16s rRNA and the RNase P gene, of the former *Eperythrozoon* spp. and *Haemobartonella* spp. and *M. pneumoniae* is 76% (Neimark et al., 2001; Tasker et al., 2003; Uilenberg et al., 2004). Additional similarities between the hemoparasites and mycoplasmas are the absence of a cell wall, small size (0.3 to 0.5 μm), pleomorphic morphology, and the presence of attachment structures (Neimark et al., 2001). Some microbiologists believe that the organisms should be placed in the *Mycoplasma* genus (Niemark et al., 2001). Others indicate that because the reclassified group attaches only to RBC (some appear free in the plasma), and the similarity is less than the similarity that occurs between other closely related genera, such a reclassification is inappropriate (Uilenberg et al., 2004).

Physiology and Growth Requirements

As a group, the mycoplasmas have lost, through genome reduction, many synthetic and physiological biochemical pathways. *Acholeplasma laidlawii, M. hominis,* and *M. mycoides* ssp. *mycoides* lack the ability to synthesize de novo purine and pyrimidine bases (Razin, 1978). In contrast, *M. mycoides* ssp. *mycoides* does have the capacity to synthesize all of the nucleotides, including adenosine triphosphate and cytosine triphosphate from the nucleosides guanine, uracil, and thymine (Razin, 1978). Analysis of the genomes for *M. genitalium, M. pneumoniae,* and *U. urealyticum* found no genes coding for amino acid metabolism and a small number of genes for lipid metabolism and energy metabolism when compared to bacteria such as *Escherichia coli* and *Bacillus subtilis* (Pollack, 2002). There are 3 pathways utilized by mycoplasmas for energy production: fermentation of sugars to lactate, oxidation of lactate or pyruvate, and the metabolism of L-arginine to ornithine (Keçeli and Miles, 2002). There is significant variation among the mycoplasma species, with some having the mechanisms for all 3 metabolic pathways and some having only 1 route of energy generation. The Embden-Meyerhoff-Parnas pathway is present to some degree in all of the Mollicutes studied to date and is believed to be the primary method of ATP generation for the group (Pollack et al., 1997). *Ureaplasma* is unique in the group because of its ability to hydrolyze urea (Razin, 1978). All of the various physiological pathways have not been identified for all mycoplasma species. The number of genes for transcription and translation are comparable to those of other bacteria (Pollack, 2002).

The result of the reduction in the physiologic capacities of mycoplasmas is the requirement for complex media when attempting to isolate or culture the organisms. The first microbial cultures with formulated media were accomplished in the 1930's by Ledingham and Gey working separately using media enriched with serum or human placental cord blood (Morton et al., 1952; Harwick et al., 1972). The identification by Klieneberger in 1935 of classical mycoplasma colonies contaminating a culture of *Streptobacillus moniliformis* as "L forms" led to years of confusion between bacterial variants lacking cell walls and *Mycoplasma* spp. (Morton et al., 1952). The confusion began to be resolved in 1961 when Liu demonstrated that the agent of atypical pneumonia was particulate (Harwick et al., 1972). Shortly thereafter, the organism was cultured on solid media and named *Mycoplasma pneumoniae* (Chanock et al., 1962). The researchers determined that both horse serum and yeast extract were required for growth of the organism. Since then many media have been developed for the isolation of mycoplasmas from animal tissues and fluids (Table 2.2).

The most basic and earliest medium used for mycoplasma culture consists of 50 g dehydrated beef heart, 10 g peptone, 5 g NaCl, and 14 g agar per liter of distilled water (Morton et al., 1952; Hayflick and Chanock, 1965). By 1951, this product was available commercially and a number of researchers were experimenting with additional supplementation in order to increase the isolation of various *Mycoplasma* spp. (Morton et al., 1952; Chanock et al., 1962; Barber and Fabricant, 1962; Hayflick and Chanock, 1965). One of the media developed by Chanock et al. (1962), Hayflick's medium, is still used, with the modification of the addition of various antibiotics, for the isolation of

Table 2.2: Media used for isolation of mycoplasmas

Component	Hayflick's	Friis'	L'Ecuyer's	SP4
Broth	Difco PPLO[1]	PPLO and BHI[2]	PPLO	PPLO
Protein	pooled horse serum	horse and pig serum	pig serum, gastric mucin, lactalbumin	digest of casein, digest of gelatin, fetal bovine serum
Sterol	yeast extract	yeast extract	yeast extract	yeast extract, yeastolate[3]
Carbohydrate		DEAE[4] dextran		
Antibiotic		bacitracin	penicllin, thallium acetate	polymyxin B, amphotericin B, penicillin
Other				CMRL 1066[5]

[1]PPLO = Pleuropneumonia-like organism media
[2]BHI = Brain heart infusion
[3]Yeastolate = the water soluble portion of autolyzed yeast
[4]DEAE = diethylamino ethanol
[5]CMRL 1066 = Connaught Medical Research Laboratories 1066, a defined medium consisting of amino acids, nucleic acids, and vitamins. An example of 1 commercial type is available from Sigma (http://www.sigmaaldrich.com/united-states.html).

Mycoplasma spp. (Table 2.2). Other media which are in common use are Friis' and SP4 (Table 2.2). It has become common practice to modify these media with the addition of various antibiotics such as thallium acetate, penicillin, ampicillin, polymyxin B, and kanamycin (Barber and Fabricant, 1962; L'Ecuyer, 1969; Harwick et al., 1972; Ciprián et al., 1988).

Mycoplasmas and Disease in Animals and Man

The impetus for the development of media to isolate mycoplasma organisms was the realization that there was a group of microorganisms causing serious diseases in animals and man which was smaller than other bacteria and not a virus. Nocard et al. were working to discover the agent of contagious bovine pleuropneumonia, a devastating disease of cattle which still has a significant economic impact in southern and eastern Africa (Thiaucourt et al., 2004). The first pathologic mycoplasma organism discovered to cause disease in man, *M. hominis*, was isolated from a Bartholin's gland abscess in 1937 (Morton et al., 1952; Harwick et al., 1972; Bébéar, 2002). This was followed by the identification in 1944 of *M. pneumoniae* as the cause of Primary Atypical Pneumonia (PAP) which was initially called Eaton's agent and thought to be a virus (Eaton et al., 1945; Harwick et al., 1972). By 1965, pathogenic mycoplasmas had been identified in 15 different animals (Hayflick and Chanock, 1965). Previously unrecognized species of mycoplasma have been identified in the past 10 years in part due to the development of molecular and DNA based methods such as PCR (Kirchhoff et al., 1997; Helmick et al., 2002; Hammond et al., 2003; Haulena et al., 2006). Table 2.3 lists the currently recognized important pathogenic mycoplasmas of animals and man as well as the clinical syndromes caused by each. The pathogenesis, epidemiology, and treatment of some of the more important mycoplasmal diseases of animals and man are reviewed.

Mycoplasma pneumoniae.

Of the 6 mycoplasmas associated with disease in man, *M. pneumoniae* is the most thoroughly studied and the most important (Waites and Talkington, 2004; Vervolet

et al., 2007). It was first cultured in 1944 but thought to be a virus because it was filterable and was not susceptible to penicillin (Eaton et al., 1945). In1961, Goodburn

Table 2.3: Major pathogens of animals and man grouped by host species

Host	Agent	Disease
Human	*M. pneumoniae*	Primary atypical pneumonia
Human	*M. hominis*	Salpingitis, pyelonephritis, pelvic inflammatory disease
Human	*U. urealyticum*	Urethritis
Cattle	*M. mycoides* ssp. *mycoides*	Pleuropneumonia
Cattle/bison/deer	*M. bovis*	Mastitis
Cattle	*M. californicum*	Mastitis
Cattle	*M. canadense*	Mastitis
Cattle/bighorn sheep	*M. arginini*	Mastitis
Cattle/bison	*M. bovigenitalium*	Infertility, mastitis
Cattle	*M. dispar*	Calf pneumonia
Goats	*M. capricolum* ssp. *capricolum*	Arthritis, mastitis, pneumonia, septicemia
Goats	*M. capricolum* ssp. *capripneumoniae*	Pleuropneumonia
Goats/sheep	*M. agalactiae*	Mastitis
Sheep/deer	*M. conjunctivae*	Keratoconjunctivitis
Sheep/bighorn sheep	*M. ovipneumoniae*	Pneumonia
Horses	*M. felis*	Pleuritis
Swine	*M. hyopneumoniae*	Pneumonia
Swine	*M. hyorhinis*	Arthritis, pneumonia
Swine	*M. hyosynoviae*	Arthritis
Mice/rats	*M. pulmonis*	Respiratory disease
Mice/rats	*M. arthritidis*	Arthritis
Turkeys/chickens	*M. gallisepticum*	Sinusitis, respiratory disease
Turkeys	*M. meleagridis*	Air sacculitis, embryo mortality
Turkeys/chickens	*M. iowae*	Embryo mortality, arthritis
Turkeys/chickens	*M. synoviae*	Synovitis
Passerine birds	*M. gallisepticum*	Conjunctivitis
Tortoises	*M. agassizii*	Sinusitis, conjunctivitis
Crocodiles	*M. crocodyli*	Arthritis

and Marmion observed the organism as a small coccobacillus on the mucous layer of bronchial epithelium and found that it was susceptible to tetracycline and erythromycin (Goodburn and Marmion, 1962). At about the same time, the organism was successfully cultured on a solid medium (Chanock et al., 1962). At present, the entire genome has been sequenced, the mechanisms of adhesion to the host cell have been elaborated, and several modes of pathogenesis and immune system modulation have been described (Waites and Talkington, 2004).

First described as the agent for PAP, *M. pneumoniae* causes a broad range of clinical manifestations in humans. The usual incubation period ranges from 1 to 3 weeks (Waites and Talkington, 2004; Vervolet et al., 2007). The earliest symptoms are non-specific and consist of fever, sore throat, hoarseness, headache, chills, myalgia, and earache (Waites and Talkington, 2004; Vervolet et al., 2007). A harsh, persistent, non-productive cough is a classic sign but it is not pathognomonic (Waites and Talkington, 2004; Vervolet et al., 2007). *Mycoplasma pneumoniae* has been determined to be the cause of 15% to 20% of the reported pneumonias (Waites and Talkington, 2004); pneumonia occurs in 3% to10% of the cases that begin with milder symptoms (Vervolet et al., 2007). Extra-pulmonary pathology occurs in 25% of infected patients (Waites and Talkington, 2004). The organism has been cultured from joints, cerebrospinal fluid, and the pericardium (Waites and Talkington, 2004). The most common extra-pulmonary complications are dermatological and consist of maculopapular and vesicular rashes, conjunctivitis, erythema multiforme, and ulcerative stomatitis (Waites and Talkington, 2004). Non-specific arthralgias, myalgias, and septic arthritis occur in 14% of patients

(Waites and Talkington, 2004). Less common sequelae are encephalitis, meningitis, polyradiculitis, pericarditis, myocarditis, hemolytic anemia, disseminated intravascular coagulation, acute glomerulonephritis, otitis externa, and otitis media (Waites and Talkington, 2004). Many of the extra-pulmonary disease syndromes have an immunologic component which is a result of the interaction of *M. pneumoniae* with immune cells and the production of various cytokines. This will be discussed further in the section on mycoplasmas and the immune system.

Disease occurs most frequently in children from 5 to 15 years of age, and young adults that are housed in group settings such as college dormitories, military barracks, and prisons (Waites and Talkington, 2004; Vervolet et al., 2007). Several studies have found that epidemics occur every 3 to 5 years (Jacobs, 2002; Waites and Talkington, 2004). Genetic research has determined that there are 8 subtypes of *M. pneumoniae* divided into 2 major groups based on variations in the P1 adhesin gene (Jacobs, 2002; Waites and Talkington, 2004). Epidemiologic studies have found that when an epidemic occurs, the causative subtype isolated is different than the 1 that had caused the most recent previous outbreak (Jacobs, 2002; Waites and Talkington, 2004). In 51% of cases, *M. pneumoniae* occurs in a co-infection with viruses and other bacteria and there is evidence that it intensifies the severity of viral infections (Waites and Talkington, 2004; Vervolet et al., 2007).

Mycoplasma pneumoniae causes disease through several different mechanisms. The first step in the process is binding to the host cell by means of several adhesin proteins which appear to attach to host sialoglyco-conjugates and sulfated glycolipids

(Waites and Talkington, 2004; Vervolet et al., 2007). The organism produces hydrogen peroxide hydroxy radicals, and superoxide anions which damage the ciliated cells of respiratory epithelium (Waites and Talkington, 2004; Vervolet et al., 2007). *Mycoplasma pneumoniae* also activates the complement system causing the production of tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8), an immune response consisting of both type 1 and type 2 cytokines (Waites and Talkington, 2004; Vervolet et al., 2007). These factors can be effective in eliminating the infection but can also exacerbate underlying chronic diseases such as asthma, and chronic obstructive pulmonary disease (COPD; Waites and Talkington, 2004; Vervolet et al., 2007). Pathologic changes consist of ulceration of airway epithelium, edema of the bronchi and bronchioles, cellular (neutrophils, lymphocytes, plasma cells, and macrophages) infiltrates of the bronchioles and alveoli, Type II pneumocyte hyperplasia, pleural effusions and in more severe cases, lung abscesses and pulmonary fibrosis (Waites and Talkington, 2004).

Due to fastidious culture requirements and the slow growth profile (21 days), diagnosis of *M. pneumoniae* by culture is difficult. Definitive species identification of positive cultures requires species specific anti-sera (Mahon et al., 2007). While there is an enzyme-linked immunosorbent assay (ELISA) available, its limit of detection is very near the typical number of organisms in a sputum sample which means that there are a number of false negatives resulting in a low sensitivity for the test (Waites and Talkington, 2004; Vervolet et al., 2007). Numerous PCR techniques have been developed and used but results vary with the specific methods and primers used (Waites

and Talkington, 2004). Polymerase chain reaction will likely become the diagnostic method of choice when a method is perfected. At present, the diagnosis is usually made with serologic methods. Complement fixation (CF), indirect immunofluorescent antibody, particle agglutination, and ELISA have all been used (Waites and Talkington, 2004; Vervolet et al., 2007). Enzyme-linked immunosorbent assay and particle agglutination detect IgM and IgG antibodies to *M. pneumoniae* (Waites and Talkington, 2004; Vervolet et al., 2007). The ELISA tests are the most widely used method for diagnosing *M. pneumoniae* infection but like all serologic tests, they are limited by the requirement for testing of acute and convalescent samples (Waites and Talkington, 2004; Vervolet et al., 2007).

Mycoplasma pneumoniae infection usually responds to treatment with macrolides, ketolides (a subset of macrolides), tetracyclines, and fluoroquinolones and treatment failures have rarely been reported (Waites and Talkington, 2004; Vervolet et al., 2007). Minimum Inhibitory Concentration (MIC) testing is generally considered unnecessary because there is little variability for in vitro results and very few antibiotic resistant *M. pneumoniae* organisms have been documented (Waites and Talkington, 2004). *Mycoplasma pneumoniae* is also susceptible to chloramphenicol, aminoglycosides, and streptogramins however these are seldom used due to the potential for side-effects (Waites and Talkington, 2004). Erythromycin has been the antibiotic of choice especially for children, however it is being supplanted by the newer macrolides such as azithromycin and clarithromycin because they are tolerated better and have once or twice a day dosing while erythromycin must be administered every 6 hours (Waites and

Talkington, 2004; Vervolet et al., 2007). For adults, the newer fluoroquinolones are often the preferred treatment because they are effective against a number of respiratory pathogens (Waites and Talkington, 2004).

Mycoplasma mycoides ssp. mycoides

Contagious bovine pleuropneumonia (CBPP) is a highly contagious and economically important disease of cattle caused by *Mycoplasma mycoides* ssp. *mycoides* Small Colony (SC) and was first described by Gallo in 1564 (Thiaucourt et al., 2004). It has occurred in every region of the world with cattle except Madagascar and South America; however it has been eradicated from North America, most of Europe, and Australia (Thiaucourt et al., 2004). It was the first mycoplasma for which Koch's postulates were satisfied and to be grown on artificial media (Nocard et al., 1896).

It causes disease in domestic cattle species (*Bos taurus*, *B. indicus*) and water buffalo (*B. bubalis*; OIE, 2009a). The course of the infection may be peracute, acute, subacute, or chronic. Acute signs consist of fever, purulent to mucopurulent nasal charge, a soft moist cough, and occasionally epistaxis. As the disease progresses, the animals become reluctant to move, and stand with outstretched necks. Calves may also have septic arthritis. With subacute infections, the signs are basically the same but milder. Animals that are chronically infected have recurrent low grade fevers, a loss of body condition, and will exhibit respiratory signs with exertion (Thiaucourt et al., 2004).

Contagious bovine pleuropneumonia is transmitted by respiratory droplets from infected animals. Respiratory secretions, urine, and uterine discharges can be infectious (Thiaucourt et al., 2004). There is some evidence for transfer of disease through

contaminated feed (Windsor and Masiga, 1977). The organism can survive in the environment for 3 days in the tropics and 2 weeks in temperate climates (Thiaucourt et al., 2004). The incubation period is usually between 3 and 6 weeks but can be as long as 6 months (Thiaucourt et al., 2004; OIE, 2009a). The long incubation period causes outbreaks to intensify slowly. As the number of organisms in the environment and infected animals increase, those animals that are still susceptible experience severe acute and peracute disease (Thiaucourt et al., 2004). Antibiotic treatment is not recommended either for the individual animal or as a means to control an epidemic because of poor clinical response and cure rates (Thiaucourt et al., 2004).

As with *M. pneumoniae* in man, *M. mycoides* ssp. *mycoides* SC infection begins with the organism adhering to the respiratory epithelium (Thiaucourt et al., 2004). The signs of the disease are caused by severe bronchiolitis, bronchopneumonia, and fibrinous pleuritis. Interlobular lung septa are widened with fluid or fibrous tissue (Kahn and Line, 2005). Microscopically, there is a severe fibrinous pneumonia, fibrinous pleurisy, thrombosis, and areas of necrosis (Kahn and Line, 2005). Sequestra form when necrotic tissue becomes encapsulated by a fibrous layer up to 1 cm thick (Thiaucourt et al., 2004). On a molecular level, only a few of the cytotoxic compounds and processes have been determined. Strains of *M. mycoides* ssp. *mycoides* SC that are more virulent produce more hydrogen peroxide through a metabolic pathway that utilizes glycerol (Pilo et al., 2007). A polysaccharide galactan, 6-O-β-D-galactofuranosyl-D-galactose, has also been found to contribute to the virulence of the organism perhaps because it is similar to galactans in the lungs of cattle (Thiacourt et al., 2004; Pilo et al., 2007). Much of the

research into the effects of *M. mycoides* ssp. *mycoides* SC has been directed at production of an effective vaccine rather than characterizing the cytokines produced during the immune response (Dedieu et al., 2006; Totté et al., 2007). Infection with *M. mycoides* ssp. *mycoides* SC is known to cause a marked inflammatory reaction and TNF-α is thought to play a role (Thiacourt et al., 2004).

Definitive diagnosis of CBPP is easier now than when Nocard et al. were using membranous pouches implanted in rabbits (Nocard et al., 1896; Thiacourt et al., 2004). The organism can be cultured in 2 to 3 days from aseptically collected pleural fluid from an animal not previously treated with antibiotics (Thiacourt et al., 2004). As with other mycoplasmas, specific identification is accomplished with biochemical tests, growth inhibition by hyperimmune serum, or PCR (Thiacourt et al., 2004). In addition, the organism can be detected using an agar-gel precipitation test directed at galactan (Thiacourt et al., 2004). A CF test and a competitive ELISA test have been recognized by the World Animal Health Organization (OIE) as valid for serological diagnosis of herds infected with CBPP (Thiacourt et al., 2004; Gonçalves et al., 2008; OIE, 2009a). Neither test detects vaccine induced antibodies so they can be used to measure the prevalence of naturally acquired infections (Thiacourt et al., 2004). However both have sensitivities that are less than 80% which means that disease rates are underestimated and low levels of disease will not be detected (Gonçalves et al., 2008). An immunoblot test was developed in Portugal in1996 and a study comparing it to the OIE approved CF test found that the CF test was also detecting *M. bovis* antibodies in addition to those against

M. mycoides ssp. *mycoides* which resulted in an overestimation of prevalence (Gonçalves et al., 2008).

The importance of CBPP to the local and national economies of the regions affected by it has directed the methods used to control or eliminate it. In most circumstances, emphasis is placed on eliminating it from or controlling it in a herd as opposed to treating the individual animal. Control methods consist of culling affected herds, vaccination, restriction of animal movement, and quarantine. Serologic testing is used in regions that border endemic areas. Importation of animals from positive countries into those free of CBPP is also prohibited (Thiacourt et al., 2004).

Mycoides Cluster Species of Sheep and Goats

Four species of mycoplasmas in the mycoides group cause disease in sheep and goats. Because these organisms are closely related genetically and immunogenically, it has been difficult until recently to clearly identify the organism causing each syndrome and accurately describe the diseases (Lefèvre and Thiacourt, 2004). Three organisms in the mycoides cluster, *M. mycoides* ssp. *mycoides* LC (large colony), *M. mycoides* ssp. *capri*, and *M. capricolum* ssp. *capricolum* and 2 other mycoplasmas, *M. putrefascens* and *M. agalactiae*, have been grouped together based on the clinical syndrome of mastitis, arthritis, keratitis, pneumonia, and septicemia (MAKePS) caused with infection (Thiacourt and Bölske, 1996). Some of the basic characteristics that differentiate the members of the mycoides cluster are listed in Table 2.4. Economically and pathologically, the 2 most important members of this group of organisms are *M. mycoides* ssp. *mycoides* LC and *M. capricolum* ssp. *capripneumoniae* (DaMassa et al.,

1992; Lefèvre and Thiacourt, 2004; Walker, 2004). Originally identified as F38, *M. capricolum* ssp. *capripneumoniae* was given a definitive name in 1992 and determined to be the causative agent of contagious caprine pleuropneumonia (CCPP, DaMassa et al., 1992; Lefèvre and Thiacourt, 2004; Walker, 2004).

Table 2.4: *Mycoplasma mycoides* and *M. capricolum* subgroup species affecting goats and sheep as primary hosts and differential qualities

Quality	*M. mycoides* subgroup		*M. capricolum* subgroup	
Ssp.	*mycoides* LC[1]	*capri*	*capricolum*	*capripneumoniae*
Host	goats, sheep, cattle	goats	goats, sheep, cattle	goats
Route	respiratory, oral	respiratory	respiratory, oral	respiratory close contact
Lung Pattern	interstitial bronchopneumonia	edema, broncho-pneumonia	inconsistent, interstitial pneumonia	interstitial edema, abscessation possible
Interlobu-lar septa	widened +/-	widened	not thickened	not thickened
Extra-pulmonary	mastitis, arthritis, peritonitis	mastitis	septicemia, fibrinous arthritis, mastitis	none

[1]LC = Large Colony

Contagious caprine pleuropneumonia is similar in many ways to CBPP. A high fever occurs at the onset of clinical disease which is followed by coughing, respiratory distress, painful respiration, and a reluctance to move. Morbidity can reach 100%, and mortality may be as high as 70% (Lefèvre and Thiacourt, 2004). In endemic regions some animals experience a subacute form with similar but less intense signs. The disease is transmitted by aerosol and requires close contact because the organism does not survive in the environment (Lefèvre and Thiacourt, 2004). The incubation period is generally between 6 and 10 days (Lefèvre and Thiacourt, 2004) but the OIE International

Animal Health code chapter 14.4 sets the incubation period at 45 days (OIE, 2009b). The disease occurs in northern, eastern, and central Africa, and the Arabian Peninsula and Turkey in the Middle East (Lefèvre and Thiacourt, 2004).

Mycoplasma capricolum ssp. *capripneumoniae* causes a fibrinous pleuropneumonia which usually affects 1 lung but can also be lobar. Serofibrinous pleuritis causes the accumulation of large quantities of serous or serosanguinous pleural fluid. Abscessation of the lungs may occur. There is no widening of the interlobular septa. The lesions are restricted to the thoracic cavity. Diagnosis of CCPP is difficult because other species of mycoplasmas and pasteurellas cause similar lesions (Thiacourt and Bölske, 1996; Lefèvre and Thiacourt, 2004). Differentiation can be made based on the distribution of lesions because *M. capricolum* ssp. *capripneumoniae* only causes pleuropneumonia (Thiacourt and Bölske, 1996; Lefèvre and Thiacourt, 2004). The organism can be cultured with some difficulty from pleural fluid and lung tissue, and metabolic or immunologic based tests are required for speciation. One of the common methods of speciation, inhibition of growth by antiserum, has reduced specificity because *M. capricolum* ssp. *capripneumoniae* shares antigens with *M. capricolum* ssp. *capricolum* and *Mycoplasma* sp. group 7 (Thiacourt and Bölske, 1996; Lefèvre and Thiacourt, 2004). Recently, PCR methods have been developed to detect organisms in tissues but even these protocols require additional steps to improve speciation such as endonuclease digestion; identification is based on the number of fragments generated from the PCR product (Thiacourt and Bölske, 1996; Lefèvre and Thiacourt, 2004).

The serological tests available for herd-level testing for CCPP are a CF test, passive hemagglutination inhibition (HI), latex agglutination (LAT), and an indirect ELISA. None of these tests have been rigorously validated with regards to specificity or sensitivity (Thiacourt and Bölske, 1996; Lefèvre and Thiacourt, 2004). Part of the problem lays with the many shared antigens among the mycoplasma species. For example, in an outbreak of CCPP reported in 1989, more goats were seropositive for *M. mycoides* ssp. *capri* than for *M. capricolum* ssp. *capripneumoniae*, the only organism isolated from diseased lungs (Thiacourt and Bölske, 1996). As with CBPP, CF is the test recognized by the OIE. A blocking-ELISA based on a monoclonal antibody (MAb) is more specific and does not cross-react with *Mycoplasma* sp. group 7 (Thiacourt et al., 1994). When the MAb is used for the detection of antigen in pleural fluid, the limit of detection is 10^7 cfu/ml, a level that occurs in acutely infected animals (Thiacourt et al., 1994). When used in a microtiter plate format, the blocking-ELISA facilitates the testing of large numbers of samples in a timely and economic manner (Thiacourt et al., 1994; Thiacourt et al., 1996).

Animals respond best to early treatment with tetracyclines, macrolides or fluoroquinolones but treatment failures with disease recurrence are common; therefore some experts have recommended measures that include strict control on animal movement to prevent the introduction of the disease into a region and rapid institution of a herd-level test and slaughter protocol when the disease occurs in a new area (Thiacourt et al., 1996). Use of an inactivated vaccine with a saponin adjuvant has been shown to create antibodies detectable for a year by CF and greater than a year by competitive

ELISA, and to result in both reduced mortality and morbidity (Thiacourt et al., 1996). It is not known if antibody levels directly correspond to the level of protection provided for this disease. A modified-live vaccine is being assessed and if proven effective would reduce the cost of immunization (Thiacourt et al., 1996).

Mycoplasma mycoides ssp. *mycoides* LC and *M. mycoides* ssp. *capri* are 99% identical genetically and while there are serologic differences, they will very likely be combined into the same subspecies (Nicholas et al., 2008). In contrast to *M. capricolum* ssp. *capripneumoniae*, *M. mycoides* ssp. *capri* causes septicemia, polyarthritis, pericarditis, peritonitis, lymphadenitis, mastitis and conjunctivitis in addition to fever and interstitial fibrinous pneumonia with widened and pale intralobular septa (DaMassa et al., 1992; Nicholas et al., 2008). The distribution of disease is worldwide but there is considerable geographic strain variation with the strains from Africa being less likely to cause mastitis and arthritis (DaMassa et al., 1992). *Mycoplasma mycoides* ssp. *capri* is the most frequently reported caprine mycoplasma in the United States (Nicholas et al., 2008). *Mycoplasma mycoides* ssp. *capri* can be transmitted orally as well as by respiratory secretions in contrast to *M. capricolum* ssp. *capripneumoniae* which is only transmitted by respiratory secretions (DaMassa et al., 1992). The growth characteristics and colony morphology make it easier to diagnose *M. mycoides* ssp. *capri* but additional metabolic and immunogenic tests are also used (DaMassa et al., 1992; Nicholas et al., 2008). Serologic testing using CF requires an array of antigens due to the similarities between the organisms of the mycoides cluster (DaMassa et al., 1992; Thiaucourt and Bölske, 1997). In Europe it is important to distinguish *M. mycoides* ssp. *capri* from *M.*

capricolum ssp. *capripneumoniae* as the occurrence of the latter would be the result of a transborder disease incursion (Nicholas et al., 2008).

Mycoplasma hyopneumoniae

The cause of enzootic pneumonia of swine was determined in 1965 (Kobisch and Friis, 1996; Wallgren, 2004). This disease is insidious and chronic and as a result the impact is primarily economic (Wallgren, 2004). Like many of the mycoplasmas that cause pneumonia, infection begins with inhalation of the organism and subsequent adhesion to the respiratory epithelium at the apex of the cilia (Kobisch and Friis, 1996; Wallgren, 2004). When it is the only infectious agent, *M. hyopneumoniae* causes high morbidity and low mortality with a dry cough which increases in intensity with exercise (Wallgren, 2004). A decrease in appetite leads to slowed weight gain which is important because the disease is most prevalent in growing pigs (Kobisch and Friis, 1996; Wallgren, 2004). In uncomplicated cases the infection resolves in 10 to 12 weeks, however, secondary infections are common (Wallgren, 2004). Co-infection by *M. hyopneumoniae* and *Pasteurella multocida* is more severe than infection by *M. hyopneumoniae* alone (Ciprián et al., 1988; Kobisch and Friis, 1996). The lesions occur most commonly in the ventral regions of the accessory and middle lung lobes and consist of alveolar interstitial thickening due to marked infiltration by inflammatory cells, giant cell proliferation, and perivascular lymphoid hyperplasia (Wallgren, 2004).

Diagnosis of *M. hyopneumoniae* with culture is complicated by slow growth characteristics and the occurrence of *M. hyorhinis* (nasal secretions) and *M. hyosynoviae* (infected tonsils) in the respiratory tract of swine (Kobisch and Friis, 1996; Wallgren,

2004). *Mycoplasma hyorhinis* and *M. hyosynoviae* both cause polyarthritis (Kobisch and Friis, 1996). Immunofluorescence and PCR are both more efficient methods than culture for identifying *M. hyopneumoniae* in tissues (Wallgren, 2004). Serology is efficient at detecting the presence of disease in herds because the antibody response is detectable for as long as 20 weeks (Kobisch and Fris, 1996). Indirect ELISA has become the preferred method of testing (Kobisch and Fris, 1996; Wallgren, 2004). Control methods for *M. hyopneumoniae* have consisted of antibiotics (tetracyclines, macrolides, and some fluoroquinolones) in the face of outbreaks, especially for naïve herds, but long term use leads to the development of resistance (Kobisch and Friis, 1996; Wallgren, 2004). Management methods such as age segregated housing, closed herds, suitable stocking densities, proper ventilation, and vaccination are effective at controlling *M. hyopneumoniae* as well as *M. hyorhinis* and *M. hyosynoviae* (Kobisch and Friis, 1996; Wallgren, 2004). Inactivated, whole-cell vaccines (bacterins) are widely available and in some countries are used in greater than 70% of herds (Maes et al., 2008). A random sample survey of swine producers in the United States found that as of 2006, one third of the producers were immunizing against *M. hyopneumoniae* (USDA, 2008). Vaccination strategies include immunizing piglets at 1 to 2 weeks of age, piglets at weaning, and sows and gilts while pregnant. Vaccination increases weight gain and reduces mortality but protection is often incomplete (Kobisch and Friis, 1996; Maes et al., 2008). Researchers are continuing to investigate innovative delivery routes, and subunit and DNA derived vaccines. A plasma membrane derived vaccine has been tested and shown to provide protection to the piglets of vaccinated sows (Kobisch et al., 1987).

Mycoplasma gallisepticum

Like *M. hyopneumoniae* in swine, losses in poultry due to *M. gallisepticum* are primarily economic because the disease caused by the organism is generally chronic and results in reduced production (Walker, 2004; Bradbury, 2005; Gaunson et al., 2006). Of the mycoplasmas that infect birds, *M. gallisepticum* has been studied the most and the genome has been completely sequenced (Bradbury, 2005). The clinical syndrome is primarily pulmonary with coughing and respiratory distress in chickens and sinusitis in turkeys but decreases in egg production can occur in both species (Walker, 2004; Bradbury, 2005). The organism binds to respiratory epithelium with a specialized adhesion structure and the prokaryotic surface proteins GapA and CrmA (Bradbury, 2005). Other molecules that may play roles both as adhesins and in evading host immune defenses by antigenic variation include the lipoprotein VlhA (formerly known as pMGA) and the membrane protein PvpA (Bradbury, 2005). *Mycoplasma gallisepticum* has the ability to penetrate non-phagocytic cells, another method for evading the host immune system (Bradbury, 2005; Kleven, 2008).

The disease is transmitted via respiratory secretions as with other mycoplasmas. *Mycoplasma gallisepticum* is also transmitted to the embryo via infected semen or in egg development (Bradbury, 2005). Like several of the other pathogenic mycoplasmas, *M. gallisepticum* has been found to act synergistically with another pathogen, *E. coli* in this case, resulting in increased pathology (Bradbury, 2005). Microscopically, there is mononuclear infiltration of the respiratory epithelium, and hyperplasia of the mucous glands (Bradbury, 2005). The immune response, especially the actions of T lymphocytes,

appears to have a role in the development of pathology (Gaunson et al., 2006). Resolution of clinical disease corresponds to the development of lymphoid follicles with B cells dominating the population and a proportional decline in T lymphocytes (Gaunson et al., 2006).

Because of the impact on production, several countries have attempted to eliminate *M. gallisepticum* from their flocks and there has been some success. The programs have utilized active surveillance, strategic use of antibiotics (tetracyclines, macrolides, quinolones, and tiamulin), and management practices. The development of serum plate agglutination and ELISA tests allows for the rapid screening of large numbers of serum samples for antibodies (Kleven, 2008). Hemagglutination inhibition tests or PCR are used for confirmation of positive screening test results because non-specific serum agglutination or ELISA reactions due to recent vaccination or exposure to other mycoplasmas occur regularly (Kleven, 2008). Reactions to vaccination induced antibodies are greatly reduced with purified antigens and species specific proteins (Stipkovits and Kempf, 1996). A blocking ELISA test that uses a MAb against 1 of the more stable *M. gallisepticum* proteins can be used to test sera or eggs of any host species (Stipkovits and Kempf, 1996). Dipping eggs in or injecting them with tylosin (a macrolide) or tetracycline is effective at eliminating vertical transmission (Stepkovits and Kempf, 1996; Kleven, 2008). While antibiotics can help to minimize losses in the face of an outbreak, continual use is not advised and may lead to the development of resistance. Management techniques such as keeping breeder flocks small, maintaining good biosecurity, and minimizing the introduction of young males appear to be more effective

at controlling disease. Vaccination is generally only employed when keeping the flock free of *M. gallisepticum* by other methods is not possible. Bacterins have been available for more than 40 years but provide minimal protection from the disease and economic losses (Kleven, 2008). There are 3 attenuated strains that have been tested for use in layers and breeding stock with varying levels of residual virulence, immunity, or adverse vaccination reactions (Whithear, 1996; Kleven, 2008). The MG 6/85 strain vaccine is available for commercial use in the United States.

Mycoplasma synoviae

Similar to *M. hyosynoviae* in pigs, *M. synoviae* primarily causes synovitis and retarded growth leading to economic losses in chickens and turkeys (Walker, 2004; Dufour-Gesbert et al., 2006). Respiratory signs in the form of air sacculitis are usually subclinical (Walker, 2004; Dufour-Gesbert et al., 2006). Infiltration of the joints and tendon sheaths by heterophils, deposition of fibrin and subsynovial infiltration by lymphocytes and macrophages causes swollen joints and tendon sheaths (Walker, 2004; Bradbury, 2005). *Mycoplasma synoviae* evades host immune response using variable surface proteins (VSP) encoded by the gene *vlhA* but by a different mechanism than that used by *M. gallisepticum* (Bradbury, 2005). The methods of control and management used for *M. gallisepticum* are also used for *M. synoviae* (Kleven, 2008).

Mycoplasma bovis

Mycoplasma bovis was first described as a cause of mastitis in 1962 (Pfützner and Sachse, 1996). Initially identified as *M. agalactiae* ssp. *bovis*, in 1976 it was elevated to a species and is now recognized as a major cause of bovine mastitis, pneumonia, and

arthritis in Europe, and North and South America (Pfützner and Sachse, 1996; Nicholas and Ayling, 2003). While *M. bovis* can cause fatal pneumonia especially in calves, the impact of infection is primarily through the loss in production that occurs with subclinical and clinical mastitis (Ghadersohi et al., 1999; Nicholas and Ayling, 2003; Wilson et al., 1997). In the United States, between 1% and 8% of herds have at least 1 cow infected with *Mycoplasma* spp. and the most frequently identified organism is *M. bovis* (González and Wilson, 2003; Fox et al., 2005). Clinically, mycoplasma mastitis is characterized by a rapid decrease in milk production, milk that is tan to grey with fibrin flakes or a gritty quality, an elevated SCC, and mastitis that shifts from quarter to quarter and is poorly responsive to antibiotics (Jasper et al., 1966; Pfützner and Sachse, 1996; González and Wilson, 2003). Generally, the affected cows show only mild signs of systemic disease (Jasper, 1977; Pfützner and Sachse, 1996; González and Wilson, 2003). Another disease syndrome caused by *M. bovis* consists of pneumonia, arthritis and sometimes otitis media primarily in calves and occasionally in mature cattle (Pfützner and Sachse, 1996; Nicholas and Ayling, 2003). Morbidity due to pneumonia ranges from 20% to 100% and the severity of disease increases when other respiratory pathogens such as *M. hemolytica*, and *P. multocida* are present (Van Donkersgoed et al., 1993; Pfützner and Sachse, 1996; Gagea et al., 2006).

Mycoplasma bovis is transmitted by latent carriers as well as clinically affected animals to susceptible individuals by direct contact (Pfützner and Sachse, 1996; Nicholas and Ayling, 2003; González and Wilson, 2003; Fox et al., 2005). Contaminated milking equipment, personnel, and intramammary infusions used for mastitis treatment have also

been implicated (Pfützner and Sachse, 1996; González and Wilson, 2003; Nicholas and Ayling, 2003; Fox et al., 2005). Laboratory studies have indicated environmental persistence under certain conditions but this has not been considered a significant factor in the transmission of disease (González and Wilson, 2003; Nicholas and Ayling, 2003).

Infection by *M. bovis* results in pathologic lesions characterized by necrosis and lymphoreticular hyperplasia. Acute mastitis is characterized by an early influx of eosinophils followed by degeneration of the alveolar epithelium (Van der Molen and Grootenhuis, 1979; Jasper et al., 1987). As the infection progresses the interstitial tissue is infiltrated by lymphocytes, macrophages, plasma cells and fibroblasts, and hypertrophy of the epithelium (Van der Molen and Grootenhuis, 1979; Jasper et al., 1987). This is in contrast to most types of bovine mastitis, where neutrophils are the predominant responding cell type (Paape et al., 2000). In severe cases, the mammary gland undergoes atrophy and fibrosis (Jasper et al., 1987). *Mycoplasma bovis*-pneumonia is characterized by similar pathologic changes of peribronchiolar infiltration by mononuclear cells, exudative bronchiolitis, lymphoreticular hyperplasia, fibrinonecrotic pneumonia and fibrinous pleuritis (Howard et al., 1987; Booker et al., 2008). Infection of the joints causes fibrinous synovitis and synovial infusion (Blood et al., 1983; Adegboye et al., 1995; Wilson et al., 2007). The molecular methods of adhesion of *M. bovis* to host epithelial tissues are not well understood (Pfützner and Sachse, 1996; Thomas et al., 2003). *Mycoplasma bovis* interaction with neutrophils causes an inhibition of chemiluminescence (Thomas et al., 2003); as noted above, neutrophils are not a major type of immune cell responding to mycoplasma mastitis. Whether the downregulation of

chemiluminescence is related to the poor neutrophil response against *M. bovis* mastitis is unknown. Studies of the interactions between *M. bovis* and cells that typically respond to it, such as eosinophils, lymphocytes, or macrophages would be of interest. Passage of *M. bovis* on artificial media results in a decrease in adhesion and a change in the expression of some of the *vsp* genes, resulting in a change in VSP expression (Thomas et al., 2003).

Diagnosis of disease due to *M. bovis* can be done at the herd or individual animal level and is accomplished through several methods. When the occurrence of mycoplasma mastitis is suspected in a dairy herd, the recommended diagnostic protocol is to sample the bulk tank milk 5 times every 3 to 4 d (González and Wilson, 2003; Fox et al., 2005; Wilson et al., 2009). The sensitivity of a single bulk tank milk culture for mycoplasmas, the ability to detect *Mycoplasma* spp. in the bulk tank when there is at least 1 infected cow in the herd, has been reported as 50%. With sensitivity of a single sample of 50%, the probability of not detecting infected cows with 5 samples collected 3 to 4 d apart = $(1/2)^5 = 1/32 = 3\%$, resulting in a sensitivity (ability to detect at least 1 positive milk sample in a mycoplasma-positive herd) of 97% (Wilson et al., 2009). *Mycoplasma bovis* can be reliably cultured from milk on modified Hayflick's medium with an atmosphere of 8 to 10% CO_2 for 7 to 10 d (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox et al., 2005). *Mycoplasma bovis* can also be cultured from samples of tissues and swabs from joints as long as care is taken to prevent drying and overgrowth by other bacteria and a transport medium favorable to mycoplasmas such as CVM is used (Pfützner and Sachse, 1996). Additional methods of detecting the organism are with ELISA and PCR (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox

et al., 2005). Polymerase chain reaction is emerging as the diagnostic method of choice due to the shortened time needed for results and the sensitivity of the method (Pfützner and Sachse, 1996; Fox et al., 2005). While an antibody response occurs with infection, serology is not effective at detecting chronic shedders and acutely infected individuals (Pfützner and Sachse, 1996; Fox et al., 2005).

Treatment of mycoplasma mastitis is unrewarding even with antibiotics that are generally effective against mycoplasmas (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox et al., 2005). Control of mycoplasma mastitis is best done through the detection and removal from the herd of infected animals (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox et al., 2005). An infected animal may stop shedding bacteria in the milk but stressors such as calving may cause the disease to undergo recrudescence and many authors suggest that adult cows should be considered infected for life (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox et al., 2005). Vaccination of cows has not been shown to be effective at eliminating disease (Pfützner and Sachse, 1996; González and Wilson, 2003; Fox et al., 2005).

In addition to causing mastitis in lactating cows, *M. bovis* is considered to be 1 of the most important causes of pneumonia in both dairy and feedlot calves (Radaelli et al., 2008; Maunsell and Donovan, 2009). *Mycoplasma bovis* can be cultured from approximately 10% to 30% of healthy calves and 80% to 92% of herds will have at least 1 calf which will culture positive with a nasal swab (Virtala et al., 1996; Wilson et al., 2007; Maunsell and Donovan, 2009). Seroconversion, which indicates infection, coincides with an increase in respiratory disease rates, a decrease in the rate of gain, and

an increase in the number of antibiotic treatments (Radaelli et al., 2008; Maunsell and Donovan, 2009). In studies of calves dying from pneumonia, *M. bovis* has been isolated alone or with other respiratory pathogens in 30% to 50% of the cases (Virtala et al., 1996; Maunsell and Donovan, 2009).

There appears to be a difference in the epidemiology of *M. bovis* pneumonia in dairy calves and beef calves; a higher proportion of dairy calves contract mycoplasma infections at an earlier age than do beef calves (Gagea et al., 2006; Booker et al., 2008; Maunsell and Donovan, 2009). *Mycoplasma bovis* can be cultured from dairy calves as young as 1 wk of age and clinical disease is generally diagnosed in calves 2 to 6 wk old (Maunsell and Donovan, 2009). In herds with mycoplasma mastitis, up to 50% of calves will have positive nasal cultures (Maunsell and Donovan, 2009). In contrast, in beef calves, only approximately 20% have a positive antibody titer against *M. bovis* upon arrival at a feedlot (after weaning at approximately 6 mo of age). The difference in ages at which mycoplasma pneumonia is seen in dairy as compared to beef calves is probably due to the calf management practices typically used in the 2 industries. The practice of feeding pooled colostrum and discard milk to dairy calves has been shown to transmit *M. bovis* to calves, with most developing positive nasal cultures within 2 wk and 37% developing otitis media (Maunsell and Donovan, 2009). In the beef industry, calves generally stay with the cow until weaning.

Similar to *M. bovis* mastitis, treatment of mycoplasma calf pneumonia is generally unrewarding; calves can improve with early treatment with antibiotics such as tulathromycin, florfenicol, tilmicosin, and tylosin, but long term prognosis is guarded and

weight gain is reduced (Rosendal and Martin, 1986; Nicholas and Ayling, 2003; Maunsell and Donovan, 2009). Management and control methods include pasteurization of milk and colostrum, segregation of new arrivals to feedlots, metaphylactic (timely mass medication of a group of animals to eliminate or minimize an expected outbreak of disease) administration of antibiotics, and possibly improving ventilation and minimizing crowding (Maunsell and Donovan, 2009). Vaccine trials have shown mixed results. In some trials, vaccinated groups have had a reduction of clinical disease, an increase in weight gain and a reduced mortality compared to control groups but in other tests, vaccination has appeared to exacerbate disease (Maunsell and Donovan, 2009). Because calves are infected at a very early age, developing an effective vaccine is a challenge (Maunsell and Donovan, 2009).

Mycoplasma dispar

Mycoplasma dispar is isolated more frequently from healthy calves (prevalence as high as 90%) than *M. bovis*, and its role as a primary pathogen is somewhat questionable (Howard, 1983; Marques et al., 2007; Maunsell and Donovan, 2009). The organism was identified and characterized in 1968 using a modification of Goodwin's media (Gourlay and Leach, 1970). In that study of both normal and pneumonic calves, the organism was not isolated from normal calves but was found in 49 of 72 of the diseased calves (Gourlay and Leach, 1970). In contrast, a study of 155 calves with respiratory disease and 146 apparently healthy calves found *M. dispar* in 35% and 9% of the calves, respectively; *M. bovis* was found only in diseased animals (prevalence 12%; Marques et al., 2007). Additional studies have found that many of the apparently healthy calves with positive *M.*

dispar cultures actually have subclinical pneumonia upon histopathologic examination of the lungs (Howard, 1983). Ultimately, it appears that *M. dispar* contributes to pathologic processes which lead to respiratory disease in calves but because the pathologic mechanism is more subtle and because the organism is fastidious in its growth requirements, that contribution has been underestimated (Howard et al., 1987; Ayling et al., 2004).

Mycoplasma dispar does not grow on conventional mycoplasma media but grows well on a medium with glucose, calf serum, lactalbumin lysate, Hartley's digest broth, and homogenized calf thymus (Gourlay and Leach, 1970). On solid media, the colonies appear lacy with a poorly defined center (Gourlay and Leach, 1970). With repeated passage, the colonies become more numerous and smaller exhibiting characteristics of other mycoplasmas (Gourlay and Leach, 1970). *Mycoplasma dispar* also grows well on Friis' medium (Table 1.2) and its use has increased the number of isolations in Britain (Ayling et al., 2004).

In 1 study of calves with pneumonia, the lesions associated with *M. dispar* were fibrinonecrotic bronchopneumonia and suppurative bronchopneumonia (Tegtmeier et al., 1999). In 84% of the cases, more than 1 organism was isolated (Tegtmeier et al., 1999). In gnotobiotic calves, the lesions have been described as subtle and primarily consisting of an alveolitis (Howard et al., 1987). Macrophages appear to be the predominant cell type found at calf lung infection sites with *M. dispar* (Howard et al., 1987). The disease course in the gnotobiotic calves was usually subclinical (Howard et al., 1987). *Mycoplasma dispar* appears to affect the course of infection by other bacteria, most often

P. multocida, by causing ciliostasis (Howard et al., 1987; Virtala et al., 1996; Maunsell and Donovan, 2009). A study of the effect of *M. dispar* on an organ culture of bovine trachea found a marked degeneration of the epithelium, loss of movement in the cilia, and markedly reduced clearance of *Serratia marcescens* (Almeida and Rosenbusch, 1994). Attempts to immunize calves against *M. dispar* have not been successful because the organism is poorly immunogenic and may even suppress the immune system (Howard et al., 1987). Management strategies consist of minimizing stress by addressing ventilation, temperature, and crowding issues and maximizing fitness with adequate nutrition, and vaccination for other respiratory diseases (Maunsell and Donovan, 2009).

Mycoplasma agalactiae

Mycoplasma agalactiae causes a disease syndrome in goats and sheep that is very similar to that of *M. bovis* in cattle. While the common name of the syndrome is contagious agalactia and mastitis predominates, keratoconjunctivitis, arthritis (especially in young animals), and occasionally pleurisy also occur (DaMassa et al., 1992; Smith and Sherman, 1994). *Mycoplasma agalactiae* has also been associated with granular vulvovaginitis of goats (DaMassa et al., 1992). Upon infection, there is a decline in milk production and the udder may become hot, swollen, and tender (Kahn and Line, 2005). The milk can have a granular consistency and be yellow in color (Smith and Sherman, 1994; Kahn and Line, 2005). The gland can atrophy and the loss of milk production can be permanent (Kahn and Line, 2005). Chronically infected sheep or goats shed organisms in milk and become carriers of the disease (Smith and Sherman, 1994; McAuliffe et al., 2008). In susceptible herds morbidity can be 30% to 60% (Kahn and

Line, 2005). Also like *M. bovis*, *M. agalactiae* can be transferred by milking equipment, workers, and by feeding infected milk (DaMassa et al., 1992; Smith and Sherman, 1994; Kahn and Line, 2005). Microbial culture and PCR can be used to detect the organism in tissues and milk (Kahn and Line, 2005). Serological tests such as CF, Western blot, and ELISA can be used to identify infected herds but not for the individual animal because the serologic response is variable and there is cross reaction with other mycoplasma species (see discussion for CCPP, Smith and Sherman, 1994). Recently developed serum ELISA tests have improved sensitivity (100%) and specificity (46% to 92% depending on the test), respectively (Pépin et al., 2003). The test with the highest specificity uses a recombinant antigen, P48, a mycoplasma surface membrane protein (Pépin et al., 2003). Management is more effective than antibiotic treatment for control of the disease (Kahn and Line, 2005). A recently developed inactivated mineral-oil adjuvanted *M. agalactiae* vaccine administered in 2 doses has been shown to prevent clinical disease in sheep challenged intranasally for up to 8 months following the second vaccination (Buonavoglia et al., 2010).

Mycoplasma spp. in Wildlife

A number of mycoplasmas have been identified in wildlife, and both newly described organisms and new hosts for already characterized organisms are being identified on a regular basis (Kirchoff et al., 1997; Helmick et al., 2002; Hammond et al., 2003; Haulena et al., 2006). The disease syndromes of conjunctivitis, bronchopneumonia, polyarthritis, and reduced fertility associated with mycoplasma infection in wildlife are similar to those that have been described for domestic animals

(Kirchoff et al., 1997; Helmick et al., 2002; Hammond et al., 2003; Haulena et al., 2006). There are several differences between the investigation of diseases in wildlife and domestic animals. The identification of outbreaks is generally more difficult because the populations of interest are more dispersed and less accessible. When death occurs, the remains are often discovered in a state of moderate to severe degeneration. The result is that isolation of the responsible agent and satisfying the requirements of Koch's postulates is difficult. The epidemiology, pathology, and population effects of *M. gallisepticum*, *M. ovipneumoniae*, and *M. agassizii* on populations of the house finch, bighorn sheep, and tortoises, respectively, are examples of the difficulties of wildlife disease investigation as well as the effects of mycoplasmas on wildlife populations (Wobeser, 1994; Rudolph et al., 2007).

Respiratory disease in free-ranging desert tortoises (*Gopherus agassizii*) was first observed in the 1970's in the Beaver Dam Slope region of southern Utah (Brown et al., 1994). At the same time, population declines had been observed in both the desert tortoise and the gopher tortoise (*G. polyphemus*). Tortoises are considered by many ecologists to be a keystone species in the biome because their dens provide support for many other species (Brown et al., 1999b). Because of their importance, captive breeding programs and research stations were established for both species. In 1988, an outbreak of respiratory disease occurred on an isolated research station in southern California (Brown et al., 1999a). There was an 86% decline in the population and a 94% decline in adult animals (Brown et al., 1999a). Investigations of free-ranging desert tortoises found a mycoplasma, later named *M. agassizii*, on the surface of the nasal mucosa of affected

tortoises (Brown et al., 1994). Experimental infection of captive tortoises with *M. agassizii* resulted in 7 of 8 animals developing clinical disease; the organism was recovered from 5 of 7 and a rising titer developed in all exposed animals (Brown et al., 1994). Studies of free-ranging tortoises have also documented rising titers in association with clinical signs and increasing prevalence levels of *M. agassizii* (Brown et al., 1999a; Dickinson et al., 2005). In addition to desert tortoises, *M. agassizii* has also been isolated from gopher tortoises and *Geochelone* spp. (Brown et al., 1999b).

Infection of tortoises with *M. agassizii* is characterized by a nasal discharge that varies from serous to mucoid to purulent, increased lacrimation, conjunctivitis, edema of the eyelids, anorexia, dehydration, and death (Brown et al., 1999b; Dickinson et al., 2005). Histologically the infection causes hyperplasia of the epithelium of the affected tissues, loss of cilia in the respiratory epithelium, and accumulation of lymphoid aggregates (Brown et al., 1994; Brown et al., 1999b). Detection of the organism is most efficient with serology via ELISA or PCR (Brown et al., 1999a; Dickinson et al., 2005). Culture is more difficult than usual for a mycoplasma with growth occurring in 4 to 6 weeks on SP4 media (Brown et al., 1999b). Continued epidemiological studies have determined that the typical course of the disease is chronic with some animals remaining infected for more than a year (Brown et al., 1994; Brown et al., 1999b). It is highly probable that stressors such as drought, changes in vegetation, and human activities impact the occurrence of disease (Brown et al., 1999a; Dickinson et al., 2005).

The impact and occurrence of *M. ovipneumoniae* in wild sheep is similar to that of *M. agassizii* in tortoises. The first documented disease outbreak occurred in a herd of

captive Dall's sheep (*Ovis dalli*), a species that is closely related to bighorn sheep (*O. canadensis*) that were maintained by the Toronto zoo (Black et al., 1988). The outbreak started when 2 ewes were returned to the herd after a stay at an outside facility where they had been housed next to a pair of domestic sheep (Black et al., 1988). Two months after they were returned to the herd, 7 of 10 individuals were coughing (Black et al., 1988). Ten days after the appearance of disease, 40% of the animals had severe respiratory distress and despite treatment with several antibiotics and flunixin meglumine, 2 individuals died (Black et al., 1988). One year after the outbreak some of the animals still had poor body condition and exercise intolerance (Black et al., 1988). Since then, *M. ovipneumoniae* has been associated with several disease outbreaks in species of bighorn sheep throughout the western United States (Rudolph et al., 2007; Besser et al., 2008).

The pathology caused by the *M. ovipneumoniae* was well described in the outbreak at the Toronto zoo and has been consistent in subsequent disease occurrences. Fibrinopurulent bronchopneumonia and fibrinous pleuritis are common with the most severe lesions occurring in the right cranial lobe (Black et al., 1988; Rudolph et al., 2007; Besser et al., 2008). Microscopically, early lesions have a hyperplastic bronchiolar epithelium (Black et al., 1988; Rudolph et al., 2007; Besser et al., 2008). As the disease progresses, lymphoid cuffing, congestion, fibrosis, and necrosis occurs (Black et al., 1988; Rudolph et al., 2007; Besser et al., 2008). Otitis media has also been documented in bighorn sheep lambs (Besser et al., 2008).

Identification of *M. ovipneumoniae* in the tissues of affected animals has been difficult and initially the outbreaks of pneumonia in wild sheep were attributed to *Pasteurella* spp. and *Mannheimia hemolytica* (Rudolph et al., 2007; Besser et al., 2008). Use of Friis' media appears to improve isolation (Black et al., 1988; Rudolph et al., 2007; Besser et al., 2008). As with other mycoplasmas which are difficult to culture, both PCR and serologic tests have been described as efficient at detecting the occurrence of *M. ovipneumoniae* in populations. However, sensitivity and specificity calculations have not been reported (Black et al., 1988; Besser et al., 2008).

The appearance of *M. gallisepticum* in house finches (*Carpodarcus mexicanus*) and other passerines in 1994 was both similar and unique in comparison to outbreaks of mycoplasmal disease in other wildlife species. The characteristics of this disease syndrome are dependent upon host and agent factors (Dhondt et al., 2005). The house finch populations of the eastern United States originated from a group of less than 300 birds imported from the western United States in the 1940s and as such have limited genetic diversity (Fischer et al., 1997; Dhondt et al., 2005). Genetic analysis of *M. gallisepticum* isolates from affected birds in 11 states found the isolates to be identical indicating a single emergence event in late 1993 or early 1994 which has been localized to the Washington D.C. area (Fischer et al., 1997; Dhondt et al., 2005). This strain differed from laboratory reference, vaccine, and poultry strains; however newer DNA sequencing methods have placed the strain in a cluster with strains isolated from poultry (Dhondt et al., 2005). The infection causes conjunctivitis that varies from mild to severe and from which some birds can recover (Luttrell et al., 1998; Dhondt et al., 2005). In

spite of this, Christmas bird counts and feeder counts carried out by thousands of bird watchers across the US documented population decreases of up to 60% in the susceptible species (Dhondt et al., 2005).

Infection of susceptible individuals by *M. gallisepticum* is characterized by mild to severe ocular swelling, conjunctivitis, nasal and ocular discharge, and decreased body condition (Fischer et al., 1997; Luttrell et al., 1998). In a natural outbreak in a group of captive birds, coughing, sneezing, and rales were not observed (Luttrell et al., 1998). Histologically, lesions are limited to the periocular tissues, nasal turbinates, and the trachea (Fischer et al., 1997; Luttrell et al., 1998). Consistent with other mycoplasma disease, lesions are characterized by mild to severe lymphoplasmacytic infiltrates, epithelial hyperplasia, and in some cases heterophilic infiltrates (Luttrell et al., 1998).

Experimentally, all finch (Fringillidae) species tested appear to be susceptible (Farmer et al., 2005). Additional susceptible species include the pine siskin (*Carduelis pinus*), tufted titmouse (*Baeolophus bicolor*), the house sparrow (*Passer domesticus*), and the budgerigar (*Melopsittacus undulates,* Farmer et al., 2005). A survey of songbirds in Auburn, Georgia detected antibodies to *M. gallisep*ticum in 20 species (Farmer et al., 2005). Polymerase chain reaction detected *M. gallisepticum* in 6 species: American goldfinch (*Carduelis tristis*), tufted titmouse (*Baeolophus bicolor*), pine grosbeak (*Pinicola enucleator*), evening grosbeak (*Coccothraustes vespertinus*), house finches (*Carpodarcus mexicanus*), and the purple finch (*Carpodacus purpureus*; Farmer et al., 2005). Factors affecting the epidemiology of the disease are the adaptation of these species to human land use patterns, seasonal migration of the eastern populations of

finches, the use of birdfeeders which increases contact between birds and allows sick birds to live longer, and the partial immunity that results from infection (Fischer et al., 1997; Dhondt et al., 2005). The persistence of the disease in the eastern United States is characterized by regional and seasonal epidemics (Hartup et al., 2000; Dhondt, et al., 2005; Farmer et al., 2005). Occasionally outbreaks occur in the western bird populations (Dhondt et al., 2005).

Summary of Mycoplasma Disease

This review of diseases caused by mycoplasmas in man and animals has demonstrated many similarities. While the course of mycoplasma infections can be acute as with CBPP and CCPP, a chronic course is more typical. Coinfection with other bacteria, such as *E. coli* or *Pasteurella* spp., or with viruses, such as influenza or circovirus, can increase the severity of the pathology observed. The pathological lesions often involve lymphoreticular hyperplasia and fibrinous or fibrinonecrotic degeneration of lungs, mammary glands, joints and other affected tissues. Identification by culture of the causative mycoplasma is made more difficult by complex and prolonged growth requirements. Serologic methods of diagnosis are often used but both the sensitivity and/or specificity of many tests frequently limits the usefulness for individual animal diagnosis. Molecular methods, such as various types of PCR, show promise as both practical and accurate diagnostic methods and many diagnostic laboratories are in the process of validating various protocols. In addition, PCR is being used to detect previously unrecognized mycoplasmas in domestic animals and wildlife.

Response

Mycoplasmas have been found to interact with many different host cell types: macrophages, polymorphonuclear (PMN) cells, monocytes, follicular dendritic cells, synovial cells, respiratory epithelial cells, and astrocytes. These interactions result in a number of immune system reactions. The mycoplasma organisms are able to resist phagocytosis unless opsonized by specific antibodies (Razin et al., 1998). Both host and organism strain differences influence the immune reaction and the persistence of the organisms. Even when evading phagocytosis, mycoplasmas can increase the destruction of other cells and bacteria by non-specifically stimulating the immune system to produce enzymes, prostaglandins, toxic oxygen moieties and peroxides, nitric oxide, proinflammatory cytokines, and chemokines (Razin et al., 1998).

An early in vitro study found that bovine anti-serum specific for *M. bovis* and *M. dispar* increased the ability of cultured bovine alveolar macrophages (BAM) and PMN to reduce the number of mycoplasmas in the culture supernatant but when rabbit anti-serum to the same organisms was used, growth of the mycoplasmas occurred (Howard et al., 1976). This indicates that opsonization of the organisms with antibodies allows immune cells to kill the organisms only when the antibodies are from the same species as the cells. Researchers soon determined that some mycoplasmas attached to and even replicated on the surface of phagocytic cells indicating that the process observed in vitro was not the same as the process in the animal (Howard et al., 1987; Almeida et al., 1992). The attachment of mycoplasmas to immune cells could allow the organisms to persist and

was hypothesized to contribute to the pathology of the disease syndromes (Howard et al., 1987). A reduction in the severity of lesions could be observed when the immune response was inhibited pharmacologically suggesting that at least part of the part of the disease syndrome was due to the host immune response (Howard et al., 1987). As immunohistochemistry for various cell types developed, researchers found that the lungs of calves infected with *M. bovis* contained few PMN cells, and large numbers of mature macrophages (Howard et al., 1987). At 2 weeks post-infection immunostaining determined that IgG1-containing plasma cells predominated with smaller numbers of cells containing IgM, IgG2, and IgA. However, by 4 weeks post-infection, more plasma cells containing IgG2 were present (Howard et al., 1987). A comparison was made with calves infected with *M. dispar*; relatively more IgA producing cells were detected especially in the cells of the submucosa of the trachea relative to calves infected only with *M. bovis* (Howard et al., 1987).

Immunosuppression has been documented in patients infected with certain species of mycoplasmas (Howard et al., 1987; Almeida et al., 1992; Razin et al., 1998). *Mycoplasma pneumoniae* causes a decrease in the response to intradermal tuberculin (Howard et al., 1987; Razin et al., 1998). *Mycoplasma dispar* reduced the serologic response of calves to *M. bovis* when the calves were simultaneously injected with both organisms (Howard et al., 1987). *Mycoplasma dispar* also reduced the lymphocyte transformation response to bovine respiratory syncytial virus when both were injected into calves (Howard et al., 1987). The lymphocytes of cattle infected with *M. mycoides* ssp. *mycoides* have a reduced ability to respond to mitogens (Howard et al., 1987).

The inhibitory effect of mycoplasmas on the immune systems of their respective hosts is accomplished through different mechanisms, a few of which have been investigated with advanced biochemical and genomic techniques. The responses of BAM in cell culture to encapsulated and unencapsulated *M. dispar* were characterized by measuring the production of tumor necrosis factor (TNF), interleukin 1 (IL-1), and glucose (Almeida et al., 1992). For all 3 products, exposure to the purified capsule or the whole encapsulated organism reduced production by BAM (Almeida et al., 1992). Moreover, the BAM were unresponsive to endotoxin in the presence of purified capsule (Almeida et al., 1992). *Mycoplasma dispar* produces a capsule in vivo as well as in culture (Almeida et al., 1992). *Mycoplasma hyorhinis* releases a protein which suppresses an interleukin-2 (IL-2) dependent cytotoxic T lymphocyte response and T cell memory (Razin et al., 1998). A deficit of IL-2 also occurs with the suppression of the in vitro lymphocyte proliferation by *M. mycoides* ssp. *mycoides* LC (Poonia and Sharma, 1998). Mycoplasmas may be able to adhere to and cause death by apoptosis of host animal T cells. Calves infected with *M. bovis* showed suppressed lymphocyte activity even though the lymphocytes were not killed as determined by trypan blue exclusion (Thomas et al., 1990; Razin et al., 1998). Subsequent research using propidium iodide incorporation, translocation of phosphatidyl serine, detection of a decrease in cell size by flow cytometry, and detection of DNA ladder generation (an indicator of DNA fragmentation) determined that there was a loss of cells occurring by apoptosis (Vanden Bush and Rosenbusch, 2002). A product of the *vsp* gene family, VSP-L, was found to suppress lymphocyte proliferation (Vanden Bush and Rosenbusch, 2004). An analogous

protein, a product of the *avgC* gene was identified for *M. agalactiae* (Santona et al., 2002).

Many mycoplasma species affect the immune system by causing effector cells to produce cytokines, both stimulatory and inhibitory, and chemoattractants (Razin et al., 1998; Yang et al., 2002; Vanden Bush and Rosenbusch, 2003; Thanawongnuwech et al., 2004; Sun et al., 2006). *Mycoplasma bovis*, *M. capricolum*, *M. gallisepticum*, *M. synoviae*, *M. hyopneumoniae*, *M. hyorhinis*, and *M. mycoides* ssp. *mycoides* induce macrophages to produce TNF-α, a cofactor for B and T lymphocyte proliferation and differentiation. Many of the mycoplasmas also increase the production of additional stimulatory cytokines such as IL-1, IL-1β, IL-6, and IFN-α (Razin et al., 1998). In contrast, production of proinflammatory TNF-α by macrophages is inhibited by *M. dispar* (Razin et al., 1998). *Mycoplasma bovis* stimulation of TNF-α and interleukin-β occurred at 100 hours post infection and continued until the end of the study (240 hours) of experimentally infected cows (via mammary gland infusion; Kauf et al., 2007). Compared to other organisms that cause intra-mammary infections such as *Streptococcus uberis* and *Escherichia coli*, the induction is delayed by as much as 60 h; this may help *M. bovis* to become established in the mammary gland, contributing to its greater likelihood of becoming a cause of chronic mastitis than *S. uberis* or *E. coli* (Kauf et al., 2007; González and Wilson, 2003). Subtle strain variations have been detected in the pattern of macrophage cytokine production (Jungi et al., 1996).

Mycoplasma pneumoniae, *M. arthritidis*, *M. fermentens*, *M. hyopneumoniae*, *M. pulmonis*, and *M. bovis* infections are also associated with production of the inhibitory

cytokine, IL-10, usually later in the course of infection (Razin et al., 1998; Thanawongnuwech et al., 2004; Sun et al., 2006; Kauff et al., 2007). Mice strains susceptible to *M. pneumoniae* expressed more IL-10 mRNA than resistant mice when exposed to antigen (Sun et al., 2006). Furthermore, the expression of IL-10 mRNA increased with increasing disease severity (Sun et al., 2006). While pulmonary alveolar macrophages harvested with bronchoalveolar lavage (BAL) demonstrated increased production of IL-10 mRNA in pigs infected with *M. hyopneumoniae*, increased amounts of IL-10 were not detected in the BAL fluid (Thanawongnuwech et al., 2004). The authors did not discuss this incongruity but it could be due to sequestration of IL-10 within the lung tissues. Cytokine IL-10 levels were increased in the supernatant of co-cultured pulmonary alveolar macrophages and tracheal rings but the IL-10 was not detected in the macrophages which suggests that epithelial cells possess the ability to produce this cytokine (Thanawongnuwech et al., 2004). *Mycoplasma bovis* increases IL-10 production in experimentally infected mammary glands 90 hours post-infection; simultaneous milk sampling determined that there were greater than 10^6 cfu/ml of *M. bovis* in milk at that time (Kauf et al., 2007). The increased production of IL-10 could be an attempt by the immune system to limit the damage or due to direct stimulation by an as yet unidentified effector molecule from the mycoplasma (Razin et al., 1998; Sun et al., 2006). The end result, however, could be impaired clearance of pathogenic organisms due to the suppression of macrophage function (Thanawongnuwech et al., 2004; Sun et al., 2006).

Mycoplasmas are capable of stimulating B cells, T cells, natural killer cells, and macrophages. Some B cells may become antibody-producing plasma cells, but the antibodies are not necessarily specific for mycoplasma antigens (Razin et al., 1998). The non-specific antibodies in combination with the upregulating cytokines further amplify the inflammatory process and as a result downregulating cytokines are then produced by effector cells while pathogenic organisms are still present (Razin et al., 1998; Vanden Bush and Rosenbusch, 2003). This process both reflects and likely contributes to the chronic nature of many mycoplasma infections (Razin et al., 1998; Vanden Bush and Rosenbusch, 2003).

Autoimmunity

An additional characteristic of mycoplasma infections is the induction of an autoimmune process. One mechanism for this effect is the occurrence of shared epitopes (the part of the macromolecule that is recognized by the immune system) between the mycoplasma and the host (Razin et al., 1998). *Mycoplasma penetrans*, which occurs as an AIDs-associated illness, causes immune system activation in humans through a complex process that includes glycolipid and lipoprotein mediators (Razin et al., 1998). *Mycoplasma penetrans* shares an epitope with CD18, an adhesin that stimulates the immune system by mediating the interactions between various macrophages and lymphocytes (Razin et al., 1998). Similar shared epitopes have been found for *M. mycoides* ssp. *mycoides*, *M. gallisepticum*, *M. hyorhinis*, *M. genitalium*, *M. pneumoniae*, and *M. arthritidis* (DeVay and Adler, 1976; Davis et al., 1981; Razin et al., 1998).

Immune Evasion by Mycoplasmas

In addition to influencing the character of the immune reaction by stimulating the production of various cytokines and enhancing the maturation of lymphocyte cell lines, several mycoplasmas possess an ability to rapidly change surface epitopes. The mechanisms of this process have been studied for several species of mycoplasmas and have been found to consist of several types of genetic recombination events (Razin et al., 1998; Beier et al., 1998; Lysnyansky et al., 2001; Nussbaum et al., 2002; Flitman-Tene et al., 2003). As different strains of *M. bovis* were subjected to antigenic analysis, researchers became aware that there was incomplete understanding of the mechanisms by which strain variation was generated (Rosengarten et al., 1994).

In an early study using MAb, researchers determined that a single epitope occurred in several different sized integral membrane proteins in several strains of *M. bovis*. It was determined by use of an immunoblot method that this variation occurred in colonies of *M. bovis* on a culture plate, thus indicating the rate of change was higher than could be accounted for by random mutation. By examining several different strains isolated from clinical cases of mastitis, pneumonia, and arthritis and 2 healthy calves it was determined that the variability of epitope expression could not be correlated with pathogenicity or the site of isolation. When a chronically infected calf was serially sampled, similar changes in the membrane proteins were detected indicating that the same process occurs in vivo (Rosengarten et al., 1994).

Research has progressed with the development of gene and protein sequencing methods. The genes that code for the VSP have been identified for *M. bovis* (*vsp*), *M.*

hyorhinis (*vlp*), *M. pulmonis* (*vsa*), and *M. agalactiae* (*avg*; Razin et al., 1998; Beier et al., 1998; Lysnyansky et al., 2001; Nussbaum et al., 2002; Flitman-Tene et al., 2003). The mechanisms used by each species to attain the maximum amount of variation from a limited genome are similar and in the case of *M. agalactiae* and *M. bovis*, homologous gene segments are present (Flitman-Tene et al., 2003). Many other bacteria have similar methods of generating phenotype variability from a single genotype but mycoplasmas are unique in the amount of variability generated (Razin et al., 1998).

The common characteristics of these gene families are an N-terminal region that contains a prokaryotic lipoprotein signaling sequence, an exposed C-terminal region of repetitive units, and an initiation codon that is subjected to site-specific insertion and deletion (Razin et al., 1998; Beier et al., 1998; Flitman-Tene et al., 2003). The result is a combination of phase and size variation of the resulting gene product (Razin et al., 1998; Beier et al., 1998; Flitman-Tene et al., 2003). Phase variation occurs in *M. bovis*, *M. pulmonis*, and *M. agalactiae* by means of site-specific intergenic recombination and inversion events (Razin et al., 1998; Lysnyansky et al., 2001; Flitman-Tene et al., 2003). This mechanism is facilitated by a DNA recombinase in the λ-integrase family (Flitman-Tene et al., 2003). Size variation of membrane proteins occurs in *M. bovis*, *M. hyorhinis*, *M. hominis*, and *M. pneumoniae* and occurs by the insertion or deletion of repetitive sequence (Razin et al., 1998; Beier et al., 1998). In *M. bovis* and *M. hyorhinis* it is independent of phase variation (Razin et al., 1998). Finally, there is a rapid ON/OFF switching of the multiple copies of the membrane protein genes by either insertion of an

initiator or promoter codon (Razin et al., 1998; Lysnyansky et al., 2001; Flitman-Tene et al., 2003).

Mycoplasmas elicit a mixture of immunostimulatory, immunosuppressive, and autoimmune reactions, as well as use surface modification to attempt to evade detection by the host's immune system in an effort to continue to reproduce while the host's immune system strives to eliminate the undesirable organisms. For many mycoplasmas, such as *M. hyorhinis*, *M. bovirhinis*, and *M. salivarium* and the host species to which they are adapted, the balance of elimination and reproduction is relatively even and the lesions that are produced, minimal (Jungi et al., 1996; Razin et al., 1998; Mahon et al., 2007). Variations in both host and pathogen characteristics can shift the balance and cause severe disease to occur. While *M. bovis* can be isolated from 30% to 50% of calves dying from pneumonia and cultured from the nasal cavity of 20% of apparently healthy calves, and 90% of mixed age herds have at least 1 calf that will culture positive, only 8% of dairy herds will have any mycoplasma, usually *M. bovis*, in adult cows (González and Wilson, 2003; Maunsell and Donovan, 2009). Variations in cytokine production by individuals may explain differences in disease susceptibility. In 1 study of experimentally induced intramammary infection, 3 out of 4 cows had equal amounts of IFN-γ and IL-4 producing cells and 1 cow had a significantly greater percentage of IL-4 producing cells. Because IFN-γ is generally classified as stimulating the immune system and IL-4 causes a downregulation in many immune functions, this difference could dramatically alter the course of disease. Similar differences have been detected between *M. pulmonis* resistant and susceptible strains of mice (Sun et al., 2006). Variations in

antigenicity, cytokine induction, and immune modulation have been documented in field isolates and laboratory cultured mycoplasmas but it has been difficult to connect these variations to changes in pathogenicity and disease syndromes (Beier et al., 1998; Thomas et al., 2003; Maunsell and Donovan, 2009). It is well recognized that mycoplasmas become less pathogenic when cultivated in the laboratory and this has been used to produce vaccines for *M. gallisepticum* (Whithear, 1996; Kleven, 2008). Another imbalance between host immunity and the invading pathogenic mycoplasma occurs when a naïve host species becomes infected as occurs with bighorn sheep and *M. ovipneumoniae*, and with house finches and *M. gallisepticum*. In both of these species, the introduction of disease was accompanied by notable population declines (Dhondt et al., 2005; Besser et al., 2008). For many of the mycoplasmas of veterinary importance, the impact of the disease lies not with the severity of disease but with the rate of morbidity and the impact on production.

Summary of literature review

In summary, mycoplasmas cause disease in a wide range of animal and bird species as well as in humans. Infection with these organisms can result in death but more often results in chronic disease, and in the case of livestock, reduced production. Mycoplasmas are often difficult to identify as the agents of the disease process because of their characteristics of growth in culture and the need for specialized media. In addition, development of antibodies by the host in response to infection is variable making serologic testing unreliable for detection of infection in individual animals. Areas in need of additional research in regards to mycoplasma infections are defining virulence factors,

improving detection of organisms which have prolonged growth curves or fastidious growth requirements, developing methods which facilitate speciation of closely related organisms, and incorporating an understanding of microbial ecology into modes of transmission and environmental persistence.

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CHAPTER 3

SURVIVAL AND REPLICATION OF *MYCOPLASMA* SPECIES IN RECYCLED BEDDING SAND AND ASSOCIATION WITH MASTITIS ON DAIRY FARMS IN UTAH[*]

Introduction

Infection with *Mycoplasma* spp., typically *M. bovis*, is an important disease complex of dairy cattle. *Mycoplasma* spp. can cause mastitis, arthritis, metritis, pneumonia, septicemia, and death of cattle (Pfützner and Sachse, 1996; Wilson et al., 2007; Wilson et al., 2009). Standard microbial cultures of milk samples do not isolate *Mycoplasma* spp.; special methods are necessary. Therefore, the presenting history associated with mycoplasma mastitis often includes a frustrating inability to determine the causative agent after clinical signs appear because of repeated negative results on standard microbial cultures of milk samples (González et al., 1992; Wilson et al., 2007). Culled or dead cows, lameness, respiratory disease in calves or adult cattle, and clinical mastitis which does not respond well to treatment can cause considerable financial loss (Pfützner and Sachse, 1996). Per case, mycoplasmal infections cause some of the largest

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[*] Originally published as: Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah. Justice-Allen A, Trujillo J, Corbett R, Harding R, Goodell G, and Wilson D. Dairy Sci. (2010) 93:192-202. Permissions for use are in the appendix.

milk production losses among all mastitis pathogens (Wilson et al., 1997). Mycoplasma infections are contagious diseases, and are primarily transmitted by milk from infected cows which contaminates milking equipment and by direct contact with respiratory secretions (Jasper, 1977; González et al., 1993; Ghazaei, 2006).

During the follow up phase of a statewide prevalence study of mycoplasma mastitis in Utah, we discovered an outbreak of clinical mycoplasma mastitis on a 4,500 cow dairy farm. This disease outbreak was associated with the isolation of *Mycoplasma* spp. from stored recycled bedding sand and from freestall bedding. An earlier reported outbreak of mycoplasma mastitis in a Florida dairy herd was associated with a mycoplasma-positive sandlot that was used for calving and to house mastitic cows (Bray et al., 1997). Additionally, seasonal variation of mycoplasma mastitis has been reported (González et al., 1992; Bray et al., 1997; González and Wilson, 2003) which could partially be attributed to seasonal cow stress, but environmental sources may play a role (Bray et al., 1997). Thus, the existence of environmental sources for *Mycoplasma* spp., including *M. bovis*, and their role in transmission and clinical disease needs further investigation.

The objectives of this study were to evaluate the concentration of *Mycoplasma* spp. in recycled bedding sand, investigate factors associated with the survival of the *Mycoplasma* spp. in recycled bedding sand and determine whether recycled bedding sand can serve as an environmental source of mycoplasmal infections of dairy cows. The null hypothesis was that *Mycoplasma* spp. would not persist in the recycled bedding sand without constant exposure to infected cows. The first alternative hypothesis was that

Mycoplasma spp. would persist for a period of time without exposure to infected cows but then numbers detected would eventually decline to zero. The second alternative hypothesis was that *Mycoplasma* spp. numbers would fluctuate over time suggesting changes in response to changing conditions. We also evaluated 2 methods of elimination of *Mycoplasma* spp. from contaminated bedding sand. Confirmation of an environmental source for *Mycoplasma* spp. in recycled bedding sand and its association with mycoplasma-related clinical disease raises possibilities for further investigation of mycoplasma disease in dairy cattle and could impact management strategies.

Materials and Methods

Study Dairies

Mycoplasma-positive dairy herds were identified in a previously performed statewide prevalence study in Utah (Wilson et al., 2009). During the follow-up portion of that study, Dairy 1 experienced an outbreak of clinical mastitis (CM) caused by *Mycoplasma* spp. which was associated with the isolation of *Mycoplasma* spp., of which 2 isolates were found by PCR to be *M. bovis*, from bedding sand and piles of recycled bedding sand that was designated to be reused. Dairy 1 had approximately 4,500 milking cows housed on dry lots and freestalls bedded with recycled sand. The endemic rate of CM attributable to *Mycoplasma* spp. was approximately 3 cases/4,500 cows per month. During the outbreak, aseptic milk samples from all CM cases were cultured from this herd by the herd health veterinarians (RH and RC). During the CM outbreak, the rate of mycoplasma mastitis cases increased to approximately 35 cases/4,500 cows per month. At this dairy, the bedding sand is recycled by scraping it out of the alleys, into a flume

where the manure is washed free. This is followed by a second washing step on a cement pad with a low slope. After drying, the sand is swept up and stored for several months in large, elongated piles (approximately 3.75 m tall and 3.75 m wide by 600 m long) prior to reuse. Samples of bedding sand from freestalls and the piles of recycled sand were collected by the herd health veterinarians. Composite bedding sand samples were collected from 20 freestall housing pens by collecting approximately 3 grams from the back third of every 10th stall. Samples were identified by date and either the pen number or pile (letter designation). Dairy 1 began using sand as the only bedding substrate in 2001. In 2004 the dairy began using recycled bedding in all pens and no new sand was imported with the exception of 1 month at the end of the described CM outbreak.

Dairies 2 through 8 also had *Mycoplasma* spp. detected in bulk tank milk as part of the prevalence study (Wilson et al., 2009). Composite bedding samples were collected (by DW and AJA) from the freestalls of those dairies by collecting approximately 3 grams of material from the back third of 6 to 10 stalls per pen. From hospital pens, 1 sample was collected from each of 6 different areas of the pen. Samples were identified with the dairy number, date, and pen number. Conventional mycoplasma culture methods identified *Mycoplasma* spp. in the freestall bedding sand and recycled sand. PCR was used to determine whether or not the organisms isolated were *M. bovis* in 4 samples from Dairy 1. The protocols for each method are described below.

Study Pile of Recycled Bedding Sand

Mycoplasma spp.-positive recycled bedding sand (18,000 kg) was transported from Dairy 1 to Utah State University (USU) and stored in a pile (approximately 3 m in

diameter and 2 m tall) outdoors, uncovered on a hard asphalt surface away from animals or their waste. The outbreak of mycoplasma mastitis at the dairy was resolving when the sand was removed from the dairy. The sand pile was composite sampled on the surface (from approximately 0 to 2 cm into the pile) and deep (from approximately 15 to 18 cm into the pile) layers on a weekly basis for 4 mo, from the beginning of April to the beginning of August. Each composite sample was collected with a disposable spoon and consisted of subsamples containing ½ of a spoonful from each of 5 sites within the pile. After 3, 4, and 5 ½ mo of study, single site samples were taken from very deep (approximately 50 cm) in the pile. For each sampling, surface and deep, a new plastic spoon was used. The superficial sand that was removed from the deep sites was replaced in the sampling site after the sample was removed. Previously sampled sites were easily identified and a new set of sampling sites were used for each collection. The temperature of the pile at the surface (2 cm) and deep (18 cm) locations was measured with a standard digital soil thermometer on each sampling day at 1600 h.

After 4 mo, the pile was composite sampled every 14 to 28 d for the next 2 mo (August to October). After 6 mo (the beginning of October), the sand was moved by a construction crew seeking access to a job site; most of the sand was stored in a dump truck bed and some was taken to a nearby storage site away from animals or their waste, and then the sand was returned after 24 h. The sand was moved again approximately 3 wk later (October 28), when it was relocated to another outdoor site where it was stored uncovered, on cement, for use in another study. Weekly sampling resumed and continued for 7 wk.

Temperature and Precipitation Records for Logan Utah

Weather data (daily highs and lows, and daily precipitation in inches) were obtained from the USU Utah Climate Center for the weather station closest to the sand pile (lat. 41°44'26", long.-111°48'37", elev. 1,460 m). This weather station is less than 3.2 km from the storage sites of the sand pile.

Sand Composition Testing

Sand nitrogen and carbon content were determined by the Utah State University Analytical lab (USUAL). A composite sample of approximately 1 kg of sand was collected from the recycled bedding sand pile 1 mo after the sand arrived at USU. Additionally, a sample of unused sand from the source sand pit and composite samples of stored recycled bedding sand from Dairy 1 were tested for total nitrogen, total carbon, and organic content.

Bedding Culture: from Dairies and from the Pile of Recycled Bedding Sand

Bedding samples were collected into new ziplock plastic bags. When freestalls were being sampled, 6 to 10 stalls were sampled from each pen; samples were taken from the back third of every tenth stall. Samples were held at 4 °C and shipped within 24 h in padded envelopes to The Dairy Authority Laboratory (TDA, Greeley, Colorado) by priority mail (2 d delivery). Samples were cultured using 1 g of bedding diluted in 1,000 ml of sterile water to make an initial 1,000 fold dilution. Three additional 1:10 dilutions were made, resulting in $1:10^4$, $1:10^5$, $1:10^6$ dilutions. For all dilutions, 50 µl of sample was plated and counted to determine colony forming units (cfu/g). Aerobic

culture was performed using MacConkey, modified Edward's agar (A70, Hardy Diagnostics, Santa Maria, CA), and Mannitol Salt agars for 48 h at 37 °C. Plates were examined at 24 and 48 h and the growth recorded. Mycoplasma culture was performed on modified Hayflick medium (G102, Hardy Diagnostics) using the same dilutions with 10% CO_2 and 80% humidity for 10 d. Plates were examined at 3, 7, and 10 d under 2X magnification with confirmation at 4X magnification and the colonies morphologically consistent with *Mycoplasma* spp. were counted. Mycoplasma plates that were overgrown with other types of bacteria were defined as contaminated.

Controlled Experiments with Recycled Bedding Sand

Temperature Trials. Bedding samples were collected from deep in the pile and incubated at -20 °C, 4 °C, 20 °C, 36 °C, and 60 °C in closed but not sealed petri dishes for 24, 48, and 72 h. At 36 °C an additional trial was run by adding 250 µl of water at 24 and 48 h of incubation in order keep the sample hydrated. An additional trial consisted of prolonged incubation for 117 d at 4 °C of 4 recycled bedding sand samples (Samples A, B, C, and D) from Dairy 1. After incubation for the allotted time, the samples were sent overnight to TDA on frozen cold packs for mycoplasma culture and cfu/g determination as described above. *Mycoplasma* spp. cfu/g were compared to those from the same samples prior to incubation.

Chemical Disinfection. A large sample was collected from deep in the pile after 2 mo and held at 4 °C for 3 d. Sample fractions (2 g) of the sand were placed in 14 ml conical tubes, and mixed with 1 of the following: 4 ml of 2% chlorhexidine gluconate solution (Durvet, Blue Springs, MO); 0.5% chlorhexidine gluconate (1:4 dilution of 2%

solution); 1:10 dilution of household bleach (Clorox, Oakland CA), 0.5% sodium hypochlorite; or 1:30 dilution of household bleach (Clorox), 0.17% sodium hypochlorite. Sterile, deionized sterile water was used as a control. The sand and disinfectant mixtures were allowed to stand undisturbed for 30 min at room temperature. The chlorhexidine or sodium hypochlorite was removed by decanting the liquid, adding 8 ml of sterile deionized water, mixing, settling and decanting of the liquid. The washing procedure was repeated 3 times. The treated samples (those treated with disinfectant) and the control samples were shipped on frozen cold packs to TDA for mycoplasma culture and cfu/g of sample determination.

Conventional PCR Protocol

Sample Preparation. Sample preparation, DNA extraction, PCR and post-PCR processing were performed at the UVDL. Two 1 g volumes of bedding sand were washed with phosphate buffered saline (PBS) and the supernatant collected. Additionally, two 100 mg volumes were placed in 5 ml of PPLO broth and incubated for 24-48 hours at 37 °C in 8% CO_2.

DNA extraction. DNA was extracted from 150 µl PPLO broth culture and from the PBS wash solution with a commercial extraction kit for tissues and fluids (Qiagen, Valencia, CA). Following the manufacturer's protocol, briefly, the sample was mixed with 180 µl of tissue lysis buffer and 20 µl of proteinase K (Qiagen, Valencia, CA) and incubated at 55 °C for 2 h. After incubation, 200 µl of AL (Qiagen, Valencia, CA) buffer were added and the mixture was incubated for 10 min at 70 °C. After the addition of 200 µl of ethanol, the mixture was transferred to the DNA binding spin column. The column

was sequentially washed with 500 µl of each of the wash buffers (Qiagen, Valencia, CA) and centrifuged at 5.9 X g for 1 min. Finally, the DNA was eluted from the column by the addition of 100 µl of AE (Qiagen, Valencia, CA) buffer and centrifugation. Elution was performed twice.

Conventional PCR. Two conventional PCR protocols were used to detect *Mycoplasma* spp. or *M. bovis* DNA in the extracted DNA (L. Li, Alabama State Veterinary Diagnostic Laboratory, Auburn, Alabama, personal communication). The *M. bovis* specific PCR master mix consisted of 35 µl of RNase free water, 5 µl of 10X PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 µl $MgCl_2$ (50 mM), 1.0 µl dNTP (10 mM), 1 µl each of the forward and reverse primers (10 pmol/µl), and 2 U of Platinum Taq (Invitrogen). The forward *M. bovis* specific primer (MYCO 5, Eurofins MWG Operon, Huntsville, AL) sequence was 5' TGATAGCAATATCATAGCGGC 3' and the reverse primer (MYCO 6, Eurofins MWG Operon, Huntsville, AL) sequence was 5' GTAGCATCATTTCCTATGCTAC 3'. The PCR reaction mix consisted of 45 µl of the PCR master mix and 5 µl of extracted DNA and produced a 400 to 420 bp amplicon when *M. bovis* was detected. Each PCR assay included a positive *M. bovis* DNA control (*M. bovis*, ATCC # 25025) and a DNA-negative control (PCR grade water). The PCR was performed using a conventional thermocycler (Eppendorf Gradient MasterCycler, Westbury, NY). Cycling conditions for PCR included an initial denaturing step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final step extension step of 72 °C for 5 min. PCR products were analyzed with a capillary electrophoresis platform (2100 Bioanalyzer, Agilent, Santa Clara, CA) or

by standard electrophoresis with a 2% agarose gel run at 100 V for 1 h and 20 min for the identification of a PCR amplicon of equal size to the *M. bovis* positive control.

The *Mycoplasma* spp. PCR protocol was a nested procedure. The flanking reaction used a forward primer, MYCP 1 (5' ACACCATGGGAGCTGGTAAT 3', Eurofins MWG Operon, Huntsville, AL) and a reverse primer MYCP 2 (5' CTT CATCGACTTTCAGACCCAAGGCAT 3', Eurofins MWG Operon, Huntsville, AL) with the same PCR master mix as in the *M. bovis* specific protocol. Each PCR reaction consisted of 5 µl of sample and 45 µl of master mix. The thermocycling conditions were as follows: 94 °C for 45 s, 29 cycles of 94 °C for 30 s, 55 °C for 2 min, and 72 °C for 1 min, finishing with 72 °C for 5 min and then a 4 °C hold. The nested reaction used the forward nested primer, MYCO3, (5' GTTCTTTGAAACTGAAT 3', Eurofins MWG Operon, Huntsville, AL) and the reverse nested primer MYCO 4 (5' GCATCCACC AAAAACTCT 3', Eurofins MWG Operon, Huntsville, AL). The PCR master mix for the nested protocol was 37.5 µl of RNase free water, 5 µl of 10X PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 2 µl forward and reverse primer (20 pmol/µl) and 2 U of Platinum Taq (Invitrogen). Each nested PCR reaction consisted of 49 µl of master mix and 1 µl of the flanking reaction product. The thermocycling conditions for this reaction were 94 °C for 45 s, then 33 cycles of the following sequence: 94 °C for 30 s, 55 °C for 2 min, and 72 °C; the final step of 72 °C for 5 min was followed by a 4 °C hold. Positive and negative controls were included as described above. The PCR products were analyzed in the manner described above for the *M. bovis* specific reaction.

Results

Culture of Bedding from Study Dairies

Results of microbial culture of *Mycoplasma* spp. in bedding sand samples from the mycoplasma-positive Dairies 1 through 8 are summarized in Table 3.1. Bedding sand from freestalls of 3 dairies, 1, 3 and 8, cultured positive for *Mycoplasma* spp. in 20 of 39 samples, 1 of 3 samples, and 6 of 6 samples, respectively (Table 3.1). Of the 2 samples of recycled bedding sand and the 2 samples of freestall bedding from Dairy 1 tested with PCR, the presence of *Mycoplasma* spp. was verified in 2 of the recycled sand samples and 1 of the freestall bedding samples. *Mycoplasma bovis* was verified by PCR in 1 sand sample from freestall pens and 1 sample of recycled sand. The fourth sample was identified as positive for *Mycoplasma* spp. by microbial culture but the PCR results were inconclusive. Except for the identification of *Mycoplasma* spp. in the straw bedding of pen 19 of Dairy 8, all of the positive samples were sand or dirt bedding. *Mycoplasma* spp. cfu/g were not determined for Dairy 1. The *Mycoplasma* spp. cfu/g for all other dairies ranged from 1.2×10^3 to 5.6×10^5.

Dairy 1 Sand Pile Culture Results and Association with Temperature and Rainfall

***Mycoplasma* spp. Culture Results.** Results of microbial culture for *Mycoplasma* spp. in samples from the pile of recycled bedding sand collected from Dairy 1 following an outbreak of clinical mycoplasma mastitis are summarized in Table 3.2. At the outset of the study, both the surface and deep samples were culture-positive with 7.7×10^5 cfu/g and 2.13×10^6 cfu/g of sand, respectively (Table 3.2). The concentration of

Table 3.1: Detection of *Mycoplasma* spp. in bedding sand from 8 Utah dairies with mycoplasma-positive bulk tanks

Date	Dairy	Counts cfu/g	Bedding type
12/2007	Dairy 1	positive[1]	sand
3/2008	Dairy 1 14/20 pens	positive[1]	sand
6/19/2008	Dairy 2	neg.	sand
7/3/2008	Dairy 3 hospital	neg.	sand
7/3/2008	Dairy 3 pen 2	1,200	sand
7/3/2008	Dairy 4	neg.	straw
7/3/2008	Dairy 5	neg.	dried manure
7/3/2008	Dairy 6	neg.	straw
7/3/2008	Dairy 7	neg.	straw
7/3/2008	Dairy 8 pen 3	72,000	sand
8/20/2008	Dairy 1 pile A	8,000	sand
8/20/2008	Dairy 1pile B	8,000	sand
8/20/2008	Dairy 1 pile C	neg.	sand
8/20/2008	Dairy 1 pile D	neg.	sand
10/18/2008	Dairy 3 hospital	neg.	sand
10/18/2008	Dairy 3 pen 2	neg.	sand
10/18/2008	Dairy 1 pile E, deep	34,200	sand
10/18/2008	Dairy 1, 5 piles, [2] superficial[3]	neg.	sand
10/18/2008	Dairy 1, 4 piles, [4] deep[5]	neg.	sand
10/21/2008	Dairy 8 pen 1	560,000	sand
10/21/2008	Dairy 8 pen 2	4,200	sand
10/21/2008	Dairy 8 pen 12	30,000	sand
10/21/2008	Dairy 8 pen 15	5,600	sand
10/21/2008	Dairy 8 pen 19	6,000	straw

[1]cfu/g were not reported
[2]Piles E, F, G, H, I
[3]Superficial samples were taken from the surface to a depth of 0 to 1 cm
[4]Piles F, G, H, I
[5]Deep samples were taken from a depth of 15 to 18 cm

Table 3.2: *Mycoplasma* spp. (cfu/g) recovered from recycled bedding sand pile and ambient maximum temperature

Date	Deep	Surface	Tmax °C[1]
3/29/2008	2,130,000	770,000	9.4
4/6/2008	990,000	720,000	6.1
4/11/2008	420,000	neg.	6.7
4/15/2008	182,000	neg.	23.3
4/29/2008	2,000	neg.	22.2
5/6/2008	68,000	neg.	23.3
5/13/2008	47,600	neg.	11.1
5/20/2008	3,400	neg.	28.9
5/27/2008	2,200	neg.	12.2
6/3/2008	7,800	neg.	23.3
6/11/2008	60,000	neg.	12.2
6/17/2008	4,000	neg.	30.0
6/19/2008	16,800	neg.	28.9
6/30/2008	2,000	neg.	33.3
7/8/2008	33,400	neg.	30.0
7/15/2008	neg.	neg.	30.6
7/22/2008	600	neg.	26.1
7/30/2008	neg.	neg.	33.9
8/5/2008	neg.	neg.	33.3
8/20/2008	neg.	neg.	31.7
9/25/2008	neg.	NR	25
10/6/2008	Sand pile moved and replaced		
10/10/2008	14,200	1,000	14.4
10/28/2008	Sand moved to calf facility		
10/30/2008	1,400	neg.	20.6
11/6/2008	32,000	neg.	3.9
11/12/2008	600	neg.	8.3
11/20/2008	200	neg.	11.1
11/28/2008	neg.	neg.	11.1
12/3/2008	neg.	neg.	NR[2]
12/11/2008	neg.	neg.	NR
12/17/2008	3,000	neg.	NR

[1]Tmax. is the maximum ambient temperature in °C.
[2]NR is not recorded.

mycoplasmas in surface samples of the recycled sand pile rapidly declined and the surface became mycoplasma culture-negative after 3 wk (Table 3.2). The samples from the deep portions of the pile became culture-negative on July 30, 2008 following apparent cyclic replication of *Mycoplasma* spp. deep within the pile as evidenced by temporal fluctuations in cfu/g recovered from the pile that corresponded to changes in temperature and precipitation. Recovery of *Mycoplasma* spp. from the sand pile appeared to be dependent on ambient temperature (Table 3.2). Not included in the table is a sample taken on July 8th from the very deep (50 cm) layer of the pile. This sample was positive at 60,000 cfu/g. Subsequent samples from this depth on August 5th and September 25th (also not included in the table) were negative.

Several factors including ambient and sand pile temperatures, precipitation and growth of other microorganisms in the pile were investigated to determine their role in persistence and replication of *Mycoplasma* spp. within the pile of recycled bedding sand. Figure 3.1 is a graphical representation of the temperature changes within deep portions of the sand pile compared to the ambient maximum temperature over the duration of the study. Initially, the entire pile was visibly and palpably damp; as the spring and summer progressed, the surface became dry and crusted but the deep interior remained moist even when the deep samples collected were culture-negative for mycoplasma.

Figure 3.2 demonstrates the association of recovery of *Mycoplasma* spp. with temperature within the pile and precipitation. Initially, recovery of *Mycoplasma* spp. in the pile declined quickly which was followed by increasing and decreasing isolation of

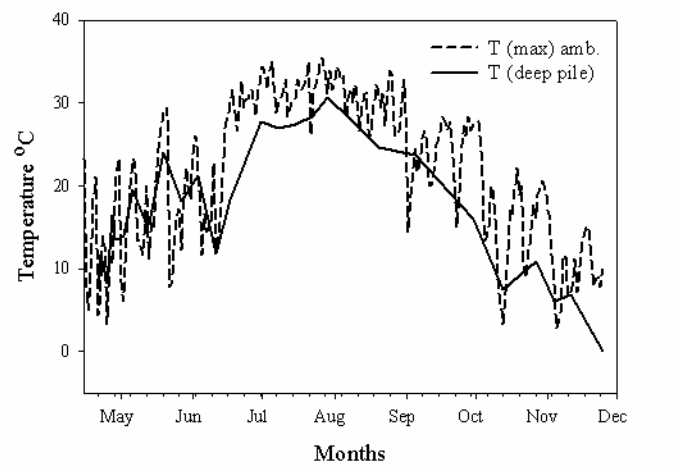


Figure 3.1: Temperature (°C) of the bedding sand at the deep level (T deep pile) compared to the ambient temperature (Tmax) of USU, Logan, Utah.

mycoplasma until the pile temperature stabilized at approximately 30 °C and measurable precipitation ceased. *Mycoplasma* spp. cfu/g increased 30-fold (from 2.0 X 10^3 to 6.0 X 10^4 cfu/g) and then decreased 3 times between May and July. With the first 2 spikes in *Mycoplasma* spp. cfu/g recovered from the pile, the pile temperature was between 15 °C and 20 °C and there were recorded events of moderate precipitation. However, the last spike in *Mycoplasma* spp. concentration recovered from the pile during early summer was not associated with a recorded precipitation event and temperature in the pile was nearly 30 °C. Following this last spike in replication of *Mycoplasma* spp., the concentration of mycoplasma within the pile rapidly declined as the temperature of the pile reached greater than 30 °C and this high temperature persisted. During the summer mycoplasmas were not recovered from the pile via culture. Measurable *Mycoplasma* spp. growth within the pile reoccurred when pile temperature decreased to 14 °C and coincided with 2 of the largest precipitation events during the study. Precipitation

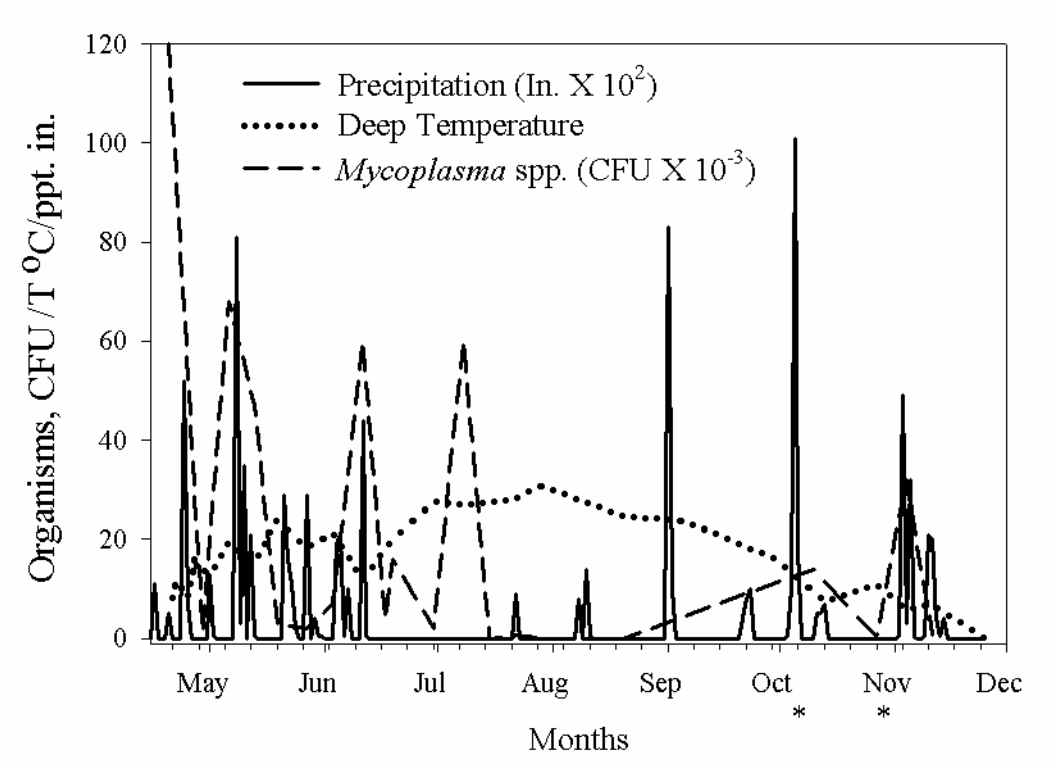


Figure 3.2: Recovery of *Mycoplasma* spp. within the recycled bedding sand pile and the cyclic growth of *Mycoplasma* spp. as it relates to precipitation and temperature. Cfu/g are reported as actual values x 10^{-3}, Deep temperature = T °C, and precipitation is reported in inches x 10^{2}. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

on Oct. 5 was followed by movement of the pile, the contents were rearranged and mixed and subsequent samples collected were culture positive for *Mycoplasma* spp. (1.4 X 10^{4} cfu/g initially deep within the pile), even though weekly samples during the 6 previous wk were culture negative (Figure 3.2). When the pile was moved a second time to another outdoor location, there was an additional 20-fold increase in cfu/g (from 1.4 X 10^{3} to 3.2 X 10^{4} cfu/g) of *Mycoplasma* spp. recovered from the deep layer of the pile which occurred 8 d following this movement.

Results demonstrate a positive association with precipitation and pile movement and increased replication and recovery of *Mycoplasma* spp. from the pile of recycled bedding sand, while demonstrating a negative association between recovery of *Mycoplasma* spp. and ambient temperatures. After 6 wk of no recovery of *Mycoplasma* spp. from the pile, *Mycoplasma* spp. could be recovered from the pile after the favorable temperature and moisture conditions returned. Results indicate that there was long-term survival of *Mycoplasma* spp. in the most protected strata of the recycled bedding sand, even during periods when none could be isolated by microbial culture of the more superficial strata.

Aerobic Culture Results. Figures 3.3 and 3.4 provide a comparison of growth patterns of *Mycoplasma* spp. to growth patterns of Gram-negative microorganisms and *Streptococcus* spp. within the pile of recycled bedding sand over the duration of the

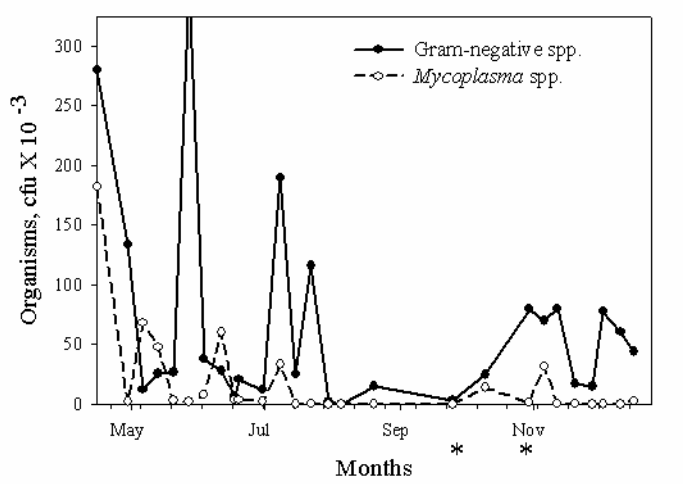


Figure 3.3: Association of the growth of *Mycoplasma* spp. and Gram-negative microorganisms in recycled bedding sand. Cfu/g are reported as actual values $x10^{-3}$. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

study. The Gram-negative microorganisms consisted primarily of *Pseudomonas* spp., and the reported cfu/g ranged from 6.8 X 10^5 in the deep layer of the bedding pile early in the year to undetectable in early August. Growth of Gram-negative microorganisms within the pile preceded (2 events) or coincided (3 events) with rises in *Mycoplasma* spp. cfu/g, but occurred at higher magnitude (Figure 3.3). *Streptococcus* spp. cfu/g ranged from 2.2 X 10^5 to undetectable with the highest counts occurring early in the year and in July. The growth of *Streptococcus* spp. within the preceded the growth of *Mycoplasma* spp. once and coincided with an increase in *Mycoplasma* spp. twice (Figure 3.4).

Sand Composition

The total nitrogen content was 0.03% and the total carbon content was 1.33% for the recycled bedding sand pile. The nitrogen and carbon contents are consistent with the presence of organic matter (Godden et al., 2008) which may play a role in biofilm

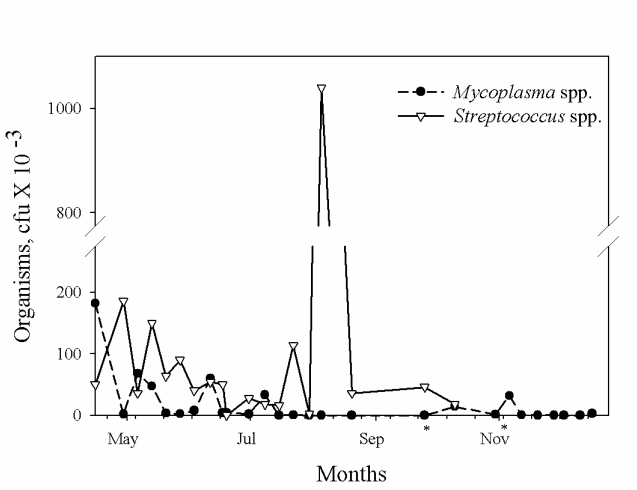


Figure 3.4: Growth patterns of *Mycoplasma* spp. and *Streptococcus* spp. in recycled bedding sand. Cfu/g are reported as actual values x10^{-3}. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

formation in recycled bedding sand. The unused bedding sand had a total nitrogen content of 0.01% and a total carbon content of 1.48%. The amount of organic material in the unused bedding sand was 0.1%. Samples of recycled bedding sand taken from the stored piles at the dairy had a mean organic content of 2.1%, mean nitrogen content of 0.185% and mean carbon content of 1.23%.

Temperature and Prolonged Refrigeration Effects on *Mycoplasma* spp. Growth and Survival

The controlled temperature experiments were conducted to obtain more data relative to survival and optimal growth temperatures for *Mycoplasma* spp. in recycled bedding sand. Results of these experiments are provided in Tables 3.3 and 3.4. *Mycoplasma* spp. survived for 24 h at all controlled temperatures tested except 60 °C (Table 3.3). For the short term experiments, the highest percent survival rate occurred at 20 °C with 32.5% mycoplasma viable after 48 h and 9.1% mycoplasma viable after 72 h. At 36 °C, the *Mycoplasma* spp. remained viable as long as the sand was kept damp

Table 3.3: Percent survival of *Mycoplasma* spp. at controlled constant temperatures in recycled bedding sand. Results are expressed as percent survival which was calculated as the number of organisms at time x divided by the number of organisms at time 0; ND—not done

Time	-20 ℃	4 ℃	20 ℃	36 ℃	36 ℃ [1] w/H20	60 ℃
24 h.	12.6	67.0	55.0	100	ND	neg.
48 h.	6.7	6.7	32.5	neg.	7.2	neg.
72 h.	2.5	4.8	9.1	neg.	37.5	neg.

[1]250 µl of deionized tap water was added at 24, 48, and 72 hours of incubation

by adding 250 µl of water every 24 h. After 72 h the cfu/g of *Mycoplasma* spp. in sand increased to 24,000 cfu/g from 4,600 cfu/g at 48 h, indicating possible replication at 36 °C.

A prolonged refrigeration (4 °C) survival experiment was carried out by storing 3 kg samples (n = 4) collected from Dairy 1 for 117 d, moist, in sealed ziplock bags (Table 3.4). Following prolonged incubation at 4 °C, *Mycoplasma* spp. increased from 8,000 cfu/g to a mean 131,000 cfu/g in 2 of the 4 samples; the other 2 samples remained negative. Replication of mycoplasma in recycled bedding sand occurred at temperatures from 4 °C to 36 °C in the laboratory setting. These data are consistent with the effects of ambient temperature and temperature deep within the sand pile on *Mycoplasma* spp. growth patterns observed in the pile of recycled bedding sand that was kept outdoors.

Chemical Disinfection Experiments

The continuing survival of the organisms in the pile of recycled sand suggested that an environmental source for *Mycoplasma* spp. could be established; we conducted experiments to evaluate the efficacy of 2 disinfectants in the reduction or elimination of mycoplasma in recycled bedding sand. Bleach (sodium hypochlorite) and chlorhexidine

Table 3.4: Recovery of *Mycoplasma* spp. following prolonged refrigeration of bedding sand from Dairy 1. Initial cfu/g: collection date 8/20/08; Ending cfu/g: collection date 12/17/08.

Source pile	Initial cfu/g	Ending cfu/g
Dairy 1 pile A	8,000	200,000
Dairy 1 pile B	8,000	62,000
Dairy 1 pile C	neg.	neg.
Dairy 1 pile D	neg.	neg.

gluconate were chosen on the basis of availability, use in the dairy industry, and for bleach, low cost. These 2 disinfectants were tested at 2 different concentrations. After incubation with the disinfectant, the sand was washed 3 times with deionized sterile water to reduce the disinfectant residue and to reduce inhibition of bacterial growth. Bedding sand treated with sterile water served as a control for these experiments.

The number of viable (cultivable) *Mycoplasma* spp. in recycled bedding sand was reduced in all treatment groups (Table 3.5). Treatment of contaminated sand with 0.5% chlorhexidine or 0.17% sodium hypochlorite resulted in 97.3% and 93.3% reductions in colony counts of *Mycoplasma* spp., respectively. Treatment with higher concentrations of disinfectants, 2% chlorhexidine and 0.5% sodium hypochlorite, resulted in 100%

Table 3.5: Concentration dependent efficacy of 2 common chemical disinfectants in reduction of *Mycoplasma* spp. in contaminated recycled bedding sand. Data represent the mean of 2 replicates. Calculated percent reduction of *Mycoplasma* spp. as compared to the control (sterile water).

Treatment	Chlorhexidine gluconate 0.5%	Chlorhexidine gluconate 2%	0.17% Na hypochlorite	0.5% Na hypochlorite[4]	Sterile water (control)
mean survival	900[1]	neg.[2]	2,200	neg.	33,000
percent reduction	97.3%	100%	93.3%	100%	0%

[1]Colony forming units
[2]neg. = *Mycoplasma* spp. not cultured
[3]1:30 bleach
[4]1:10 bleach.

reduction of mycoplasma colony counts from contaminated bedding sand – none could be isolated by culture. Results demonstrate the initial efficacy of 2 common disinfectants in reducing *Mycoplasma* spp. in contaminated recycled bedding sand.

Discussion

Classically, recognized modes of transmission of mycoplasma associated disease in dairy cattle include direct cow to cow transmission by inhalation and respiratory secretions and through contaminated milking equipment (Jasper, 1977; González and Wilson, 2003; Nicholas and Ayling, 2003). Others have found mycoplasma pathogens in cooling ponds and dry lots in association with mycoplasma disease in dairy herds but the clinical significance of these environmental sources of this infectious agent remains undetermined (Bray et al., 1997; González and Wilson, 2003). Previous laboratory studies reported that *M. bovis* survived up to 236 d in manure and 23 d in drinking water at 23 °C to 28 °C (González and Wilson, 2003). The finding of multiple *Mycoplasma* spp.-positive bedding samples associated with an outbreak of *Mycoplasma* spp. mastitis initiated the investigation reported here into the survival of mycoplasma in recycled bedding sand and factors associated with the persistence of this possible environmental source of *Mycoplasma* spp. Previous studies demonstrated that exposure of the teat ends to microorganisms in bedding can result in the development of mastitis (Kristula et al., 2005; Godden et al., 2008). The amount of mycoplasma organisms required to induce mastitis is not well defined but researchers have successfully induced mastitis by direct teat infusion of 70 cfu of *M. bovis* (Bennet and Jasper, 1980). In this study the amounts of *Mycoplasma* spp. recovered from the recycled bedding sand ranged from 10^3 to 10^6cfu/g,

suggesting that this environmental source may play a role in the epidemiology of mycoplasma-associated disease.

Moreover, the present results showed that *Mycoplasma* spp. remained viable in recycled bedding sand intermittently over a prolonged period of time (8 mo), and that the concentration of mycoplasma in the sand was associated with temperature, precipitation (moisture), and possibly, the growth of Gram-negative microorganisms within the pile. There was renewed isolation of *Mycoplasma* spp. in the pile of recycled bedding sand after it had been repeatedly culture-negative for some time. This new isolation of mycoplasma occurred following the return of favorable growth conditions and movement of the pile. Utilization of a pooled sampling method should have minimized the variability in the counts due to location within the pile, so that increases of at least 10-fold can be interpreted as true increases in the numbers of organisms per gram. In addition, the smaller samples that were stored at 4 °C and 36 °C also demonstrated similar increases. Therefore, it is likely that there was indeed growth of mycoplasma after the sand pile was moved and conditions were more favorable for growth. A previous study of aerated and unaerated leachfield soil found that the active microbial population was 5 times higher in aerated soil (Amador et al., 2006).

Mycoplasma spp. possess a unique combination of traits including: a genome that has undergone major reduction which includes loss of cell wall production, a resistance to some antibiotics as a result of the absence of a cell wall, reduced sensitivity to freezing but increased sensitivity to desiccation and chemical agents, and growth requirements for exogenous sources of sterols and lipids (Razin et al., 1998; González and Wilson, 2003;

Walker, 2004). In our recycled sand pile study, we identified the occurrence of factors in recycled bedding sand over time that supported the establishment of an environmental source for *Mycoplasma* spp. Factors included sand pile temperature, precipitation (moisture), and low levels of organic material. Organic matter will support more bacterial growth than clean sand and recycled bedding sand contains a low level of organic material (Godden et al., 2008). The variability of different bedding materials to support of growth microorganisms has been investigated (Kristula et al., 2005; LeJeune and Kauffman, 2005; Godden et al., 2008). Depending on the recycling process and storage conditions, recycled bedding sand will vary in the amount and composition of organic material that will provide support for bacterial growth (Kristula et al., 2005).

Given that isolation and growth of mycoplasmas in the laboratory requires complex media that include sterols, lipids and nucleotides, it is unlikely that low levels of nitrogen and organic compounds would support prolonged survival of mycoplasmas. The formation of a biofilm community that included mycoplasmas and Gram-negative organisms could account for the survival and replication of mycoplasmas in what at first appears to be a growth factor deficient environment. Biofilms are defined as a heterogeneous but highly organized community of microbial cells collected into microcolonies embedded in extracellular polymeric substance matrix (Donlan, 2002; Halverson, 2005). In the cycle of replication and cell death, the other less fastidious organisms could supply the nutrients required by the mycoplasmas. Sand has been previously demonstrated as a matrix for biofilm development for *Escherichia coli* (Landini and Zehnder, 2002) and *Pseudomonas aeruginosa* (Holden et al., 2002; Robleto

et al., 2003). A previous study (McAuliffe et al., 2006) found that many strains of *M. bovis* were capable of forming biofilms and in a biofilm *M. bovis* was more resistant to desiccation and temperature extremes. Our results support this observation and it may explain why *Mycoplasma* spp., including *M. bovis* can persist in a pile of recycled bedding sand over the hot, dry summer months.

In this study, the ideal temperature for survival and growth of *Mycoplasma* spp. in recycled bedding sand ranged from 15 °C to 20 °C. *Mycoplasma* spp. replicated in the sand at 4 °C when stored for 117 d and remained viable at temperature ranges from 4 °C to 27 °C. Our results are consistent with previous investigations of the survivability of *M. pneumoniae* (Wright et al., 1968). That study tested the viability of this organism in an aerosol at controlled temperatures; *M. pneumoniae* survived best at 15 °C. Another study found that some species of mycoplasma, including *M. bovis*, *M. bovirhinis*, *M. arginini*, remained viable from 50 to more than 100 d in liquid media at 4 °C but only remained viable for 7 to 28 d at outdoor temperatures on paper discs (Nagatomo et al., 2001).

The present study demonstrated that *Mycoplasma* spp. occurred in high numbers in bedding sand from a dairy concurrently experiencing a clinical outbreak of mycoplasma mastitis and that an environmental source for *Mycoplasma* spp. can be established in recycled bedding sand. Therefore, we conducted experiments to evaluate the efficacy of 2 common disinfectants in the reduction or elimination of *Mycoplasma* spp. in recycled bedding sand. Reportedly, the advantage of using sand as a bedding material is the potential to remove or inactivate most of the microbial contamination

through washing (Kristula et al., 2005; Godden et al., 2008); however, in our study, *Mycoplasma* spp., including some isolates confirmed by PCR as *M. bovis*, and other microorganisms could be recovered from washed (recycled) bedding sand. Following treatment with 2% chlorhexidine and 0.5% sodium hypochlorite, no *Mycoplasma* spp. could be isolated from previously positive bedding sand, demonstrating the efficacy of 2 common disinfectants in reducing or eliminating mycoplasma, or at least making it such that none could be isolated thereby reducing the potential for the transmission of disease.

In summary, *Mycoplasma* spp. was discovered in high numbers in recycled bedding sand originating from a dairy experiencing an outbreak of clinical mycoplasma mastitis, and was also found in bedding from 2 other farms with mycoplasma-positive cows. *Mycoplasma* spp. survived in recycled bedding sand for 8 months and replication was associated with temperature and precipitation. Recycled bedding sand appeared to be an environmental source of *Mycoplasma* spp. Results warrant investigation of the contribution of biofilm formation in the establishment of this environmental source and the epidemiological role of this source of mycoplasma-associated disease. Chemical disinfection appeared to be effective, but practicality and cost-effectiveness require further study. Moisture appears to contribute to the survival and replication of the organism in bedding sand. This is also an avenue for further investigation because 1 method of reducing the numbers of all bacteria in recycled bedding sand could be to improve drying by repeatedly turning the piles, at least in arid climates such as in the western United States.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The first mycoplasma was identified as a disease agent by Nocard et al. in 1896 when the field of microbiology was in its infancy. Although the organism was too small to visualize with the optics of the day, they were able to determine that a biologic agent was the cause of contagious bovine pleuropneumonia, to culture it in a membranous pouch which resulted in broth solution becoming cloudy in appearance, and to use that culture solution to cause disease in a susceptible calf (Nocard et al., 1896). The story of our developing understanding of mycoplasmas illustrates the development of scientific techniques, methods, and technological advancements: from utilizing chick embryo cultures to undefined media incorporating animal products to the use of PCR to detect the presence of mycoplasmas in tissues and fluids (Eaton et al., 1945; Brown et al., 1999; Bashiruddin et al., 2005); from the identification of the cell types involved in the pathologic processes to the identification of the cytokines produced in response to the presence of mycoplasmas (Howard et al., 1987; Razin et al., 1998; Vanden Bush and Rosenbusch 2003); from the classification of organisms based on morphology and disease syndrome to reclassification of hemoplasmas based on matching DNA sequences (Rikihisa et al., 1997). Research of the basic biology of mycoplasmas has revealed that this group of organisms is both simple and complex.

Many mycoplasmas cause similar disease syndromes and have a tropism for related tissues. While some of the diseases are acute, such as contagious bovine

pleuropneumonia and contagious caprine pleuropneumonia, many more present with a chronic course which heavily impacts production parameters (Wallgren, 2004; Gaunson et al., 2006; Wilson et al., 2007). Most of the diseases are characterized by lymphoproliferative infiltrates in the affected tissues which is in contrast to the polymorphonuclear response seen with many bacterial infections (Jasper et al., 1987; Brown et al., 1994; Paape et al., 2000). Identification of mycoplasmas as the causative agent is often difficult, especially in the individual animal because many of the organisms grow slowly or have complex requirements for growth in culture (Thiacourt and Bölske, 1996; González and Wilson, 2003). Antibody tests are improving in both sensitivity and specificity but ultimately these tests rely on the animal response to disease which is highly variable because of the effects on the host immune system (Thiacourt and Bölske, 1996; Wallgren, 2004). Mycoplasmas have mechanisms to evade the host immune system through rapid variations in immunogenic proteins thus causing the production of an inconsistent antibody population. By causing the host's immune system to produce cytokines that affect the ratio of the T lymphocyte cell populations, they also affect the level of antibody production, the overall level of activation, and the effectiveness of the response (Razin et al., 1998). Finally, many of the mycoplasmas share epitopes which makes separation of species with polyclonal antibodies difficult (DaMassa et al., 1992; Thiaucourt and Bölske, 1996).

For many of the mycoplasma diseases, the organisms persist within the host, therefore diagnostic tests which identify the organism itself may be more reliable. Methods for antigen detection are usually either based on antibodies directed toward the

organism such as ELISA, or DNA based such as PCR. Some new tests have been developed using MAb which have the benefit of being adaptable to field diagnostics (Thiacourt et al., 1994; Stipkovits and Kempf, 1996). The literature suggests however that the methods that show real promise for specificity and sensitivity are PCR methods. Several techniques, such as restriction fragment length polymorphism and denaturing gel gradient electrophoresis, have been used to analyze the amplicon generated in order to determine the species, something that is often needed with mycoplasmas as the amplified genes are often highly conserved but vary in size (Foddai et al., 2005; McAuliffe et al., 2005). Another method used is to analyze the melt temperature, the temperature at which the double stranded DNA separates, of amplicons generated. This real-time PCR method works by detecting the fluorescent marker which is released with the separation of the DNA. This has been used to detect *Mycoplasma* spp. contamination of mammalian cell cultures (Ishikawa et al., 2006).

Another problem associated with mycoplasma infections lies with development of effective vaccines. An ideal vaccine stimulates an immune response that efficiently removes organisms from the host without damaging tissues. Because of the immunomodulatory effect of many mycoplasmas including the production of upregulating cytokines, the risk of negative vaccine reactions is highly probable with whole cell bacterins and attenuated vaccines (Whithear, 1996; Kleven, 2008; Maunsell and Donovan, 2009). Many attenuated mycoplasma vaccines have been developed by repeated passages in culture but reversion to virulence can occur as the vaccine strain circulates in the host population (Whithear, 1996; Kleven, 2008). An additional

challenge to vaccine development is the phase and size variation of cell surface antigens that occurs in the population of organisms infecting a group of animals; this creates a moving target for the immune response. As mycoplasma cell surface proteins have been identified, researchers have begun testing vaccines based on subunits as well as whole proteins. Some of these have been effective experimentally such as the 1 developed for *M. hyopneumoniae* (Kobisch et al., 1987).

For almost all mycoplasmal diseases, the source of infection has generally been recognized as chronically or latently infected carriers. Documented routes of transmission include direct contact between animals, indirect contact via contaminated milking equipment and personnel, and for preweaned animals, the consumption of contaminated colostrum and milk. Because mycoplasmas lack a cell wall which appears to increase susceptibility to drying, elevated environmental temperatures, and to some disinfectants, environmental contamination and exposure have not been considered significant factors in the disease process. However, a number of researchers have documented experimental survival of pathogenic mycoplasmas on various substrates at temperatures ranging from 4 °C to 28 °C (González and Wilson, 2003).

The original research in presented in this thesis describes the survival and replication of *Mycoplasma* species, confirmed with PCR as *M. bovis* in 2 samples, in recycled bedding sand. The study began with the investigation of an outbreak of mycoplasma mastitis at a dairy farm in Utah. It was discovered that recycled bedding sand used in freestalls was positive for *M. bovis* by microbial culture and conventional PCR. In order to determine if the presence of *M. bovis* in the recycled bedding could

contribute to the persistence of mycoplasma mastitis at the dairy, 18,000 kg was moved to Utah State University and serially and quantitatively cultured for 8 mo.

While there were times at which mycoplasma organisms could not be recovered from the pile, samples were intermittently culture positive over a period of 8 months. There were several factors that appeared to influence the number of mycoplasma organisms recovered. The project started in the spring with an initial marked decline in the number of all types of organisms recovered from the deep sampling stratum (15 to 18 cm from the surface). As environmental temperatures climbed above 25 °C and the weather became drier, the number of mycoplasmas declined to 10^2 organisms per gram of sand. Two, one hundred-fold increases were seen during the early part of the summer and both coincided with moderate precipitation events. As the weather cooled in the fall, *Mycoplasma* spp. were again recovered from the bedding pile. An additional factor appeared to play a role in this increase. Serendipitously and due to factors beyond the control of the researchers, the recycled bedding sand was removed from the study site, temporarily stored in a dump truck and then replaced. An additional precipitation event also occurred. The result was that the number of mycoplasma organisms recovered increased from undetectable to greater than 10^4 cfu/g of bedding. This phenomenon recurred when the pile was purposefully moved by the researchers a month later. These results suggest that a *Mycoplasma* spp. which was identified as *M. bovis* at the outset of the study was able to replicate in recycled bedding sand, an event previously considered unlikely based on the culture requirements of the organism.

Research has determined that *Mycoplasma* spp. and *M. bovis* in particular are capable of forming single species biofilms (a heterogeneous but highly organized community of microbial cells) in the laboratory (McAuliffe et al., 2006). Sand has been previously demonstrated as a matrix for biofilm development for *Escherichia coli* (Landini and Zehnder, 2002) and *Pseudomonas aeruginosa* (Holden et al., 2002; Robleto et al., 2003). Organisms within a biofilm are more resistant to desiccation, changes in temperature, and some disinfectants (McAuliffe et al., 2006). While it is not clear from the results, there could be an interaction occurring between the *Mycoplasma* spp. and either the Gram-negative organisms, the Gram-positive organisms or both.

The clinical significance of the recovery of *M. bovis* for 8 months in a pile of recycled bedding sand is uncertain. Experimentally, mycoplasma mastitis can be caused with the infusion of as few as 70 cfu into the teat end (Bennet and Jasper, 1980). Previous studies have shown that exposure of the teat ends to microorganisms in bedding can result in the development of mastitis (Kristula et al., 2005; Godden et al., 2008). The amount of *Mycoplasma* spp. recovered from the recycled bedding sand ranged from 10^3 to 10^6 cfu/g, suggesting that this environmental source may play a role in the epidemiology of mycoplasma-associated disease. Considered together, these points suggest that contaminated bedding sand could have a role in the perpetuation of a mastitis outbreak and with seasonal fluctuations in the number of organisms per gram of stored bedding, could even initiate an outbreak. The results presented in this thesis suggest that research including investigation of biofilm formation by mycoplasmas and of

transmissibility of mycoplasma infections from environmental sources to ruminants including dairy cattle is warranted.

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APPENDIX

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February 23, 2010
Susan Pollock
Managing editor
Journal of Dairy Science
2441 Village Green Place
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Dear Ms. Pollock:

I am preparing my thesis in order to complete the requirements for a Master's Degree from the Animal, Dairy and Veterinary Sciences Department at Utah State University (USU). I hope to complete my degree in the spring of 2010.

An article, Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah, of which I am first author and which appeared in the Journal of Dairy Sciences in January of 2010 (93:192-202) is an essential part of my thesis. I would like permission to reprint it as a chapter in my thesis (some revision may be needed to satisfy the graduate school requirements). Please note that USU send theses to Bell and Howell Dissertation Services to be made available for reproduction.

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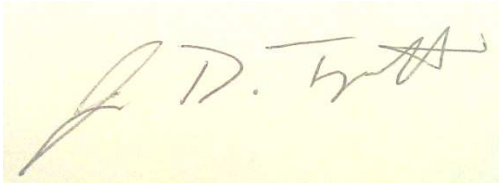
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Sincerely,



Jessie Trujillo, DVM, PhD
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Sincerely,

Robert B Corbett DVM

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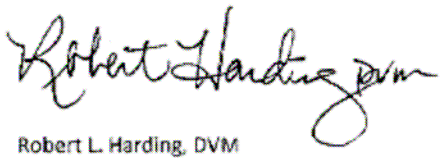
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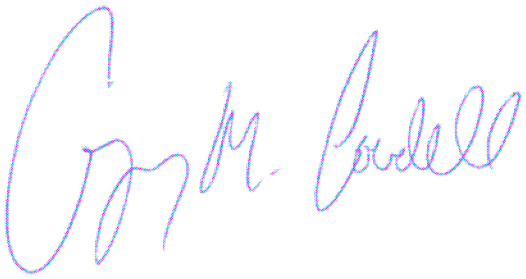
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