MOLECULAR EPIDEMIOLOGY OF MYCOPLASMA MASTITIS OUTBREAK

By

VEERASAK PUNYAPORNWITHAYA

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

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

MAY 2010

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of VEERASAK PUNYAPORNWITHAYA find it satisfactory and recommend that it be accepted.

____
Lawrence K. Fox, Ph.D., Chair

____
Dale D. Hancock, Ph.D.

____
John M. Gay, Ph.D.

____
J. Richard Alldredge, Ph.D.

ACKNOWLEDGEMENT

I would like to thank Dr. Larry Fox, committee chair, for his guidance and support. I thank my other committee members, Dr. Dale Hancock, Dr. John Gay and Dr. Rich Alldredge for their guidance and suggestions on the various aspects of my research.

This research would not have been possible without the many people who provided technical assistance both in the laboratory and on the farm. First, I thank Dorothy Newkirk for her expert assistance and patience. I also wish to thank John Swain at the WSU dairy for his assistance. I thank Nancy Hanson and Dr. Allan Britten at Udder Health System for their assistance. I thank Dr. John Wenz for his suggestions. I also would like to thank all dairy operators for their cooperation.

I thank my wife, Sukolrat, for her patience, understanding, and assistance. I would like to express my gratitude to my parents, Chaiyaporn and Anongnart for their support and encouragement throughout my academic endeavors. I thank my grandparent, Pieng for her support and encouragement. Finally, I would also like to thank Faculty of Veterinary Medicine, ChiagMai University and Thai Government for their financial support over the last six years.

MOLECULAR EPIDEMIOLOGY OF MYCOPLASMA MASTITIS OUTBREAK

Abstract

by Veerasak Punyapornwithaya, Ph.D.
Washington State University
May 2010

Chair: Lawrence K. Fox

Objectives of this research were: 1) determine the association between mycoplasma mastitis and colonization of mycoplasma organisms at body sites of asymptomatic carriers; 2) determine whether concentration of milk via centrifugation prior to conventional culture could increase the ability to detect *Mycoplasma spp.*; 3) determine factors associated with the clearance of mycoplasma mastitis from a dairy herd; and 4) determine the effect of segregation of mycoplasma mastitis cows into a hospital pen on incidence rate and transmission of the disease in a commercial dairy herd. Colonization of the mastitis outbreak mycoplasma strain at accessible mucosal surfaces of cows and replacements was concurrent with the start of the outbreak, in a dairy herd. However, isolation of *Mycoplasma bovis* of any strain type from these mucosal surfaces did not appear to precede mastitis. The ability to detect *Mycoplasma spp.* in milk samples was enhanced using centrifugation followed by resuspension in a reduced volume of fluid before agar plating. Detection of *Mycoplasma spp.* after centrifugation and resuspension was improved and appeared to be best when the concentration was at, or below, the minimum concentration for detection using standard direct culture techniques. Culling of mycoplasma mastitis cows did not have a significant effect on time to clearance of mycoplasma mastitis in outbreak herds. Fifty percent of herds cleared the disease within 1 month without culling the mycoplasma mastitis cows, while an equal number of herds did not preferentially cull cows and had similar times to clearance of this disease. The segregation of *Mycoplasma bovis* mastitis cows in a hospital pen was associated with a higher risk of *Mycoplasma bovis* mastitis incidence in an outbreak herd.

Every episode of mycoplasma mastitis transmission in the hospital pen corresponded to the introduction of a *Mycoplasma bovis* mastitis cows from the milking pens and only one strain caused mycoplasma mastitis and other mycoplasma diseases. Evidence indicates that *Mycoplasma bovis* mastitis cows from milking pens were the source of transmission of the disease in the hospital pen suggesting that the hospital pen appeared to be a risk factor for transmission *Mycoplasma bovis* mastitis.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENT……………..…………………………………………………….iii

ABSTRACT…………………………………………………………..……….................iv

LIST OF TABLES…………………………………………………………………………ix

LIST OF FIGURES…………………………………………………………….................x

CHAPTER

1. INTRODUCTION…………………………………………………………………1
2. LITERATURE REVIEW…………………………………………………………7
3. MATERIALS AND METHODS……………………………………….................44
4. ASSOCIATION BETWEEN AN OUTBREAK STRAIN CAUSING MYCOPLASMA BOVIS MASTITIS AND ITS ASYMTOMATIC CARRIAGE IN THE HERD: A CASE STUDY FROM IDAHO, USA…………………………………………………………………………55
5. THE EFFECT OF CENTRIFUGATION AND RESUSPENSION ON THE RECOVERY OF *MYCOPLASMA SPP.* FROM MILK………………………….71
6. A ROLE OF CULLING ON TIME TO CLEARANCE OF MYCOPLASMA MASTITIS………………………………………………………………………83
7. INCIDENCE AND TRANSMISSION OF *M. BOVIS* MASTITIS IN A HOSPITAL PEN…………………………………………………………..98
8. CONCLUSION…………………………………………………………………117

APPENDIX

A. MEDIUM AND PROCEDURES………………………………………………119

A.1 Mycoplasma enrichment media ingredients and procedures…………...119

A.2 Modified Hayflicks agar ingredients and procedures……….................120

A.3 Phosphate buffered saline solution ingredients and procedures………..122

A.4 Pulsed field gel electrophoresis (PFGE) solution ingredients and procedures ……………………………………………………...……….123

A.5 A nested polymerase chain reaction (PCR) procedures………………..128

B. RAW DATA…………………………………………………...……….130

B.1 Study 1 (Chapter 4) Raw Data……………………………………….130

B.2 Study 2 (Chapter 5) Raw Data……………………………………….144

B.3 Study 3 (Chapter 6) Raw Data……………………………………….147

B.4 Study 4 (Chapter 7) Raw Data……………………………………….158

LIST OF TABLES

CHAPTER 4

1. Frequency of mycoplasma isolation from milk and various body site samples of cows with *Mycoplasma bovis* ………………………………………………………….…68
2. Frequency of mycoplasma isolation from various body sites of replacements with *Mycoplasma bovis*……………………………………………………………..…69

CHAPTER 5

1. Contrast of means (±SE) of Log $_{10}$ mycoplasma counts (cfu/mL) in treatment and control groups……………………………………………………………………………….81

CHAPTER 6

1. Number of herds by time to clearance of mycoplasma mastitis and culling strategy……………………………………………………………………………..96
2. Number of herds and the performing of milking time hygiene practices……………….96

CHAPTER 7

1. Incidence rate of have *Mycoplasma bovis* mastitis for milking pen and hospital pen by month………………………………………………………………………………114

LIST OF FIGURES

CHAPTER 4

1. Pulsed-field gel electrophoretograms of 4 strains of *Mycoplasma bovis* and one strain of *Mycoplasma californicum* following digestion …………………...................................70

CHAPTER 5

1. The relative difference in mycoplasma counts from all treatment vs. control group samples by high, medium, low and very low concentrations………................................82

CHAPTER 6

1. A Kaplan-Meier survival curve showing the time to clearance of mycoplasma mastitis in studied herds ………………………………………………………………………….....97

CHAPTER 7

1. Pulsed-field gel electrophoretograms of *M. bovis* strain. Isolates in lane 2, lanes 4 to 8 and lanes 10 to 12 were strains obtained from clinical mastitis mycoplasma cow…………………………………………………………………………………...114
2. A Kaplan-Meier survival curve showing the time to detection of *Mycoplasma bovis* mastitis for cows entering the hospital pen free of the agent…………………………..…..115
3. Incidence rate of *Mycoplasma bovis* mastitis for milking pen and hospital pen by month………………………………………………………………………………116

CHAPTER ONE

INTRODUCTION

This dissertation is written in the alternative format. It consists of this introduction, a review of the pertinent literature, materials and methods, four manuscripts and a conclusion. The candidate was the major contributor and writer of all four manuscripts and this dissertation. Committee members (co-authors) have reviewed the manuscripts and the comments and advice of my committee members have been included as deemed appropriate. All changes were the final decision of the candidate.

Background

Mycoplasma mastitis is widely distributed and a cause of significant economic loss to the dairy industry (Nicholas and Ayling, 2003). The spread of disease is believed to most often occur during milking time as the organisms are contagious mastitis pathogens (Fox et al., 2005). An outbreak of mycoplasma mastitis is frequently associated with the introduction of carriers replacement animals (Gonzalez and Wilson, 2003). However, asymptomatic carriers in a herd, not newly purchased animals, have been reported as a nidus of outbreaks (Jackson et al., 1981, Jackson and Boughton, 1991, Mackie et al., 2000)

Regular bulk tank milk cultures are suggested as a good tool to monitor a herd's mycoplasma mastitis status. A positive result indicates that at least one cow has mycoplasma mastitis. However, the sensitivity of single bulk tank milk culture is approximately 60% (Gonzalez and Wilson, 2003). This indicates that a herd with a cow with mycoplasma mastitis would not be identified 40% of the time. Low sensitivity of bulk tank milk cultures to detect

mycoplasma mastitis herds may be the result of a low number of mycoplasma microorganisms in bulk tank milk. Theoretically, the threshold of detection is 10 CFU/mL when 0.1mL of a bulk tank milk sample is directly plated on an agar plate. Thus, for mycoplasma organisms to be detected in bulk tank milk samples, the concentration of *Mycoplasma spp.* should be equal or greater than 10 CFU/mL of bulk tank milk. Biddle and others (2003) estimated that 39% of the time cows with mycoplasma mastitis would not shed sufficient numbers of this pathogen so that a bulk tank sample would have a concentration of mycoplasma organisms exceeding this threshold. A technique that is more sensitive is needed to overcome this false negative problem. Sensitivity may be improved by increasing the concentration of microorganisms cultured. This can be achieved by sedimentation of bacteria by centrifugation of milk samples and then resuspension in a smaller volume that is cultured. The cultures from sedimented milk for *Staphylococcus aureus* isolation increased the number of positive results by 145.5%, depending on herd (Zecconi et al., 1997). It has not been shown whether such concentration of centrifugation improves sensitivity of detection of *Mycoplasma spp.* from bulk tank milk samples.

There have been several investigations into the risk factors for herd mycoplasma mastitis outbreaks (Thomas et al., 1981, Bayoumi et al., 1988, Brown et al., 1990, Gonzalez et al., 1992, Fox et al., 2003). However, no one has investigated the factors associated with recurrence of mycoplasma mastitis after a first occurrence. Primarily there are two recommended strategies for controlling mycoplasma mastitis when an outbreak exists. Some suggest that eradication of all infected animals is the best control strategy for mycoplasma mastitis (Bushnell, 1984, Bayoumi et al., 1988). Alternatively, some support the strategy that culling all infected animals is not required (Jackson and Boughton, 1991, Mackie et al., 2000) and infected animals can remain in the herd but need to be segregated and milked last. If a

test and slaughter means of control of mycoplasma mastitis is not always required then there must be other cow and herd factors that contribute to the control of the disease. Assuming that the recovery of *Mycoplasma spp.* from bulk tank milk is indicative of mycoplasma mastitis in the herd then identification of factors associated with recurrence of *Mycoplasma spp.* in bulk tank milk could aid in the development of practical strategies to control the disease.

Studies undertaken

The objective of the studies presented here were: 1) Determine the role of asymptomatic carriage in a mycoplasma mastitis outbreak by studying the association between mycoplasma mastitis and colonization of mycoplasma organisms at body sites of asymptomatic cattle; 2) Determine whether centrifugation of milk with *Mycoplasma spp.* prior to culture could increase the ability to detect this pathogen; 3) Determine factors associated with the clearance of mycoplasma mastitis from a herd; and 4) Determine the effect of segregation of mycoplasma mastitis cows into a hospital pen on the incidence rate and transmission of this disease within a herd.

A study of epidemiology of mycoplasma mastitis in a herd with no history of mycoplasma mastitis was made to complete objective 1. One third of cows and replacements were colonized with *Mycoplasma bovis* at the beginning of an outbreak in a local dairy herd. The association between the outbreak strain and asymptomatic carriage of *Mycoplasma bovis* by lactating cows and their replacements during the outbreak was determined. Findings from this study confirm the role of asymptomatic carriage is ancillary to the outbreak, but such carriage might not contribute to the longevity of the outbreak. This indicated that there were some

unknown factors that might be associated with the cessation of the occurrence of the disease. In an effort to improve the sensitivity of herd mycoplasma mastitis detection the second objective was pursued. The fulfillment of Objective 2 enhanced herd mycoplasma mastitis detection and when applied, improved the ability to identify management factors associated with clearance of mycoplasma mastitis from a herd (Objective 3). A last study developed as a case from a herd enrolled in the third study. In this case the incidence and the transmission of mycoplasma mastitis in a pen of infirm cows, hospital pen, were studied.

References

Bayoumi, F. A., T. B. Farver, B. Bushnell, and M. Oliveria. 1988. Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. J. Am. Vet. Med. Assoc. 192(7):905-909.

Biddle, M. K., L. K. Fox, and D. D. Hancock. 2003. Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. J Am Vet Med Assoc 223(8):1163-1166.

Brown, M. B., J. K. Shearer, and F. Elvinger. 1990. Mycoplasmal mastitis in a dairy herd. J. Am. Vet. Med. Assoc. 196(7):1097-1101.

Bushnell, R. B. 1984. Mycoplasma mastitis. Vet Clin North Am Large Anim Pract 6(2):301-312.

Fox, L. K., D. D. Hancock, A. Mickelson, and A. Britten. 2003. Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. J Vet Med B Infect Dis Vet Public Health 50(5):235-240.

Fox, L. K., J. H. Kirk, and A. Britten. 2005. Mycoplasma mastitis: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health 52(4):153-160.

Gonzalez, R. N., P. M. Sears, R. A. Merrill, and G. L. Hayes. 1992. Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. Cornell Vet 82(1):29-40.

Gonzalez, R. N. and D. J. Wilson. 2003. Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19(1):199-221.

Jackson, G., E. Boughton, and S. G. Hamer. 1981. An outbreak of bovine mastitis associated with Mycoplasma canadense. Vet Rec 108(2):31-32.

Jackson, G. and E. Boughton. 1991. A mild outbreak of bovine mastitis associated with Mycoplasma bovigenitalium. Vet Rec 129(20):444-446.

Mackie, D. P., D. Finlay, N. Brice, and H. J. Ball. 2000. Mixed mycoplasma mastitis outbreak in a dairy herd. Vet Rec 147(12):335-336.

Nicholas, R. A. and R. D. Ayling. 2003. Mycoplasma bovis: disease, diagnosis, and control. Res. Vet. Sci. 74(2):105-112.

Thomas, C. B., P. Willeberg, and D. E. Jasper. 1981. Case-control study of bovine mycoplasmal mastitis in California. Am. J. Vet. Res. 42(3):511-515.

Zecconi, A., R. Piccinini, A. Zepponi, and G. Ruffo. 1997. Recovery of Staphylococcus aureus from centrifuged quarter milk samples. J. Dairy Sci. 80:3058-3063.

CHAPTER TWO

LITERATURE REVIEWS

Introduction

Mycoplasma spp. are microorganisms that lack a cell wall (Razin et al., 1998). These organisms have been associated with a variety of diseases in dairy cows such as mastitis (Jasper et al., 1966, Bushnell, 1984), pneumonia (Ayling et al., 2004), arthritis (Henderson and Ball, 1999, Wilson et al., 2007), otitis (Walz et al., 1997) and keratoconjuctivitis (Gonzalez and Wilson, 2003). *Mycoplasma spp.* can infect cows of any age and any stage of lactation. Mastitis caused by *Mycoplasma spp.* may be subclinical or clinical. The disease is generally characterized by the edematous swelling of udder and the presence of small flakes or sandy sediment in milk. In later stages of mastitis, the milk may change to a yellow brown fluid and becomes purulent (Jasper et al., 1966, Bushnell, 1984,). The species of *Mycoplasma* of greatest concern to the dairy manager is *Mycoplasma bovis*. This species was the most frequently found in mycoplasma mastitis cases and also appeared to be the most pathogenic (Bushnell, 1984, Gonzalez et al., 1992). Mycoplasma mastitis can cause considerable economic losses due to decreased milk yield (Pfutzner and Sachse, 1996), an elevation of milk somatic cell count (Ghadersohi et al., 1999, Biddle et al., 2003,) and culling of infected animals (Fox et al., 2005).

Characteristics

Mycoplasma spp. are the smallest living organisms capable of self-replication. Mycoplasmas are grouped under the class *Mollicutes* which also contain ureaplasma, acholeplasma and

siroplasma. They are highly fastidious, difficult to culture, and slow growing organisms (Razin et al., 1998). These organisms are characterized by small size, no cell wall, fastidious in vitro and a tendency to form centered colonies on solid medium. The lack of cell wall indicates that *Mycoplasma spp*. are inherently resistant to the class of antibiotics that target the cell wall such as beta-lactam drugs (Nicholas and Ayling, 2003, Byrne et al., 2005).

The most commonly used method to identify *Mycoplasma spp.* in milk is a microbiological procedure (Kirk et al., 1997). A potentially positive sample should be cultured on modified Hayflicks media and incubated at 37°C under 10% CO_2 for 7-10 days (Gonzalez and Wilson, 2003). The media usually contains a rich protein base, serum, yeast extract, glucose and/or pyruvate and selective agent such as ampicillin (Razin et al., 1998). The appearance of fried-egg morphology on a solid media is a characteristic of *Mycoplasma spp.* Mycoplasma colony has a central dense convex core surrounded by lighter peripheral zone.

The prevalence of mycoplasma mastitis

The recognition of the importance of mycoplasma mastitis as a dairy industry problem has been increasing. The disease has been reported in many dairy farms throughout the United States and the prevalence of this disease appears to be increasing in many locations throughout the world.

<u>The prevalence of mycoplasma mastitis in US</u>

The disease was first reported in the United States in Connecticut in 1961. Subsequently, *Mycoplasma spp.* have been isolated from mammary glands of cows in many states in the US

including New York, California, Oregon, Washington, Idaho, Alaska, Florida, Texas, Arizona, Arkansas, Hawaii, Pennsylvania, Alabama, Wisconsin, Ohio and Utah (Jasper, 1981, Kunkel, 1985, Brown et al., 1990, Kirk et al., 1997, Fox et al., 2003, Gonzalez and Wilson, 2003, Wilson et al., 2009). An increase in the percentage of herds testing positive for *Mycoplasma spp.* in bulk tank milk was seen in a study in New York (Gonzalez et al., 1992). The frequency of mycoplasma positive herds increased steadily from 1972 to 1981 and then remained fairly constant from 1982 to 1990. During a 6 year investigation, the prevalence of mycoplasma mastitis varied from 1.8% to 5.8% (Kirk et al., 1997). It was expected that about 1% to 8% of all dairies in the US have at least one cow with mycoplasma mastitis (Fox et al., 2005). Recently the herd-level prevalence of mycoplasma mastitis in Utah dairy herds based on the presence of *Mycoplasma spp.* in bulk tank milk was reported to be 7% (Wilson et al., 2009).

<u>The prevalence of mycoplasma mastitis outside the US</u>

The disease is also known as a worldwide mastitis problem (Nicholas and Ayling, 2003) and has been documented by the investigators from Europe, Britain, Canada, Australia and Asia. In a British survey conducted between 1990 and 2000, postmortem materials and blood serum samples from dairy cows were examined to identify the infections. *Mycoplasma bovis* appeared to be the most common species causing bovine disease. The overall percentage of *Mycoplasma bovis* positive sample was 52% from postmortem cultures and between 19% and 24% from serum samples tested by ELISA (Ayling et al., 2004). An investigation conducted by monitoring bulk tank milk weekly for three consecutive weeks from 258 dairy farms in Canada indicated that the cumulative prevalence of *Mycoplasma spp.* was 1.9% (Olde Riekerink et al., 2006). The high prevalence of udder infection with *Mycoplasma bovis* in

dairy cows in Australia is reported when testing milk samples from cows with high somatic cell count (Ghadersohi et al., 1999). A study in Northern Greece examining quarter milk samples from clinical mastitis cases indicated that the prevalence of *Mycoplasma bovis* mastitis was 8.2% (Filioussis et al., 2007). In a herd outbreak in Saudi Arabia, the incidence rate of mycoplasma mastitis was 1.8% of the total mastitis cases (Al-Abdullah and Fadl, 2006). The survey to detect *Mycoplasma bovis* in New Zealand showed that none of 244 bulk tank milk samples tested using a nested polymerase chain reaction (PCR) contained *Mycoplasma bovis* (McDonald et al., 2009). The authors concluded that Mycoplasma *bovis* is not present in the dairy cattle population in New Zealand.

Risk factors for mycoplasma infection

It is thought that an outbreak of mycoplasma mastitis is caused by the introduction of carrier replacement animals to a herd (Bushnell, 1984, Gonzalez and Wilson, 2003). However, risk factors including herd size, climate, improper milking management, people and milking equipment are recognized as potential factors.

<u>The introduction of newly purchased infected heifers or cows</u>

Purchase of new animals is considered the most important factor influencing a mycoplasma mastitis outbreak (Bicknell et al., 1983, Bushnell, 1984, Gonzalez and Wilson, 2003). In the state of New York and Pennsylvania, it has been frequently found that purchased heifers were the origin of severe mycoplasma mastitis in previously mycoplasma free herds (Gonzalez and Wilson, 2003). In a survey study, mycoplasma mastitis appeared in several herds started shortly after purchased heifers entered herds (Gonzalez et al., 1992). Quality Milk Production

Service investigated 140 herds with mycoplasma mastitis problems between January 1989 and December 1995. In almost all the herds, mycoplasma organisms were introduced to herds when replacements including virgin heifers, pregnant heifers or cows were purchased and commingled with the existing herd without quarantine and bacteriological testing (Gonzalez and Wilson, 2003). In a recent report, the outbreak of mastitis and arthritis caused by *Mycoplasma bovis* in a 120 cow dairy herd in UK occurred in three weeks after arrival of newly purchased cows (Houlihan et al., 2007).

Herd size

Herd size is one of factors most associated with mycoplasma mastitis. A case control study in California showed that large herds that have more than 350 cows were 15 times more likely to have mycoplasma positive in bulk milk than small herds (Thomas et al., 1981). An investigation in Washington suggested that herds that sell more milk are more likely to have *Mycoplasma spp.* cultured from the bulk tank (Fox et al., 2003). Perhaps the larger herds are more likely to purchase the new animals that may carry *Mycoplasma spp.* into the herd. In an additional study, the National Animal Health Monitoring System (NAHMS) surveyed the prevalence of *Mycoplasma spp.* in bulk tank milk between February 25, 2002, and June 30, 2002. Dairy operations with at least 30 milking cows from 21 states were visited by animal health officials. Timing of bulk tank sample collection from 871 herds ensured that at least 70 percent of lactating cows were represented in each herd's sample. Data showed that 7.9 percent of dairies tested positive for *Mycoplasma spp.* when a single bulk tank milk sample was cultured using standard culture methods. Large herds with 500 cows or more were more likely to have positive mycoplasma cultures than medium size herds with 100 to 499 cows or small herds with less than 100 cows (USDA, 2003). In contrast, some reports showed that

herd size was not a risk factor (Gonzalez et al., 1992, Hoblet and Iqbal, 1996). Thus, it is possible to assume that the disease can occur in any dairy herd regardless of the herd size, but the larger herds that purchase replacement heifers without quarantine or testing for mycoplasma infection may be more at risk for the disease.

Season

Some studies found a seasonal trend of mycoplasma mastitis with the majority of cases reported during winter months. In a California study, new mycoplasma infections were diagnosed most commonly during the rainy, winter season (Bayoumi et al., 1988). It was suspected that cold weather increases a susceptibility to disease by decreasing resistance of animals to pathogenic microorganisms. Additionally, the difficulty in maintaining optimal hygiene during the rainy season may be a predisposing factor for mastitis. In New York dairy herds the highest number of mycoplasma mastitis cases was found to occur during the winter, starting late in the fall, peaking in January, and decreasing by mid-spring (Gonzalez et al., 1992). This would suggest that temperature is associated with the high prevalence of mycoplasma infection because *Mycoplasma spp.* can survive outside the host at 4° C nearly for 2 months (Kirk and Lauerman, 1994). One study showed that ideal temperatures for replication of *Mycoplasma spp*. occurred between 15 and 20°C (Justice-Allen et al., 2010). In contrast, the study in Washington indicated no seasonal trend for the first isolation of mycoplasma infection at the herd level by culturing bulk milk tank (Fox et al., 2003). The difference in finding may be associated with the difference in year of study and geographical location.

Management practices

Management practices that provide an opportunity of a close contact between infected animals and healthy animals play an important role for mycoplasma transmission. *Mycoplasma spp.* may be shed in nasal discharge or vaginal discharge of infected animals (Bushnell, 1984). Thus, an exposure to contaminated discharges may cause an infection in animals that are in contact with contaminated secretions. In several herds in New York an occurrence of clinical mastitis with *Mycoplasma spp.* in lactating cows was associated with exposure to calves, heifers and cows with signs of respiratory disease that share the same barn (Gonzalez et al., 1993).

Faulty milking machines and management practices are risk factors for the spread of mycoplasma mastitis in a herd (Bushnell, 1984, Gonzalez and Wilson, 2003). Once mycoplasma mastitis is established in a dairy herd, the organism can be transmitted during milking. Poor udder preparation, faulty milking machine function and improper milking procedures have an effect on increasing the number of infected animals. Gonzales and Wilson (2003) indicated that an outbreak of mycoplasma mastitis in large dairy herds occurred when proper udder preparation before milking is neglected or herds where milkers put less emphasis on teat dipping. Wilson et al. (2009) reported that using the same towel to clean multiple udders was frequently found for mycoplasma mastitis positive herds.

Asymptomatic carriers

Calves and young cattle also play an important role in the spread of *Mycoplasma bovis* (Gonzalez and Wilson, 2003) since calf respiratory systems can be commonly colonized by *Mycoplasma spp.* (Woldehiwet et al., 1990). Young animals may be exposed to the disease during calving by direct contact with the urogenital tract and from nasal discharge of the dam.

They may initially develop clinical signs of respiratory disease but they can recover and become carriers. Subsequently, carriers potentially shed the organism to other young animals or lactating cows via aerosol route or direct contact (Gonzalez and Wilson, 2003). Outbreaks of mycoplasma mastitis occurred after a high prevalence of respiratory infection in young cattle (Kirk and Lauerman, 1994).

Calves feeding with mycoplasma contaminated milk

Feeding waste milk or mycoplasma contaminated milk to calves can be a potential risk factor (Gonzalez and Wilson, 2003). In a Michigan study, five preweaned dairy calves from a 600 cow dairy developed clinical signs of otitis media after they were fed waste milk from mastitis cows (Walz et al., 1997). The investigation of prevalence of mycoplasma infection in young dairy animals in mycoplasma mastitis herds showed that all of the calves fed *Mycoplasma spp.* contaminated milk become infected with the disease. Importantly, the organism can colonize nasal passages of young animals for several months (Bennett and Jasper, 1977). The consumption of waste milk from mycoplasma infected cows introduces organisms to the calf, which can become colonized in the respiratory tract and various body sites. Thus, it is recommended to avoid feeding waste milk from mastitis cows to calves to reduce the risk of the disease (Walz et al., 1997).

People and fomites

People who were in contact with infected milk such as milkers, owners and veterinarians are also involved in the transmission of mycoplasma mastitis (Gonzalez et al., 1992). In some epizootic areas, it was suspected that the disease could have been transmitted between

adjacent farms by carriers, people who were in contact with infected milk, such as a milker, herd owner, truck driver or veterinarian (Bushnell, 1984). In addition, contaminated equipment, treatment device or any type of improperly cleaned material may serve as a fomite for mycoplasma transmission (Bushnell, 1984).

The introduction and colonization of mycoplasma mastitis

The introduction of *Mycoplasma spp.* to dairy herds

The most common scenario for the transmission of mycoplasma mastitis is shedding from purchased animals to other animals in a naive farm (Bushnell, 1984, Gonzalez and Wilson, 2003); or the disease can originate from carriers in the herd (Jackson and Boughton, 1991). Several studies showed that a first outbreak of mycoplasma mastitis frequently occurred after purchased heifers entered a herd (Jasper et al., 1974, Gonzalez et al., 1992, Gonzalez et al., 1993). Replacement cows may be the source of first occurrence of mycoplasma mastitis since *Mycoplasma spp.* can persist in mammary glands for several months without clinical signs of mastitis (Byrne et al., 2005). The introduction of an infected cow into a dairy herd can result in the organism spreading to a large proportion of the lactating cows in the herd (Pfutzner and Sachse, 1996).

Mycoplasma mastitis outbreaks can originate not only from newly purchased animals but also from resident animals in a herd (Jackson and Boughton, 1991, Wilson et al., 2007). The investigation of a closed herd indicated that the outbreak of *Mycoplasma bovigenitalium* mastitis originated from dry cows and periparturient cows. The colonization of mycoplasma organisms in the vagina was suspected as a source of the disease (Jackson and Boughton,

1991). Wilson et al. (2007) also reported on the occurrence of *Mycoplasma bovis* mastitis and arthritis in a commercial closed herd. Thus an outbreak of mycoplasma mastitis outbreak may be a result of transmission from animals that are newly introduced to the herd or resident in the herd (Fox et al., 2005).

The colonization of *Mycoplasma spp.*

Mycoplasma spp. can colonize the mammary gland for several months. The repeated isolation from natural intramammary infected cows at intervals several months apart suggested that cows can shed organisms for as long as 8 months (Ruhnke et al., 1976) to 12 months (Jasper et al., 1966). Later, this finding was confirmed in an additional experimental study that cows were inoculated with *Mycoplasma bovis* and milk sample were collected more frequently than those previous reports. The result showed that mycoplasma mastitis cows persistently shed organisms from current lactation through the dry periods and into next lactation (Byrne et al., 2005). However, the mammary gland is not the only site of mycoplasma colonization but other sites such as the mucosal surface of eyes, nasal cavities, ears and vestibular fossa of animals can also be colonized (Biddle et al., 2005). *Mycoplasma bovis* can colonize the respiratory tract of young calves for several months (Bennett and Jasper, 1977). It also has been isolated from urogenital tract (Nakamura et al., 1977, Mackie et al., 2000), joint (Gonzalez et al., 1993) and musculoskeletal systems (Jasper, 1981).This suggests that the transmission pathway might occur with spreading via non-mammary sites from asymptomatic carriers to lactating cows.

Since *Mycoplasma spp.* can colonize the mammary gland and various body sites such as respiratory tract and urogenital tract of cattle, young animals and cows can be carriers for the

disease. A common reservoir of *Mycoplasma spp.* is young cattle (Bushnell, 1984, Gonzalez and Wilson, 2003). Calves may be infected with *Mycoplasma spp.* when they have contact with their dam that may shed the organism during parturition and post-partum period. Transmission may be through direct contact or an aerosol route. The calves may develop clinical signs of respiratory disease and subsequently recover and become a reservoir for this pathogen. The organisms can colonize the nose of young infected animals up to one year (Bennett and Jasper, 1977). An outbreak of mycoplasma mastitis in several herds was associated with nasal transmission from the reservoir of young animals (Jasper, 1981).

Lactating cows infected with *Mycoplasma bovis* can consistently shed the organism into milk throughout the lactating and dry period, and into their next lactation (Byrne et al., 2005). Thus these reservoir animals may shed the organism to other lactating cows during milking time and may shed the pathogens to their calves during parturition. Additionally, dry cows may act as reservoirs for the disease. Jackson and Boughton (1991) report that an outbreak of *Mycoplasma bovigenitalium* in 16 of 99 cows in a dairy herd originated in 13 dry cows and one periparturient cow.

The transmission of *Mycoplasma spp.*

Mycoplasma spp. is a contagious pathogen. A transmission of *Mycoplasma spp.* likely occurs during milking time. Spread of the organism can be controlled by using proper milking hygiene (Fox et al., 2005). However, despite implementation of proper milking hygiene the prevalence of intramammary mycoplasma infections continues to increase suggesting other mechanisms of pathogen transfer. In contrast to other contagious mastitis pathogens, mycoplasma infection can spread from respiratory and urogenital system from cow to cow.

Infected animals may spread the pathogen by aerosol and droplets that may contaminate feed and water or by direct contact (Gonzalez and Wilson, 2003).

The mechanism of transmission can be internal or external transfer. The external transfer is the transmission from one animal to other animals. Internal transfer is defined as the transmission within the same animal normally by lymphatic or hematogenous spread (Fox et al., 2005).

External transmission

The main sources of external transmission are new animals and lactating cows infected with the disease (Gonzalez and Wilson, 2003). Transmission of the disease can be characterized by the transfer from non lactating animals to a herd mate, from infected lactating cows to other lactating animals during milking, and from dam to calf.

The introduction of newly purchased healthy animals infected with *Mycoplasma spp.* is thought to be the most important source of external transfer (Jasper, 1982, Bushnell, 1984). Aerosol spread of mycoplasma organisms from respiratory locations are one important route of transmission for the disease, as well as, direct contact with nasal or vaginal discharge contaminated with the organism (Gonzalez and Wilson, 2003). Outbreaks of mycoplasma mastitis in several herds occurred after the high prevalence of respiratory infection (Kirk and Lauerman, 1994). Additionally, transmission from urogenial tract to mammary gland was suggested in a herd with mycoplasma mastitis outbreak, the investigator suspected that organisms spread from vagina to mammary gland among freshly calved cows (Mackie et al., 2000).

The organism is a contagious mastitis pathogen because it may be readily transmitted from cow to cow during milking time (Fox and Gay, 1993). *Mycoplasma spp.* can be carried from cow to cow during milking by milker's hand and parlor equipment such as milking machines and teat cups (Jasper, 1981, Gonzalez et al., 1992). Mycoplasma mastitis cows can shed the organism into milk from the current lactation to their next lactation (Byrne et al., 2005). The persistent shedding from one lactation to the next may pose a risk for the transference among animals. Milking cows in a herd have a chance to become infected with this pathogen during milking time through the milking process. In addition, calves may be at risk of transmission from the dam by contact with the dam at birth or by consuming contaminated milk (Gonzalez and Wilson, 2003). Outbreaks of respiratory disease, polyarthritis and ear infections were found in calves fed milk from cows with mycoplasma mastitis (Bennett and Jasper, 1977, Butler et al., 2000).

Internal transmission

There is some evidence that mycoplasma mastitis can result from infection or colonization of body sites other than the mammary gland and spread of the organisms via the circulatory or lymphatic system.

Cows with mammary glands inoculated with *Mycoplasma spp.* had the organism subsequently isolated from blood and other organ systems. After *Mycoplasma bovis* was inoculated into one mammary quarter, the organisms could be isolated from all quarters within 17-19 days despite the use of strict milking hygiene (Bennett and Jasper, 1978). The organisms can be isolated from blood and other organ after they were inoculated into

mammary gland (Jain et al., 1969). This would suggest the hematogenous spread from mammary glands to other organs via blood circulation.

In one additional study, Biddle et al. (2005) investigated various samples of cows with chronic infection with mycoplasma mastitis. Seven cows infected with mycoplasma mastitis were sampled before and after slaughter. Milk samples and swab specimens from the mucosal surface of eye, nasal cavities, ears, and genital tracts were collected for 4 week before slaughter. Post mortem samples were the surface of eyes, ears, respiratory organs and urogenital organs. All organisms isolated from each cow from milk, mammary gland parenchyma and supramammary lymph nodes had the same pulsed-field gel electrophoresis (PFGE) pattern. All cows had at least one isolate from nonmammary tissues that had the identical PFGE pattern as isolated from the mammary systems indicating they were the same strain. Also, more than 90% of the mycoplasma isolates from all samples were the same strain. These findings might suggest that the same strain of *Mycoplasma spp.* at mammary gland becomes disseminated throughout the body, although dissemination from other organ systems to the mammary gland is also a very possible route of internal transmission.

The evidence of vertical transmission is suggested in a study demonstrating that the same strain of mycoplasma that causes mastitis in lactating cows was the cause of mastitis in young replacement calves (Fox et al., 2008). The strain of *Mycoplasma bovis* found in pustular mass from mammary gland of calf was undistinguishable from the strain isolated from the dam of such calf. This finding supports the report that *Mycoplasma bovigenitalium* was a cause of mastitis in a 7 week old Holstein calf (Roy et al., 2008).

The transmission of *Mycoplasma spp.* can be characterized as a cycle. The cycle may begin with mycoplasma infection of the respiratory tract or urogenital tract of calves and heifers. Infected animals may develop respiratory disease and typically recover later. However those animals still carry the organism in their body. They act as reservoirs and may spread the organism to herd mates. The most critical source is female animals. After infected heifers freshen, they may develop mastitis and begin shedding organisms in the milk. The cycle starts again when calves are infected with the organism through consumption of contaminated milk or contact with the dam.

Duration of mycoplasma mastitis positive status in herd

There is evidence to suggest that the dynamics of mycoplasma mastitis transmission begins with a high prevalence, followed by a lower prevalence, suggesting the period of spread is short. Fox et al. (2003) investigated the change of mycoplasma mastitis from positive to negative status in 79 herds through analysis of monthly bulk milk tank cultures every month for one year. Of the 50 herds tested during the first month after initial mycoplasma isolation, only 16 herds (32%) herds were still positive with mycoplasma mastitis. Only 13 herds from 41 (32%) herds tested during the second month after first isolation were mycoplasma mastitis positive. Regarding herds that were tested at least one time during the first 3 months after first positive test, as determined by bulk tank culture, only 26 of 70 herds (37%) were positive to *Mycoplasma spp.* Sixty-three herds were tested one or more time between 3 and 12 months after first isolation and only 15 (27%) of these were positive for mycoplasma mastitis. Similar findings were reported in other studies. In a survey of four herds, the duration of mycoplasma outbreak was less than 2 months (Ruhnke et al., 1976). An outbreak of mixed mycoplasma mastitis from *Mycoplasma californicum* and *Mycoplasma canadense*

in a dairy herd was limited to approximately 2 months after first identification (Mackie et al., 2000). However, others studies reported that the duration of infection is much longer (Jasper et al., 1966, Bicknell et al., 1983, Bayoumi et al., 1988). The elimination program for mastitis caused by *Mycoplasma bovis* in a dairy herd in Great Britain was successful after implementation of an eradication program, as the duration of the outbreak lasted approximately 15 months (Bicknell et al., 1983). One report showed that mycoplasma mastitis could be a problem in a dairy herd that purchased nearly 25% of their replacements for several years (Bayoumi et al., 1988). Thus, the reports in the literature clearly demonstrate that the duration of herd prevalence of mycoplasma mastitis is very variable. Although speculation as to the reasons for this variability exists, the risk factors for re-occurrence of this disease are largely unknown.

Diagnosis of mycoplasma mastitis by culturing of bulk tank milk

<u>Advantage and disadvantage</u>

The common diagnosis method for *Mycoplasma spp.* causing mastitis is microbiological culture of milk samples taken from individual cows or herd bulk tank (Gonzalez and Wilson, 2003). Culturing of bulk tank milk is a good procedure for screening and surveillance of this disease on a herd basis. A positive result suggests that at least one cow in a herd has mycoplasma mastitis. However, a negative result does not necessarily indicate the absence of mycoplasma infection in a herd because cows may stop shedding the organism or shed the organism at such a low level that the organism can not be detected in bulk tank samples by standard culture techniques (Guterbock and Blackmer, 1984, Biddle et al., 2005). Sensitivity of a single culture of bulk tank milk samples for *Mycoplasma spp.* ranges from 33%

(Gonzalez and Wilson, 2002) to 59% (Gonzalez et al., 1988). Multiple samplings of bulk tank milk should be performed to more accurately monitor herd mycoplasma prevalence. A serial culture is recommended to detect the presence of contagious pathogens in bulk tank milk (Gonzalez and Wilson, 2003). If at least three bulk tank milk samples collected 3-4 days apart are cultured for *Mycoplasma spp*., and are all negative, the probability is 70% that cows contributing milk to the tank are negative for mycoplasma mastitis (Gonzalez and Wilson, 2003). Seven consecutive daily cultures are better in assurance that bulk tank milk is negative to mycoplasma organisms (Gonzalez and Wilson, 2003). A false positive result from culture of bulk tank on farms is not likely because non-pathogenic *Mycoplasma spp.* rarely cause mastitis (Kirk and Lauerman, 1994). False negative results of mycoplasma isolation from bulk tank milk are more common. A false negative result for *Mycoplasma spp.* may relate to the intermittent shedding from infected cows (Biddle et al., 2003, Gonzalez and Wilson, 2003) or through withholding of abnormal milk from mycoplasma mastitis cows for sale by dairy operators (Jasper, 1979, Thomas et al., 1981).

Although culture of the bulk tank is not generally accepted as a gold standard test for mastitis prevalence (Godkin and Leslie, 1993), it is usually used in survey studies to determine the prevalence of mastitis infection in a population of herds (Fox et al., 2003, Jayarao and Wolfgang, 2003, Jayarao et al., 2004), and it is a common method for rapid assessment of the mastitis status of a dairy herd (Godkin and Leslie, 1993, Jayarao and Wolfgang, 2003, Jayarao et al., 2004). The culture of a sample of bulk tank milk is a useful technique to screen dairy herd for mycoplasma mastitis. Samples of bulk milk are readily obtainable and can be rapidly and inexpensively cultured and used as a screen to determine a herd’s mastitis infection status (Godkin and Leslie, 1993). Bulk tank milk culturing is useful for screening herds and pens of cows to determine the presence of cows with mycoplasma mastitis after the

outbreak (Gonzalez and Wilson, 2003, Fox et al., 2005). A positive result from bulk milk culturing indicates that at least one animal in a herd or group that represents the pool of milk in a bulk tank sample, is infected with *Mycoplasma spp*. Thus selective sampling of the bulk tank can be used to narrow the number of cows in a group to sample individually to determine which cows are infected. Routine culture of bulk tank milk should be used to monitor the positive status of the herd. A bulk tank milk sample that is negative to *Mycoplasma spp*. after an outbreak may be a result of some animals infected with *Mycoplasma spp.* that are shedding the organism intermittently. Routine bulk tank milk culturing should be done as a monitoring program for efficient control of the disease.

Methods to improving the sensitivity

The low sensitivity of single bulk tank milk culture is the major limitation for detection mycoplasma organisms. Approximately 30-40% of bulk tank milk samples were no *Mycoplasma spp.* are cultured are false negative indicating that a herd would errorously considered free of mycoplasma mastitis (Gonzalez and Wilson, 2003). Theoretically, the threshold of detection is 10 CFU/mL, if 0.1 mL of a bulk tank milk sample is directly plated on an agar plate. Thus, for mycoplasma organisms to be detected in bulk tank milk samples, the concentration of *Mycoplasma spp.* should be equal or greater than 10 CFU/mL. Biddle and others (2003) estimated that 39% of the time cows with mycoplasma mastitis would not shed sufficient numbers of this pathogen so that a bulk tank sample would have a concentration of mycoplasma organisms exceeding this threshold. A higher sensitivity technique is needed to overcome a false negative problem.

Several studies suggest that the sensitivity for detection bacteria in milk sample can be increased by adding pre-enrichment media or using centrifuged milk samples. Biddle and authors (2003) compared the percentage of positive samples for mycoplasma organism from two procedures including a standard method and an enrichment method, for standard method a 100 µL of milk was plated on mycoplasma medium. For enrichment method, 5 mL of mycoplasma enrichment medium is inoculated with 100 mL of the milk sample. Samples were incubated for 4 days at 37° C in 10% CO_2 before plating. For 71 of 104 (68%) samples, mycoplasma organisms were isolated from both following direct plating and following enrichment. However, for 24 of 104 (23%) samples, mycoplasma organisms were isolated only following enrichment, and for 9 of 104 (9%) samples, mycoplasma organisms were isolated only after direct plating and not after enrichment. This indicates the advantage of an enrichment medium to enhance the recovery of *Mycoplasma spp.* from a milk sample. This result is similar to the previous study in which the probability of isolation for mycoplasma organism with enrichment medium was increased 1.7 fold when compared with direct plating (Thurmond et al., 1989).

Results from one study showed the benefit of centrifugation for detecting microorganisms from centrifuged milk sample (Zecconi et al., 1997). The frequency of isolation of *Staphylococcus aureus* from quarter milk samples was compared with the frequency of recovery of *Staphylococcus aureus* from sediment after centrifugation of those same samples. Overall, 776 quarter milk samples from 194 cows in 6 dairy herds were studied. For each milk sample, a 0.01 mL of milk was plated directly on agar. The remaining milk was centrifuged at 2000 x g for 15 min, supernatant discarded, and the bottom layer was resuspended in 0.05 mL of sterile saline. All of the resuspension was spread on agar. Overall, a culture that was positive for *Staphylococcus aureus* was obtained from 82 quarter milk

samples; 71 sediments from 694 quarters that were negative for the microorganism from quarter milk samples were found to be positive for *Staphylococcus aureus.* This represents an 87% increase in the detection of for *Staphylococcus aureus* by centrifugation and concentration of the inoculation prior to plating. For the cow level, cows were identified as being positive for *Staphylococcus aureus* when at least one quarter was positive for the microorganism. Interestingly, the finding from the cow level is consistent with the result from the quarter level. The number of positive cows to *Staphylococcus aureus* from centrifugation technique was 80.5% higher than cows identified from cultures of quarter milk sample without centrifugation. Clearly, the concentration of *Staphylococcus aureus* in a mammary quarter milk sample was frequently below the detection limit for the standard bacteriological method (100 cfu/mL); therefore, the microorganism could be detected more effectively with a method such as the culturing of sediment of milk sample which increases the sensitivity up to 1 cfu/mL.

Another method for enhancing the sensitivity is to use a larger volume of milk for plating. It was suggested that the inoculation with 0.1mL of milk sample enhances the recovery for several organisms, including environmental streptococci and coliform bacteria (Dinsmore et al., 1992). However, increasing from 0.01 to 0.05 ml did not improve the sensitivity of culture in the diagnosis of *Streptococcus agalactiae* mastitis (Dinsmore et al., 1991).

Enrichment of milk suspected of having *Mycoplasma spp.* adds several days to the standard procedure. The apparent success of the centrifugation, and concentration, of *Staphylococcus aureus* suggests that it might be an option worthy of study for the enhancement of *Mycoplasma spp.* from bulk tank milk.

Control of mycoplasma mastitis

Methods to control mycoplasma mastitis are identification, culling and segregating the infected animals and strict milking hygiene (Bushnell, 1984, Gonzalez and Wilson, 2003, Fox et al., 2005). Generally, the first step to control mycoplasma mastitis is to identify infected lactating cows. When an outbreak occurs, it may be best controlled by an intensive identification and culling of infected cows. Culturing a pooled sample of milk from several cows can be used to narrow the number of cows that require individual milk cultures. Additionally, cows with high somatic cell counts and cows with clinical mastitis should be sampled. Along with identifying the infected animal, most of researchers agree that good milking hygiene practices during milking time can limit the transmission of *Mycoplasma spp.* from mastitis cows to other lactating cows (Jasper et al., 1966, Bicknell et al., 1983, Bushnell, 1984, Gonzalez and Wilson, 2003, Fox et al., 2005).

Role of milking time hygiene practice

Good milking hygiene and procedures can reduce the spread of mycoplasma mastitis (Gonzalez and Wilson, 2003). Milking time hygiene practices that include disinfecting the udder before milking, using of smooth surface rubber gloves, use of post-dipping disinfectant and using backflush are recommended to be employed to control the spread of *Mycoplasma spp.* during milking time (Bushnell, 1984, Gonzalez and Wilson, 2003). Use of disinfectant for back flushing helps to control the transmission of the disease from one animal to others during milking time (Thomas et al., 1981, Bushnell, 1984). Teat dipping with disinfectant before and after milking is also beneficial in preventing contamination and spreading of *Mycoplasma spp.* from cow to cow (Bushnell, 1984). However, good milking hygiene

practices may not be sufficient to control mycoplasma mastitis in a herd because *Mycoplasma spp.* may have a different epidemiology from other contagious mastitis pathogens (Fox et al., 2003). Data showed that herds with *Staphylococcus aureus* and/or *Streptococcus agalactiae* problems did not have a higher risk of mycoplasma mastitis than herds without problems with those pathogens. The similar bulk tank concentration of *Staphylococcus aureus* and the parallel prevalence of *Streptococcus agalactiae* in mycoplasma positive and mycoplasma negative herds suggest the transmission of mycoplasma mastitis may be different from those contagious pathogens (Fox et al., 2003). Hence the control of mycoplasma mastitis should not be limited to milking hygiene practices but should be expanded to include additional intervention strategies. An employment of strict milking time hygiene practices may be done in parallel with the identification and segregation (or culling) of infected animals when an outbreak occurs (Fox et al., 2005).

<u>Culling strategy</u>

There are two different strategies to control mycoplasma mastitis outbreak after mycoplasma mastitis cows are identified. Some researchers suggest that dairy operators should cull all infected animals as soon as possible from the herd (Bushnell, 1984, Bayoumi et al., 1988). Alternatively, some argue that it is not necessary to cull infected animals. The outbreak of mycoplasma mastitis can be controlled by segregation and milking infected cows last (Jackson and Boughton, 1991).

Data from an 8-year study in a large dairy herd suggests the identification and immediate culling of infected cows, as well as a good sanitation, is a strategy to control mycoplasma mastitis (Bayoumi et al., 1988). A program for individual milk culturing was implemented to

identify and monitor infected animals during the entire the period of study. A composite milk sample from freshened cows and cows recently added to the herd as well as milk samples from all quarters of cows with clinical mastitis were cultured weekly. All cows with mycoplasma positive cultures were culled immediately. Freshened heifers and cows were separated from hospitalized cows to limit the transmission of the disease between these animals in sick cow pens. The parlor and milking equipment were cleaned after milking each string. Freshened cows were not allowed to be in contact with hospitalized cows. All cows with *Mycoplasma spp.* positive cultures were culled from a herd immediately. However, a new infection of mycoplasma mastitis was found for many years. It could not be assumed that the culling of all infected cows is not an effective program to control the disease because about 25% of replacements in the herd were normally purchased from other farms. These purchase replacement may carry *Mycoplasma spp.* to the herd because they have never been checked for mycoplasma infection before entering to the herd. Another reason that why the new infection exists although all infected animals were culled is that mastitis was spreading in the herd before the infected cows were identified.

The advantage of culling all mycoplasma mastitis cows is that the transmission between mastitis cows and other lactating cows during milking time is terminated. However, the culling of infected milking animals may be benefit in controlling the spread of the disease only during milking time via milking procedures. The culling of mycoplasma mastitis animals will not guarantee that the disease is eradicated from the herd. It is possible that asymptomatic carriers may shed the microorganism to their herd mates before their clinical signs are shown. Sick animals can shed the microorganism not only during milking time but also via other routes of transmission, such as direct contact and aerosol.

Replacements and non lactating animals as asymptomatic carriers may subsequently develop as carriers in the herd. It is possible that these animals may intermittently shed the microorganism which can be transmitted to their herd mates. Susceptible animals may clinically develop the disease. For this reason, the culling of mastitis cows may not be of benefit if some animals that remain in a herd asymptomatically carry the pathogen at an extramammary site. Perhaps a better method of control is to segregate cows with mycoplasma mastitis and milk them last.

Segregation strategy

Segregation of the infected animals including milking infected milking cows last and separation those animals into areas that minimize their exposure to uninfected cows may be an alternative strategy to control mycoplasma mastitis. Culling infected animals is one of the control methods. However, there was a report that indicated that culling is not a necessary method to control mycoplasma mastitis (Jackson and Boughton, 1991). A mild outbreak of mycoplasma mastitis with *Mycoplasma bovigenitalium* was apparently resolved without resorting to the segregation and culling of infected animals. The main factor introduced to the herd was the cleaning of milking equipment. However, successful control of the disease may be associated with the type of *Mycoplasma spp.* because in general *Mycoplasma bovigenitalium* mastitis is a less severe type of mycoplasma mastitis (Jasper, 1982), and that the shedding of *Mycoplasma bovigenitalium* may not be as prolonged as for *Mycoplasma bovis* (Jackson and Boughton, 1991). The control of mycoplasma mastitis due to *Mycoplasma bovis* in four dairies was successful by segregation infected cows rather than culling (Gonzalez and Sears, 1994). The dairy operator decided to keep several infected cows after identification of the infected animals in the herd due to their high genetic value, milking them

either last or with a separated milking unit. Eighteen of 28 infected cows apparently eliminated the infection spontaneously and 15 of the 18 infected cows fully recovered milk production. Bulk tank milk cultures from all four herds were negative to *Mycoplasma spp.* within a year. Cows that were milked last, although sharing the same barn, or cows milked with separate milking unit, seemed not to have posed a risk for transmission of the microorganism during milking time to the remaining cows in the herd.

The combination of culling and segregation strategy

A combination program of identification, segregation and culling of infected animals was used to eradicate *Mycoplasma bovis* infection in a dairy herd in Great Britain (Bicknell et al., 1983). The herd contained 370 milking cows. At the beginning of outbreak, a total of 19 dry cows were infected with *Mycoplasma bovis* causing clinical mastitis. When the eradication program started thirty seven cows were known to have had clinical mastitis during the dry period. The eradication program started with collection of milk samples from whole herd to identify shedder cows. Then, a series of group and whole milk herd tests was carried out over a period of 15 months. Bulk tank milk was sampled at weekly intervals during the two year period. Nasal swabbing samples from the entire herd were examined for *Mycoplasma bovis* approximately 2 years after starting the investigation. The majority of *Mycoplasma bovis* shedding animals were found in the first 12 to 14 weeks after the start of the program with 38 infected cows identified. Subsequently, approximately one to three cows were positive to mycoplasma infection during a series of subsequent samplings. Most infected cows were removed from the herd as soon as confirmation of infection had been obtained. However, some mycoplasma positive cows were kept in the herd but they were segregated and were tested routinely. They were to be kept in the herd if the test showed a negative result. At the

end of study, eight of fifty nine infected cows identified as shedder cows during the entire study remained in the herd, having had negative milk culture test for *Mycoplasma spp.* in at least eight, and up to 12 milk culture examinations. Also, nasal swabbing samples were collected. *Mycoplasma spp.* was not cultured from nasal swabbings from all cows remaining in herd. Results from the study would indicate that bulk milk tank monitoring program is a good tool for investigators and farmers to identify the status of the disease during the study. Results showed that one or more bulk tank samples were positive during first eighteen weeks. Subsequently, they were intermittently positive (week 20 to 60) and finally they were continuously negative until the end of study. The authors stated that judging by test results the eradication of the disease was successful. Monitoring of bulk milk samples continued at monthly intervals but no further evidence of infection was found. Eight previously infected cows were later regarded as free of infection.

A similar finding was reported by Mackie et al. (2000). In that report a mixed mastitis infection of *Mycoplasma californicum* and *Mycoplasma canadense* caused an outbreak but was limited by good management which was highlighted by segregation and rigorous hygienic practices during and after milking. The outbreak of clinical mastitis initially appeared in freshly calved cows and the source of the outbreak was unknown. Eight milk samples from these animals were positive to both *Mycoplasma californicum* and *Mycoplasma canadense*. The problem had been ongoing for several weeks and was not responding to the control procedures that included treatment of infected quarters with an intramammary antibiotic after each milking. All suspect and treated animals were segregated and milked last. Strict hygiene was applied to limit the spread of disease. Only a small number of animals during the early stages of the outbreak were culled. Indeed, with the intensive segregation of

cases and rigorous application of hygiene the problem was solved approximately 2 months after the first case of mycoplasma mastitis.

The result from a field study in a large Florida dairy had a similar outcome. The dairy herd had 126 cows of 1,535 that were positive for *Mycoplasma bovis.* Infected cows were maintained in a mycoplasma mastitis subherd and were milked at the end of each milking session. Of the 126 cows, 66 cows were culled due to mastitis or low production or other reasons. Of the remaining 60 cows, only 16 cows remained culture positive for *Mycoplasma bovis* but these cows never developed clinical signs of mastitis (Brown et al., 1990)

It has been suggested that the most rational approach for controlling mycoplasma mastitis on a dairy is immediately culling of known infected cows (Bushnell, 1984, Bayoumi et al., 1988). Culling these animals limits the exposure between mastitis cows and other animals. Contrarily, some studies suggested that the mycoplasma mastitis problem can be controlled although infected animals still remain in a dairy. The comparison of the probability of success in controlling mycoplasma mastitis between culling method versus keeping, but segregating mycoplasma positive animals, needs to be investigated. Case reports sometime found culling, sometimes found segregating, to be successful. In addition to culling or segregation of infected animals, the apparent strategy to control mycoplasma mastitis using strict milking time hygiene practices that included disinfecting the udder prior to milking, use of rubber gloves with milker hand cleansing between cow milkings, use of post-milking teat disinfectant, and milking unit backflush between cow milkings is always warranted (Jasper, 1981, Kirk and Lauerman, 1994, Gonzalez and Wilson, 2003, Fox et al., 2005).

Recurrence of mycoplasma mastitis

The change of mycoplasma mastitis from positive to negative status in 79 herds was investigated by culturing bulk tank milk every month in one study (Fox et al., 2005). Of the 50 herds tested during the first month after initial mycoplasma isolation, only 16 herds (32%) herds were still positive with mycoplasma mastitis. Only 13 herds from 41 (32%) herds tested during the second month after first isolation were mycoplasma mastitis positive. It is not understood why some herds become negative within one month, and why some herd continue to have a problem with mycoplasma mastitis as evidenced by recurrent mycoplasma isolation from bulk tank samples. This may indicate that there are some unknown factors triggering the occurrence of mycoplasma mastitis and some factors are associated with the cessation of the occurrence of the disease. Identifying the risk factors is an important first step in developing control and prevention strategies for mycoplasma mastitis.

Summary

Mycoplasma spp. is an important contagious mastitis pathogen. Transmission may often occur by contact with naïve replacement animals or from asymptomatic carriers such as calves or cows. *Mycoplasma spp*. colonize not only mammary glands but also mucosal surface of various body sites. A transmission of *Mycoplasma spp.* likely occurs during milking time. Many studies reported that outbreak of mycoplasma mastitis is caused by the introduction of carrier replacement. However, risk factors including herd size, climate, improper milking management, people and milking equipment are recognized as potential factors. The common diagnosis method for *Mycoplasma spp.* causing mastitis is microbiological culture of milk samples taken from individual cows or herd bulk tank Culturing of bulk tank milk is also a good procedure for screening and surveillance of this disease on a herd basis, although the sensitivity of this method is not high. Methods to control

mycoplasma mastitis are identification, culling and segregating the infected animals and strict milking hygiene. Some factors associated with the recurrence of this disease remain unknown. Identifying such factors will impact to control and prevention strategies for this disease.

References

Al-Abdullah, H. A. and E. A. Fadl. 2006. Mycoplasma mastitis in dairy cattle herds in Saudi Arabia. Vet Rec 159(3):88-89.

Ayling, R. D., S. E. Bashiruddin, and R. A. Nicholas. 2004. Mycoplasma species and related organisms isolated from ruminants in Britain between 1990 and 2000. Vet Rec 155(14):413-416.

Bayoumi, F. A., T. B. Farver, B. Bushnell, and M. Oliveria. 1988. Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. J Am Vet Med Assoc 192(7):905-909.

Bennett, R. H. and D. E. Jasper. 1977. Nasal prevalence of Mycoplasma bovis and IHA titers in young dairy animals. Cornell Vet 67(3):361-373.

Bennett, R. H. and D. E. Jasper. 1978. Immunologic and pathologic response of cows to naturally occurring *Mycoplasma bovis* mastitis. Vet. Micro. 2:325-340.

Bicknell, S. R., R. F. Gunning, G. Jackson, E. Boughton, and C. D. Wilson. 1983. Eradication of Mycoplasma bovis infection from a dairy herd in Great Britain. Vet Rec 112(13):294-297.

Biddle, M. K., L. K. Fox, and D. D. Hancock. 2003. Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. J Am Vet Med Assoc 223(8):1163-1166.

Biddle, M. K., L. K. Fox, M. A. Evans, and C. C. Gay. 2005. Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. J Am Vet Med Assoc 227(3):455-459.

Brown, M. B., J. K. Shearer, and F. Elvinger. 1990. Mycoplasmal mastitis in a dairy herd. J Am Vet Med Assoc 196(7):1097-1101.

Bushnell, R. B. 1984. Mycoplasma mastitis. Vet Clin North Am Large Anim Pract 6(2):301-312.

Butler, J. A., S. A. Sickles, C. J. Johanns, and R. F. Rosenbusch. 2000. Pasteurization of discard mycoplasma mastitic milk used to feed calves: thermal effects on various mycoplasma. J Dairy Sci 83(10):2285-2288.

Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of Mycoplasma bovis infection in the mammary glands of lactating cows inoculated experimentally. Vet Rec 156(24):767-771.

Dinsmore, R. P., P. B. English, R. N. Gonzalez, P. M. Sears, and H. F. Schulte. 1991. Evaluation of methods for the diagnosis of Streptococcus agalactiae intramammary infections in dairy cattle. J Dairy Sci 74(5):1521-1526.

Dinsmore, R. P., P. B. English, R. N. Gonzalez, and P. M. Sears. 1992. Use of augmented cultural techniques in the diagnosis of the bacterial cause of clinical bovine mastitis. J Dairy Sci 75(10):2706-2712.

Filioussis, G., G. Christodoulopoulos, A. Thatcher, V. Petridou, and E. Bourtzi-Chatzopoulou. 2007. Isolation of Mycoplasma bovis from bovine clinical mastitis cases in Northern Greece. Vet. J. 173(1):215-218.

Fox, L. K. and J. M. Gay. 1993. Contagious mastitis. Vet Clin North Am Food Anim Pract 9(3):475-487.

Fox, L. K., D. D. Hancock, A. Mickelson, and A. Britten. 2003. Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. J Vet Med B Infect Dis Vet Public Health 50(5):235-240.

Fox, L. K., J. H. Kirk, and A. Britten. 2005. Mycoplasma mastitis: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health 52(4):153-160.

Fox, L. K., F. J. Muller, M. L. Wedam, C. S. Schneider, and M. K. Biddle. 2008. Clinical Mycoplasma bovis mastitis in prepubertal heifers on 2 dairy herds. Can Vet J 49(11):1110-1112.

Ghadersohi, A., R. G. Hirst, J. Forbes-Faulkener, and R. J. Coelen. 1999. Preliminary studies on the prevalence of Mycoplasma bovis mastitis in dairy in cattle in Australia. Vet Microbiol 65(3):185-194.

Godkin, M. A. and K. E. Leslie. 1993. Culture of bulk tank milk as a mastitis screening test: A brief review. Can Vet J 34(10):601-605.

Gonzalez, R. N., D. E. Jasper, T. B. Farver, R. B. Bushnell, and C. E. Franti. 1988. Prevalence of udder infections and mastitis in 50 California dairy herds. J Am Vet Med Assoc 193(3):323-328.

Gonzalez, R. N., P. M. Sears, R. A. Merrill, and G. L. Hayes. 1992. Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. Cornell Vet 82(1):29-40.

Gonzalez, R. N., B. M. Jayarao, and S. P. Oliver. 1993. Pneumonia, arthritis and mastitis in dairy cows due to *Mycoplasma bovis*. Pages 178-186 in Proc. National Mastitis Council, Kansas City, MO.

Gonzalez, R. N. and P. M. Sears. 1994. Persistence of Mycoplasma bovis in the mammary gland of naturally infected dairy cows. The bovine proceedings 26:184-186.

Gonzalez, R. N. and D. J. Wilson. 2002. Realistic milk culture programs for herd expansion. Proceedings of the National Mastitis Council, Orlando FL. Madison (WI): National Mastitis Council:118-124.

Gonzalez, R. N. and D. J. Wilson. 2003. Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19(1):199-221.

Guterbock, W. M. and P. E. Blackmer. 1984. Veterinary interpretation of bulk-tank milk. Vet Clin North Am Large Anim Pract 6(2):257-268.

Henderson, J. P. and H. J. Ball. 1999. Polyarthritis due to Mycoplasma bovis infection in adult dairy cattle in Northern Ireland. Vet Rec 145(13):374-376.

Hoblet, K. and M. Iqbal. 1996. Mycoplasma mastitis: survey of Ohio herds. in Proc. National mastitis council annual meeting proceedings. Pages 77-81

Houlihan, M. G., B. Veenstra, M. K. Christian, R. Nicholas, and R. Ayling. 2007. Mastitis and arthritis in two dairy herds caused by Mycoplasma bovis. Vet Rec 160(4):126-127.

Jackson, G. and E. Boughton. 1991. A mild outbreak of bovine mastitis associated with Mycoplasma bovigenitalium. Vet Rec 129(20):444-446.

Jain, N. C., D. E. Jasper, and J. D. Dellinger. 1969. Serologic response of cows to Mycoplasma under experimental and field conditions. Am J Vet Res 30(5):733-742.

Jasper, D. E., N. C. Jain, and L. H. Brazil. 1966. Clinical and laboratory observations on bovine mastitis due to Mycoplasma. J Am Vet Med Assoc 148(9):1017-1029.

Jasper, D. E., J. M. Al-Aubaidi, and J. Fabricant. 1974. Epidemiologic observations on mycoplasma mastitis. Cornell Vet 64(3):407-415.

Jasper, D. E. 1979. Bovine mycoplasmal mastitis. J Am Vet Med Assoc 175(10):1072-1074.

Jasper, D. E. 1981. Bovine mycoplasmal mastitis. Adv Vet Sci Comp Med 25:121-157.

Jasper, D. E. 1982. The role of Mycoplasma in bovine mastitis. J Am Vet Med Assoc 181(2):158-162.

Jayarao, B. M. and D. R. Wolfgang. 2003. Bulk-tank milk analysis. A useful tool for improving milk quality and herd udder health. Vet Clin North Am Food Anim Pract 19(1):75-92

Jayarao, B. M., S. R. Pillai, A. A. Sawant, D. R. Wolfgang, and N. V. Hegde. 2004. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J Dairy Sci 87(10):3561-3573.

Justice-Allen, A., J. Trujillo, R. Corbett, R. Harding, G. Goodell and D. Wilson. 2010. Survival and replication of Mycoplasma species in recycled bedding sand and association with mastitis on dairy farms in Utah. J Dairy Sci 93(1): 192-202

Kirk, J. H., K. Glenn, L. Ruiz, and E. Smith. 1997. Epidemiologic analysis of Mycoplasma spp isolated from bulk-tank milk samples obtained from dairy herds that were members of a milk cooperative. J Am Vet Med Assoc 211(8):1036-1038.

Kirk, J. H. and L. L. Lauerman. 1994. Mycoplasma mastitis in dairy cows. Compend Contin Educ 16(4):541-551.

Kunkel, J. R. 1985. Isolation of Mycoplasma bovis from bulk milk. Cornell Vet 75(3):398-400.

Mackie, D. P., D. Finlay, N. Brice, and H. J. Ball. 2000. Mixed mycoplasma mastitis outbreak in a dairy herd. Vet Rec 147(12):335-336.

McDonald, W. L., T. G. Rawdon, J. Fitzmaurice, I. Bolotovski, H. Voges, S. Humphrey, K. Fernando, Y. Canagasebey, R. N. Thornton, and L. McIntyre. 2009. Survey of bulk tank milk in New Zealand for Mycoplasma bovis , using species-specific nested PCR and culture. N Z Vet J 57(1):44-49.

Nakamura, R. M., M. L. Walt, and R. H. Bennett. 1977. Studies of bovine genital tracts: Mycoplasma isolations from dairy cows. Theriogenology 7(6):351355.

Nicholas, R. A. and R. D. Ayling. 2003. Mycoplasma bovis: disease, diagnosis, and control. Res Vet Sci 74(2):105-112.

Olde Riekerink, R. G., H. W. Barkema, S. Veenstra, D. E. Poole, R. T. Dingwell, and G. P. Keefe. 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. Can Vet J 47(6):567-572.

Pfutzner, H. and K. Sachse. 1996. Mycoplasma bovis as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. Rev Sci Tech 15(4):1477-1494.

Razin, S., D. Yogev, and Y. Naot. 1998. Molecular biology and pathogenicity of mycoplasmas. Micro. Mol. Biol. Rev 62:1094-1156.

Roy, J. P., D. Francoz, and O. Labrecque. 2008. Mastitis in a 7-week old calf caused by Mycoplasma bovigenitalium. Vet. J. 176(3):403-404.

Ruhnke, H. L., D. Thawley, and F. C. Nelson. 1976. Bovine mastitis in Ontario due to Mycoplasma agalactiae subsp. bovis. Can J Comp Med 40(2):142-148.

Thomas, C. B., P. Willeberg, and D. E. Jasper. 1981. Case-control study of bovine mycoplasmal mastitis in California. Am J Vet Res 42(3):511-515.

Thurmond, M. C., J. W. Tyler, D. M. Luiz, C. A. Holmberg, and J. P. Picanso. 1989. The effect of pre-enrichment on recovery of Streptococcus agalactiae, Staphylococcus aureus and mycoplasma from bovine milk. Epidemiol Infect 103(3):465-474.

USDA. 2003. Mycoplasma in bulk tank milk on U.S. dairies. Department of Agriculture. Animal and Plant Health Inspection Service.

Walz, P. H., T. P. Mullaney, J. A. Render, R. D. Walker, T. Mosser, and J. C. Baker. 1997. Otitis media in preweaned Holstein dairy calves in Michigan due to Mycoplasma bovis. J Vet Diagn Invest 9(3):250-254.

Wilson, D. J., G. Goodell, A. Justice-Allen, and S. T. Smith. 2009. Herd-level prevalence of Mycoplasma spp mastitis and characteristics of infected dairy herds in Utah as determined by a statewide survey. J. Am. Vet. Med. Assoc. 235(6):749-754.

Wilson, D. J., R. T. Skirpstunas, J. D. Trujillo, K. B. Cavender, C. V. Bagley, and R. L. Harding. 2007. Unusual history and initial clinical signs of Mycoplasma bovis mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. J Am Vet Med Assoc 230(10):1519-1523.

Woldehiwet, Z., B. Mamache, and T. G. Rowan. 1990. Effects of age, environmental temperature and relative humidity on the colonization of the nose and trachea of calves by Mycoplasma spp. Br Vet J 146(5):419-424.

Zecconi, A., R. Piccinini, A. Zepponi, and G. Ruffo. 1997. Recovery of Staphylococcus aureus from centrifuged quarter milk samples. J Dairy Sci 80(11):3058-3063.

CHAPTER THREE

MATERIALS AND METHODS

Specific aim #1 (Chapter 4): Determine the association between the outbreak strain of *Mycoplasma spp.* causing mastitis and asymptomatic carriage of *Mycoplasma spp.*

Mycoplasma spp. have been considered contagious mastitis pathogens and the transmission of *Mycoplasma spp.* has been postulated to occur primarily during milking time (Fox and Gay, 1993). Despite implementation of contagious mastitis control methods the herd prevalence of mycoplasma infection has increased, suggesting that transmission may more commonly occur from pathways other than those associated with milking (Fox et al., 2003). The mucosal surface of eyes, nasal cavities, ears and vestibular fossa are colonization sites for *Mycoplasma spp.* in dairy cattle (Fox et al., 2005) and may be associated with mastitis (Biddle et al., 2005). Transmission of *Mycoplasma spp.* from extramammary sites of asymptomatic carriers, to the mammary gland, has been hypothesized.

Study herd

A dairy herd of approximately 80 Holstein lactating cows with the first outbreak of mycoplasma mastitis was investigated. Replacements born at this herd, aged 4-6 months, were routinely transported to a state dairy institutional herd, raised, and returned just prior to first parturition.

Sampling scheme

Milk and body site samples were collected quarterly with the start of the outbreak. All

animals present in the herd were sampled once during each sampling period. Composite milk samples were collected aseptically from lactating cows. Swabs, moistened by mycoplasma enrichment media (appendix 1), were in contact and rotated over the mucosal surfaces of the right and left eyes, the right and left nasal cavities, the right and left ears, the vestibular fossa and vaginal wall from all animals which included calves, young heifers, heifers, dry cows and lactating cows. Swabs were returned to the mycoplasma enrichment media tubes after collection.

Microbiological culturing of samples and examination

Milk samples were vortexed, and 100 µL of milk were inoculated into 10 mL of mycoplasma enrichment medium. The mycoplasma enrichment media tubes from both milk samples and body site samples were incubated at 37 ^{0}C, 10% CO_2, for 4 days. A 100 µL portion of incubated media was plated on modified Hayflicks agar (appendix 1) for 10 days, then examined with a 15X dissecting microscope, to identify colonies with the distinctive "fried egg" appearance (Hogan et al., 1999). Results were considered positive if any mycoplasma colonies were seen and negative if there was no evidence of growth of *Mycoplasma spp.*

Species and strain identification

Pulsed field gel electrophoresis (PFGE) of chromosomal digests was used to fingerprint the mycoplasma isolates as described (Biddle et al., 2005). In brief, mycoplasma samples were centrifuged at 1,200 X g at 4°C for 10 minutes to pellet the microorganism. The supernatant was discarded, and pellet was suspended in 200 µL of buffer solution. Ten microliters of proteinase K was added, and the DNA was embedded in 200 µL of agarose. Plugs were cast

and lysed. Chromosomal DNA was digested with the restriction enzyme *Sal* I. Electrophoresis was performed at 14°C for 20.2 hours at a setting of 6V/cm of gel and a linear pulse ramp of 1 to 12.9 seconds. After electrophoresis, gels were stained with ethidium bromide for 30 minutes, washed twice in distilled water, and then photographed under UV light. Isolates were considered to be the same strain when chromosomal digestion produced the same number of bands of the same size (Figure 1). Samples were speciated by polymerase chain reaction technique (Tang et al., 2000).

Statistical analysis

Proportional data were arranged into 2x2 contingency tables. The χ^2 - test was used to test difference in proportions. In cases where a contingency table cell had an expected value less than 5, Fisher's exact test was used. Statistical analysis was performed using PROC FREQUENCY (SAS version 9.1, SAS institute, Inc., Cary, NC, USA). Level of significance was set at $\alpha = 0.05$.

Specific aim # 2 (Chapter 5): Determine if concentrating *Mycoplasma spp.* from milk could enhance detection after standard culture

A highly sensitive test is required to diagnosis whether a dairy herd has at least one cow with mycoplasma mastitis. However, the sensitivity of single bulk tank milk culturing is approximately 60% which indicates false negatives are 40%. One study showed that centrifugation of milk samples and resuspension and concentration of the bacterial pellet before culture improved the ability of detection of *Staphylococcus aureus* (Zecconi et al.,

1997). This method was applied to mycoplasma to determine if it improved the sensitivity of *Mycoplasma spp.* detection.

Experimental design

A completely randomized design with a 2x2x4 factorial arrangement was used to test the factors: 1) Two different treatments (centrifugation vs. direct agar plating); 2) Two different test species (*Mycoplasma bovis* and *Mycoplasma californicum*; four different strains for each species were used); and 3) Four different concentrations of *Mycoplasma spp.* (1,000 cfu/mL, 100 cfu/mL, 10 cfu/mL and 1 cfu/mL).

Test media, centrifugation and colony examination

Fresh bulk tank raw milk from the herd with no history of mycoplasma mastitis during a 20-year period was used as the test media. Broth cultures of mycoplasma strains in the logarithmic phase of growth were centrifuged at 5,000 x g for 30 min. The cell pellet was suspended in phosphate buffered saline solution (PBS). The mycoplasma suspension was diluted to achieve an optical density (OD) of 2.0 at 540 nm corresponding to a theoretical concentration of 10^8 colony forming units (cfu)/mL. This mycoplasma suspension was then serially diluted with PBS (appendix 1) to achieve theoretical concentrations of 10,000 cfu/mL, 1,000 cfu/mL, 100 cfu/mL and 10 cfu/mL. At each concentration, one of the duplicates was randomly allocated as the treatment tube, the other as the control tube.

A 5 mL portion of mycoplasma suspensions from each control and treated tube were added to 45 mL of fresh bulk tank milk to achieve a theoretical concentration of 1,000 cfu/mL, 100

cfu/mL, 10 cfu/mL and 1 cfu/mL. Treatment tube milk samples were vigorously mixed and centrifuged at 5000 x g for 30 min. After centrifugation the fat layer was removed and the skim decanted. Then 400 µL of PBS was added to resuspend the centrifuged pellet. This resuspension was vigorously mixed and 200 µL removed and spread on Modified Hayflick agar plate (Hogan and NMC, 1999). Control tube milk samples which were not centrifuged were vigorously mixed and 200 µL of mycoplasma inoculated milk was removed and plated on Modified Hayflick agar plate in duplicate. The actual concentration of *Mycoplasma spp.* used to inoculate the milk was determined by plating and culturing 200 µL of the original PBS suspension of 1,000 cfu/mL, and the colonies enumerated as described. All plates were incubated at 37°C with 5% CO_2 for 5 days. Plates were examined using 15 X microscope for evidence growth of mycoplasma microorganism with the distinctive fried-egg appearance and colonies counts made. Plates were classified as a positive culture if at least one colony of mycoplasma organism was found. Bulk tank milk samples were also plated and cultured by conventional method to determine that samples were free from *Mycoplasma spp.*

Statistical analysis

The difference in mean of recovery rate of mycoplasma organism between standard method and centrifugation technique was tested by using analysis of variance. The level of significance was set at $\alpha = 0.05$.

Specific aim #3 (Chapter 6): Identify risk factors that were associated with the decrease time to clearance of mycoplasma mastitis from a herd after the initial outbreak

The change of herd mycoplasma mastitis status from positive to negative in 79 herds was investigated through monthly culture of bulk tank milk (Fox et al., 2005). Approximately 30 per cent of herds were still positive to mycoplasma mastitis after one month. It is not understood why some herds became negative and why other herds continued to have a mycoplasma mastitis problem with monthly recurrent mycoplasma isolation from bulk tank milk samples. Some dairy operators might employ management factors that quickly lead to the elimination of herd mycoplasma mastitis while others fail to employ the necessary strategies that lead to elimination.

There are two commonly recommended strategies to control mycoplasma mastitis outbreak after mycoplasma mastitis cows are identified. Some researchers suggest that dairy operators should cull all infected animals as soon as possible from the herd (Bushnell, 1984, Bayoumi et al., 1988). Alternatively, some argue that it is not necessary to cull infected animals. The outbreak of mycoplasma mastitis can be controlled by segregation and milking infected cows last (Brown et al., 1990, Jackson and Boughton, 1991).

Sample herd and herd survey

Dairy herds that ship milk to Northwest Dairy Association (NDA) were eligible for this study. Enrollment criteria were a willingness to participate and a BTM sample that was positive to *Mycoplasma sp.* by standard culture where herd BTM had been previously negative for 6 months prior. A herd visit was scheduled within 2 weeks after the dairy operator agreed to enroll. A questionnaire was used to obtain herd and management data from the dairy operator during the herd visit (Appendix 2).

Bulk milk sampling and culturing

Bulk Milk Tank (BTM) samples from enrolled dairies were routinely collected to monitor the mycoplasma mastitis status. Each BTM sample was collected at 7-10 day intervals. Milk samples (50 mL) were centrifuged at 5,000 x *g* for 30 min before culture to enhance mycoplasma recovery. Sediment was resuspended with 200 mL phosphate buffer saline. A 200 µL of resuspension was plated on modified Hayflicks agar. Plates were kept in the incubator at 37 ^{0}C with 10% CO_2 for 10 days then examined with a 15X dissecting microscope, to identify colonies with the distinctive "fried egg" appearance (Hogan and NMC, 1999). Results were considered positive if any mycoplasma colonies were seen and negative if there was no evidence of growth of *Mycoplasma* spp. All positive samples from first isolation (n=18) were speciated by polymerase chain reaction technique (Tang et al., 2000).

Statistical Analysis

Time to clearance of mycoplasma mastitis referred to the time interval from the first isolation of BTM sample to *Mycoplasma spp.* to the first negative of three consecutive negative of BTM samples to *Mycoplasma spp.* A Kaplan-Meier survival curve was made using PROC LIFETEST in SAS program (SAS version 9.1, SAS institute, Inc., Cary, NC, USA) to determine the survival function of mycoplasma mastitis in the dairy herd. Herds with time to clearance mycoplasma mastitis less than 3 months were defined as cases. Other herds were defined as censors.

Specific aim # 4 (Chapter 7): Determine the incidence and the transmission of mycoplasma mastitis in a herd that segregated diseased animals in a hospital pen

Mycoplasma mastitis can be controlled by: Identification of the infected cows followed by segregation and milking the infected cows last (Jackson and Boughton, 1991); 2) by identifying and then culling diseased cows (Bushnell, 1984, Bayoumi et al., 1988). During approximately 7-10 day period between identification of cows suspected of having mycoplasma mastitis and the confirmation by culture, dairy managers may prefer to keep suspected cows with clinical mycoplasma mastitis penned with healthy milking cows. Other dairy managers may group cows with clinical mastitis into a pen of infirm cows, termed the hospital pen, and milk hospital pen cows separately from other milking cows (Bayoumi et al., 1988). The incidence and the transmission of mycoplasma mastitis in a herd that segregated diseased animals in a hospital pen were determined in this study.

Study herd and herd visiting

A commercial Washington State herd of approximately 650 lactating cows was investigated for the suspected mycoplasma mastitis outbreak. Two months after the first case of mycoplasma mastitis the herd operator decided to create a hospital pen for diseased cows. Transmission of the outbreak mycoplasma pathogen among mastitis cows in the hospital pen was suspected.

Sampling and Microbiological culturing

Milk samples were aseptically collected from all functional mammary quarters of clinical

mastitis cows immediately before antibiotic treatment on the day they entered the hospital pen. A second milk sample for culture was collected on the day they exited the hospital pen. Additional samples were taken from hospital pen cows if a new mammary quarter developed clinical mastitis or if the same quarter was unresponsive to antibiotic treatment, deemed an abnormal udder or having abnormal milk. Joint fluid samples were aseptically collected from arthritis cows. A weekly BTM sample was collected to monitor the mastitis status throughout the study.

Cultures of milk and joint fluid were made to determine the presence of *Mycoplasma spp*. In brief, a 50 µl aliquot of milk or joint fluid was plated on modified Hayflick's agar plate and incubated at 37°C, 10% CO_2, for 10 days. Plates were examined with a 15X dissecting microscope to identify colonies with the distinctive "fried egg" appearance (Hogan et al., 1999).

Species and strain identification

Pulsed field gel electrophoresis (PFGE) of chromosomal digests was used to fingerprint the mycoplasma isolates as described (Biddle et al., 2005). In brief, mycoplasma samples were centrifuged at 1,200 X g at 4°C for 10 minutes to pellet the microorganism. The supernatant was discarded, and pellet was suspended in 200 µL of buffer solution. Ten microliters of proteinase K was added, and the DNA was embedded in 200 µL of agarose. Plugs were cast and lysed. Chromosomal DNA was digested with the restriction enzyme *Sal* I. Electrophoresis was performed at 14°C for 20.2 hours at a setting of 6V/cm of gel and a linear pulse ramp of 1 to 12.9 seconds. After electrophoresis, gels were stained with ethidium

bromide for 30 minutes, washed twice in distilled water, and then photographed under UV light. Samples were speciated by polymerase chain reaction technique (Tang et al., 2000).

Data and Statistical Analysis

Individual cow productivity, management and health records were obtained from the herd software program, Dairy Plan C21 version 5.2 (WestfaliaSurge, Inc., Naperville, IL, USA). Incidence rates of mycoplasma mastitis for lactating pen cows and hospital pen cows were made. A Kaplan-Meier survival curve of mycoplasma mastitis in the hospital pen was made using PROC LIFETEST in SAS program (SAS version 9.1, SAS institute, Inc., Cary, NC, USA). An event was defined as the occurrence of clinical case mycoplasma mastitis. A hospital pen cow that developed mycoplasma mastitis was defined as case. A cow that never developed mycoplasma mastitis in the hospital pen was considered as censored.

References

Bayoumi, F. A., T. B. Farver, B. Bushnell, and M. Oliveria. 1988. Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. J Am Vet Med Assoc 192(7):905-909.

Biddle, M. K., L. K. Fox, M. A. Evans, and C. C. Gay. 2005. Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. J. Am. Vet. Med. Assoc. 227(3):455-459.

Brown, M. B., J. K. Shearer, and F. Elvinger. 1990. Mycoplasmal mastitis in a dairy herd. J. Am. Vet. Med. Assoc. 196(7):1097-1101.

Bushnell, R. B. 1984. Mycoplasma mastitis. Vet Clin North Am Large Anim Pract 6(2):301-312.

Fox, L. K. and J. M. Gay. 1993. Contagious mastitis. Vet Clin North Am Food Anim Pract 9(3):475-487.

Fox, L. K., D. D. Hancock, A. Mickelson, and A. Britten. 2003. Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. J Vet Med B Infect Dis Vet Public Health 50(5):235-240.

Fox, L. K., J. H. Kirk, and A. Britten. 2005. Mycoplasma mastitis: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health 52(4):153-160.

Hogan, J. S. and NMC. 1999. Laboratory handbook on bovine mastitis. Rev. ed. . National Mastitis Council, Madison, WI.

Jackson, G. and E. Boughton. 1991. A mild outbreak of bovine mastitis associated with Mycoplasma bovigenitalium. Vet Rec 129(20):444-446.

Tang, J., M. Hu, S. Lee, and R. Roblin. 2000. A polymerase chain reaction based method for detecting Mycoplasma/Acholeplasma contaminants in cell culture. J. Microbiol. Methods 39(2):121-126.

Zecconi, A., R. Piccinini, A. Zepponi, and G. Ruffo. 1997. Recovery of Staphylococcus aureus from centrifuged quarter milk samples. J. Dairy Sci. 80(9406099):3058-3063.

CHAPTER FOUR

Association between an outbreak strain causing *Mycoplasma bovis* mastitis and its asymptomatic carriage in the herd : A case study from Idaho, USA

V. Punyapornwithaya [a], L. K. Fox [a]*, D.D. Hancock [a], J.M. Gay [a], J. R. Alldredge [b]

[a] Department of Veterinary Science,

Washington State University, Pullman, WA, 99164-6610 USA

[b] Department of Statistics,

Washington State University, Pullman, WA 99164-3144, USA

This manuscript was accepted to be published in

Preventive Veterinary Medicine.

ABSTRACT

The objective of this study was to determine the association between mycoplasma mastitis and colonization of mycoplasma organisms at body sites of asymptomatic carriers. The investigation was done in a dairy herd with a first outbreak of mycoplasma mastitis. Milk and swab solution specimens from accessible mucosal surfaces of body sites from cows and replacements were sampled at quarterly intervals (Herd Samplings 1-4). Samples were cultured and *Mycoplasma spp.* were isolated, speciated and fingerprinted. During Herd Sampling 1 two cows with mycoplasma bovis mastitis were identified and all swabbing solutions of body site samples from 18 of 84 cows and 36 of 77 replacements were positive to *Mycoplasma bovis* and fingerprinted as the same strain. A case of clinical *M. bovis* mastitis developed during Herd Sampling 3. During Herd Samplings 2-4, 4 lactating cows and 12 replacements were positive to *M. bovis* at various body sites with 4 different strains. Three isolates of *M. californicum* were found from swabbing solutions of 3 cows during Herd Samplings 3 and 4. Only one strain of *M. bovis* caused mastitis although four strains were isolated from body sites of animals. Isolation of *M. bovis* from a body site never preceded mastitis. No lactating cow developed mastitis during Herd Sampling 4 although some animals were colonized with the organism. It appears that during the initial outbreak of *M. bovis* mastitis colonization of body sites by the outbreak strain may be common. However, the prevalence of colonization subsides and colonization does not appear to precede mastitis.

Keywords: Mastitis, *Mycoplasma bovis*, Fingerprinting, Pulsed Field Gel Electrophoresis

1. Introduction

Mycoplasma spp. have been considered contagious mastitis pathogens and thus the transmission of *Mycoplasma spp.* has been postulated to occur primarily during milking time and can be controlled by use of proper milking time hygiene practices (Fox and Gay, 1993; Gonzales and Wilson, 2003). Despite implementation of contagious mastitis control methods the prevalence of mycoplasma infection has increased, suggesting that transmission may more commonly occur from pathways other than those associated with milking (Fox et al., 2003). The mucosal surface of eyes, nasal cavities, ears and vestibular fossa are colonization sites for *Mycoplasma spp.* in dairy cattle (Fox et al., 2005) and may be associated with mastitis (Biddle et al., 2005). Transmission of *Mycoplasma spp.* from extramammary sites of asymptomatic carriers, to the mammary gland, has been hypothesized. Such internal transmission may account in part for the inability of herds to eradicate mycoplasma mastitis despite employment of excellent milking time hygiene practices.

In this paper we describe the epidemiology of an outbreak of mycoplasma mastitis in a herd that had no known history of this disease. The association between the outbreak strain and asymptomatic carriage of *Mycoplasma bovis* mastitis by lactating cows and their replacements was made.

2. Materials and Methods

2.1.1 Herd history

During a twenty year period the University of Idaho (UI) dairy herd of approximately 80 Holstein lactating cows had no history of mycoplasma mastitis based upon cultures of both bulk tank milk and milk from mammary quarters of cows with clinical mastitis. Thus the herd had been considered mycoplasma mastitis free, a naïve herd, but then reported their first case of mycoplasma mastitis that was initially detected through microbiological culture of bulk tank milk on August 3, 2004. One cow with clinical mastitis (Case 1) was identified as having an intramammary infection with *Mycoplasma spp.* from milk culture by the Washington Animal Disease Diagnosis Laboratory Pullman, WA. This cow was culled from the herd before the investigation began and no samples were obtained by our laboratory. Bulk tank milk culture results were negative to *Mycoplasma spp.* during the next 3 week period after the infected animal was removed. Approximately two weeks thereafter a bull calf was diagnosed with mycoplasma pneumonia and was culled. *Mycoplasma spp.* was again isolated from a bulk tank milk sample on September 22, 2004. Replacements born at UI herd, aged 4-6 months, were routinely transported to a state dairy institutional herd, raised, and returned just prior to first parturition. The last batch of replacements returned to the herd approximately 2 months before the first case of mycoplasma mastitis developed.

2.1.2 Definitions

A cow with clinical mastitis from which *Mycoplasma spp.* was indentified in milk was considered a case. Cows are animals that were born and raised at the UI herd and never left the premises. Replacements included 1) Calves and heifers that were born and raised at the UI herd and never left the premises, 2) Calves and heifers born at the UI herd but partially raised at the institutional herd; and 3) Primiparous cows that were purchased from the institutional herd. The outbreak refers to the time when the second case of mycoplasma

mastitis was found which was associated with a high prevalence of colonization in both replacements and cows. Sampling periods were the time that all animals in the herd were sampled and were conducted at quarterly intervals (Herd Samplings 1-4) for 1 year. Exposed animals were raised partially or entirely at the institutional herd. All other animals were considered restricted.

2.2 Sampling scheme

Milk and body site samples were collected quarterly with the start of the outbreak. All animals present in the herd were sampled once during each sampling period. The collection of milk samples and other body site samples were done as described (Biddle et al., 2005). In brief, composite milk samples were collected aseptically from lactating cows. Swabs, moistened by mycoplasma enrichment media, were in contact and rotated over the mucosal surfaces of the right and left eyes, the right and left nasal cavities, the right and left ears, the vestibular fossa and vaginal wall (vulvovaginal tract) from all animals which included calves, young heifers, heifers, dry cows and lactating cows. Swabs were returned to the mycoplasma enrichment media tubes after collection.

2.3 *Microbiological culturing of samples and Examination*

Milk samples were vortexed, and 100 µL of milk were inoculated into 10 mL of mycoplasma enrichment medium. The mycoplasma enrichment media tubes from both milk samples and body site samples were incubated at 37 ^{0}C, 10% CO_2, for 4 days. A 100 µL portion of incubated media was plated on modified Hayflicks agar for 10 days, then examined with a 15X dissecting microscope, to identify colonies with the distinctive "fried

egg" appearance (Hogan et al., 1999). Results were considered positive if any mycoplasma colonies were seen and negative if there was no evidence of growth of *Mycoplasma spp.*

2.4 Species and strain identification

Pulsed field gel electrophoresis (PFGE) of chromosomal digests was used to fingerprint the mycoplasma isolates as described (Biddle et al., 2005). In brief, mycoplasma samples were centrifuged at 1,200 X g at 4°C for 10 minutes to pellet the microorganism. The supernatant was discarded, and pellet was suspended in 200 µL of buffer solution. Ten microliters of proteinase K was added, and the DNA was embedded in 200 µL of agarose. Plugs were cast and lysed. Chromosomal DNA was digested with the restriction enzyme *Sal* I. Electrophoresis was performed at 14°C for 20.2 hours at a setting of 6V/cm of gel and a linear pulse ramp of 1 to 12.9 seconds. After electrophoresis, gels were stained with ethidium bromide for 30 minutes, washed twice in distilled water, and then photographed under UV light. Isolates were considered to be the same strain when chromosomal digestion produced the same number of bands of the same size (Figure 1). Samples were speciated by the method outlined previously (Tang et al., 2000).

2.5 Statistical analysis

Proportional data were arranged into a 2x2 contingency table. The χ^2 - test was used to test :1) differences in proportions of colonization at any body site between replacements and cows, 2) differences in proportions of colonization at any body site between exposed animals and restricted animals and, 3) differences in proportions of colonization at multiple body sites between replacements and cows and 4) differences in proportions of colonization

at multiple body sites between exposed animals and restricted animals. In cases where a contingency table cell had an expected value less than 5, the Fisher's exact test was used. Statistical analysis was done using PROC FREQUENCY (SAS version 9.1, SAS institute, Inc., Cary, NC, USA). Level of significance is set at $\alpha = 0.05$.

3. Results

All *Mycoplasma spp.* were fingerprinted using PFGE and strain differences were distinguishable (Figure 1). *M. bovis* is the only species that was found during Herd Sampling 1, and it was the only species that caused mycoplasma mastitis cases. The prevalence of *Mycoplasma spp.* colonization at body sites of all replacement and cows decreased from 34 % (n=54/161) at Herd Sampling 1 to 1.5 % (n=2/135), 5.6 % (n=8/143) and 4.4 % (n=6/137) in Herd Samplings 2, 3 and 4. The variation in number of animals available to be sampled by period was a function of the herd's culling practices.

At Herd Sampling 1, body site swabbing solution samples were collected from 84 cows and 77 replacements. *M. bovis* strain O, the outbreak strain, was found to colonize 18 cows (21%) and 36 replacements (47%), with 3 cows and 8 replacements colonized at multiple body sites (Tables 1 and 2). Nasal mucosa was the area most likely to be colonized for cows and replacements. The proportion of cows that had *M. bovis* at any body site was less than the proportion of replacements (P=0.003). Similarly, the proportion of animals colonized by *M. bovis* at any body site in exposed animals (46%; n=33/72) was almost 2 times greater than that of restricted animals (24 %; n=21/89) (P<0.001). The proportion of multiple-site colonization between cows (3.6%; n=3/84) and replacements (10.4%; n=8/77) was not significantly different (P=0.11). Similarly, the proportion of multiple-site

colonization between exposed animals (9.7%; n=7/72) and restricted animals (4.5%; n=4/89) was not significantly different (P=0.22).

Clinical manifestations of mycoplasma infections occurred during Herd Sampling 1. One lactating cow was found to have a clinical case of *M. bovis* mastitis (Case 2) and was culled. One male calf had polyarthritis with *M. bovis* infection approximately one month later and was culled. A third case of *M. bovis* mastitis (Case 3) developed two months after Case 2 and this cow was isolated from the herd and milked last. Eight months later the Case 3 cow began a new lactation and appeared free of mycoplasma mastitis. Body site colonization of the agent was not detected in samples collected during Herd Samplings 3 and 4 from the Case 3 cow. All *M. bovis* isolates from clinical disease were the same strain as the outbreak strain (strain O) as determined by PFGE (Figure 1).

During Herd Samplings 2-4, 4 lactating cows (5.1 %) and 12 replacements (2.4%) were found colonized with *M. bovis* at body sites. One cow had *M .bovis* at the vulvovaginal tract and eye. In contrast to the Herd Sampling 1, most colonization was at the vulovaginal site (Tables 1 and 2). Isolation of *M. bovis* from a body site of an individual at one herd sampling was never repeated at another herd sampling. The fourth case of clinical mycoplasma mastitis (Case 4) occurred at the time of the Herd Sampling 3. However, this cow was not colonized with *M. bovis* at any body site during this or any prior herd sampling. This cow remained with the herd and was deemed free of mycoplasma mastitis and mycoplasma colonization at the last herd sampling. *Mycoplasma californicum* was isolated from swabbing solution samples from vulvovaginal tract from 2 cows at Herd Sampling 3 and from 1 replacement at Herd Sampling 4.

Electrophoretograms of all 66 isolates from milk and body sites samples from replacements, and cows at the Herd Sampling 1 were indistinguishable and were deemed to be the same strain and were considered outbreak isolates (strain O) (Figure 1, lanes 2 and 3). A representative isolate was selected for depiction in Figure 1 when multiple isolates of the same strain were found. Isolates were determined to be *M. bovis* and *M. californicum*. Based on criteria outlined by Tenover et al. (1995), two strains of *M. bovis* were closely related to strain O and were designated as O1 and O2 (lane 5, 6, 7, 8, 12 and 15). The isolate from mastitis Case 4 (lane 4) had a PFGE pattern that was indistinguishable to the outbreak strain (lane 3). A new strain (strain A) was found from one cow during Herd Sampling 4 (lane 11). Representative strain O isolates from cows at Herd Samplings 3 and 4 presented at lane 9, 10 and 13. Of 17 animals positive with *M. bovis* during Herd Samplings 2-4, four animals had Strain O and 10 animals had strains that were closely related to strain O including O1 (n=7), O2 (n=3). A strain of *M. californicum* is depicted at lane 14.

All mastitis cases were caused by strain O, the last case of mastitis occurred at Herd Sampling 3. The herd continues to monitor mycoplasma mastitis monthly by culturing bulk tank milk and has not reported a new case although replacements and cows colonized with *Mycoplasma spp.* at body sites are still in the herd.

4. Discussion

The key feature of this study is that it is the first investigation examining the epidemiology of the mycoplasma mastitis through culture of mycoplasma organisms from body site samples from all animals in a herd as well as individual milk samples from all lactating cows, for an extended period, 1 year. Isolates were fingerprinted to distinguish

between strains that caused mastitis and those that colonized body sites. An important finding of this current study is that although the outbreak strain did not cause any new cases of mastitis after Herd Sampling 3, the strain remained in the herd for more than a year, associated with asymptomatic colonization of body sites.

Colonization of multiple body sites with the same strain of *Mycoplasma spp.* is common, and isolates from these sites are very frequently the same type as isolates obtained from the mammary gland (Biddle et al., 2005). This suggests that there is a potential for internal transmission of mycoplasma within the animal and that an association between the development of mycoplasma mastitis and the colonization of this microorganism at body sites exists. Yet, the findings from this study did not demonstrate that mastitis strain O colonized at a body site prior to intramammary infection. Three cows that developed mycoplasma mastitis during the study did not have detectable *M. bovis* in body sites before the occurrence of mastitis. Therefore it does not appear that colonization of body site is a risk factor for intramammary infection; although the limited number of mastitis cases suggests that future studies will be need to test the significance of this risk factor.

M. bovis strain O was the only strain found at body sites during Herd Sampling 1 and it remained in the herd and appeared to colonize body sites of replacements or cows during the entire study. It was the only strain associated with mastitis although during other sampling periods related strains were isolated from body sites but did not cause mastitis. This might suggest that strain O possessed virulence factors that enabled it to cause intramammary infection while apparent emergent strains (O1 and O2) lacked this ability. An understanding of the *Mycoplasma spp.* virulence factors that lead to mastitis is largely unknown. But the identification of what appears to be two emergent strains from an outbreak strain might be

very useful in future studies on the virulence factors of the pathogens that are associated with mycoplasma mastitis. *Mycoplasma californicum* was initially isolated at Herd Sampling 3 but never caused mastitis. It colonized the body sites of a small proportion of animals (< 2%).

This is a first study in the investigation of the epidemiology of mycoplasma mastitis through fingerprinting of the outbreak strain and examination of the relationship between strains that colonized other body sites of both cows and replacements. The percentage of colonization by any strains appeared to decrease after Herd Sampling 1. Replacements and cows that were first positive to *M. bovis* at body sites were negative to the organism at all accessible mucosal surface samples at the next herd sampling and no replacements or cows were intermittently positive to the organism throughout the study. Perhaps the replacements and cows developed natural immunity to the outbreak strain and spontaneously eliminated the mycoplasma colonization. Alternatively, colonization was at site not sampled, or shedding of the microorganism at the site sampled was at level that could not be detected. A follow up study to test the hypothesis that natural immunity develops during an outbreak to outbreak strains in herds would be worthy of initiation.

It was reasonable to hypothesize that mycoplasma mastitis in the UI herd originated from animals in the institutional herd and was transmitted to the previously naïve UI herd by direct contact with the institutional herd animals. Such contact could be from exposed animals. Alternatively, mycoplasma mastitis might have originated from an animal in the UI herd and that replacements from the institutional herd contracted the agent after their introduction to the UI herd from either a symptomatic or an asymptomatic carrier, colonized by the outbreak strain, or both. Two bull calves suspected as having mycoplasma pneumonia might have been the source of the infection. Logically, if the source of the *M. bovis* strain was

exposed animals then it might be expected that a greater proportion of exposed animals would be carriers than restricted animals. The proportion of exposed animals positive to *M. bovis* strain O was significantly higher than the proportion of restricted animals. This could suggest mastitis cases began with the introduction of exposed animals to the UI herd, which led to infection and colonization of *M. bovis* strain O to restricted animals.

5. Conclusion

Mycoplasma mastitis in this herd was caused by one strain of *M. bovis* although five strains of the microorganism colonized body sites of animals. However, the strain that caused mastitis never colonized an animal before a mastitis case and it was never isolated from cows that recovered from mastitis. The risk of future mastitis from asymptomatic carriage may not be high. The outbreak strain appeared to be transmitted by exposure of naive animals from others previously raised at another dairy herd.

Acknowledgements

This study was supported in part by The Field Disease Investigation Unit (10A 3072 0858), the Safe Food and Emerging Disease Fund (10U 3922 0001) and the authors gratefully acknowledge the assistance of University of Idaho faculty and staff, Dr. Mark McGuire, Dr. Christopher Schneider, Dr. Samuel Mosley and Mr. Ed Wagner.

References

Biddle, M.K., Fox, L.K., Evans, M. A., Gay, C.C., 2005. Pulsed-field gel electrophoresis patterns of mycoplasma isolates from various body sites in dairy cattle with mycoplasma mastitis. J Am Vet Med Assoc. 227, 455-459.

Fox, L.K., Gay J.M., 1993. Contagious mastitis. Vet Clin North Am Food Anim Pract. 9, 475-487.

Fox, L.K., Hancock, D.D., Mickelson, A., Britten, A., 2003. Bulk tank milk analysis: factors associated with appearance of *Mycoplasma sp.* in milk. J Vet Med B Infect Dis Vet Public Health. 50, 235-240.

Fox, L.K., Kirk, J.H., Britten, A., 2005. Mycoplasma mastitis: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health. 52, 153-160.

Gonzalez, R.N., Wilson, D.J., 2003. Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract. 19, 199-221.

Hogan, J.S., National Mastitis Council (U.S.)., 1999. Laboratory handbook on bovine mastitis. Rev. ed. National Mastitis Council, Madison, WI.

Tang, J., Hu, M., Lee, S., Robin, R. 2000. A polymerase chain reaction based method for detecting Mycoplasma/ Acheoleplasma contaminants in cell culture. J. Microbiol. Methods 39, 121-126

Tenover, F.C., Arbeit, R.D., Goering, R.V., 1995. Interpreting chromosomal DNA restriction

patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 33, 2233-2239.

Table 1

Frequency of mycoplasma isolation from milk and various body site samples of cows with *Mycoplasma bovis.*

Herd Sampling[a]	Ear	Eye	Nose	vulvovaginal tract	Milk	% positive [b]
1	0	3	17	1	1	24 (n=18/84)
2	0	0	1	0	1	1.7 (n=1/59)
3	0	0	0	3	1	5.56 (n=3/54)
4	0	0	0	0	0	0 (n=0/42)

[a] Herd Sampling 1= October, 04-February, 05; Herd Sampling 2= March-May, 05; Herd Sampling 3 = June-August, 05; Herd Sampling 4 = September-December, 05

[b] % positive = number of positive animals divided by total number of animals sampled at each Herd Sampling. The numerator may be smaller than the sum of site positives in any row as some animals had multiple sites with mycoplasma isolates.

Table 2

Frequency of mycoplasma isolation from various body sites of replacements with *Mycoplasma bovis*.

Herd Sampling[a]	Ear	Eye	Nose	vulvovaginal tract	% positive [b]
1	1	6	34	3	47 (n=36/77)
2	0	1	0	0	1.3 (n=1/76)
3	0	1	4	0	5.6 (n=5/89)
4	0	1	1	5	6.3 (n=6/95)

[a] Herd Sampling 1= October, 04-February, 05; Herd Sampling 2= March-May, 05; Herd Sampling 3 = June-August, 05; Herd Sampling 4 = September-December, 05

[b] % positive = number of positive animals divided by total number of animals sampled at each Herd Sampling. The numerator may be smaller than the sum of site positives in any row as some animals had multiple sites with mycoplasma isolates.

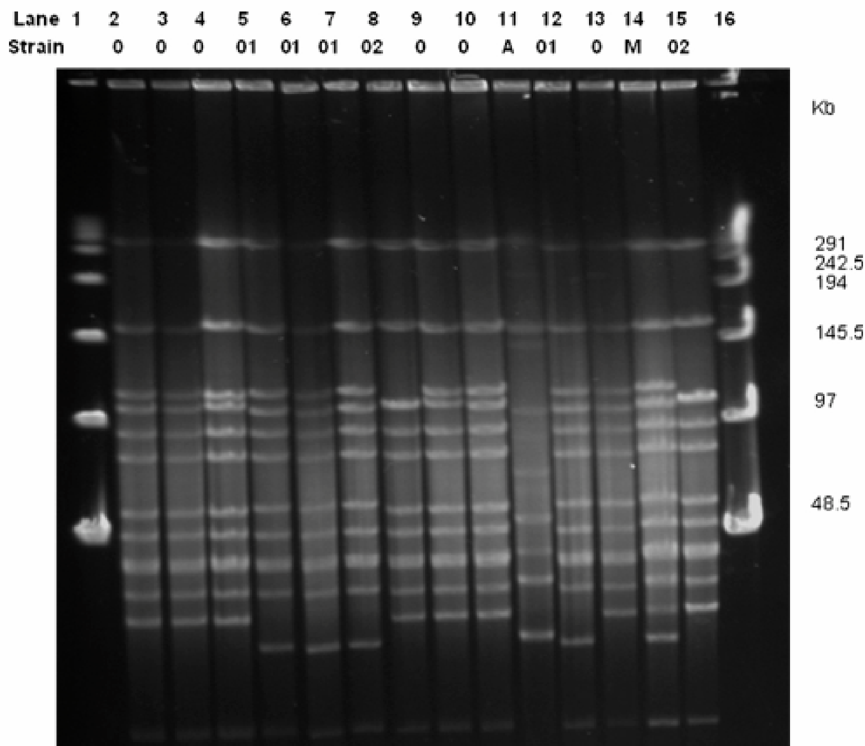

Figure1. Pulsed-field gel electrophoretograms of 4 strains of *Mycoplasma bovis* and one strain of *Mycoplasma californicum* following digestion. The isolate depicted at lane 2 (strain O) was the representative strain obtained from all animals at the first sampling in Herd Sampling 1. Strain O isolates at lane 3 was from a bulk tank milk sample at Herd Sampling 1. The strain O isolate at lane 4 was the strain from a cow with clinical mastitis in Herd Sampling 3. Strain O1 isolates at lane 5, 6, 7 and 12 were the representative strains from body site samples from 5 replacements and 2 cows in Herd Sampling 2, 3 and 4. Strain O isolates at lane 9, 10 and 13 were from body site samples from 3 replacement at Herd Sampling 3 and 4. Strain O2 isolate at lane 15 was a representative strain from 2 body site samples from 2 replacements at Herd Sampling 2 and 4. Strain O2 isolate at lane 8 was from 1 replacement at Herd Sampling 3. Isolate at lane 11 was strain A from 1 replacement at Herd Sampling 4. Isolate at lane 14 was *M. californicum* strain. Lane 1 and lane 16 depict the lamdar ladder with DNA digestion fragments of 48.5, 97, 145.5, 194, 242.5 and 291 kb.

CHAPTER FIVE

The effect of centrifugation and resuspension on the recovery of *Mycoplasma spp.* from milk

V. Punyapornwithaya*, L.K. Fox*[1], G.M. Gay*, D. D. Hancock* and J. R.Alldredge†

∗ College of Veterinary Medicine,

Washington State University, Pullman 99164-6610

†Department of Statistics,

Washington State University, Pullman 99164-3144

This manuscript was accepted to be published in

the Journal of Dairy Sciences.

ABSTRACT

Low sensitivity of a single bulk tank milk culture is a major limitation for detection mycoplasma organisms. We hypothesized that sedimentation of *Mycoplasma spp.* in a milk sample by centrifugation followed by resuspension in a small volume of fluid before agar platting would increase the ability to detect *Mycoplasma spp.* as compared with the direct conventional culture. The experiment was conducted to determine recovery of *Mycoplasma spp*. from milk as effected by: 1) treatment (centrifugation vs. conventional method; 2) two different species (*M. bovis* and *M. californicum* and 4 different strains for each species); and 3) four different concentrations of *Mycoplasma spp.* (1,000, 100, 10, and 1 cfu/mL). A 5 mL portion of mycoplasma suspensions from each strain were inoculated into 45 mL of fresh bulk tank milk to achieve concentrations of 1,000, 100, 10, and 1 cfu/mL. Treatment samples were vigorously mixed and centrifuged at 5,000 X *g* for 30 min. Control samples were vigorously mixed. All samples were plated on modified Hayflick agar. Plates were incubated at 37°C, 5% CO_2, for 5 d. Mean (± SE) $\log_{10}$ number of mycoplasma counts (cfu/mL) in the treatment groups (1.91±0.15) were higher than in the control groups (1.70±0.16). Recovery of at least 1 mycoplasma colony on agar culture was 100% in both treatment and control groups at high, medium and low concentrations. Yet, at the lowest concentration, recovery of at least 1 mycoplasma colony on agar culture in treatment and control groups was 75% (n = 12/16) and 18.75% (n = 3/16), respectively. Centrifugation of milk followed by suspension in a smaller volume of saline prior to conventional culture increased the ability to detect mycoplasma microorganisms in the milk sample, as compared to controls. Recovery by centrifugation appeared best at the lowest concentration where detection of a positive sample was 4 times more likely than when conventional methods were used.

Key words: centrifugation, *Mycoplasma spp.*, mastitis, bulk tank milk

INTRODUCTION

The conventional method of diagnosis of mycoplasma mastitis is microbiological culture of milk samples taken from individual cows or from herd bulk tank milk and the direct agar plating and culture of those samples (Gonzalez and Wilson, 2003). It was suggested that culture of bulk tank milk be used for screening and surveillance of this disease as a positive result usually indicated that at least 1 cow in a herd has mycoplasma mastitis. Still, a negative result is not necessarily indicative of the absence of mycoplasma infection in a herd (Guterbock and Blackmer, 1984; Biddle et al., 2003) because the test is approximately 60% sensitive.

Low sensitivity of a single bulk tank milk culture is a major limitation for detection mycoplasma organisms. Approximately 30 to 40% of bulk tank milk samples from herds with mycoplasma mastitis were mycoplasma negative, indicating a herd would be erroneously considered free of mycoplasma mastitis (Gonzalez and Wilson, 2003). The most common explanation for a false negative bulk tank mycoplasma culture result is that the concentration of the pathogen in the milk sample is lower than the threshold of detection. Biddle and coworkers (2003) estimated that approximately one-third of the time a cow with mycoplasma mastitis would shed the pathogen in her milk at a low level such that when combined with milk from the rest of the herd, the concentration would be below the level of detection. One study showed that centrifugation of milk samples and resuspension of the bacterial pellet before culture improved the sensitivity of detection of *Staphylococcus aureus* (Zecconi et al., 1997).

We hypothesized that sedimentation of *Mycoplasma spp.* in a milk sample by centrifugation followed by resuspension in a small volume of fluid before agar plating would increase the ability to detect *Mycoplasma spp.* as compared with the conventional culture.

MATERIALS AND METHODS

A completely randomized design with a 2x2x4 factorial arrangement was used to test the factors: 1) 2 treatments (centrifugation vs. direct agar plating; 2) 2 test species (*M. bovis* and *M. californicum*; 4 strains for each species were used); and 3) 4 concentrations of *Mycoplasma spp.* (1,000, 100, 10, and 1 cfu/mL). Sixty-four milk samples were randomly assigned to receive the combination of these treatment factors.

Fresh bulk tank raw milk from the Washington State University dairy farm was used as the test media. The herd had no history of mycoplasma mastitis as determined from periodic cultures of cows with clinical and subclinical mastitis, and from bulk tank milk cultures over a 20 yr period. Broth cultures of mycoplasma strains in the logarithmic phase of growth were centrifuged at 5,000 x g for 30 min. Four strains of *M. bovis* and 4 of *M. californicum* were used. Strains were from cows with mastitis and had different genotypes as determined by pulsed field gel electrophoresis (**PFGE**; Biddle et al., 2005). The cell pellet was suspended in PBS. The mycoplasma suspension was diluted to achieve an optical density of 2.0 at 540 nm corresponding to a theoretical concentration of 10^8 colony forming units (**cfu**)/mL. This mycoplasma suspension was serially diluted with PBS to achieve theoretical concentrations of 10,000, 1,000, 100, and 10 cfu/mL. At each concentration, 1 of the duplicates was randomly allocated as the treatment tube, the other as the control tube.

A 5 mL portion of mycoplasma suspensions from each control and treated tube was added to 45 mL of fresh bulk tank milk to achieve a theoretical concentration of 1,000, 100, 10, and 1 cfu/mL. These concentrations correspond to what was termed high, medium, low and very low, respectively. Treatment tube milk samples were vigorously mixed and centrifuged at 5000 x g for 30 min. After centrifugation the fat layer was removed and the skim decanted. Then 400 µL of PBS was added to resuspend the centrifuged pellet. This resuspension was vigorously mixed and 200 µL removed and spread on Modified Hayflick agar plate (Hogan et al., 1999). Control tube milk samples which were not centrifuged were vigorously mixed and 200 µL of mycoplasma inoculated milk was removed and plated on Modified Hayflick agar plate in duplicate. The actual concentration of *Mycoplasma spp.* used to inoculate the milk was determined by plating and culturing 200 µL of the original PBS suspension of 1,000 cfu/mL, and the colonies enumerated as described. All plates were incubated at 37°C with 5% CO_2 for 5 d. Plates were examined using 15 X microscope for evidence growth of mycoplasma microorganism with the distinctive fried-egg appearance and colonies counts made. Plates were classed as a positive culture if at least 1 colony of mycoplasma organism was found. Bulk tank milk samples were plated and cultured by these conventional methods (Hogan and NMC, 1999) to determine that samples were free from *Mycoplasma spp.*

The differences in mean number of mycoplasma counts (cfu/mL) between treatment tube milk and control tube milk were analyzed using ANOVA, mixed linear model (PROC MIXED; SAS, 2002). Treatment, concentration and species were defined as fixed effects. Strain nested within species was defined as a random effect. Mycoplasma counts were transformed to $\log_{10}$ values to satisfy normal distributional requirements of ANOVA. Multiple comparisons were done using least significant difference method. A trend line was

created by Excel® (Microsoft Corp.) to note the recovery of *Mycoplasma spp.* by concentration level (Figure 1).

RESULTS

Mean (± SE) $\log_{10}$ number of mycoplasma counts in the treatment groups (1.91±0.15) were higher than in the control groups (1.70±0.16) ($P < 0.05$). At each theoretical concentration level (1,000, 100, 10 and 1 cfu/mL) the mean treatment recoveries were significantly higher than controls (Table 1). The counts of all mycoplasma species and strains were pooled to create the means. Moreover, the trend in the increase in recovery of *Mycoplasma spp.* was linear but negatively associated with concentration. The relative increase in recovery was lowest at the high concentration and highest at the lowest concentration (Figure 1). Recovery of at least 1 mycoplasma colony on agar culture was 100% in both treatment and control groups at high, medium and low concentrations. At the very low concentration, recovery of at least 1 mycoplasma colony on agar culture in treatment and control groups was 75% (n = 12/16) and 18.75% (n = 3/16), respectively. Species and strain had no significant effect on mycoplasma counts. No significant interactions were found among factors.

DICUSSION

Evidence from the current study supports the hypothesis that centrifugation of milk with *Mycoplasma spp.* followed by resuspension in a small volume of PBS before culture increases the ability to detect the organisms. Centrifugation improved the recovery of mycoplasma for both species tested, all strains within species, and at all levels of initial

concentration. Moreover, the procedure was able to detect a mycoplasma positive milk sample 75% of the time at a very low concentration, 1 cfu/mL. This concentration is below the normal threshold of detection as the minimum level of detection would range from 10 to 20 cfu/mL corresponding to plating volumes of 100 to 200 µL of milk (Farnsworth, 1993).

This ability to detect a positive sample at the lowest concentration occurred 4 times more frequently than if the sample was cultured using the conventional (control) technique. Thus, the technique through a theoretical 250 fold increase in cell concentration improved the recovery at all levels (Figure 1). Still, recovery was not nearly as high as the theoretical expectation. Possibly not all mycoplasma organisms in milk samples were recovered after centrifugation in the pellet. The organisms may be partitioned into the skim and fat portion. For example, *Mycobacterium avium* subsp. *paratuberculosis* was partitioned into cream and whey portion as well as the pellet, leading to a lower bacterial count than expected (Gao et al., 2005). Alternatively, centrifugation could have resulted in aggregation of mycoplasma colonies so that when plated, individual cfu could not be distinguished after growth of the colony. The trend in the recovery of mycoplasma colonies increased with a decreasing concentration of inoculum (Figure 1). This is evidence to support the contention that aggregation of colonies decreased as concentration decreased and thus, the percentage recovery of colonies improved. The advantage of centrifugation was consistent with the previous report that showed that the centrifugation procedure increased the recovery of *Staphylococcus aureus* from milk samples (Zecconi et al., 1997). Recovery of mycoplasma organisms was increased by inoculation of enrichment medium with milk and incubation for 4 d (Biddle et al., 2003). Although the enrichment method increased the recovery of the organisms from 24 of 104 samples (24%), it failed to recover mycoplasma organisms from 9 of 104 samples (9%) that were positive using the conventional method. The centrifugation

method described herein has the advantage over the preincubation with enrichment medium. The centrifugation method does not require additional time for incubation.

Single sampling is commonly used during herd monitoring programs (Jayarao et al., 2004), although multiple sampling and culture of bulk tank milk increased the sensitivity of detection of *Mycoplasma spp.* (Gonzales and Wilson, 2003). The concentration of mycoplasma organisms may often be lower than the threshold of detection using the conventional culture method. At times a cow or cows will shed mycoplasma intermittently and there can be variation in the amount of organism shed when daily comparisons are made. (Biddle et al., 2003).The results from the current study indicate recovery of mycoplasma organisms can be improved so that single rather than multiple consecutive samples can be used to monitor bulk tank milk. Moreover, the technique was not affected by species or strain of mycoplasma organism tested and the technique appeared to be most efficient when concentration of mycoplasma was lowest.

In conclusion, centrifugation of milk followed by suspension in a smaller volume of saline prior to conventional culture increased the ability to detect mycoplasma microorganisms in the milk sample as compared to controls. Recovery appeared best at the very low concentration (1 cfu/mL), where detection of a positive sample was 4 times more likely than when conventional methods were used.

REFERENCES

Biddle, M. K., L. K. Fox, and D. D. Hancock. 2003. Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. J Am Vet Med Assoc 223: 1163-1166.

Biddle, M.K., L.K. Fox, M.A., Evans, and C.C., Gay. 2005. Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. J Am Vet Med Assoc 227: 455- 459

Fransworth, T.J. 1993. Microbiological examination of bulk tank milk. Vet Clin North Am Food Anim Pract 9: 469-474

Gao, A., J. Odumeru, M. Raymond, and L. Mutharia. 2005. Development of improved method for isolation of Mycobacterium avium subsp. Paratuberculosis from bulk tank milk : Effect of age of milk, centrifugation, and decontamination. Can J Vet Res. 69: 81-87.

Gonzalez, R. N., and D. J. Wilson. 2003. Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19: 199-221.

Guterbock, W. M., and P. E. Blackmer. 1984. Veterinary interpretation of bulk-tank milk. Vet Clin North Am Large Anim Pract 6: 257-268.

Jayarao, B. M., S. R. Pillai, A. A. Sawant, D. R. Wolfgang, and N. V. Hegde. 2004. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J Dairy Sci 87: 3561-3573.

Hogan, J. S. and NMC.1999. Laboratory handbook on bovine mastitis. Rev. ed. National Mastitis Council, Madison, WI.

SAS.2002. SAS User's Guide: Statistics, Version 9.1.SAS Inst. Inc., Cary, NC.

Zecconi, A., R. Piccinni, A. Zepponi, and G. Ruffo. 1997. Recovery of *Staphylococcus aureus* from centrifuged quarter milk samples. J Dairy Sci. 80:3058-3063

Table 1. Contrast of means[1] (±SE) of Log_{10} mycoplasma counts (cfu/mL) in treatment and control groups. Treatment was centrifugation followed by resuspension of small volume of fluid before agar plating. Control was conventional direct plating.

Concentration[2]	Treatment	Control
High	3.07±0.28[a]	2.97±0.45[b]
Medium	2.18±0.07[a]	1.93±0.10[b]
Low	1.36±0.1[a]	1.14±0.13[b]
Very low	1.02±0.06[a]	0.77±0.32[b]

[1] Least squares means of all mycoplasma counts from all replicates, of all strains and species, within group (treatment or control).Values in a row with different superscripts were significantly different ($P < 0.01$).

[2] Concentration of high, medium, low and very low represent theoretical concentrations of 1,000,100, 10, and 1 cfu/mL.

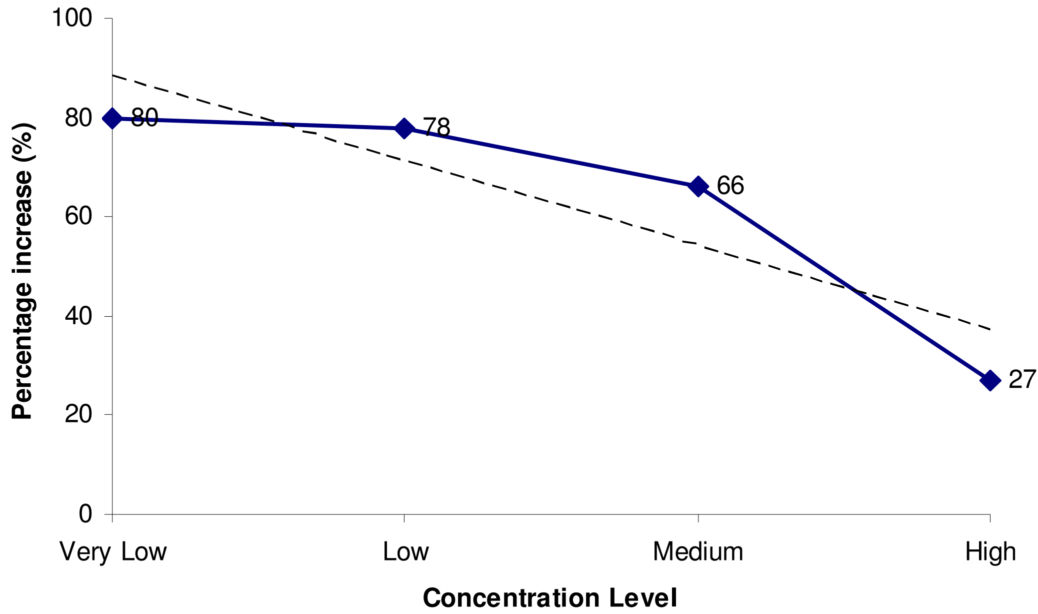

Figure 1. The relative difference in mycoplasma counts from all treatment vs. control group samples by high, medium, low and very low concentrations. The number above the point corresponding to each concentration level is the percentage increase in counts of the treatment mean compared to control mean. Trend line is the dashed line. Treatment was centrifugation followed by resuspension of small volume of fluid before agar plating.

CHAPTER SIX

A role of culling on time to clearance of mycoplasma mastitis

V. Punyapornwithaya [a], L. K. Fox [a]*, D.D. Hancock [a], J.M. Gay [a],

J. R. Alldredge [b]

[a] Department of Veterinary Clinical Sciences,

Washington State University, Pullman, WA, 99164 USA

[b] Department of Statistics,

Washington State University, Pullman, WA 99164, USA

Formatted for submission to

the Veterinary Records as a paper.

SUMMARY

The objective of this study was to determine factors associated with time to clearance of mycoplasma mastitis. Eighteen herds with a bulk tank milk sample that was positive to *Mycoplasma sp.* by standard culture were investigated. Bulk tank milk samples from enrolled dairies were routinely collected every 7-10 days to monitor the mycoplasma mastitis status. Milk samples were centrifuged at 5000 X *g* for 30 mins before culturing. The dairy operator was interviewed and milking time hygiene practices were observed. The second interview was made when bulk tank milk samples were negative to *Mycoplasma spp.* for 3 consecutive samplings or at 3 months. Herds were classified as fast recovery (n=14), slow recovery (n=2) and non-recovery (n=1) groups based on time to clearance. A Kaplan-Meier survival curve showed that approximately 75% of participating herds cleared mycoplasma mastitis within 1 month and 90% of herds cleared mycoplasma mastitis within 2 months. Greater than 50 per cent of study herds culled mycoplasma mastitis cows preferentially in an effort to eradicate mycoplasma mastitis but only 38 per cent of study herds were successful in eradicating this disease within 1 month. Thus, there was no evidence to suggest an advantage of preferential culling in control of mycoplasma mastitis.

INRODUCTION

Mycoplasma mastitis is widely distributed and a cause of significant economic loss to the dairy industry (Nicholas and Ayling 2003). The spread of mycoplasma mastitis most often occurs during milking time as the organisms are contagious pathogens (Fox and others 2005). Culture of bulk tank milk (BTM) samples has been used to monitor mycoplasma mastitis and the culture of *Mycoplasma sp*. from BTM samples has been assumed to be indicative of the

presence of mycoplasma mastitis in the herd (Gonzalez and Wilson 2003). There have been several investigations into the risk factors for herd mycoplasma mastitis outbreaks (Thomas and others 1981; Gonzalez and others 1992; Fox and others 2003). The change of herd mycoplasma mastitis status from positive to negative in 79 herds was investigated through monthly culture of bulk tank milk (Fox and others 2005). Approximately 30 per cent of herds were still positive to mycoplasma mastitis after one month. It is not understood why some herds became negative and why other herds continued to have a mycoplasma mastitis problem with monthly recurrent mycoplasma isolation from BTM samples. Some dairy operators might employ management factors that quickly lead to the elimination of herd mycoplasma mastitis while others fail to employ the necessary strategies that lead to elimination. One of the most important factors is culling the infected cows.

There are two different strategies to control mycoplasma mastitis outbreak after mycoplasma mastitis cows are identified. Some researchers suggest that dairy operators should cull all infected animals as soon as possible from the herd (Bushnell 1984; Bayoumi and others 1988). Alternatively, some argue that it is not necessary to cull infected animals. The outbreak of mycoplasma mastitis can be controlled by segregation and milking infected cows last (Brown and others 1990; Jackson and Boughton 1991). However, dairy operators are often encouraged to cull cows with mycoplasma mastitis in effort to control the disease. Thus, we hypothesized that culling is a strategy that has a major impacts in eradication of mycoplasma mastitis.

The objective of this study was to determine factors associated with time to clearance of mycoplasma mastitis. Major factors of interest were strategies to control mycoplasma mastitis and milking time hygiene practices.

MATERIALS AND METHODS

Sample herd and herd survey

Dairy herds that shipped milk to Northwest Dairy Association during October 2008-October, 2009 were eligible for the study. Enrollment criteria was a willingness to participate and a BTM sample that was positive to *Mycoplasma sp.* by standard culture where herd BTM had been previously negative for 6 months prior. A herd visit was scheduled within 2 weeks after the dairy operator agreed to enroll. A questionnaire was used to obtain herd and management data from the dairy operator during the herd visit. In brief, the questionnaire covered the following areas: herd population; milk production; bedding type; frequency of milking machine evaluation; milking procedures; dairy animal addition, if herds were currently not purchasing replacements nor rearing replacement off-premise (closed herd); methods used to identify mastitis problems; mastitis control programs and culling policy. Milking practices and milking time hygiene were observed by one veterinarian with checklists during the survey. In brief, the observations covered the following areas: udder preparation (foremilk-stripping, method of pre-milking teat asepsis); cleanliness of milking parlor; number of milking stalls/milker; postmilking teat asepsis; use of postmilking unit backflush and milking system evaluation. Based on National Institute for Research in Dairying classification (Bramley, 1980), herds with complete milking time hygiene practices were those: using disinfectant for udder washing, using a single service towel for udder cleaning, milkers wearing latex gloves, use of post-dipping and use of a milking unit backflush. All other herds were classified as using partial milking time hygiene practices. The second interview was made when BTM samples from the herd were negative to *Mycoplasma sp.* for 3 consecutive

samplings or at 3 months for all other herds. Information about how operators managed the mycoplasma mastitis since the first interview was noted.

Bulk tank milk monitoring

BTM samples from enrolled dairies were routinely collected to monitor the mycoplasma mastitis status. Each BTM sample was collected at 7-10 day intervals. Results from 3 consecutive BTM samples were used to interpret a negative status. Gonzales and Wilson (2003) estimated that 40 per cent of herds would be misidentified as free of cows with mycoplasma intramammary infection based on a single bulk tank milk culture negative to this pathogen. Therefore using 3 consecutive bulk tank milk cultures would have a sensitivity of detection of 93 per cent.

Microbiological culturing

Milk samples (50 mL) were centrifuged at 5000 X *g* for 30 min before culture to enhance mycoplasma recovery (Punyapornwithaya and others 2009). Sediment was resuspended with 200 mL phosphate buffer saline. A 200 µL of resuspension was plated on modified Hayflicks agar. Plates were kept in an incubator at 37 ^{0}C with 10per cent CO_2 for 10 days then examined with a 15X dissecting microscope, to identify colonies with the distinctive “fried egg” appearance (Hogan and NMC 1999). Results were considered positive if any mycoplasma colonies were seen and negative if there was no evidence of growth of *Mycoplasma sp.* All positive samples from first isolation (n=18) were speciated by polymerase chain reaction technique (Tang et al., 2000).

Statistical Analysis

A Kaplan-Meier survival curve was made using PROC LIFETEST in SAS program (SAS version 9.1, SAS institute, Inc., Cary, NC, USA) to determine the survival function of mycoplasma mastitis in the dairy herd. Herds with time to clearance of mycoplasma mastitis of less than 3 months were defined as cases. Other herds were defined as censored. Survival time was the time to clearance of mycoplasma mastitis which refers to the time interval from the first isolation of BTM sample to *Mycoplasma sp.* to the first negative of three consecutive negatives of BTM samples.

RESULTS

Herd characteristics

Eighteen herds were enrolled. The number of milking cows per herd ranged from 100 to 2800 with a mean of 680. Mean milk production from all herds was 30 kg/cow/day (range 20 to 41). Nine herds were closed signifying that cattle raised off the premise were not incorporated into the herd.

Time to clearance

Fourteen herds cleared mycoplasma mastitis within 1 month (Fast Recovery group) as determined by 3 consecutive negative BTM cultures. Six herds in this group never had a positive BTM samples to *Mycoplasma sp.* after the first isolation. Two herds cleared within 2 months and one herd cleared mycoplasma mastitis within 3 months (Slow Recovery group). Only one herd could not eliminate the disease within 3 months (Non Recovery group).The

Kaplan-Meier survival curve (Figure 1) showed that approximately 75% of participating herds cleared mycoplasma mastitis within 1 month and 90% of herds cleared mycoplasma mastitis within 2 months. The median and mean time to clearance were 3 and 4.2 weeks respectively. *M. bovis* were found in all samples. One of these samples had both *M. bovis* and M. *arginini*.

Mastitis sampling and culling strategies

Operators in 11 herds preferentially culled mycoplasma mastitis cows. These operators collected milk from cows exhibiting signs of mastitis either clinical or subclinical cows deemed to have elevated milk somatic cell counts. In contrast, operators in 6 herds did not have individual cow milk samples cultured for *Mycoplasma sp.* One herd operator intended to preferentially cull cows found to be infected with *Mycoplasma sp.* but all of the cultured milk samples submitted from suspected cows were negative. Fifty per cent of herds in Fast Recovery group tested and culled mycoplasma cows from the herd. All operators of herds in Slow and Non-recovery group detected mycoplasma cows and culled them (Table 1).

Biosecurity practices

Operators of 9 herds separated mastitis cows from the healthy milking cows and place them in an area with infirm cows, a hospital pen. Fifty percent of the study herds were closed herds. Operators in 5 herds fed non-pasteurized milk to calves. Operators in 10 herds performed a complete milking time hygiene practices whereas the other 8 operators performed partial milking time hygiene practices. The number of operators for each category

of milking time hygiene practices is in Table 2. All of operators performed post dipping and more than 80% of them used backflush.

DISCUSSION

This is a first report relating management factors associated with time to clearance of herd mycoplasma mastitis. The assumption is that bulk tank milk culture is an effective monitor for the mycoplasma mastitis status in a herd. The majority of dairy herds cleared mycoplasma mastitis within 1 month. There was no evidence to suggest an advantage of preferential culling in control of mycoplasma mastitis. Greater than 50 per cent of study herds culled mycoplasma mastitis cows preferentially in an effort to eradicate mycoplasma mastitis but only 38 per cent of study herds were successful in eradicating this disease within 1 month. Given that mycoplasma mastitis is a contagious mastitis pathogen where diagnosis is often slowed by conventional culture methods, preferential culling of cows with mycoplasma mastitis to eradicate the disease is suggested (Bayoumi and others 1988). Culling is a strategy that has been advocated to have a major impact on a herd's ability to rapidly eradicate mycoplasma mastitis (Bushnell 1984). Thus we would have expected that the majority of herds in the Fast Recovery group would be herds where operators culled cows with mycoplasma intramammary infections preferentially. Contrary to expectation this study revealed that culling was only an associated practice in half of the Fast Recovery group herds. Moreover operators of herds in the Slow and Non Recovery groups failed to clear mycoplasma mastitis within a month although all of them elected to test cows they suspected might have mycoplasma mastitis and to cull those cows if confirmed.

The other half of operators in Fast Recovery group did not cull any mycoplasma mastitis cows. They chose to solely continue to monitor their bulk tank milk in an effort to note changes in the mycoplasma mastitis status of the herd. Three consecutive weekly BTM samples after first isolation from 7 herds became negative to mycoplasma mastitis. Operators of these herds indicated in the second interview that they did not know how and why mycoplasma mastitis occurred as evidenced by a positive bulk tank culture. Nor did they have an explanation as to why within a short interval, 1 month, mycoplasma mastitis apparently disappeared. Either the first bulk tank sample was a true positive, indicating that at least one cow had a mycoplasma mastitis infection, or it was a false negative indicator. We isolated the *Mycoplasma sp.* from 17 bulk tanks and found them to be *M. bovis*. Both *M. bovis* and *M. arginini* was found in 1 bulk tank. This indicates that the initial finding of *Mycoplasma sp.* from bulk tank milk was not falsely attributed to the isolation of a mollicute other than *Mycoplasma sp*. The initial bulk tank positive samples could have resulted from contamination(s) from a cow or cows that were asymptomatically colonized at an extramammary site by this pathogen, and not reflective of a cow or cows with intramammary mycoplasma mastitis. The latter explanation suggesting a false positive test as a result of a non-mammary source isolation although possible, seems unlikely given that it occurred on nearly half of all dairies studied. We suspect that the second negative result of BTM sample from herds that did not cull mycoplasma mastitis cows was the result of a spontaneous recovery from subclinical mycoplasma mastitis signifying that the initial result was a true positive and subsequent samples yielded true negative results. Alternatively, the first positive sample could have yielded a true result and subsequent bulk tank milk samples could have been falsely negative as cows were still diseased but not shedding detectable levels of mycoplasma as noted previously by Biddle and coworkers, 2003. The latter two explanations, either spontaneous recovery or low shedding cows, seem most plausible. Either explanation

does suggest that the contagious nature of the disease had passed and thus the risk of new mycoplasma intramammary infections was not significant. The advantage of continuous BTM samples culturing was clearly demonstrated in this study. Herd operators used this method to monitor mycoplasma herd status. Most herds (14/18) were free of this disease within 1 month and 17 of 18 herds were free within 3 months. This finding was closely consistent with a previous report (Fox and others 2003). It should be noted that there are individual herds where time to clearance maybe approximately a year or longer (Bicknell and others 1983, Bayoumi and others 1988; Biddle and others 2003).

Approximately forty-five per cent of study herds (n=8/18) with complete milking time hygiene practices were able to clear mycoplasma mastitis within 1 month. The majority of herds (n=10) used 5 complete milking time hygiene practices, 7 herds used 4 of the 5 complete milking time hygiene practices and only 1 herd used less than 4. Postmilking teat asepsis was practiced by all herds and this practice has been attributed as the most fundamental strategy to control contagious mastitis (Oliver and others 1989). Thus, we suspect that there was not enough herd variation in contagious mastitis control practices to fully analyze the effect of management strategies that were associated with time to clearance of mycoplasma mastitis.

In conclusion, testing and preferential culling of cows with mycoplasma intramammary infections did not appear to hasten the clearance of mycoplasma mastitis from the herd as determined by bulk tank cultures. More than 75 per cent of herds were able to become mycoplasma mastitis free within one month regardless of culling strategies. Monitoring the mycoplasma mastitis status by culturing bulk tank milk samples is recommended.

References

BAYOUMI, F. A., FARVER, T. B., BUSHNELL, B. & OLIVERIA, M. (1988) Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. J Am Vet Med Assoc 192, 905-909

BICKNELL, S. R., GUNNING, R. F., JACKSON, G., BOUGHTON, E. & WILSON, C. D. (1983) Eradication of Mycoplasma bovis infection from a dairy herd in Great Britain. Vet Rec 112, 294-297

BIDDLE, M. K., FOX, L. K. & HANCOCK, D. D. (2003) Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. J Am Vet Med Assoc 223, 1163-1166

BROWN, M. B., SHEARER, J. K. & ELVINGER, F. (1990) Mycoplasmal mastitis in a dairy herd. J Am Vet Med Assoc 196, 1097-1101

BUSHNELL, R. B. (1984) Mycoplasma mastitis. Vet Clin North Am Large Anim Pract 6, 301-312

FOX, L. K., HANCOCK, D. D., MICKELSON, A. & BRITTEN, A. (2003) Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. J Vet Med B Infect Dis Vet Public Health 50, 235-240

FOX, L. K., KIRK, J. H. & BRITTEN, A. (2005) Mycoplasma mastitis: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health 52, 153-160

GONZALEZ, R. N., SEARS, P. M., MERRILL, R. A. & HAYES, G. L. (1992) Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. Cornell Vet 82, 29-40

GONZALEZ, R. N. & WILSON, D. J. (2003) Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19, 199-221

HOGAN, J. S. & NMC (1999) Laboratory handbook on bovine mastitis. Rev. ed. . Madison, WI., National Mastitis Council

JACKSON, G. & BOUGHTON, E. (1991) A mild outbreak of bovine mastitis associated with Mycoplasma bovigenitalium. Vet Rec 129, 444-446

NICHOLAS, R. A. & AYLING, R. D. (2003) Mycoplasma bovis: disease, diagnosis, and control. Res Vet Sci 74, 105-112

OLIVER, S. P., KING, S. H., TORRE, P. M., SHULL, E. P., DOWLEN, H. H., LEWIS, M. J. & SORDILLO, L. M. (1989) Prevention of bovine mastitis by a postmilking teat disinfectant containing chlorous acid and chlorine dioxide in a soluble polymer gel. J Dairy Sci 72, 3091-3097

PUNYAPORNWITHAYA, V., FOX, L. K., GAY, G. M., HANCOCK, D. D. & ALLDREDGE, J. R. (2009) Short communication: The effect of centrifugation and resuspension on the recovery of Mycoplasma species from milk. J Dairy Sci 92, 4444-4447

THOMAS, C. B., WILLEBERG, P. & JASPER, D. E. (1981) Case-control study of bovine mycoplasmal mastitis in California. Am J Vet Res 42, 511-515

Table 1 Number of herds by time to clearance of mycoplasma mastitis and culling strategy

	Fast Recovery group [b]	**Slow and Non-recovery group**
Culling [a]	7	4
No culling	7	0

[a] Culling was defined as herd operators culled cows identified with mycoplasma mastitis in an effort to eradicate the disease. No culling was defined as herd operators did not cull cows identified with mycoplasma mastitis in an effort to eradicate the disease.

[b] Fast Recovery group had time to clearance of mycoplasma mastitis $\leq$ 1 month. Slow and Non-recovery group had time to clearance of mycoplasma mastitis $>$ 1 month.

Table 2. Number of herds and use of milking time hygiene practices (yes/no)

Milking time hygiene practice	**Yes**	**No**
Disinfectant udder wash	14	4
Individual cloths	17	1
Rubber gloves	16	2
Post-dipping	18	0
Use of backflush	15	3

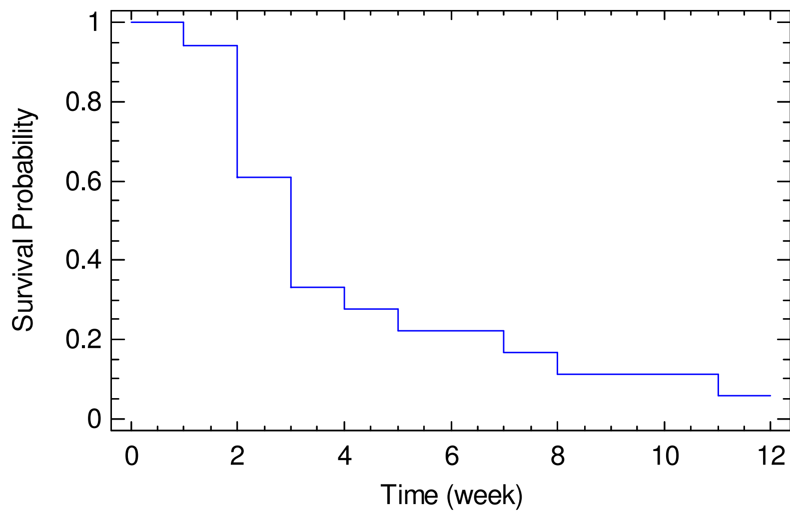

Figure 1. A Kaplan-Meier survival curve showing the time to clearance of mycoplasma mastitis in studied herds

CHAPTER SEVEN

Incidence and transmission of *M.bovis* mastitis in a hospital pen

V. Punyapornwithaya [a], L. K. Fox [a]*, D.D. Hancock [a], J.M. Gay [a], J.R. Wenz [a],

J. R. Alldredge [b]

[a] Department of Veterinary Clinical Sciences,

Washington State University, Pullman, WA, 99164 USA

[b] Department of Statistics,

Washington State University, Pullman, WA 99164, USA

Formatted for submission to

Preventive Veterinary Medicine as a paper.

Abstract

The objective was to determine the incidence and transmission of mycoplasma mastitis in the hospital pen in a dairy herd of 650 lactating cows after a hospital pen was established following an outbreak of this disease. Mycoplasma mastitis status was monitored for 3 months through repeated collection of milk samples from cows with clinical mastitis (CM) and from bulk tank milk. During the outbreak 13 cows were diagnosed with *Mycoplasma bovis* (*M. bovis*) CM and 8 cows showed signs of arthritis, 3 of which were confirmed as having *M. bovis* arthritis. *M.bovis* isolates (n=19) from cows with CM, arthritis and bulk tank milk had indistinguishable chromosomal digest pattern fingerprints. Incidence rates of *M. bovis* CM cases in the milking and hospital pens were 0.01 and 1.6 cases per 100 cow-days at risk, the incidence in the hospital pen was 106 times the incidence rate in the milking pens. Approximately 70% of cows with *M. bovis* CM became infected within 12 days of entering the hospital pen. Transmission of *M. bovis* in the hospital pen occurred as 3 episodes. Each episode corresponded to the introduction of a cow with *M. bovis* CM from a milking pen. Evidence indicates that cows with *M. bovis* CM from milking pens were the source of transmission of the disease in the hospital pen and thus the hospital pen appeared to be a risk factor for transmission of *M. bovis* mastitis.

.

1. Introduction

Mycoplasma spp. have been considered contagious mastitis pathogens with transmission postulated to occur primarily during milking time (Fox and Gay, 1993). It is believed that mycoplasma mastitis can be controlled by identifying the infected cows followed by segregation and milking infected cows last (Brown et al., 1990; Jackson and

Boughton, 1991) or by identifying and then culling diseased cows (Bayoumi et al., 1988). If this is true, then time to detection of a mycoplasma mastitis case is critical given that isolation of the infected cow is a primary means to control this disease. However, the current microbiological culture procedures used to detect *Mycoplasma* spp. require 7 to 10 days (Hogan and NMC, 1999). During the period between identification of cows suspected to have mycoplasma mastitis and culture confirmation, some dairy managers may keep these cows penned with healthy milking cows. Other dairy managers may move cows with clinical mastitis (CM) into a pen of infirm cows, termed the hospital pen, and milk cows in this pen separately (USDA, 2008).

It has been presumed that creation of a hospital pen will help control contagious diseases by isolating diseased from healthy cows. Yet anecdotal reports suggest that the hospital pen is a nidus for the spread of *Mycoplasma* spp.

In this report, we determined the incidence of *Mycoplasma bovis* (*M.* bovis) CM for milking pen cows and hospital pen cows of a dairy herd with no prior history of mycoplasma mastitis during the last 8 years. The occurrence and transmission of *M. bovis* CM in the herd was investigated.

2. Materials and Methods

2.1 Background and Herd descriptions

A commercial Washington State herd of approximately 650 lactating cows routinely submitted bulk tank milk (BTM) samples to a local veterinary service for culture in an effort

to monitor the presence of contagious mastitis pathogens. We were contacted by the veterinary service after *Mycoplasma* spp. were detected in one of these samples and we visited the farm to obtain a history of the suspected outbreak.

The dairy had no history of mycoplasma mastitis during the last 8 years. Milking cows were housed in 5 pens based on milk production and parity. Cows in the high production pen were milked 4 times per day. Cows in the remaining pens were milked 3 times per day. Cows were milked through a double16 herringbone parlor by 2 milkers. Milking time hygiene practices included fore-milk stripping by milkers who wore latex disposable gloves, pre-dipping and post-dipping with 0.5% iodine, use of single service towels to clean and remove iodine solution before machine attachment, and use of automatic backflush. Milk from cows with CM was not fed to calves. A pen for infirm cows was created after the first *Mycoplasma* spp. was detected in bulk tank milk. Most cows transferred from their home milking cow pen to the hospital had clinical mastitis.

2.3 Sampling and Microbiological culturing

Milk samples were aseptically collected by farm personnel from all functional mammary quarters of cows with CM immediately before antibiotic treatment on the day they entered the hospital pen. Cows with clinical mastitis were held in the hospital pen for the prescribed withholding period, a function of the antibiotic used. A second milk sample for culture was collected on the day they exited the hospital pen. Additional samples were taken from hospital pen cows if a new mammary quarter developed clinical mastitis or if the same quarter was unresponsive to antibiotic treatment, abnormal udder or abnormal milk. Joint fluid samples were aseptically collected from 3 arthritis cows. A weekly BTM sample was

collected to monitor for the presence of *Mycoplasma* spp. throughout the study.

Cultures of milk and joint fluid were made to determine the presence of *Mycoplasma* spp.. In brief, a 50 µl aliquot of milk or joint fluid were plated on modified Hayflick's agar plate and incubated at 37°C, 10% CO_2, for 10 days. Plates were examined with a 15X dissecting microscope to identify colonies with the distinctive "fried egg" appearance (Hogan and NMC, 1999). Results were considered positive if any colonies with this appearance were detected and negative if none were.

2.4 Species and strain identification

Pulsed field gel electrophoresis (PFGE) of chromosomal digests was used to fingerprint the mycoplasma isolates as described (Biddle et al., 2005). In brief, mycoplasma samples were centrifuged at 1,200 X g at 4°C for 10 minutes to pellet the microorganism. The supernatant was discarded, and pellet was suspended in 200 µL of buffer solution. Ten microliters of proteinase K was added, and the DNA was embedded in 200 µL of agarose. Plugs were cast and lysed. Chromosomal DNA was digested with the restriction enzyme *Sal* I. Electrophoresis was performed at 14°C for 20.2 hours at a setting of 6V/cm of gel and a linear pulse ramp of 1 to 12.9 seconds. After electrophoresis, gels were stained with ethidium bromide for 30 minutes, washed twice in distilled water, and then photographed under UV light. Isolates were considered to be the same strain when chromosomal digestion produced the same number of bands of the same size (Figure 1). Samples were speciated by the method outlined previously (Tang et al., 2000).

2.5 Definitions

Mycoplasma mastitis cows were those cows with clinical signs of mastitis that were confirmed to be infected by *M. bovis* and defined as a *M. bovis* mastitis case. The *M. bovis* mastitis incidence rate was the number of new cases of *M. bovis* mastitis divided by number of cow days at risk multiplied by 100 (Dohoo et al., 2003). The incidence rate ratio was the ratio of incidence rate of hospital pen *M. bovis* mastitis and the incidence rate of milking pen *M. bovis* mastitis (Dohoo et al., 2003).

2.6 Data and Statistical Analysis

Individual cow productivity, management and health records were obtained from the herd software program, Dairy Plan C21 version 5.2 (WestfaliaSurge, Inc., Naperville, IL, USA). The following parameter were calculated; (1) incidence rate of mycoplasma mastitis for milking pen each month from March to August; (2) incidence rate of *M. bovis* mastitis for hospital pen each month from May to August; (3) incidence rate of *M. bovis* mastitis for milking pen and hospital pen when time at risk was defined as a three month period of May through July; and (4) incidence ratio of *M. bovis* mastitis for milking pen cows and hospital pen cows pen when time at risk was defined as a three month period of May through July. A Kaplan-Meier survival curve of *M. bovis* mastitis in the hospital pen was made using PROC LIFETEST in SAS program (SAS version 9.1, SAS institute, Inc., Cary, NC, USA). An event was defined as the occurrence of a clinical case *M.bovis* mastitis. A hospital pen cow that developed *M. bovis* mastitis was defined as case. A cow that never developed mycoplasma mastitis in the hospital pen was considered as censored.

3. Results

3.1 Descriptive Results

The first outbreak case of *M. bovis* mastitis was reported on March 12, 2009 in a first lactation cow purchased from another farm as a heifer on October 9, 2008. At 3 days in milk, she was noted with clinical signs of mastitis. This cow remained in her home pen, for 3 weeks, until culture results were obtained and was then immediately culled when mycoplasma mastitis was diagnosed. The second clinical *M. bovis* mastitis case was observed on April 23, 2009 and this cow was later culled when the culture results confirmed mycoplasma mastitis. One cow developed pneumonia on April 29, 2009. Although she did not have clinical signs of mastitis, a composite milk sample was collected and was positive for *M. bovis*. All cows with clinical disease remained in their home pens until the herd manager decided to form a hospital pen on April 30. Beginning May 1 all cows diagnosed with CM were moved to the hospital pen. The number of new *M. bovis* mastitis cases in the hospital pen during May, June and July were 4, 4, and 1 respectively. In contrast, only one cow per month developed *M. bovis* mastitis in the milking pens during May through July. All *M. bovis* mastitis cows were culled from the herd within a day after the herd manager received the laboratory report.

Milk samples were collected from a cow with a swollen hind leg carpal joint on May 20; and culture revealed *M. bovis*. In June, five cows had clinical signs of arthritis similar to the first case but only 2 cows were sampled. Joint fluid samples were collected from one and a milk sample from another even though she did not exhibit signs of clinical mastitis. *M. bovis* was isolated from both samples. *M. bovis* was isolated from joint fluid samples of two cows that developed arthritis during the first week of August. In summary, a total of 8 cows were diagnosed with arthritis, 3 of which were confirmed as having *M. bovis* arthritis. All

cows with arthritis developed clinical signs when they stayed in their home milking pen. They were culled within a day of diagnosis and collection of a sample for mycoplasma culture.

The first BTM sample was *M. bovis* positive on April 21. Weekly BTM samples were continuously positive for *M. bovis* from April 22 to May 5. Then weekly BTM samples from May 6 to May 26 became negative to *Mycoplasma* spp. However, weekly BTM samples were positive to *M. bovis* on May 27 and June 5. Weekly BTM samples from the rest of June through July 12 were negative to *Mycoplasma* spp. The last positive BTM sample was found on July 13.

3.2 Species and strain

All isolates (n=19) were confirmed as *M. bovis.* The chromosomal digest patterns, representative isolates shown in Figure 1, were indistinguishable. However, 5 isolates from quarter milk samples initially submitted to their local laboratory that were presumptively identified as mycoplasma were not defined further.

3.3 Incidence rate and incidence ratio

Incidence rates of *M. bovis* mastitis for milking pen and hospital pen each month were shown in Table 1. For the period of May through July, incidence rates of *M. bovis* mastitis in the milking and hospital pens were 0.015 and 1.6 cases per 100 cow-days at risk, respectively, with an incidence rate ratio of 106.

3.4 Transmission of *M. bovis* in the hospital pen

Approximately 95% of cows entering the hospital pen were clinical mastitis cows. These clinical mastitis cows were classified into 3 groups. The negative (NEG) group were cows (n=76) that entered the pen for clinical mastitis other than *M .bovis* mastitis and were negative to *M. bovis* at exit. The positive (POS) group were cows (n=3) that were positive to *M. bovis* at entry day. The third group were cows (n= 9) that were negative to *M. bovis* at entry day but positive to *M. bovis* at some time during their stay in the hospital pen (NEG/POS). These cows acquired *M. bovis* mastitis while in the hospital pen group. POS cows were from 3 different milking pens each containing approximately 270 other cows. No cows with diseases other than clinical mastitis developed *M. bovis* mastitis while in the hospital pen. The NEG/POS cows that developed mycoplasma mastitis in the hospital pen were detected after 4 days residence (Figure 2); approximately 70% of hospital pen cows that become *M. bovis* mastitis cows did so before 12 days (Figure 2). The mean of time to *M. bovis* mastitis occurrence post hospital pen entry was 13 days.

Hospital pen transmission of *M. bovis* was classified into 3 episodes (A-C) corresponding to the introduction of a POS cow from one of the milking pens (Figure 3). Episode A began with the occurrence of mycoplasma mastitis POS/NEG cow 1563 after the introduction of the POS cow 1596. Shortly afterwards NEG/POS cows 178, 1262 and 11 developed clinical cases. For the second episode, we judged that POS cow 1484 spread the disease to NEG/POS cow 1510 as cow 1484 was the only mycoplasma mastitis cow in the hospital pen at the time. During episode C a case of mycoplasma mastitis occurred with NEG/POS cow 475 after mycoplasma POS cow 601 entered the hospital pen. No cows

developed other forms of mycoplasma disease, arthritis or pneumonia, while resident in the hospital pen.

4. Discussion

This is a first study demonstrating that the hospital pen may be a risk factor for transmission of mycoplasma mastitis. Whereas Bayoumi et al (1988) investigated cases of mycoplasma mastitis in the hospital pen, no study has examined the incidence of new cases in the hospital pen. Only one strain caused mycoplasma mastitis in the herd suggesting a common primary source of the pathogen. The increased rate of mycoplasma mastitis transmission in the hospital pen may be the result of increased contact time between mycoplasma mastitis cows and other cows compared to the milking pen and impaired immune function of hospital pen cows.

Occurrence of a new clinical mastitis cases is associated with contact time between mastitis cows and susceptible cows (Lam et al., 1996). In this study herd, the contact time between mycoplasma mastitis cows and other milking pen cows was of short duration relative to contact time in hospital pen. Cows with clinical mastitis were removed from their home pen within a day of detection and moved to the hospital pen during antibiotic therapy and the withholding periods. In contrast, hospital pen *M. bovis* mastitis cows were milked with other mastitis cows at least 5 days given the aggregate antibiotic treatment protocol and milk withholding times. The minimum five-fold increase in exposure time a *M. bovis* mastitis cow has with a naïve cow increases the opportunity for *M. bovis* transmission in the hospital pen. The risk of exposure to *M. bovis* mastitis pathogens might increase as the density ratio of *M. bovis* clinical mastitis cows to non-infected *M. bovis* cows increases. The transmission of

M. bovis mastitis might occur during milking time when *M. bovis* mastitis cows were milked together with cows without *M. bovis* mastitis. Mastitis milk from mammary glands with *M. bovis* mastitis may contain a high number of bacteria, greater than 10^6 CFU/ml (Biddle et al., 2003; Byrne et al., 2005). The pathogen might contaminate fomites such as teat cup liners, other surfaces of milking equipment (Bushnell, 1984) or even milker's rubber gloves (Gonzalez and Wilson, 2003) and result in transfer of the pathogen despite the milking hygiene techniques used to isolate fomites.

Also infirm cows are more susceptible than healthy cows to a new disease (Grohn et al., 2003) because they may be immuno-compromised. Some viral diseases were found to be associated with an increased risk of mastitis (Zecconi et al., 1994; Zadoks et al., 2001; Wellenberg et al., 2002). Additionally it is known that cows with intramammary infection in 1 quarter are more susceptible to intramammary infection with a different pathogen in a different quarter (Nickerson and Boddie, 1994). Increased exposure to mycoplasma mastitis cows while in an immuno-compromised state from clinical mastitis might explain the augmented transmission of this disease in the hospital pen compared to the home pen.

It appears that cows with clinical mycoplasma mastitis carried *M. bovis* into the hospital pen and were the nidus of three episodes of infection. All episodes of the *M. bovis* mastitis was caused by one strain, similar to that reported in a previous description of a herd outbreak (Punyapornwithaya et al., 2010), and appeared to begin with the introduction of a *M. bovis* mastitis cow into the hospital pen. The first case of each episode (cow 1563, cow 1510 and cow 475) occurred at 5 days (cow 1563 and cow 1510) and 7 days (cow 475) after the introduction of a cow with *M. bovis* mastitis into the hospital pen (Figure 3). Byrne et al. (2005) reported that clinical mastitis signs were first observed 3 to 4 days after intramammary

inoculation with 10^8 CFU/mL of *M. bovis*. The time that we observed between exposure to an infectious dose from a *M. bovis* mastitis cow and development of clinical signs of mycoplasma is consistent with this experimental observation.

It may be argued that NEG/POS cow acquired intramammary infection in milking pen but developed clinical mycoplasma mastitis in the hospital pen. If this were the case then we would have expected some new cases of clinical mycoplasma mastitis to occur in apparent NEG/POS cows before the entry of a POS cow. However, this never appeared to occur (Figure 3).

To control mycoplasma mastitis a dairy operator may keep suspected mycoplasma mastitis cows in their home pens and milk them last in that pen. Alternatively, operators may move suspected mycoplasma mastitis cows to a hospital pen and milk them with other sick cows presumably after all other cows are milked., Data from this study suggests that keeping *M. bovis* mastitis cows in a hospital pen was associated with a higher risk of *M. bovis* mastitis incidence. In addition, the incidence of *M. bovis* mastitis cows for each month in milking pens, before and after the hospital pen was created was similar (Table 1). Only one cow per month from March through July developed *M. bovis* mastitis in the milking pens. This suggests that segregation of *M. bovis* mastitis cows by transferring infected cows to the hospital pen had no advantage on controlling *M. bovis* mastitis in milking pens. However, this study was limited to one herd. The differences in virulence of *M. bovis* strains and variation in milking management practices for hospital pen cows between dairy farms may be associated with different outbreak or disease patterns. This study provided evidence to support the hypothesis that formation of a hospital pen in an effort to control *M. bovis* mastitis by segregation of cows with clinical mastitis leads to an initial increase an *M. bovis*

mastitis. However, this hypothesis would need to be tested in a study where more *M. bovis* mastitis herds are enrolled to account for herd and agent effects. In addition, the effect of keeping infected cows in their home pen and either milking them last or disinfecting the milking unit and workers' hands after mycoplasma cows are milked, needs to be compared to moving infected cows to a hospital pen and milking them separately.

5. Conclusion

The role of a hospital pen for control of *M. bovis* clinical mastitis was studied. The incidence rate of mycoplasma mastitis in the hospital pen was more than 100-fold greater than the incidence rate in the milking pens. Although it is possible that cows had subclinical mycoplasma intramammary prior to their entry into the hospital pen, the evidence from this study suggested that *M. bovis* mycoplasma mastitis from the milking pen was carried into the hospital pen. Thus addition of cows with clinical mycoplasma mastitis was the nidus for additional infections in the hospital pen and may have prolonged the disease in this herd.

References

Bayoumi, F.A., Farver, T.B., Bushnell, B., Oliveria, M., 1988. Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. J Am Vet Med Assoc 192, 905-909.

Biddle, M.K., Fox, L.K., Hancock, D.D., 2003. Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. J. Am. Vet. Med. Assoc. 223, 1163-1166.

Biddle, M.K., Fox, L.K., Evans, M. A., Gay, C.C., 2005. Pulsed-field gel electrophoresis patterns of mycoplasma isolates from various body sites in dairy cattle with mycoplasma mastitis. J Am Vet Med Assoc. 227, 455-459.

Brown, M.B., Shearer, J.K., Elvinger, F., 1990. Mycoplasmal mastitis in a dairy herd. J Am Vet Med Assoc 196, 1097-1101.

Bushnell, R.B., 1984. Mycoplasma mastitis. Vet Clin North Am Large Anim Pract 6, 301-312.

Byrne, W., Markey, B., McCormack, R., Egan, J., Ball, H., Sachse, K., 2005. Persistence of Mycoplasma bovis infection in the mammary glands of lactating cows inoculated experimentally. Vet Rec 156, 767-771.

Dohoo, I., Martín, W., Stryhn, H., 2003. Veterinary epidemiologic research. AVC Inc. Charlottetown.

Fox, L.K., Gay, J.M., 1993. Contagious mastitis. Vet Clin North Am Food Anim Pract 9, 475-487.

Gonzalez, R.N., Wilson, D.J., 2003. Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19, 199-221.

Grohn, Y.T., Rajala-Schultz, P.J., Allore, H.G., DeLorenzo, M.A., Hertl, J.A., Galligan, D.T., 2003. Optimizing replacement of dairy cows: modeling the effects of diseases. Prev. Vet. Med. 61, 27-43.

Hogan, J.S., NMC, 1999. Laboratory handbook on bovine mastitis. Rev. ed. . National Mastitis Council Madison, WI.

Jackson, G., Boughton, E., 1991. A mild outbreak of bovine mastitis associated with Mycoplasma bovigenitalium. Vet Rec 129, 444-446.

Lam, T.J., DeJong, M.C., Schukken, Y.H., Brand, A., 1996. Mathematical modeling to estimate efficacy of postmilking teat disinfection in split-udder trials of dairy cows. J. Dairy Sci. 79, 62-70.

Nickerson, S.C., Boddie, R.L., 1994. Effect of naturally occurring coagulase-negative staphylococcal infections on experimental challenge with major mastitis pathogens. J. Dairy Sci. 77, 2526-2536.

Punyapornwithaya, V., Fox, L.K., Hancock, D.D., Gay, J.M., Alldredge, J.R., 2010. Association between an outbreak strain causing mycoplasma bovis mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. Prev. Vet. Med. 93, 66-70.

Tang, J., Hu, M., Lee, S., Robin, R. 2000. A polymerase chain reaction based method for detecting Mycoplasma/ Acheoleplasma contaminants in cell culture. J. Microbiol. Methods 39, 121-126

USDA, 2008. Prevalence of contagious mastitis pathogens on U.S. dairy operation, 2007. Department of Agriculture. Animal and Plant Health Inspection Service

Wellenberg, G.J., Bruschke, C.J., Wisselink, H.J., Barkema, H.W., Van Oirschot, J.T., 2002. Simultaneous intramammary and intranasal inoculation of lactating cows with bovine herpesvirus 4 induce subclinical mastitis. Vet. Microbiol. 86, 115-129.

Zadoks, R.N., Allore, H.G., Barkema, H.W., Sampimon, O.C., Wellenberg, G.J., Grohn, Y.T., Schukkent, Y.H., 2001. Cow- and quarter-level risk factors for Streptococcus uberis and Staphylococcus aureus mastitis. J. Dairy Sci. 84, 2649-2663.

Zecconi, A., Bronzo, V., Piccinini, R., Spreafico, G., Ruffo, G., 1994. Phagocytic activity of bovine polymorphonuclear neutrophil leucocytes. J. Dairy Res. 61, 271-279.

Table 1. Incidence rate of have *Mycoplasma bovis* mastitis for milking pen and hospital pen by month. Unit was 100 cow-days at risk.

	March	April	May	June	July	August
Milking pen	0.013	0.012	0.012	0.012	0.012	0.00
Hospital pen[a]	-	-	3.3	1.3	0.5	0.00

[a] Hospital pen was formed on April 1.

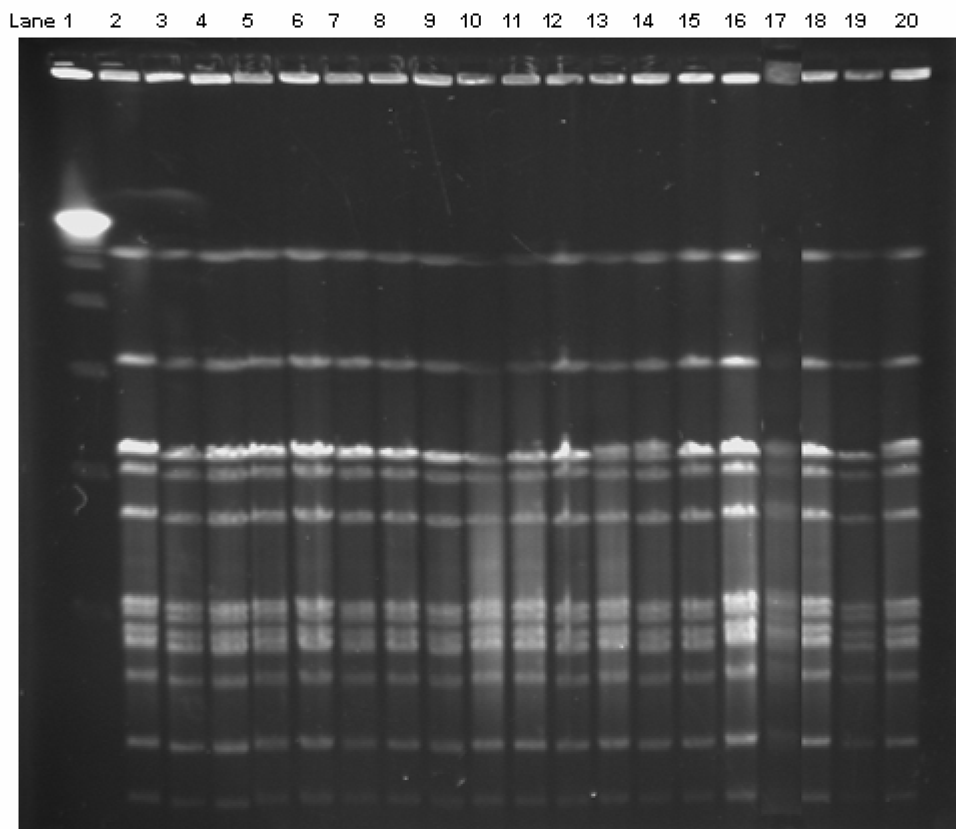

Figure 1. Pulsed-field gel electrophoretograms of *M.bovis* strain. Isolates in lane 2, lanes 4 to 8 and lanes 10 to 12 were strains obtained from clinical mastitis mycoplasma cows. Isolates

in lanes 3 and 9 were obtained from non-clinical mammary glands from a pneumonia cow and an arthritis cow respectively. Isolates in lanes 13 to 15 were obtained from joint fluid samples from arthritis cows. Isolates in lanes 16 to 20 were obtained from bulk tank milk samples. Lane 1 contains the Lamda ladder.

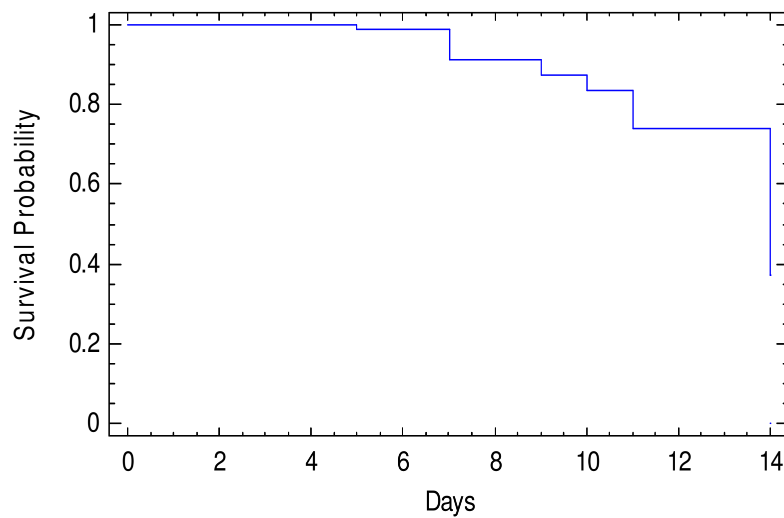

Figure 2. A Kaplan-Meier survival curve showing the time to detection of *Mycoplasma bovis* mastitis for cows entering the hospital pen free of the agent.

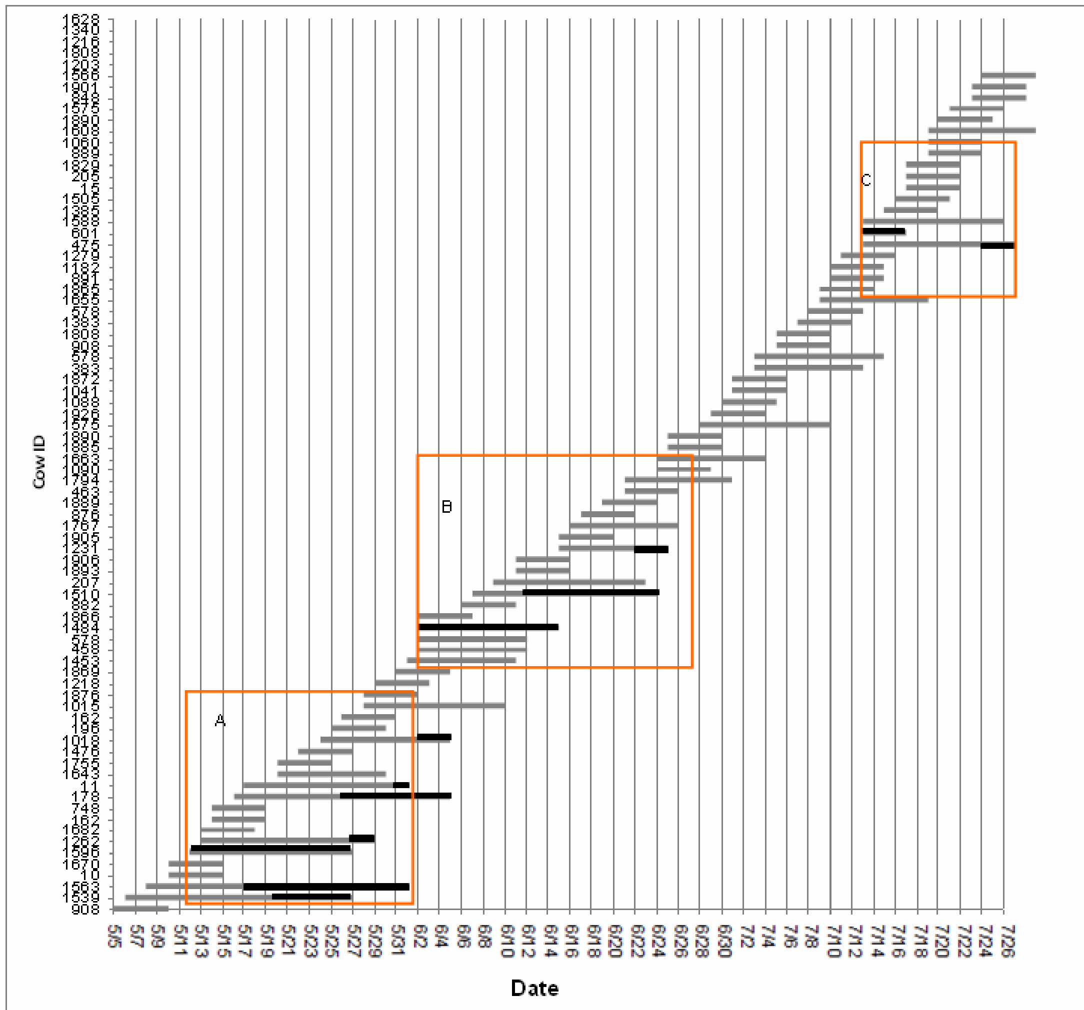

Figure 3. The total time that a cow remained in the hospital pen is depicted by a horizontal bar. The time a cow was not considered to have *Mycoplasma bovis* mastitis is depicted in gray and the time a cow was considered to have *M. bovis* mastitis is depicted in black. The boxes with the letters A, B and C indicate first, second and third episodes of mycoplasma transmission, respectively.

CHAPTER EIGHT

CONCLUSIONS

An association exists between mycoplasma strains causing intramammary infection and extramammary mucosal surface colonization as shown in the first study. A strain that caused an outbreak of mycoplasma mastitis remained in the herd and colonized body sites of cows and replacements during a year of study. One third of cows and replacements were colonized by this *Mycoplasma spp.* strain at the start of the outbreak, but less than 5% were colonized by one year after the outbreak, suggesting that animals spontaneously recovered from mycoplasma colonization. New strains were found to colonize cattle during the latter three quarters of the study. These new strains might have emerged from the parent outbreak strain. However, emerging strains never caused mastitis suggesting that these strains may have lost virulent factors enabling mycoplasma intramammary infections.

The third study showed that preferential culling of mycoplasma mastitis cows did not reduce the time to clearance of mycoplasma mastitis in outbreak herds. This finding challenges the common understanding that the most direct method to control mycoplasma mastitis is intensive detection and culling of infected cows. Evidence from an ancillary investigation to Study 3 indicated that *Mycoplasma bovis* mastitis cows from milking pens were sources of transmission of this mastitis to infirm cows housed in a hospital pen. This suggests that the hospital pen was a risk factor for the transmission of *Mycoplasma bovis* mastitis. All cases of mycoplasma mastitis and mycoplasma diseases were caused by one outbreak strain in this case study. This finding was consistent with the first study that the outbreak of mycoplasma mastitis began with one predominant strain and disseminated to susceptible animals.

In aggregate the findings from studies reported herein improve the understanding of the epidemiology of mycoplasma mastitis. *Mycoplasma spp.* colonization of different body sites can be concomitant with intramammary infection. Such colonization appears to wane as intramammary infections diminish. However, extramammary colonization did not appear to precede intramammary infection and thus extramammary colonization could not be used as a predictor of mastitis. The emergence of different strains, the majority appeared to be outgrowths from the parent strain, during the waning months of the outbreak signifies genetic changes in the outbreak strain. Yet only the outbreak strain caused mastitis. Perhaps the genetic changes lead to a loss of virulence factors related to the ability to cause IMI. Alternatively or additionally, to animal exposure 1) these strains have no genetic property to cause the mastitis but they still have ability to sustain in a host by colonization at extramammary sites and 2) animals developed a natural immunity that can protect them from these strains.

The role of the test and culling methods to control mycoplasma mastitis outbreaks was questioned. This method was not practiced by operators who had equivalent success in resolving the outbreak as those that did practice test and slaughter. Segregation of infected cows did not appear be an effective control measure, and might actually perpetuate the disease. It should be realized that mycoplasma strain differences may necessitate use of different control measures for the disease in the dairy.

APPENDIX 1

MEDIUM AND PROCEDURES

A.1 Mycoplasma enrichment media ingredients and procedures

Ingredients used

Base Medium

Mycoplasma Broth Base, CRITERION........................ 32 g

Phenol Red... 18 mg

Water, Distilled or deionized ....1000 ml

Supplements

Horse sera..55ml

5% Yeast Extract solution...35ml

Penicillin 200,000 IU/m...l2ml

Thallium acetate 1% 7ml

Procedures

1. Stir to mix thoroughly and heat to dissolve completely.

2. Put 250 ml into 500ml Nalgene bottles.

3. Autoclave. Cool to 45-50 C before adding supplements.

A.2 Modified Hayflicks agar ingredients and procedures

Ingredients used

Thallium acetate

Dissolve 1 g of thallium acetate in 100 ml distilled deionized water for 1% stock solution. Pass through a sterile disposable 0.22 µm pore size filter, dispense in 7 ml amounts, label, and store in a freezer (-30 to -70 C).

Fresh yeast extract

Purchase or prepare as follows: Heat 500 ml of distilled deionized water to 80 C on a hot plate, and slowly add while stirring continuously 125 g of Fleischmann's dry yeast, type 2040. Keep the mixture at 80 C for 20 minutes. After cooling, centrifuge at 1000 x *g* for 45 minutes. Remove the supernatant fluid and filter several times through decreasing pore size on filters (1.2, 0.8, 0.45 µm), dispense in convenient aliquots and autoclave at 10 lb pressure (115 C) for 5 minutes or pass through a sterile disposable 0.22 µm pore size filter. Label and store in a freezer (-30 to -70 C).

Penicillin

Dissolve 5 million IU of potassium penicillin G in 25 ml of deionized distilled water. Pass through a sterile disposable 0.22 µm pore size filter, dispense in 2-ml amounts, label, and store in a freezer (-30 to -70 C).

DNA solution

Dissolve 0.2 g DNA (sodium salt from salmon testes) in 100 ml of deionized distilled water. Dispense in 5-ml aliquots and autoclave at 15 lb pressure (121 C) for 15 minutes. Label and store in a freezer (-30 to -70 C).

Dextrose solution

Dissolve 50 g of dextrose in 100 ml of deionized distilled water. Pass through a sterile disposable 0.22 µm pore size filter, dispense in 5-ml amounts, label, and store in a freezer (-30 to -70 C).

Horse serum

Purchase or prepare from whole blood as follows: Allow blood to clot at room temperature for several hours or overnight at 4 C. Break clots and separate serum by centrifugation at 2000 x *g*. Pass through a sterile disposable 0.22 µm pore size filter, label and store in sterile bottles in a freezer at -30 C.

Procedures

1. Prepare stock agar ingredients

Mycoplasma Agar Base .... 36 g

Distilled water..1000 ml

or

PPLO Broth w/o crystal violet .... 21 g

Agar No.1 (Oxoid) .... 10 g

Distilled water ....1000 ml

2. Boil to dissolve the agar and distributed in 250 ml amounts into flasks (500 ml) large enough to hold additives.

3. Sterilize by autoclaving at 15 lb pressure (121 C) for 20 minutes. Store at 4 C.

4. Melt 250ml of stock agar and then cooled to 50 C.

5. Add (warmed to 37 C):

Horse serum ....55 ml

Fresh yeast extract solution ....35 ml

Thallium acetate 1% (w/v) solution ....7 ml

DNA 0.2% (w/v) solution ....5 ml

Penicillin 200,000 IU/ml2ml

Dextrose 50% (w/v) solution ....5 ml

6. Pour into 15 x 100 mm petri plates, approximately 15ml/plate.

7. Ready to use when agar surface is dry.

8. Store inveted at 4 C in plastic bag.

A.3 Phosphate buffered saline solution ingredients and procedures

Solution used

Solution 1

Sodium phosphate.. 0.69 g

Distilled water..500 ml

Solution2

Sodium phosphate (dibasic)...2.84

Distilled water..2000 ml

Add to final solution

NaCl .... 17 g

Procedures

1. Make solution 1 and solution 2, heat to dissolve crystals.

2. Mix 380 ml of solution 1 with 1620 ml solution 2

3. Add the NaCl and heat to dissolve

4. Aliquot to four 500 ml bottles and autoclave for 15 min.

A.4 Pulsed field gel electrophoresis (PFGE) solution ingredients and procedures

Solution used

TE Buffer for 1 liter

Tris 1M, pH 8.0..10 ml

EDTA 0.5M, pH 8.0 ....2 ml

Distilled water ....988 ml

Autoclave and store at Room Temp

Cell Sus'pension Buffer for 0.5L

Tris 1 M, ph 8.0..50 ml

EDTA 0.5M, pH 8.0 ....100 ml

Distilled water ....350 ml

Autoclave and store at Room Temp

Proteinase K

Proteinase K ....200 mg

Distilled water ....5 ml

Glycerol...1.5ml

Aliquot 1ml/tube

Heat to 50°C to dissolve. Store at -20°C.

Sarcosyl 10%

Sarcosyl... 10 g

Distilled water ....100 ml

Heat to dissolve. Store at Room Temp

Cell Lysis Buffer

Tris 1 M, ph 8.0.. 25 ml

EDTA 0.5M, pH 8.0 ....50 ml

SDS 20%

SDS ....20 g

Distilled water ....100 ml

Heat to dissolve. Store at Room Temp

1.6 % Chromosomal Grade Agarose

Chromosomal Grade Agarose......................................0.8g

TE Buffer ....46.7 ml

SOS 20%.. 2.5 ml

Heat to dissolve.

Aliquot 10ml/tube

Blue colored dye .... 15 drops/tube

Store at Room Temp

<u>Mycoplasma PFGE Plug Procedure</u>

1. Grow pure culture from isolate in PPLO Broth for 4 days at 37°C in 10 % CO_2. Make at least 2 tubes per sample to make a good sized pellet when washing (2-3 mm diameter).
2. Centrifuge tubes at 4000 RPM for 30 min to pellet Mycoplasma.

3. Melt Chromosomal Grade Agarose for plugs and keep at 55-65°C

4. Label plug mold. Make 2-3 plugs/ sample

5. Remove supernatant from pelleted Mycoplasma with vacuum apparatus. Be careful not to disturb pellet.

6. Add 250 µl PBSS to pellet in broth tube and resuspend Mycoplasma. Combine duplicate samples.

7. Transfer suspension to labeled conical (pointed bottom) microcentrifuge tubes, spin for 1 min in microcentrifuge to pellet sample. Discard supernatant take care not to disturb pellet. Add 500 µl PBSS, resuspend and repeat wash. Discard supernatant, take care not to disturb pellet.

8. Add 200 µl of Cell Suspension Buffer and 20 µl of Proteinase K. to pellet and gently resuspend. Mix well.

8. Add 200 µl melted Chromosomal Grade Agarose, mix by pipetting gently and make plugs. Use about 100 µl /plug

9. Cool for ~10 min to allow plug to solidify.

10. Add 1.5 ml Cell Lysis Buffer and 50 ul Proteinase K to labeled microcentrifuge tubes. Make one tube per sample. Use 2 ml microcentrifuge tubes with a nearly flat bottom.

11. Add all 2 or 3 plugs to solution, put tubes into foam floaters and incubate in a water bath for 4 hours at 50°C. Warm TE Buffer at the same time for next step.

12. Transfer plugs to sterile tissue capsules (all plugs for each sample in one capsule, ie. 20 samples=20 capsules, 1 capsule = 2-3 plugs) and put in 1000 ml Nalgene bottles with 500 ml warm (50 ºC) TE Buffer. Put Nalgene bottle in 50 ºC shaking waterbath or heated shaking apparatus set to 50 rpm. Wash for 30 min. Decant wash solution from plugs by pouring off solution. Add fresh warm TE Buffer. DO NOT OPEN CAPSULES. Wash 2X more (3X wash total) with 500 ml fresh warm TE Buffer each time.

13. Transfer plugs to labeled tubes with 5-10 ml TE Buffer and store at 4°C. Plugs are good for about a year.

Mycoplasma PFGE Gel Procedure

1. Mix master digestion solution according to this recipe (add 2 to total # samples to allow for pipetting errors):

# of Samples + 2	μl /sample		Total μl for Master Mix
N + 2	X	3 ul *Sal I* (1000U/μl)	1.5 ul *Sal I* if using 2000U/μl
N + 2	X	10 μl Enzyme Buffer (included with enzyme by manufacturer)	
N + 2	X	87 μl H_2O	
Total per sample =	100 μl		

Example

# of Samples + 2		μl /sample	Total μl for Master Mix
15 + 2 = 17	X	3 μl *Sal I*	51 μl
15 + 2 = 17	X	10 μl Enzyme Buffer	170 μl
15 + 2 =17	X	87 μl H_2O	1479 μl
Total for 15 + 2=17=		100 μl	1700 μl master mix

2. Put 100 μl digestion solution into labeled microcentrifuge tubes (pointed bottom).
3. Sterilize glass slide and gel plug cutting tool (pointed spatula) by dipping in ethanol and flaming.

4. Take gel plug out of TE Buffer, put on slide and cut about a 2mm slice off of the end. Put slice in labeled tube with digestion solution. Replace unused portion of gel plug in TE Buffer.
5. Incubate gel slices at 37°C for 4 hours.
6. Make TBE Buffer for running gel.

   105 ml 10X TBE

   1995 ml d H_2O

   2 ml Thiourea (concentration =50 µl M)
7. Save out 100 ml running buffer to make 1% gel. Use 1 g PFGE certified agarose.
8. Put the rest of the TBE buffer in the PFGE-CHEF unit to chill.
9. Microwave agarose for 2 min, swirl to mix and reheat for 30 sec. Cool to 65°C before pouring.
10. Assemble gel mold. After incubation is finished, place gel slices on comb. Leave 1st & 20th lanes for Pulse Markers or other DNA size marker. Record gel layout.
11. Let plug slices dry on comb for about 15-20 min.
12. Set comb upright in gel mold and carefully pour agarose into mold. (Pour in at bottom corner and allow to spread upwards toward comb.) Use a couple of drops of low melting point agarose to fill wells after gel sets.
13. Remove gel from mold and wipe off any excess gel pieces. Put into PFGE-CHEF unit with wells toward the back of the unit.
14. Set these parameters on control unit:

    6 Volt/cm

    0.5-12.9 sec Ramp time

    20.2 hours Run time

15. Shut lid and press start button. Make sure there are bubbles rising from the electrode wires.
16. Stain gel in ethidium bromide solution for 30 min. Wash with d H_2O 2X for 30 min each
17. Photograph gel.

A.5 A nested polymerase chain reaction (PCR) procedures

Procedures

1. PCR was performed in a total volume of 50 µL containing 1x PCR buffer (20 mM Tris-HCl, 2 mM MgCl2, and 50 mM KCl; pH 8.4), 50 µM of each of deoxynucleoside triphosphates (dNTPs), 20 pmol of each primer, and 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA).
2. For the first-stage PCR, 5 µL of DNA extract was used as a template by adding into a 45 µL of reaction mixture with outer primer pair (F1 and R1).
3. For the second-stage PCR, 1 µL of the first-stage PCR product was added into 49 µL of reaction mixture with the inner primer pair (F2 and R2). Sequences of primers are shown in Table below.

Primer	Sequence
F1	5’-ACACCATGGGAG(C/T)TGGTAAT-3’
R1	5’-CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT-3’
F2	5’-GTG(C/G)GG(A/C)TGGATCACCTCCT-3’
R2	5’-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3’

4. The thermal cycling protocol involving initial denaturation at 94 °C for 30 s, followed by 30 cycles for the first-stage PCR (or 35 cycles for the second-stage PCR) of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 2 min, and extension at 72 °C for 2 min.

5. A final extension was performed at 72 °C for 5 min. The final PCR products were electrophoresed on a 2% agarose gel and DNA bands was visualized by UV fluorescence. To identify different species of *Mycoplasma spp.*, the different patterns of DNA fragments was obtained after the digestion of a restriction enzyme, AseI. Different fragment sizes in digested PCR products was separated using electrophoresis on a 2% agarose gel and DNA bands was visualized by UV fluorescence.

APPENDIX 2

RAW DATA

B.1 First study (Chapter 4) raw data

Animal data, samples and microbiological results

Data were collected for four periods. Period 1 was the outbreak period. Composite milk samples were collected aseptically from lactating cows. Swabs, moistened by mycoplasma enrichment media, were in contact and rotated over the mucosal surfaces of the right and left eyes, the right and left nasal cavities, the right and left ears, the vestibular fossa and vaginal wall (vulvovaginal tract) from all animals which included calves, young heifers, heifers, dry cows and lactating cows.

Period 1

Codes, definition and classification were shown in the following table.

Code	**Definition & Classification**
Cow	Cow identification number
Source	Origin of animals UI = UI born and never raise off-farm UIW = UI born animals but raise at institution herd W = Purchase animals from institution herd
Type	Type of animal C=cow R=replacement
Milk	Milk sample was positive to *Mycoplasma spp.* Y= yes

	N= no
Ear	Swab specimen from ear surface was positive to *Mycoplasma spp.* Y= yes N= no
Eye	Swab specimen from eye mucosal surface was positive to *Mycoplasma spp.* Y= yes N= no
Nose	Swab specimen from nose mucosal surface was positive to *Mycoplasma spp.* Y= yes N= no
Vul	Swab specimen from vulvovaginal tract surface was positive to *Mycoplasma spp.* Y= yes N= no
Post	Swab specimens from any body site surfaces were positive to *Mycoplasma spp.* Y= yes N= no
Multi	Swab specimens from more than one site were positive to *Mycoplasma spp.* Y= yes N= no

Raw data

Cow	Source	Type	Milk	Ear	Eye	Nose	Vul	Post	Multi
1765	UI	C	N	N	N	N	N	N	N
1884	UI	C	N	N	N	Y	N	Y	N
1904	UI	C	N	N	N	N	N	N	N
1931	UI	C	N	N	N	N	N	N	N
1933	UI	C	N	N	N	N	N	N	N
1944	UI	C	N	N	N	N	N	N	N
1946	UI	C	N	N	N	N	N	N	N
1981	UI	C	N	N	N	N	N	N	N
1984	UI	C	N	N	N	N	N	N	N
1998	UI	C	N	N	N	N	N	N	N
2019	UI	C	N	N	Y	Y	N	Y	Y
2020	UI	C	N	N	N	N	N	N	N
2030	UI	C	N	N	N	N	N	N	N
2036	UI	C	N	N	N	N	Y	Y	N
2038	UI	C	N	N	N	Y	N	Y	N
2042	UI	C	N	N	N	N	N	N	N
2043	UI	C	N	N	N	Y	N	Y	N
2045	UI	C	N	N	N	N	N	N	N
2046	UI	C	N	N	N	N	N	N	N
2052	UI	C	Y	N	N	N	N	N	N
2056	UI	C	N	N	N	N	N	N	N
2061	UI	C	N	N	N	N	N	N	N
2062	UI	C	N	N	N	Y	N	Y	N
2066	UI	C	N	N	N	N	N	N	N
2067	UI	C	N	N	N	N	N	N	N
2070	UI	C	N	N	N	N	N	N	N
2076	UI	C	N	N	N	N	N	N	N
2085	UI	C	N	N	N	N	N	N	N
2087	UI	C	N	N	N	N	N	N	N
2090	UI	C	N	N	N	N	N	N	N
2091	UI	C	N	N	N	N	N	N	N
2092	UI	C	N	N	N	N	N	N	N
2093	UI	C	N	N	N	N	N	N	N
2097	UI	C	N	N	N	N	N	N	N
2099	UI	C	N	N	N	N	N	N	N
2100	UI	C	N	N	N	N	N	N	N
2103	UI	C	N	N	N	N	N	N	N
2106	UI	C	N	N	N	N	N	N	N
2107	UI	C	N	N	N	N	N	N	N
2109	UI	C	N	N	N	N	N	N	N
2113	UI	C	N	N	N	N	N	N	N
2118	UI	C	N	N	N	N	N	N	N
2121	UI	C	N	N	N	N	N	N	N
2123	UI	C	N	N	N	N	N	N	N
2127	UI	C	N	N	N	Y	N	Y	N
2128	UI	C	N	N	N	N	N	N	N
2129	UI	C	N	N	N	N	N	N	N

2130	UI	C	N	N	N	N	N	N	N
2132	UI	C	N	N	N	N	N	N	N
2133	UI	C	N	N	N	N	N	N	N
2134	UI	C	N	N	N	Y	N	Y	N
2136	UI	C	N	N	N	N	N	N	N
2138	UI	C	N	N	N	N	N	N	N
2139	UI	C	N	N	N	Y	N	Y	N
2140	UI	C	N	N	N	N	N	N	N
2141	UI	C	N	N	N	N	N	N	N
2143	UI	C	N	N	N	Y	N	Y	N
2145	UI	C	N	N	N	Y	N	Y	N
2146	UI	C	N	N	N	N	N	N	N
2147	UI	C	N	N	N	N	N	N	N
2148	UI	C	N	N	N	N	N	N	N
2150	UI	C	N	N	N	N	N	N	N
2152	UI	C	N	N	N	Y	N	Y	N
2153	UI	C	N	N	N	N	N	N	N
2154	UI	C	N	N	N	N	N	N	N
2155	UI	C	N	N	N	N	N	N	N
2156	UI	C	N	N	N	N	N	N	N
2158	UI	C	N	N	N	N	N	N	N
2160	UI	C	N	N	N	N	N	N	N
2161	UI	C	N	N	Y	Y	N	Y	Y
2162	UI	C	N	N	N	N	N	N	N
2164	UI	C	N	N	N	Y	N	Y	N
2165	UI	C	N	N	N	N	N	N	N
2166	UI	C	N	N	N	N	N	N	N
2168	UI	C	N	N	N	N	N	N	N
2169	UI	C	N	N	Y	Y	N	Y	Y
2170	UI	C	N	N	N	N	N	N	N
2171	UI	C	N	N	N	N	N	N	N
2172	UI	C	N	N	N	N	N	N	N
2173	UI	C	N	N	N	N	N	N	N
2174	UI	C	N	N	N	Y	N	Y	N
2175	UI	C	N	N	N	N	N	N	N
2178	UI	C	N	N	N	Y	N	Y	N
2179	UI	C	N	N	N	Y	N	Y	N
2180	UI	R	N	N	N	N	N	N	N
2181	UI	R	N	N	Y	Y	N	Y	Y
2182	UI	R	N	N	N	N	Y	Y	N
2183	UI	R	N	N	N	N	N	N	N
2184	UI	R	N	N	N	Y	N	Y	N
2186	UIW	R	N	N	N	N	N	N	N
2188	UIW	R	N	N	N	N	N	N	N
2191	UIW	R	N	N	N	N	Y	Y	N
2192	UIW	R	N	N	N	Y	N	Y	N
2199	UIW	R	N	N	N	N	N	N	N
2201	UIW	R	N	N	N	N	N	N	N
2205	UIW	R	N	N	N	N	N	N	N
2206	UIW	R	N	N	N	N	N	N	N
2207	UIW	R	N	N	N	N	N	N	N
2212	UIW	R	N	N	N	N	N	N	N

2214	UIW	R	N	N	N	N	N	N	N
2222	UIW	R	N	N	N	N	N	N	N
2224	UIW	R	N	N	N	N	N	N	N
2226	UIW	R	N	N	N	N	N	N	N
2228	UIW	R	N	N	N	N	N	N	N
2229	UIW	R	N	N	N	N	N	N	N
2230	UIW	R	N	N	N	N	N	N	N
2231	UIW	R	N	N	N	N	N	N	N
2233	UIW	R	N	N	N	N	N	N	N
2273	UIW	R	N	N	N	Y	N	Y	N
2274	UIW	R	N	N	N	Y	N	Y	N
2275	UIW	R	N	N	N	Y	N	Y	N
2276	UIW	R	N	N	N	N	N	N	N
2277	UIW	R	N	N	Y	Y	N	Y	Y
2278	UIW	R	N	N	N	Y	N	Y	N
2279	UIW	R	N	N	Y	Y	N	Y	Y
2280	UIW	R	N	N	N	Y	N	Y	N
2281	UIW	R	N	N	N	Y	N	Y	N
2282	UIW	R	N	N	Y	Y	N	Y	Y
2283	UIW	R	N	N	N	N	N	N	N
2284	UIW	R	N	N	Y	Y	N	Y	Y
2285	UIW	R	N	N	Y	Y	N	Y	Y
2286	UIW	R	N	N	N	Y	N	Y	N
2287	UIW	R	N	N	N	Y	N	Y	N
2288	UIW	R	N	N	N	Y	N	Y	N
2289	UIW	R	N	N	N	Y	N	Y	N
2290	UIW	R	N	N	N	Y	N	Y	N
2956	W	R	N	N	N	N	N	N	N
3050	W	R	N	N	N	Y	N	Y	N
3144	W	R	N	N	N	N	N	N	N
3151	W	R	N	N	N	Y	N	Y	N
3153	W	R	N	N	N	N	N	N	N
3178	W	R	N	N	N	Y	N	Y	N
3190	W	R	N	N	N	N	N	N	N
3191	W	R	N	N	N	Y	N	Y	N
3194	W	R	N	N	N	N	N	N	N
3195	W	R	N	N	N	Y	N	Y	N
3199	W	R	N	N	N	N	N	N	N
3201	W	R	N	N	N	Y	N	Y	N
3206	W	R	N	N	N	N	N	N	N
3207	W	R	N	N	N	Y	N	Y	N
3208	W	R	N	N	N	N	N	N	N
3214	W	R	N	N	N	Y	N	Y	N
3226	W	R	N	N	N	Y	N	Y	N
3228	W	R	N	N	N	N	N	N	N
3229	W	R	N	N	N	N	N	N	N
3708	W	R	N	N	N	N	N	N	N
5001	W	R	N	N	N	N	N	N	N
5002	W	R	N	N	N	N	N	N	N
5004	W	R	N	N	N	Y	N	Y	N
5006	W	R	N	N	N	N	N	N	N
5007	W	R	N	N	N	N	N	N	N

5013	W	R	N	N	N	N	N	N	N
5014	W	R	N	N	N	N	N	N	N
5037	W	R	N	N	N	N	N	N	N
5055	W	R	N	N	N	N	N	N	N
6012	W	R	N	N	N	Y	Y	Y	Y
6013	W	R	N	N	N	Y	N	Y	N
6014	W	R	N	Y	N	Y	N	Y	Y
6015	W	R	N	N	N	Y	N	Y	N
6016	W	R	N	N	N	Y	N	Y	N
6017	W	R	N	N	N	N	N	N	N

Period 2 to 4

Codes, definition and classification were shown in the following table.

Code	**Definition & Classification**
Period	Herd Sampling Period
id	Animal identification number
type	Type of animal cow=cow repl=replacement
Body site	Body site that was positive to *Mycoplasma spp.* 0= none of body sites were positive to *Mycoplasma spp.* nose = swab specimen from nose was positive to *Mycoplasma spp.* eye = swab specimen from eye was positive to *Mycoplasma spp.* vg = swab specimen from vulvovaginal tract was positive to *Mycoplasma spp.*

Raw data

Period	id	type	Body site
2	153	cow	0
2	1765	cow	0
2	1944	cow	0
2	1946	cow	0

2	1981	cow	0
2	1984	cow	0
2	1998	cow	nose
2	2022	cow	0
2	2030	cow	0
2	2042	cow	0
2	2045	cow	0
2	2046	cow	0
2	2056	cow	0
2	2061	cow	0
2	2062	cow	0
2	2070	cow	0
2	2076	cow	0
2	2085	cow	0
2	2087	cow	0
2	2091	cow	0
2	2099	cow	0
2	2107	cow	0
2	2109	cow	0
2	2113	cow	0
2	2121	cow	0
2	2123	cow	0
2	2127	cow	0
2	2128	cow	0
2	2129	cow	0
2	2130	cow	0
2	2136	cow	0
2	2138	cow	0
2	2139	cow	0
2	2141	cow	0
2	2147	cow	0
2	2148	cow	0
2	2152	cow	0
2	2154	cow	0
2	2155	cow	0
2	2156	cow	0
2	2160	cow	0
2	2161	cow	0
2	2162	cow	0
2	2164	cow	0
2	2165	cow	0
2	2166	cow	0
2	2169	cow	0
2	2170	cow	0
2	2173	cow	0
2	2174	cow	0
2	2175	cow	0
2	2178	cow	0
2	2179	cow	0
2	2180	cow	0
2	2182	cow	0
2	2183	cow	0

2	2184	cow	0
2	2185	cow	0
2	2187	cow	0
2	2191	repl	0
2	2192	repl	0
2	2195	repl	0
2	2198	repl	0
2	2199	repl	0
2	2201	repl	0
2	2205	repl	0
2	2206	repl	0
2	2207	repl	0
2	2208	repl	0
2	2209	repl	0
2	2210	repl	0
2	2212	repl	0
2	2213	repl	0
2	2214	repl	0
2	2216	repl	0
2	2218	repl	0
2	2219	repl	0
2	2220	repl	0
2	2221	repl	0
2	2223	repl	0
2	2225	repl	eye
2	2232	repl	0
2	2258	repl	0
2	2261	repl	0
2	2262	repl	0
2	2263	repl	0
2	2264	repl	0
2	2265	repl	0
2	2266	repl	0
2	2267	repl	0
2	2268	repl	0
2	2269	repl	0
2	2270	repl	0
2	2271	repl	0
2	2272	repl	0
2	2273	repl	0
2	2274	repl	0
2	2275	repl	0
2	2276	repl	0
2	2277	repl	0
2	2278	repl	0
2	2279	repl	0
2	2280	repl	0
2	2281	repl	0
2	2283	repl	0
2	2306	repl	0
2	2307	repl	0
2	2308	repl	0

2	2309	repl	0
2	2310	repl	0
2	2311	repl	0
2	2312	repl	0
2	2313	repl	0
2	2314	repl	0
2	2315	repl	0
2	2316	repl	0
2	2317	repl	0
2	2318	repl	0
2	2319	repl	0
2	2321	repl	0
2	2323	repl	0
2	2324	repl	0
2	2325	repl	0
2	3144	repl	0
2	3151	repl	0
2	3153	repl	0
2	3191	repl	0
2	3194	repl	0
2	3199	repl	0
2	3208	repl	0
2	3218	repl	0
2	5007	repl	0
2	6020	repl	0
2	6021	repl	0
2	6022	repl	0
3	1765	cow	0
3	1946	cow	0
3	1994	cow	0
3	1998	cow	0
3	2020	cow	0
3	2030	cow	0
3	2042	cow	0
3	2045	cow	0
3	2045	cow	0
3	2046	cow	0
3	2061	cow	0
3	2062	cow	0
3	2070	cow	0
3	2076	cow	0
3	2085	cow	0
3	2087	cow	0
3	2091	cow	0
3	2093	cow	0
3	2109	cow	0
3	2118	cow	0
3	2121	cow	0
3	2123	cow	0
3	2126	cow	0
3	2127	cow	0
3	2128	cow	0

3	2130	cow	0
3	2136	cow	0
3	2138	cow	0
3	2139	cow	0
3	2143	cow	vg
3	2147	cow	0
3	2152	cow	0
3	2152	cow	0
3	2153	cow	0
3	2154	cow	0
3	2156	cow	0
3	2160	cow	0
3	2161	cow	0
3	2164	cow	0
3	2165	cow	0
3	2169	cow	0
3	2171	cow	0
3	2171	cow	0
3	2171	cow	0
3	2172	cow	0
3	2174	cow	vg
3	2175	cow	0
3	2179	cow	0
3	2180	cow	vg
3	2181	cow	0
3	2182	cow	0
3	2184	cow	0
3	2185	cow	0
3	2186	cow	0
3	2187	repl	eye
3	2195	repl	0
3	2198	repl	0
3	2199	repl	0
3	2201	repl	0
3	2205	repl	0
3	2206	repl	0
3	2207	repl	0
3	2208	repl	0
3	2209	repl	0
3	2210	repl	0
3	2213	repl	0
3	2214	repl	0
3	2216	repl	0
3	2217	repl	0
3	2218	repl	0
3	2219	repl	0
3	2220	repl	0
3	2221	repl	0
3	2222	repl	0
3	2223	repl	0
3	2225	repl	0
3	2230	repl	0

3	2232	repl	0
3	2233	repl	0
3	2234	repl	0
3	2240	repl	0
3	2241	repl	0
3	2244	repl	0
3	2245	repl	0
3	2248	repl	0
3	2253	repl	0
3	2260	repl	0
3	2261	repl	0
3	2262	repl	0
3	2263	repl	0
3	2264	repl	0
3	2265	repl	0
3	2266	repl	0
3	2267	repl	0
3	2268	repl	0
3	2269	repl	0
3	2271	repl	0
3	2272	repl	0
3	2281	repl	0
3	2282	repl	0
3	2284	repl	0
3	2285	repl	nose
3	2286	repl	0
3	2288	repl	0
3	2289	repl	0
3	2290	repl	0
3	2291	repl	nose
3	2292	repl	0
3	2292	repl	0
3	2293	repl	0
3	2294	repl	0
3	2295	repl	0
3	2296	repl	0
3	2297	repl	0
3	2298	repl	0
3	2299	repl	0
3	2300	repl	0
3	2301	repl	0
3	2302	repl	0
3	2303	repl	0
3	2304	repl	0
3	2305	repl	0
3	2306	repl	0
3	2307	repl	0
3	2308	repl	0
3	2309	repl	0
3	2310	repl	0
3	2311	repl	nose
3	2312	repl	0

3	2315	repl	0
3	2327	repl	nose
3	3151	repl	0
3	3153	repl	0
3	3191	repl	0
3	3194	repl	0
3	3195	repl	0
3	3199	repl	0
3	3208	repl	0
3	5007	repl	0
3	C1	repl	0
3	C2	repl	0
3	C3	repl	0
3	C4	repl	0
4	148	cow	0
4	172	cow	0
4	175	cow	0
4	1944	cow	0
4	1984	cow	0
4	2020	cow	0
4	2030	cow	0
4	2045	cow	0
4	2046	cow	0
4	2061	cow	0
4	2062	cow	0
4	2076	cow	0
4	2085	cow	0
4	2093	cow	0
4	2099	cow	0
4	2109	cow	0
4	2118	cow	0
4	2123	cow	0
4	2127	cow	0
4	2128	cow	0
4	2134	cow	0
4	2136	cow	0
4	2139	cow	0
4	2143	cow	0
4	2148	cow	0
4	2153	cow	0
4	2154	cow	0
4	2156	cow	0
4	2160	cow	0
4	2161	cow	0
4	2164	cow	0
4	2165	cow	0
4	2165	cow	0
4	2171	cow	0
4	2172	cow	0
4	2174	cow	0
4	2175	cow	0
4	2178	cow	0

4	2179	cow	0
4	2180	cow	0
4	2181	cow	0
4	2182	cow	0
4	2184	cow	0
4	2185	cow	0
4	2187	repl	0
4	2191	repl	0
4	2192	repl	0
4	2198	repl	0
4	2199	repl	0
4	2201	repl	0
4	2207	repl	vg
4	2207	repl	eye
4	2208	repl	0
4	2209	repl	vg
4	2210	repl	0
4	2214	repl	vg
4	2217	repl	0
4	2221	repl	0
4	2222	repl	0
4	2223	repl	0
4	2224	repl	0
4	2225	repl	0
4	2226	repl	0
4	2230	repl	0
4	2231	repl	0
4	2232	repl	0
4	2233	repl	0
4	2235	repl	0
4	2238	repl	0
4	2240	repl	0
4	2242	repl	0
4	2243	repl	0
4	2244	repl	0
4	2245	repl	nose
4	2248	repl	0
4	2251	repl	0
4	2255	repl	vg
4	2257	repl	0
4	2260	repl	0
4	2261	repl	0
4	2264	repl	0
4	2265	repl	0
4	2267	repl	0
4	2268	repl	0
4	2269	repl	0
4	2271	repl	0
4	2272	repl	0
4	2273	repl	0
4	2274	repl	0
4	2276	repl	0

4	2277	repl	0
4	2279	repl	0
4	2280	repl	0
4	2281	repl	0
4	2282	repl	0
4	2284	repl	0
4	2285	repl	0
4	2288	repl	0
4	2290	repl	0
4	2291	repl	0
4	2294	repl	0
4	2296	repl	0
4	2297	repl	0
4	2298	repl	0
4	2299	repl	0
4	2300	repl	0
4	2301	repl	0
4	2303	repl	0
4	2304	repl	0
4	2305	repl	0
4	2306	repl	0
4	2307	repl	0
4	2308	repl	0
4	2312	repl	0
4	2313	repl	0
4	2314	repl	0
4	2315	repl	0
4	2316	repl	0
4	2318	repl	0
4	2319	repl	0
4	2320	repl	0
4	2321	repl	0
4	2322	repl	0
4	2323	repl	0
4	2334	repl	vg
4	2336	repl	0
4	2339	repl	0
4	2340	repl	0
4	2341	repl	0
4	2346	repl	0
4	2347	repl	0
4	2348	repl	0
4	2348	repl	0
4	2349	repl	0
4	2367	repl	0
4	3191	repl	0
4	3218	repl	0
4	5007	repl	0
4	5008	repl	0

B.2 Second study (Chapter 5) raw data

A completely randomized design with a 2x2x4 factorial arrangement was used to test the factors: 1) 2 treatments (centrifugation vs. direct agar plating; 2) 2 test species (*M. bovis* and *M. californicum*; 4 strains for each species were used); and 3) 4 concentrations of *Mycoplasma spp.* (1,000, 100, 10, and 1 cfu/mL). Sixty-four milk samples were randomly assigned to receive the combination of these treatment factors. Codes, definition and classification were shown in the following table.

Code	Definition & Classification
Group	Treatment or Control tx= treatment ct = control
Dil	Dilution tested h= high m= medium l = low vl = very low
Sp	Species of mycoplasma microorganism
Str	Strain of mycoplasma microorganism
cfu_mL	Mycoplasma count (cfu/mL)
log_cfu_ml	Log $_{10}$ cfu/mL of mycoplasma count

Raw data

Group	Dil	Sp	Str	cfu_mL	log_cfu_ml
tx	h	c	2146	1245	3.10
tx	m	c	2146	180	2.26
tx	l	c	2146	17.5	1.24

tx	vl	c	2146	12.5	1.10
ct	h	c	2146	1085	3.04
ct	m	c	2146	55	1.74
ct	l	c	2146	7.5	0.88
ct	vl	c	2146	5	0.70
tx	h	c	33461	1247.5	3.10
tx	m	c	33461	262.5	2.42
tx	l	c	33461	70	1.85
tx	vl	c	33461	17.5	1.24
ct	h	c	33461	1075	3.03
ct	m	c	33461	187.5	2.27
ct	l	c	33461	55	1.74
ct	vl	c	33461	7.5	0.88
tx	h	b	m2	1417.5	3.15
tx	m	b	m2	192.5	2.28
tx	l	b	m2	12.5	1.10
tx	vl	b	m2	7.5	0.88
ct	h	b	m2	575	2.76
ct	m	b	m2	70	1.85
ct	l	b	m2	10	1.00
ct	vl	b	m2	5	0.70
tx	h	b	ucd1	1367.5	3.14
tx	m	b	ucd1	275	2.44
tx	l	b	ucd1	57.5	1.76
tx	vl	b	ucd1	12.5	1.10
ct	h	b	ucd1	1075	3.03
ct	m	b	ucd1	187.5	2.27
ct	l	b	ucd1	55	1.74
ct	vl	b	ucd1	7.5	0.88
tx	h	b	27368	1380	3.14
tx	m	b	27368	92.5	1.97
tx	l	b	27368	22.5	1.35
tx	vl	b	27368	7.5	0.88
ct	h	b	27368	1150	3.06
ct	m	b	27368	45	1.65
ct	l	b	27368	10	1.00
ct	vl	b	27368	5	0.70
tx	h	b	21528	815	2.91
tx	m	b	21528	85	1.93
tx	l	b	21528	12.5	1.10
tx	vl	b	21528	7.5	0.88
ct	h	b	21528	587.5	2.77
ct	m	b	21528	35	1.54
ct	l	b	21528	7.5	0.88
ct	vl	b	21528	5	0.70
tx	h	c	2146b	1062.5	3.03
tx	m	c	2146b	120	2.08
tx	l	c	2146b	20	1.30
tx	vl	c	2146b	17.5	1.24
ct	h	c	2146b	1180	3.07
ct	m	c	2146b	147.5	2.17
ct	l	c	2146b	10	1.00

ct	vl	c	2146b	7.5	0.88
tx	h	c	5689	1077.5	3.03
tx	m	c	5689	115	2.06
tx	l	c	5689	15	1.18
tx	vl	c	5689	7.5	0.88
ct	h	c	5689	980	2.99
ct	m	c	5689	87.5	1.94
ct	l	c	5689	7.5	0.88
ct	vl	c	5689	5	0.70

B.3 Third study (Chapter 6) raw data

Questionnaires

Herd visits were scheduled within 2 weeks after dairy operators agreed to enroll. A questionnaire was used to obtain herd and management data from the dairy operator during the herd visit. In brief, the questionnaire covered the following areas: herd populations; milk production; bedding type; frequency of milking machine evaluation; milking procedures; dairy animal addition, if herds were currently not purchasing replacements nor rearing replacement off-premise (closed herd); methods used to identify mastitis problems; mastitis control programs and culling policy. Milking practices and milking time hygiene were observed by one veterinarian with checklists during the survey. In brief, the observations covered the following areas: udder preparation (foremilk-stripping, method of pre-milking teat asepsis); cleanliness of milking parlor; number of milking stalls/milker; postmilking teat asepsis; use of postmilking unit backflush and milking system evaluation. The second interview was made when BTM samples from the herd were negative to *Mycoplasma spp.* for 3 consecutive samplings or at 3 months for all other herds. Information about how operators managed the mycoplasma mastitis since the first interview was noted.

Part 1. Herd characteristics and Management

Herd surveys	
Date :	
Farm ID :	
Owner name :	
Herd Characteristics	
Cow population	
Number of milking cows	=

Number of dry cows	=
Number of heifers	=
Number of young animals	=
Milk production / cow/ day	=
Mastitis cow handling	
How to mange mastitis cows 1= keep in hospital pen 2= keep in milking cow pen	
Criteria for returning mastitis cow to herd after treatment 1= no physical sign of mastitis & normal milk appearance 2= normal milk appearance only 3 = no physical sign only 4= others ……………………………….	
Number of mastitis cows for the previous month	= ……….. cows
Detection & handling of <u>mycoplasma mastitis cows</u> 1= yes & separate milking by keeping in hospital pen 2= yes & keeping in milking herd and milk last 3= yes & culling 4= no detection 5 = other …………………………………………………	

Number of cow culled from mycoplasma	
Number of cow in hospital pen due to mastitis or high somatic cell count	= ……….. days
Number of cows culling from mastitis of high somatic cell count in this month	
Bulk tank milk hygiene	
Somatic cell count	=………………………….
Milking Management & milking machine	
Method for grouping cows for milking 1= by day in milk 2= by lactation 3= other ……………………	
Bedding type for milking cows	=………………………….
Frequency of bedding change	=………………………….
Type of pre-dipping / post-dipping disinfectant	
Vacuum level	=………………………..
Frequency of milking machine checking	=……………………..
Dry cow managements& Early postpartum	
Dry cow therapy 1= blanket therapy	

2 = selective therapy 3 = no 4 = others ……………………………..	
Feed mastitis milk to calve 1= yes and no pasteurization 2= yes but pasteurization 3= no	
Herd expansion & Security	
Purchasing animals 1= yes type = …………………………. 2= no	
Quarantine purchase animals before entering to herd 1= yes & method …………………………………………… …………………………………………… 2= no 3= others ……………………………..	
Number of purchased animals in previous 3 months	=……………… heads
Time between last purchasing and bulk tank positive to mycoplasma mastitis	=……………… …… weeks

Part 2. Milking time hygiene practices

Date :	
Farm ID :	
Milking Practices	Note
Prepare pre-milking udder by cleaning udder with water 1= yes 2= no	
Consistency of udder cleaning with water, if water is used 1= all cows 2= not all cow but >= 75% 3= not all cow but <75% 4= no cleaning	
Use of towel or paper to clean udder 1= Paper /Single per cow 2= Paper/ One paper per more than one cow 3= Towel/ Single per cow 4= Towel/ One towel per more than one cow	
Selection of cows that are dry wiped 1= dirty cow only 2= all cows	
Use of pre-dipping 1= yes	

type of disinfectant = ……………………… 2= no	
Method of pre-dipping, if use 1= spray 2= teat cup	
Consistency of pre-dipping, if use 1= all cows 2= not all cow but >= 75% 3= not all cow but <75%	
Contact time of pre-dipping solution on teats before attachment of milking unit 1= less than or equal to 30 seconds 2= greater than 30 seconds	
If no pre-dipping and water is used for cleaning, the condition of udder before attachment milking unit is 1= dry 2= wet	
Check foremilk before attaching milking unit 1= yes / stripping to the floor 2= yes /stripping to teat cup 3= not check	

Consistency of foremilk checking 1= all cows 2= not all cow but >= 75% 3= not all cow but <75% 4= no checking	
Time between drying the udder to attachment of milking unit	= ……… min
Number of milking unit	= ……… units
Number of milker in milking parlor	=………………..
Milker wear rubber gloves 1= yes / change every string 2= yes / change but every string 3= no	
Number of cows leaking of milk before milking and total cow observed	= ………………….
Method for cleaning milk presenting on the floor 1= clean with water immediately 2= clean with water after finishing milking	
Method for attachment milking unit if it is dropped to the floor	

1= re-attach without cleaning 2= re-attach with cleaning with water or disinfectant	
Use of automatic take off 1= yes 2= no	
Use of backflush 1= yes 2= no	
Use post dipping 1= yes 2= no	
Method for post dipping, if use 1= spray 2 = teat cup 3= other …………………………	
Consistency of post-dipping 1= all cows 2= not all cow but >= 75% 3= not all cow but <75% 4= no post-dipping	
The allocation of post dipping on teats 1=completely 2= partially	

Cleaning the floor after milking 1= yes 2= no	

Data summary

Data were summarized from a spreadsheet file. The explanation of codes are shown below.

No. = Variable number

Code = abbreviation for variables

Description = the definition of code

Type = type of data, continuous or categorical data (class data). For class data, the class level and its definition were shown

Summary = Mean was shown for continuous data and frequency was shown for categorical data.

No.	Code	Description	Type	Summary
1	MC	Number of milking cow	continuous	mean=680
2	DC	Number of dry cows	continuous	mean=94
3	HF	Number of heifers	continuous	mean=283
4	YNG	Number replacement (<6 mths)	continuous	mean=76
5	PROD	Average milk production	continuous	mean=67
6	MMMA	Method of handling mastitis cow	1 = keep in hospital pen 0= keep in home pen	n=9 n=9
7	NUMMAC	Number of mastitis cows in last months	continuous	
8	DH	Detection and handling mycoplasma Mastitis cow	1 = culling 0= no	n=11 n=7

			detection / no culling	
9	NUM	Number of mycoplasma mastitis cows that were culled	continuous	
10	VCC	Vacuum level	continuous	
11	FMMCHK	Frequency of milking machine checking (system)	continuous	
12	MLCLF	Fed mastitis milk to calve	1= yes and no pasteurization 0= no or fed with pasteurized milk	n=5 n=13
13	BED	Bedding type		
14	PUR	Purchase replacements	1= yes 0 = no	n=9 n=9
15	WT	Clean udder with water at the beginning of udder preparation	1=yes 0=no	n=13 n=5
16	TW	Use 1 towel per cow	1=yes 0=no	n=17 n=1
17	PRDIP	Use pre-dipping	1=yes 0=no	n=14 n=4
18	MPDIP	Method of pre dipping	1= spray 2= teat cup 3 = no	n=11 n=3 n=4
19	CONPDIP	Consistency of pre dipping	1= dip all cow 0 = not all	n=14 n=0
20	CF	Fore milk stripping	1=yes 0= no	n=3 n=15
21	DRF	Contact time of pre-dipping solution on	1=dry	n=11

		teats before attachment of milking unit	0= not dry	n=7
22	TMIN	Time between udder dry cleaning and milking unit attachment	continuous	mean=1.6
23	NUMU	Number of milking units	continuous	mean=26
24	NUMMLK	Number of milkers	continuous	mean=1.3
25	UMRATI	Ratio of milking units and milkers	continuous	mean=20
26	GLOVE	Milker wear glove	1=yes 0= no	n=16 n=2
27	METFL	Clean milk presented on the floor immediately	1=yes 0= no	n=14 n=4
28	BFLUSH	Use backflush	1=yes 0= no	n=15 n=3
29	POSTD	Use post dipping	1=yes 0= no	n=18 n=0
30	MPOSTD	Method of Post dipping	1= teat cup 0= spray	n=10 n=8
31	CONPOSTD	Consistency of post dipping	1= dip all cow 0 = not all cow	n=18
32	CPOSDIP	The allocation of post dipping on teats	1=completely 2= partially	n=8 n=10
33	CLFA	Clean parlor floor after milking for each turn	1=yes 0= no	n=18 n=0

B.4 Fourth Study (Chapter 7) raw data

All cows that entered to hospital pen and their duration in hospital pen were recorded.

Code :

ID= cow identification number

Start_risk = Entry Date

End_risk = Exit Date

Duration = number of days in hospital pen

Raw data

ID	Start_risk	End_risk	Duration
1549	26-Apr-09	1-May-09	5
1763	28-Apr-09	3-May-09	5
1841	28-Apr-09	3-May-09	5
1071	29-Apr-09	4-May-09	5
1410	30-Apr-09	5-May-09	5
1873	1-May-09	6-May-09	5
1262	13-May-09	27-May-09	14
1453	4-May-09	14-May-09	10
1476	4-May-09	9-May-09	5
908	5-May-09	10-May-09	5
1539	6-May-09	20-May-09	14
1563	8-May-09	17-May-09	9
10	10-May-09	15-May-09	5
1670	10-May-09	15-May-09	5
1682	13-May-09	18-May-09	5
162	14-May-09	19-May-09	5
748	14-May-09	19-May-09	5
11	17-May-09	31-May-09	14
1643	20-May-09	30-May-09	10
1755	20-May-	25-May-	5

	09	09	
1476	22-May-09	27-May-09	5
196	25-May-09	30-May-09	5
162	26-May-09	31-May-09	5
178	16-May-09	26-May-09	10
1015	28-May-09	31-May-09	3
1876	28-May-09	31-May-09	3
1218	29-May-09	31-May-09	2
1018	24-May-09	31-May-09	7
1869	31-May-09	31-May-09	0

ID	Start_risk	End_risk	Duration
1453	1-Jun-09	11-Jun-09	10
458	2-Jun-09	12-Jun-09	10
578	2-Jun-09	12-Jun-09	10
1866	2-Jun-09	7-Jun-09	5
882	6-Jun-09	11-Jun-09	5
207	9-Jun-09	23-Jun-09	14
1893	11-Jun-09	16-Jun-09	5
1906	11-Jun-09	16-Jun-09	5
1510	7-Jun-09	12-Jun-09	5
1231	15-Jun-09	22-Jun-09	7
1905	15-Jun-09	20-Jun-09	5
1767	16-Jun-09	26-Jun-09	10
876	17-Jun-09	22-Jun-09	5
1889	19-Jun-09	24-Jun-09	5
463	21-Jun-09	26-Jun-09	5
1794	21-Jun-09	30-Jun-09	9
1090	24-Jun-09	29-Jun-09	5
1663	24-Jun-09	30-Jun-09	6
1885	25-Jun-09	30-Jun-09	5
1890	25-Jun-09	30-Jun-09	5
1575	28-Jun-09	30-Jun-09	2
1926	29-Jun-09	30-Jun-09	1
1088	30-Jun-09	30-Jun-09	0

ID	Start_risk	End_risk	Duration
1041	1-Jul-09	6-Jul-09	5
1872	1-Jul-09	6-Jul-09	5
383	3-Jul-09	13-Jul-09	10
578	3-Jul-09	15-Jul-09	12
908	5-Jul-09	10-Jul-09	5
1808	5-Jul-09	10-Jul-09	5
1383	7-Jul-09	12-Jul-09	5
578	8-Jul-09	13-Jul-09	5
1655	9-Jul-09	19-Jul-09	10
1865	9-Jul-09	14-Jul-09	5
891	10-Jul-09	15-Jul-09	5
1182	10-Jul-09	15-Jul-09	5
1279	11-Jul-09	16-Jul-09	5
475	13-Jul-09	24-Jul-09	11
1588	13-Jul-09	26-Jul-09	13
1385	15-Jul-09	20-Jul-09	5
1505	16-Jul-09	21-Jul-09	5
15	17-Jul-09	22-Jul-09	5
205	17-Jul-09	22-Jul-09	5
1829	17-Jul-09	22-Jul-09	5
889	19-Jul-09	24-Jul-09	5
1060	19-Jul-09	24-Jul-09	5
1608	19-Jul-09	29-Jul-09	10
1890	20-Jul-09	25-Jul-09	5
1575	21-Jul-09	26-Jul-09	5
848	23-Jul-09	28-Jul-09	5
1901	23-Jul-09	28-Jul-09	5
1566	24-Jul-09	29-Jul-09	5
1203	26-Jul-09	31-Jul-09	5
1808	26-Jul-09	31-Jul-09	5
1216	29-Jul-09	31-Jul-09	2
1340	29-Jul-09	31-Jul-09	2
1628	30-Jul-09	31-Jul-09	1

Cows that developed mycoplasma mastitis in hospital pen were recorded for their disease status, date of entry, exit date, microbiological result and the occurrence of new mammary infection.

Code :

ID = cow identification number

Date_Entry = entry date to hospital pen

Date_Culling = date of culling

Mastitis = mammary gland that developed mastitis

Result = microbiological culture result relative to Mastitis

New_Mastitis = mammary gland that developed mastitis when cows stayed in hospital pen

Result_2 = microbiological culture result relative to New_Mastitis

Strep = *Streptococcus spp.*

Staph= *Staphylococcus spp.*

Cory = *Corynebacterium spp.*

Myco= *Mycoplasma spp.*

E.coli = *Escherichia coli.*

ID	Date_Entry	Date_Culling	Mastitis	Result	New_Mastitis	Result
1539	6-May-09	27-May-09	RF	Strep	RR	Myco
1563	8-May-09	1-Jun-09	LF	Strep	LR	Myco
1262	13-May-09	29-May-09	RF	Staph	RR	Myco
178	16-May-09	5-Jun-09	RF	Staph	RR	Myco
11	17-May-09	1-Jun-09	LF	Cory	RR	Myco
1018	24-May-09	5-Jun-09	LF	E.coli	LR	Myco
1510	7-Jun-09	24-Jun-09	RF,RR	Strep	LF	Myco
1231	15-Jun-09	25-Jun-09	LF	Strep	RR	Myco
475	13-Jul-09	27-Jul-09	LR	Strep	LF	Myco