ASSAY DEVELOPMENT AND CHARACTERIZATION OF *MYCOPLASMA OVIS*

by

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To all who helped make this happen

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CI	Confidence interval
C_q	Quantification cycle
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

ABSTRACT

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The hemotrophic mycoplasma, *Mycoplasma ovis*, is found in sheep and goats throughout the world. This pathogenic bacterium is capable of causing an acute, life-threatening infection as well as chronic or subclinical infections in these animals. The purposes of the present studies were to develop *M. ovis*-specific assays for detection of this hemoplasma, and to better understand infection dynamics within pregnant ewes and lambs. The first study describes the development and validation of a SYBR® Green quantitative PCR (qPCR) assay, which was subsequently used to determine the prevalence of *M. ovis* infection within a population of goats and to evaluate risk factors for infection. This highly sensitive and specific assay consistently detected as few as 10 copies of plasmid/reaction. Convenience-based sampling of 362 goats from 61 farms located in Indiana revealed a prevalence of infection of 18% (95% confidence interval (CI), 14% to 22%). Bacterial loads of *M. ovis* ranged from 1.05×10^3 to 1.85×10^5 copies/mL of blood with a mean of 1.31×10^4 copies/mL of blood. The only risk factor associated with hemoplasma infection was the production use of the goat; dairy goats had a 3.3 fold increase compared with the prevalence in goats used for meat. This study not only demonstrates that *M. ovis* infection is common in goats in Indiana, but shows the variability of bacterial loads that can be found in chronically-infected animals. While sub-clinically infected goats may have a bacteremia, levels are characteristically less than 2.0×10^5 copies/mL.

The second project utilized a combination of cross-sectional and longitudinal studies to estimate the prevalence of *M. ovis* infection from a cohort of naturally-infected pregnant ewes, assess changes in their bacterial loads, and determine the incidence of *M. ovis* in lambs pre- and post-weaning. The prevalence of *M. ovis* infection in ewes was not found to be significantly different during pregnancy, and before and after weaning of the lambs, with prevalence estimates of 45% (95% CI, 23.1 – 68.5), 36% (95% CI, 17.9 – 57.4), and 44%, (95% CI, 24.4 – 65.1),

respectively. Bacterial loads of the ewes from the cross-sectional study ranged from 10^4 to 10^9 copies/mL of blood, with the median bacterial load at 10^5 copies/mL of blood. While higher bacterial loads are typical of an acute infection, none of the ewes in this study had overt clinical signs. The data suggest that *M. ovis* loads may be higher in pregnant sheep, particularly in ewes half-way through pregnancy. Most of the *M. ovis* infections in the study lambs were detected post-weaning which suggests that transplacental or transmammary infection of *M. ovis* are unlikely routes.

In the third study, a subset of *M. ovis* genes for use in a multi-locus sequence typing assay (MLST) were evaluated. Next-generation sequencing was performed to generate data from pooled DNA amplicons in order to identify single nucleotide polymorphisms (SNPs) of *M. ovis* from five genes. Evaluation of the quality and depths of coverage for the reads and SNPs indicated that the pooled DNA amplicons produced reads and SNPs having high quality and sufficient depth. This pooling technique is a cost-effective alternative to whole-genome sequencing. While the MLST has good discriminatory power and may be used to identify genetically distant and divergent clusters of *M. ovis* from different geographical origins, within a herd the discrimination power is low, which may hamper its usefulness in transmission studies.

The fourth and final study was the development of a loop-mediated isothermal amplification (LAMP) assay targeting the dnaK gene of *M. ovis*, with comparison of the assay to conventional PCR (cPCR). The metal ion indicator hydroxynaphthol blue (HNB) was added prior to the reaction, which allowed for visual detection of LAMP-positive samples as indicated by a color change from violet to sky blue. *Mycoplasma ovis* was consistently detected in 45 minutes with the LAMP assay at a reaction temperature of 64°C, with more infected sheep being detected than by cPCR. Therefore, the LAMP assay is fast and reliable in the detection of *M. ovis*. The developed LAMP assay may have applications in diagnostics, surveillance and disease management as well as prevalence studies. However, a more robust molecular technique is necessary for *M. ovis* isolate or stain discrimination to investigate transmission or disease spread in an outbreak.

In conclusion, three new molecular tools for the detection of *M. ovis* in goats and sheep were developed as results of these studies. We have shown that the qPCR assay is an efficient tool for detection and quantification of *M. ovis* loads in blood from both of these species. On the other hand, the value of the LAMP assay is for reliable detection of infection (not quantification),

especially in resource-limited situations. The five-locus MLST protocol developed herein, a typing assay based on the polymorphism of five gene sequences, is a laborious technique requiring DNA extraction, PCR amplification, purification and sequencing of target loci. The value of this technique is not as a routine diagnostic, but rather it may be used to better understand the genetic diversity of *M. ovis* and investigate strain variations. Most importantly, the scheme is sufficiently robust to allow direct genotyping of *M. ovis* in total blood DNA extracts without culture isolation. The MLST approach may prove useful as a tool for future investigations of transmission and disease spread. These studies have also expanded our understanding of the infection dynamics of *M. ovis* in pregnant sheep and lambs. It is shown herein that despite the high prevalence and sometimes high bacterial loads in pregnant ewes, *M. ovis* does not appear to be transmitted to the lambs in utero or during the perinatal period. The lambs become infected mostly after weaning; this may suggest a protective effect during the pre-weaning period and/or subsequent exposure/infection from their environment.

CHAPTER 1. LITERATURE REVIEW

1.1 Sheep Industry

1.1.1 Worldwide, United States, Indiana

Sheep (*Ovis aries*) are considered a multi-purpose animal and are bred for meat, wool and to a lesser degree, milk (USDA). They are useful in the control of invasive and noxious weeds by grazing on vegetation, and help reduce wildfires by eating grass and brush that would be a fuel source for fires. Worldwide, in 2016, there were over 1.1 billion sheep. China and Australia have the largest populations with approximately 162 and 67 million sheep, respectively (FAOSTAT). The number of sheep in the United States in 2016 was 5.3 million (FAOSTAT) with the majority of farms considered to be small livestock operations, since most had 100 head or fewer. Sheep within the United States are also sold internationally with the majority of exports going to Canada and Mexico (USDA). In 2012, there were an estimated 52,169 sheep in Indiana from 2,109 farms with 70% of the farms having less than 24 sheep (USDA 2012). Although the majority of United States sheep production originates from small operations, over $5,900,000 was generated in Indiana alone between animal sales and wool production (USDA 2012).

1.1.2 Use of Sheep in Biomedical Research

The quality and selection of an appropriate research model is crucial for obtaining meaningful data. Ideally, model selection should be based on accurate representation of the condition being studied, allow for procedures to be performed and ensure availability of reagents for analyses (Scheerlinck, Snibson et al. 2008). Sheep are often used in biomedical research due in part to their quiet nature, size, anatomy, and cost effectiveness to house and feed (Scheerlinck, Snibson et al. 2008). Sheep and lambs have proven useful models for studying lung diseases in humans (Polejaeva, Rutigliano et al. 2016) and infants (Derscheid and Ackermann 2012) as well as cardiovascular research, vaccine development and wound repair and surgical techniques (Scheerlinck, Snibson et al. 2008). Ewes are invaluable not only for orthopedic research (Oheim, Amling et al. 2012), but as a model for the study of placental and fetal physiology and nutrient transfer (Barry and Anthony 2008). Animals used for research are not in their natural habitat and it can be assumed are experiencing stress whether from handling, their health status, environmental

issues or from procedures. While sheep have proven to be critical for a variety of research fields, their use may be impacted by a common, yet often asymptomatic ovine infection. The hemotrophic mycoplasma, *Mycoplasma ovis,* is notorious for establishing chronic infections among sheep. Its spread and clinical signs associated with infection may be exacerbated in animals that are stressed. Considering the wide geographic distribution of *M. ovis* (Jensen 1943, Ohder 1967, Campbell, Sloan et al. 1971, Weikel and Graunke 1995, Hornok, Meli et al. 2009, Tagawa, Takeuchi et al. 2012, Johnson, do Nascimento et al. 2016) and prevalence as high as 80% (Nascimento 2011, Hampel, Spath et al. 2014) in research sheep, it is likely *M. ovis*-infected sheep are unknowingly used in biomedical research. Chronically infected sheep when stressed may experience reversion to an acute, life-threatening infection often characterized by hemolytic anemia and hypoglycemia (Messick 2004). The literature is replete with articles describing reactivation of hemotrophic mycoplasma infections among research animals, resulting in the invalidation of entire studies (Baker, Cassell et al. 1971, Kemming, Messick et al. 2004). While hemotrophic mycoplasma infections are no longer considered a problem in derived mice and rat populations raised under rigorous conditions, it is well known that other animals used for research such as dogs, pigs, goats, and sheep are not free of these infections. The prevalence of hemotrophic mycoplasma infections among animals raised in confined or commercial facilities is quite high (Tagawa, Yamakawa et al. 2013). Since research data may be influenced by an existing sub-clinical infection, it is recommended that sheep and other animals used for research should be screened for species-specific hemotrophic mycoplasma infections using PCR assays. This assay is far more sensitive and specific than blood smear examination for the presence of these bacteria (Hampel, Spath et al. 2014, Johnson, do Nascimento et al. 2016); a recently developed qPCR assay was validated for detection of *M. ovis* in sheep (Johnson, do Nascimento et al. 2016). This is the first step to gain a better understanding of the extent of infection and risk factors for it as well as potential problems that such infections pose to performing research using sheep.

1.1.3 Hemotrophic Mycoplasmas

General Characteristics

Hemotropic *Mycoplasma* spp. (hemoplasmas) are epierythrocytic gram-negative bacteria lacking cell walls which have been found in a range of mammalian species and exhibit an affinity for red blood cells (RBCs) (Messick 2004). Previously known as *Hemobartonella* and

Eperythrozoon spp., work done by Neimark and colleagues (Neimark, Johansson et al. 2001, Neimark, Hoff et al. 2004) based on phylogenetic analyses of several 16S rRNA gene sequences led the way to reclassification of these bacteria from the order *Rickettsiales* to members of the *Mycoplasmataceae* family. The mycoplasmas (class *Mollicutes*) are found on the mucous membranes and the joint surfaces of mammalian and avian species. Most mucosal mycoplasmas and hemoplasmas have a unique codon usage of UGA, encoding for tryptophan instead of the stop codon (Smiley and Minion 1993). Unlike the mucosal and joint surface-associated mycoplasmas, hemoplasmas depend upon the host red blood cell for nutrients (Messick 2004).

Microscopic examination of stained blood smears using Romanowsky-type stains for detection of hemoplasmas demonstrates pleomorphic morphology ranging from coccoid, ring, rod-shaped, and small clusters (Neimark, Johansson et al. 2001) of bacterial organisms on red blood cells, which may result in morphologic changes to the host cells, possibly attributable to mechanical damage (Neitz 1934). While hemoplasmas exhibit a tropism for RBCs, the bacteria have also been found free within the plasma. Electron microscopy of hemoplasmas has shown coccoid or round bacteria with diameters of 0.3 to 3 μM, which lack cell walls and are found within the depressions of the erythrocyte's surface (Messick 2004). Short fibrils visible only on transmission or scanning electron microscopy extend from the bacterial cell to the cell surface of the erythrocyte (Neimark, Hoff et al. 2004). It is hypothesized that the fibrils may be involved with attachment to the host's cell, but their function remains unknown.

Hemoplasma infections can display a range of signs from asymptomatic to hemolytic anemia and death. Animals with latent hemoplasma infections often are asymptomatic, but some hemoplasma species are pathogenic and cause overt disease even with an immunocompetent host (Neimark, Johansson et al. 2001). *Mycoplasma haemofelis* (Willi, Boretti et al. 2007) and *M. ovis* (Neimark, Johansson et al. 2001) are considered primary pathogens, since they are capable of causing overt disease in immunocompetent and non-splenectomized animals. However, many animals that survive acute infection, thereafter become chronic asymptomatic carriers.

Stress, illness, pregnancy, age or immunosuppression can cause reversion of a chronic infection to an acute infection. Cytoadherance phase and antigenic variation of surface proteins to avoid detection by the host's immune system is believed to play a major role in maintaining infection (Citti, Nouvel et al. 2010, Santos, Guimaraes et al. 2011) . The hemoplasma genomes have numerous paralog genes and large paralog gene families whose role is believed to be involved

with evasion of the host's immune system (Santos, Guimaraes et al. 2011, do Nascimento, Dos Santos et al. 2013). Since the hemoplasmas do not have cell walls, antibiotics targeting such are useless. Unfortunately, treatment using tetracycline does not clear the infection, and often the animal becomes chronically infected. Sub-clinically infected animals are believed to comprise the main source of infection within a herd. A previous study by Guimaraes and colleagues (Guimaraes, Vieira et al. 2011) suggested that a pig negative for *Mycoplasma suis* became positive as detected by qPCR after being housed with a *Mycoplasma suis* chronically infected pig.

Hemoplasmas have not been successfully maintained in culture which has made it difficult to elucidate how the bacteria are transmitted. Transmission routes are poorly understood. Blood-feeding arthropods appear to play a role, but experimental transmission of hemoplasmas using arthropods has been inconsistent. A role for fleas, flies, lice, ticks and mosquitoes has been implicated in the transmission of the hemoplasmas in various animals (Foggie and Nisbet 1964, Seneviratna, Weerasinghe et al. 1973, Prullage, Williams et al. 1993, Willi, Boretti et al. 2007, Fyumagwa, Simmler et al. 2008, Hornok, Micsutka et al. 2011, Reagan, Clarke et al. 2017). The transfer of infected RBCs from one animal to another, whether from fighting, vaccination, ear tagging or other blood-producing procedures, may allow for the infection to transfer among herd members (Messick 2004).

1.1.4 Taxonomy

Classification and Phylogeny

The hemoplasmas have been transferred, to the genus *Mycoplasma* within the class *Mollicutes*, following a formal proposal by Neimark (Neimark, Johansson et al. 2001, Neimark, Hoff et al. 2004). Phylogenetic analyses of several 16S rRNA genes of organisms previously classified in the genera *Haemobartonella* and *Eperythrozoon* showed the hemotrophic bacteria were more closely related to members of the genus *Mycoplasma*. However, these bacteria now form a group of erythrocyte-associated organisms within a genus that was associated solely with mucosal and joint surfaces. Since the hemoplasmas remain incompletely characterized and whose broad taxonomic relationships are undefined, they have been given the name *incertae sedis*, within the family *Mycoplasmataceae*.

Based on phylogenetics using the 16S rRNA genes the hemoplasmas are members of the Mycoplasma-Ureaplasma clade (Volokhov, Simonyan et al. 2012) and separate into two clusters

(suis and haemofelis) (Guimaraes, Santos et al. 2014), with a 24 bp deletion of the 16S rRNA genes for members of the haemofelis group; *M. ovis* is a member of the suis cluster (Volokhov, Norris et al. 2011). The hemotrophic mycoplasmas have also been found to be more closely related to members of the pneumoniae group (Messick, Walker et al. 2002) than the hominis and mycoides phylogenetic groups of *Mycoplasma*. However, the pneumoniae group contains five subgroups with the hemotrophic mycoplasmas comprising a cluster that is distinct from other members of the pneumoniae group, but more closely related to three *Mycoplasma* species (*M. cavipharyngis*, *M. insons*, and *M. fastidiosum*) (Volokhov, Simonyan et al. 2012).

1.1.5 Aspects of controversial taxonomy

Uilenberg (Uilenberg, Thiaucourt et al. 2004) took issue with the criteria used to justify the transfer of members of the *Haemobartonella* and *Eperythrozoon* genera to the *Mycoplasma* genus (comparison of only the 16S rRNA genes), and that a broader comparison of genes was warranted. A sequence identity of 77.3% obtained between *M. wenyonii* and *M. fastidiosum*, a close member of the mucosal mycoplasmas, was lower than identities for bacteria from established different genera and below the cut-off for microorganisms of the same genus (Uilenberg, Thiaucourt et al. 2004). Also, the biological differences (ecological niche, transmission, and cultivability) between the mucosal-mycoplasmas and hemoplasmas were further justification for the argument that the hemoplasmas should not be placed within the *Mycoplasma* genus. Additionally, the issue of priority of nomenclature was of concern to Uilenberg and colleagues (Uilenberg, Thiaucourt et al. 2006), since the genus, *Eperythrozoon* was created before the *Mycoplasma* genus, and therefore should have priority.

1.2 Genomic structure and metabolism

In 2014, the complete genome sequence of *M. ovis* strain Michigan was reported (Deshuillers, Santos et al. 2014). A single, circular chromosome comprised of 702, 511 bp was described and falls within the range of genomes published for other hemoplasmas (Santos, Guimaraes et al. 2011, do Nascimento, dos Santos et al. 2014, Guimaraes, Santos et al. 2014). Interestingly, two distinct, but dissimilar copies of the 16S rRNA genes were discovered, which corresponded to sequences previously reported as two distinct species of hemoplasmas infecting sheep (Hornok, Meli et al. 2009). Over 800 protein-coding sequences (CDSs) were predicted, with

approximately 60% of the CDSs encoding hypothetical proteins. The putative function of most of the proteins identified in this study were involved with energy metabolism via the glycolytic pathway and ATP synthesis; both common mechanisms for energy production in hemoplasmas, while 32.4% of the genome was dedicated to duplicate genes organized into paralog families. It has been suggested that this "paralog pool" may be a rich source of different antigenic epitopes that can be varied to elude the host's immune system and establish chronic infection (Santos, Guimaraes et al. 2011). The hemoplasmas are exceptional in that they possess small genomes and are not free living, rather they are extracellular red cell-associated pathogens, openly exposed to a hostile immune environment. Thus, the paralogs provide these organisms with the physiological plasticity they need to survive.

To date, there are no published reports on the computational analysis of the biochemical pathways of *M. ovis*. Therefore, generalizations are based on data from other published hemoplasma research. Guimaraes (Guimaraes, Santos et al. 2014) reported conserved metabolic pathways and only three metabolic pathways which differed among the compared hemoplasmas. Hemoplasmas are glycolytic species that generate energy by ATP-synthase and fermentation of sugars. The Embden-Meyerhof-Parnas Pathway (EMP or glycolysis) is present in the genomes of *M. haemofelis* (Fard 2014)and *M. suis* (Guimaraes, Santos et al. 2011). Hemoplasmas are dependent upon the host (RBCs and plasma) for amino acids, fatty acids, cholesterol, and vitamins (Messick 2004). The enzymes of the pentose-phosphate pathway (PP), pyruvate dehydrogenase complex (PDC) and coenzyme A (CoA) metabolism are not present in the hemoplasmas; believed to be a result of the nutrient-rich environment that blood provides (Guimaraes, Santos et al. 2014).

1.3 Mycoplasma ovis infection

1.3.1 History

Sheep infected with *M. ovis* have been identified throughout the world, being first described in South Africa in 1934 (Neitz 1934). Peculiar, ring-shaped bodies that were 0.5 µm to 1.0 µm in diameter were found incidentally on Giemsa-stained blood smears from a splenectomized sheep infected with *Ehrlichia ruminantium*. Initially, thought to be stained artifacts, they were subsequently determined to be organisms located on the red blood cells and within the plasma. After identifying that organisms were present and not artifacts, the authors

mentioned observing them previously and with some frequency. They proposed the name *Eperythrozoon ovis* after taking into consideration the organism's morphology, association with red blood cells (RBCs), Giemsa staining reaction, and disease outcomes within sheep.

1.4 Epidemiology

Several studies analyzing risk factors of hemoplasma infection in cats have shown that age, breed, outdoor access, location and health status were correlated with significant differences between infected and non-infected cats. Specifically, a significantly higher risk of hemoplasma infection in cats from multi-cat households, particularly male cats that have outdoor access, as well as cats with FIV infection and abortive FeLV infections have been identified (Tasker, Binns et al. 2003, Willi, Boretti et al. 2006, Sykes, Terry et al. 2008, Bergmann, Englert et al. 2017). A prevalence and risk factor analysis of bovine hemoplasma infections in Japan by Tagawa and colleagues (Tagawa, Ybanez et al. 2012) found sampling location, living conditions and age influenced observed significant differences. Cattle one year of age or older were found to be at higher risk for a hemoplasma infection than cattle less than a year old. A study conducted in Australia on 1,820 sheep from 91 farms used a serological assay to detect sheep with *M. ovis*-specific antibodies. They found significantly more farms with sheep producing *M. ovis*-specific antibodies in the Great Southern region, (Kabay, Richards et al. 1991) but were unable to determine risk factors for a farm with a sheep producing antibodies to *M. ovis.* The sample size of sheep tested per farm may have made risk factor analysis difficult as the authors mentioned that the number of sheep tested per farm was too small to estimate the prevalence of *M. ovis* infection with certainty. In another study, a sheep flock in Hungary experienced two hemoplasma infection outbreaks nine years apart. Risk factors for fatal disease outcome were analyzed; it was found that local sheep were less frequently diseased than sheep introduced from an outside source, and sheep born prior to the first outbreak had significantly more animals survive the second outbreak (Hornok, Meli et al. 2009). A cross-sectional study estimating the prevalence of *M. ovis* infection in goats found the production use of the goat (dairy) to be the only statistically significant risk factor when comparing infected and uninfected goats (Johnson, do Nascimento et al. 2016). Dairy goats had a 3.3 fold increase in hemoplasma positivity compared to meat goats.

1.5 Transmission

Several hematophagous vectors, including sheep keds, lice, ticks, mosquitoes, and stable flies, have been implicated in the transmission of *M. ovis* (Foggie and Nisbet 1964, Nikol'skii and Slipchenko 1969, Overas 1969, Howard 1975, Daddow 1980, Hornok, Hajtos et al. 2012). However, their role in the natural spread of infection has not been established. Blood-borne transmission of *M. ovis* associated with procedures such as docking, castrating, and disbudding or fighting among herd members is suspected. Ticks are known to transmit a variety of pathogens to vertebrates, particularly to species of veterinary importance. While reports of humans infected with *Bartonella henselae* after possible exposure to tick bites have been documented, (Maggi, Ericson et al. 2013) the extent of ticks as potential vectors of hemoplasmas has not been sufficiently studied. Two veterinarians were reported as being infected not only with hemoplasmas, but with other bacteria (*Bartonella henselae*, *Anaplasma platys*) possibly transmitted through tick bites (Sykes, Lindsay et al. 2010, Maggi, Ericson et al. 2013, Maggi, Mascarelli et al. 2013). In Brazil, an immunocompromised HIV-positive man was found co-infected with *Mycoplasma haemofelis* and *Bartonella henselae* (dos Santos, dos Santos et al. 2008). Although it was unknown how he became infected with bacteria from cats, this illustrates the zoonotic potential of hemoplasmas, especially in immunocompromised individuals. A report by Maggi and colleagues (Maggi, Compton et al. 2013) showed a greater risk for hemotrophic *Mycoplasma* sp. infection for people in the United States with extensive arthropod and animal contact. Nine of the 11 patients that had amplification of the 16S rRNA gene of a hemotropic *Mycoplasma* species using PCR were found to be infected with a *M. ovis*-like bacterium. In 1973, Seneviratna and colleagues (Seneviratna, Weerasinghe et al. 1973) demonstrated transmission of a dog hemoplasma from the tick, *Rhipicephalus sanguineus*, to splenectomized dogs. Transstadial and transovarial transmission of the hemoplasma in the tick was also demonstrated in this study. A Russian study reported the transmission of *M. ovis* in sheep by the ticks *Hyalomma plumbeum* and *Rhipicephalus bursa*, but evidence beyond this single report is lacking (Nikol'skii and Slipchenko 1969). Sheep co-infected with *M. ovis* and *Anaplasma ovis* believed to have been transmitted through an arthropod vector were documented in a flock having fatal hemolytic anemia (Hornok, Meli et al. 2009).

The stable fly (*Stomoxys calcitrans*) and the sheep ked (*Melophagus ovinus*) have not been confirmed as *M. ovis* vectors, but ground-up lice injected intravenously from an infected sheep to an uninfected sheep led to a transient *M. ovis* infection (Foggie and Nisbet 1964). On the other

hand, lice that fed directly on bacteremic goats were found to be PCR negative and were considered unlikely to be a competent vector for *M. ovis* in this host species (Hornok, Hajtos et al. 2012). The mosquitoes, *Culex annulirostris* (Daddow 1980) and *Aedes camptorhynchus* (Howard 1975) have been reported as *M. ovis* competent vectors under experimental conditions. Numerous sheep studies have anecdotally noted the rise in *M. ovis* outbreaks during the months when blood-feeding arthropods are present. Overas (Overas 1969) set up a series of transmission experiments exposing lambs to *M. ovis*. Urine and feces from heavily infected sheep were given by stomach tube to susceptible lambs with no subsequent infection. He also wiped the mucous membranes of the eyes and mouth of a heavily infected sheep with a tampon and then touched the mucous membranes of several susceptible sheep. No infection was detected. Only when lambs were given heavily infected blood through a stomach tube was subsequent infection detected, but without a pronounced anemia. Interestingly, when newborn lambs from infected ewes were immediately tested after birth and for a while after, no infection was detected until the lambs were 5 months old; thus implying that transplacental and transmammary transmission may not occur. Blood from the newborn lambs from the *M. ovis*-infected ewes was also injected intravenously into uninfected lambs without causing infection. Further strengthening the argument against transplacental or transmammary transmission of *M. ovis*. It is assumed the lambs were not splenectomized or given glucocorticoids prior to the inoculum since the study did not indicate this.

1.6 Host Specificity

The hemoplasmas are primarily host-specific, but *M. ovis* can cross-infect into goats and possibly humans (Sykes, Lindsay et al. 2010, Maggi, Compton et al. 2013). In 1960, Foggie and Nisbet (Foggie and Nisbet 1964) took *M. ovis*-infected blood from a sheep and inoculated it intravenously into 14 mice, seven of which had been splenectomized. Examination of blood smears every four days for 33 days failed to show *M. ovis* bacteria. Hoyte reported that hemoplasmas of cattle, *M. wenyoni* and *M. teganodes,* were not able to survive in a splenectomized sheep that was experimentally infected (Hoyte 1971). However, that same study reported that *M. ovis* infected blood was inoculated into a calf which was then able to transmit viable *M. ovis* to a susceptible sheep nine days after the initial experimental infection. This indicates the bacterium was capable of surviving in a host other than a sheep or goat. A limitation of this study was that detection of infection was based solely on blood smear examination, which is often fraught with false positive

results. In 1963, experimental transmission of *M. ovis* to a splenectomized goat and splenectomized deer (*Dama virginiana*) were reported (Kreier and Ristic 1963). Kreier and Ristic then intravenously inoculated *M. wenyoni* into a splenectomized goat and a splenectomized deer (*Dama virginiana*) , but neither animal developed infection based on Giemsa-stained blood smears (Kreier and Ristic 1963). Daddow reported that blood taken from an aged doe having a low titer on a complement fixation test was used to experimentally infect a susceptible goat (Daddow 1979). This goat developed a bacteremia 25 days post-inoculation and blood was then inoculated intravenously into a sheep that did not have prior exposure to *M. ovis*. Eleven days post-inoculation organisms believed to be *M. ovis* were visible on Giemsa-stained blood smears from the sheep. Transmission studies have shown that sheep and goats are susceptible to experimental infection of *M. ovis*-infected blood from each other (Daddow 1979, Mason, Corbould et al. 1989). In Japan, a study was undertaken to test 19 free-living Japanese serows (*Capricornis crispus*) for the presence of hemoplasma infections (Ohtake, Nishizawa et al. 2011). These even-toed ungulate mammals are considered a Japanese goat-antelope. Phylogenetic studies have shown that members of the genus *Capricornis*, phylogenetically are more closely related to goats and sheep than cattle (Jass and Mead 2004). Positive samples were re-analyzed using conventional PCR which targeted the 16S rRNA gene of hemoplasmas. Sequencing of an amplicon revealed the 16S rRNA gene sequence of the hemoplasma found in the Japanese serows, when compared to the 16S rRNA gene sequence of *M. ovis*, ranged between 99.5 to 99.8% identity (Ohtake, Nishizawa et al. 2011). Additional studies have reported *M. ovis*-like bacteria in South American deer (Grazziotin, Santos et al. 2011) and white-tailed deer (Boes, Goncarovs et al. 2012) having high sequence identity (>98%) of the 16S rRNA gene to *M. ovis* in sheep. A study involving hemoplasma infection in goats in Indiana found positive samples with 98 to 100% identity of the dnaK gene of *M. ovis* among sheep; this genomic marker provides similar data on identity reported for the 16S rRNA in sheep (Grazziotin, Santos et al. 2011).

1.7 Immunity and Susceptibility of sheep and goats to *M. ovis*

Sheep and goats are susceptible to *M. ovis*. However, prevalence in goats has been reported to be lower than that for sheep (Gulland, Doxey et al. 1987, Mason, Corbould et al. 1989, Mason and Statham 1991, Rjeibi, Darghouth et al. 2015). The reason for this discrepancy between prevalence in similar hosts remains unknown. Possible explanations for low prevalence could be

due to a higher resistance to *M. ovis* within goats (biological advantage), too small of a sample size for accurate estimation, poor sensitivity of assay to detect infections, insect vector host preference, or differences within management practices (Mason and Statham 1991, Rjeibi, Darghouth et al. 2015). Whether or not premunition (protecting against high numbers of organisms and illness without eliminating the infection) exists for *M. ovis*-infected animals remains up for debate. Foggie (Foggie and Nisbet 1964) reported on a sheep that had been experimentally infected with *M. ovis* and was reinoculated, three weeks later the bacteremia was still not detectable. Weekly blood smears, up to four week post-reinoculation, did not show reoccurrence of the bacteria. However, it is important to remember that chronically infected animals often have no detectable bacteria on blood smear examination. Foggie also demonstrated the infectious nature of blood from sheep having undetectable *M. ovis* bacteremias on blood smear examination (Foggie and Nisbet 1964). Hornok (Hornok, Meli et al. 2009) reported on the possibility of an immunoprotective effect in some sheep that had been exposed to an earlier outbreak of *M. ovis* within a flock, resulting in greater survival rate of those established sheep compared with new herd members. Ohder (Ohder 1967), after experimentally infecting and re-infecting, sheep reported that the duration of immunity fluctuated considerably.

1.8 Interaction with other infectious agents

While it is known that a resurgence of *M. ovis* may occur during times of stress, the effect a latent infection may have on other infectious agents and ultimately the research data is rarely mentioned in articles. In 1967, Ohder (Ohder 1967) described an initial suppression of Bovine Petechial Fever in sheep that were co-infected with *M. ovis*. Bacteremia levels subsequently increased and the *M. ovis* infection became severe. Foggie and Nisbet (Foggie and Nisbet 1964) reported that sheep co-infected with *M. ovis* and Tick-borne fever (TBF) exhibited differences in the *M. ovis* bacteremia levels among sheep having prior exposure to TBF versus non-exposed sheep. *Mycoplasma ovi*s bacteremias appeared lower in the non-immune TBF sheep and were believed to be due to elevation of body temperature after initial exposure to TBF. In 2013, a case report was published regarding a goat diagnosed with parasitic gastro-enteritis concurrent with *M. ovis* infection (Jesse A, Abdullah et al. 2013). The goat was anemic and required two blood transfusions and interestingly, the authors' mentioned the animal was also stressed due to its malnourished state. Gulland (Gulland, Doxey et al. 1987) reported the experimental infection of

sheep with *Dermatophilus congolensis* during the same time as a *M. ovis* bacteremia was present; implying *M. ovis* may have overwhelmed the immune system of the animals, permitting a recrudescence of a preexisting, chronic *M. ovis* infection. A study on listeriosis in sheep looked at the possibility of *M. ovis*-induced blood changes (hemolytic anemia and acidosis) as a cause of clinical listeriosis (Gronstol and Overas 1980). They found that *M. ovis*-induced blood changes resulted in longer illnesses of *Listeria monocytogenes* in sheep. The animals recovered slower, had a delay in development of antibody titers and reduced daily weight gains compared with sheep that were exposed only to *M. ovis or L. monocytogenes*.

1.9 Clinical signs

1.9.1 Natural infection

Acute disease

Numerous reports of sheep displaying hemolytic anemia, exercise intolerance, staggering, enlarged spleens, jaundice, fever, hemoglobinuria and mortality are present within the literature. Littejohns (Littlejohns 1960) reported mortality in 10% of a flock of lambs with infected lambs showing severe clinical anemia and difficulty walking. Blood smear showed hypochromasia and poikylocytosis in addition to the presence of *M. ovis* on red blood cells. In contrast to other reports, Littlejohn did not find body temperature to be a value or reliable indicator of infection. Maxwell (Maxwell 1969) reported on an outbreak of *M. ovis* infection involving 2-4 month old lambs. Clinical signs among the lambs were loss of condition, paleness of the skin, collapse and death. However, the ewes in the flock were not clinically affected.

Chronic infection

Often adult sheep with sub-clinical infections do not display signs such as lethargy, pallor, low hematocrit, or exercise intolerance. Ganter (Ganter, Bickhardt et al. 1993) described a natural infection of *M. ovis* in ten sheep that experienced only transient bacteremias and transient, mild anemia. He then splenectomized one of the ten sheep which then developed a high bacteremia, hemolytic anemia and hemoglobinuria was detected. Sub-clinically infected sheep from a university were found to have changes to several complete blood count parameters (Hampel, Spath et al. 2014). Although they mentioned that the control group was older than the infected group and

some of the observed changes may reflect age-related differences, rather than differences related *to M. ovis* infection per se.

1.9.2 Experimental infection

Generally, sheep experimentally infected with *M. ovis* develop anemia and fever which can last for weeks. While Neitz (Neitz 1934) failed to detect fever in experimentally infected sheep, he determined that as anemia developed so did the bacteremia. However, as the anemia worsened, few bacteria were detectable. Slight icterus was observed in a few sheep and no deaths were noted. Foggie (Foggie and Nisbet 1964) and Sutton (Sutton and Jolly 1973) were both in agreement that the length of the incubation period depended upon the concentration of the inoculum. Incubation time was inversely proportional to the inoculation dose. However, Sutton (Sutton and Jolly 1973) observed that the time to reach the greatest degree of anemia (21-25 days post-infection) was similar for all of the infecting doses and did not appear to be related to the incubation time. There was also a significant retardation of growth in lambs that had been kept on pasture vs. housed lambs fed *ad lib*, but inappetance occurred with the house lambs that had been experimentally infected. In contrast to this, Foggie (Foggie and Nisbet 1964) did not detect a significant difference in weight gain between infected and control sheep. Ohder (Ohder 1967) splenectomized an infected sheep in order to recover a strain of *M. ovis* that had been maintained through several passages in sheep, but that had gone below detection level. The spleen was found to be very enlarged and was subsequently ground up and inoculated into susceptible sheep. Those sheep developed high temperatures, anemia, pale mucous membranes, increased pulse and respirations, but they did not die.

1.10 Pathology associated with *M. ovis* infection

Clinical Pathology

In 1958, an outbreak associated with *M. ovis* infection occurred in 2-3-month-old lambs. (Littlejohns 1960). Mortality reached 10% of the 390 unweaned lambs. Post-mortem examination of a lamb showed pronounced jaundice, liver lesions, blackening of the subcapsular region of the kidneys, an enlarged spleen, excess pericardial fluid and pallor of several muscles. Gram-negative bacillary bodies were observed from smears of the liver lesions, but these organisms were unable to be cultured aerobically and anaerobically. A 14-week-old intact male lamb housed at a

university sheep research facility in Michigan developed severe anemia with small, basophilic organisms visible on stained blood smear examination (Martin, Chrisp et al. 1988). Despite treatment with oxytetracycline the lamb died. Necropsy examination showed an underweight male lamb with severe icterus of all tissues. The spleen was noted as unusually small, but the cause of the atrophy was not determined. Alpha-hemolytic streptococci were isolated from the left lung and partial autolysis of the kidneys was noted. Extramedullary hematopoiesis was not observed in the spleen, however many hemosiderin-containing macrophages were present. Lambs less than four months of age showed icterus less frequently, but severe anemia was commonly found during post-mortem examinations. (Campbell, Sloan et al. 1971) A pregnant, five-year old ewe from Scotland with clinical signs of ill-thrift, jaundice and anemia was found to have basophilic bodies believed to be *M. ovis* in blood smears (Philbey, Barron et al. 2006). The ewe was treated with 8 mg/kg oxytetracycline given IM daily for 16 days showing improvement in PCV and no bacteria visible on blood smears for up to four months. After lambing, the ewe became ill with suspected bacterial pneumonia and was treated with two doses of 20 mg/kg oxytetracycline. Unfortunately, the anemia became more severe and *M. ovis*-suspect organisms reappeared in blood smears. The ewe was euthanized and upon histological examination hemosiderin was seen accumulated in the proximal convoluted tubular epithelial cells and interstitial macrophages in the kidneys, as well as within other organs (Philbey, Barron et al. 2006). The removal of infected erythrocytes via erythrophagocytosis leads to anemia and hemosiderin accumulation in the tissues (Philbey, Barron et al. 2006). A study by Sutton (Sutton 1978) looked into the pathological changes associated with experimental *M. ovis* infection in male sheep 12 months old or less. He found significant increases in weights of the spleen and liver compared to the control sheep. Increases as much as 250% and 36% at the peak of bacteremia were noted for the spleen and liver, respectively. The enlargement of the spleens were due to lymphoid hyperplasia. The infected sheep had hemosiderin in their kidneys, livers, and spleens when examined at the peak and late stages of bacteremia. However, hemosiderin was also found within the spleens of the control sheep. Intravascular hemolysis was assumed to be the main mode of removing infected erythrocytes due to the presence of hemosiderin in the kidneys. A study by Foggie and Nisbet (Foggie and Nisbet 1964) examined kidneys from 26 sheep either experimentally or naturally infected and found hemosiderin in the largest amounts in the proximal convoluted tubules. In contrast to other reports, no macroscopic abnormalities were

observed in the livers and no evidence of iron in the livers of a subset of the necropsied sheep (Foggie and Nisbet 1964).

1.10.1 Pathophysiology of anemia

Intra and extravascular hemolysis

Intravascular hemolysis in *M. ovis* infection is possibly due to the production of antibodies binding to the RBCs (Fitzpatrick, Barron et al. 1998) resulting from damage to the erythrocytes caused by the bacteria. Anemia due to erythrophagocytosis of infected RBCs has been documented in sheep (Philbey, Barron et al. 2006).

Immune-mediated hemolysis

Positive Coomb's tests have been reported for hemoplasma infected animals (Messick 2004). Sialoglycoconjugates on the red cell membrane have cold-reacting agglutinins directed towards them leading to destruction of the erythrocytes.

Decreased resistance to oxidant damage

Sutton (Sutton 1979) reported findings that showed *M. ovis* had an effect on glutathione which hampered the function of the erythrocytes. Nutrient competition between *M. ovis* and RBCs is believed to reduce available energy resources leading to oxidative stress and reduced life span of the RBCs (Santos, Guimaraes et al. 2011).

1.11 Treatment and Control of *M. ovis*

Reports on the recrudescence of *M. ovis* bacteremias following treatment in sheep are common in the literature. In 1958, an outbreak of suspected *M. ovis* infection in lambs occurred in southern New South Wales (Littlejohns 1960). This outbreak was the catalyst for a series of experiments studying the effects of *M. ovis* on sheep. One experiment assessed the effect of different treatments on eliminating the bacteria. Five different treatments (Stovarsol, Babesan, neoarsphenamine, antimosan, and Rovimix E-vitamin E) were administered as a single dose and only antimosan and neoarsphenamine reduced the bacteremia levels (Littlejohns 1960). However, 100% elimination of the bacteria did not occur and bacteremia levels began to increase as soon as seven days post-treatment. In 1968, oxytetracycline given IM at a dose of 10mg/kg, once daily for

three days, to a subset of lambs having severe anemia was reported as having no effect (Campbell, Sloan et al. 1971). However, the immune status of the flock may have been compromised as moderate numbers of gastrointestinal parasites were reported. Sheriff (Sheriff 1973) reported the use of a sulfur-containing arsenobenzene called Spirotrypan Forte. A large decrease in the bacteremia was observed within an hour of administration of the drug. Unfortunately, half the sheep displayed toxic effects when given a dose of 5 mL IV per 18 to 45 kg/sheep. In another study, non-splenectomized Cheviot and Soay sheep were experimentally infected with *M. ovis* and subsequently treated with two doses of 4 mg/kg of imidocarb dipropionate 24 hours apart (Hung 1986). A drop in bacteremias was noted within 24 hours of the first injection and no bacteria were seen on blood smears 24 hours after the second injection. However, recrudescence of the bacteremias was observed two weeks and four weeks post-treatment for the Soay and Cheviot sheep, respectively. Oxytetracycline given at 20 mg/kg bodyweight IM every third day for two weeks reduced the amount of *M. ovis* bacteria as assessed by blood smear examination from a flock of research sheep, (Martin, Chrisp et al. 1988) but treatment did not eliminate the infection. Researchers in another study used oxytetracycline in a single dose at 20 mg/kg bodyweight subcutaneously and found alleviation of clinical signs took longer in the ewes than the lambs (Weikel and Graunke 1995). Since the majority of infected sheep develop lifelong chronic infection, maintaining immunocompetent animals becomes essential in preventing the resurgence of *M. ovis*.

1.12 Classic diagnosis of *M. ovis* infection and preservation of organisms

1.12.1 Light microscopy

Mycoplasma ovis has been described as having numerous shapes. In 1934, while studying *Ehrlichia ruminantium* infection in sheep, Neitz and colleagues (Neitz 1934) observed small, ring-shaped bodies approximately 0.5-1.0 µm in diameter on some of the red blood cells in Giemsa-stained blood smears. Initially these mysterious bodies were assumed to be artifacts or stain precipitate, but upon repeat blood sampling, based on the findings of chain-formations of these structures on many red cells, it was determined these were organisms. In addition to the frequently observed ring shape, they observed ovoid forms, irregular triangular forms, and rod, dumbbell, and comma-shaped forms. Most commonly the bacteria were found in clusters or chains toward

the cell center, but they were also located on the cell periphery. Extracellular forms were also noted. Kreier and Ristic (Kreier and Ristic 1963) observed *M. ovis* on Giemsa-stained blood smears from an experimentally infected sheep. The most common forms were rods and spheres along the periphery of the erythrocyte. The bacteria appeared to be attached to the surface of the cell when fluorescein-labeled serum from hyper-immunized *M. ovis*-infected animals was used to stain the bacteria. A study by Gulland and colleagues (Gulland, Doxey et al. 1987) using light microscopy found a decrease in the proportions of the sphere and rod forms as the bacteremia increased, but an increase in the proportions of multiple other forms was observed. In another study, ring forms were reduced as the bacteremia increased (Sheriff, Clapp et al. 1966).

1.12.2 Electron microscopy

McKee (McKee, Ziegler et al. 1973), using transmission and scanning electron microscopy, noted *M. ovis* on the surface of erythrocytes and free within the plasma. Other studies using transmission and/or scanning electron microscopy have also identified a single membrane, granular material within the cytoplasm and thin filamentous structures between bacterial cells and the erythrocyte surface (McKee, Ziegler et al. 1973, Neimark, Hoff et al. 2004). Gulland, (Gulland, Doxey et al. 1987) using scanning electron microscopy, reported finding *M. ovis* within erythrocyte surface depressions and chains of the bacteria projecting from the surface. Interestingly, pedunculated protrusions were commonly noted on erythrocytes from *M. ovis*-infected sheep, but rarely seen in uninfected blood. Upon further analysis using transmission microscopy no internal structures were found within the membrane protrusions. In agreement with these findings, Martin (Martin, Chrisp et al. 1988) described finding extracellular spherical bodies and Fitzpatrick (Fitzpatrick, Barron et al. 1998) also concluded that *M. ovis* organisms were not intraerythrocytic. The organisms had a peripheral dense region assumed to be a membrane, while clumps of finely granular material were seen towards their center.

1.12.3 Culture

To date, the hemoplasmas have not been successfully cultured, despite attempts using a variety of media and experimental conditions. Foggie and Nisbet's (Foggie and Nisbet 1964) attempt to grow *M. ovis* using blood or plasma from an infected sheep onto sheep-blood agar or sheep-kidney monolayers failed to produce evidence of growth. Ohder (Ohder 1967) tried to

propagate *M. ovis* using mixed hamster kidney bovine lymphatic tissue culture as well as biological media, but both methods failed. Additionally, inoculations of *M. ovis* in to small laboratory animals, fertile eggs and even subinoculations with allantoic or yolk fluid and embryo tissue in to sheep resulted in no detectable infection. However, Ershaduzzaman (Ershaduzzaman 2001) reported in his thesis that *in vitro* culture and maintenance of *M. ovis* was successfully done up to 17 days using tissue culture media. He also inoculated *M. ovis* into the yolk sac, chorioallantoic membrane and allantoic sac resulting in large numbers of *M. ovis* bacteria seen within the yolk sac membrane. Rouse and Johnson also (Rouse 1966) failed to culture *M. ovis* using a wide range of media and atmospheric conditions.

1.13 Preservation

1.13.1 Storing infected blood

Ohder (Ohder 1967) inoculated *M. ovis*-in

fected blood, stored with and without 30% glycerine, at -20°C in to susceptible sheep. Infection was detected in susceptible sheep that had been inoculated using infected blood within two weeks after collection while freeze-dried blood failed to produce infection. Gulland (Gulland, Doxey et al. 1987) inoculated 17 sheep and two goats with *M. ovis*-positive heparinized blood that had been stored in dimethyl sulfoxide at -113°C. Less than half of the sheep and none of the goats developed a *M. ovis* infection. Baker (Baker, Cassell et al. 1971) reported *Mycoplasma*-infected whole blood became noninfectious after incubation for 3 hours at 37°C, while plasma or whole blood frozen at -3°C retained infectivity for up to 10 days and infectivity extended to several months when frozen at -70°C.

1.13.2 Heat inactivation

Jensen (Jensen 1943) using a sample size of only six sheep determined the thermal death point for *M. ovis* to be greater than 48°C and less than or equal to 50°C. Tubes of blood were heated to either 45°C, 48°C, 50°C or 54°C in a water bath for 10 minutes. However, the report does not include information pertaining to the storage of the heated blood or duration of time between heating and injecting intravenously into the sheep. Infection was diagnosed by observing the bacteria in stained blood smears. From a limited number of trials, it was suggested that thermal death of *M. ovis* occurs after being held for 10 minutes at temperatures above 48^{o}C.

1.14 Detection of DNA of *M. ovis*

1.14.1 Conventional PCR

In the mid-1980's, Kary Mullis developed a method capable of producing millions of copies of a specific portion of DNA from a small sample volume (Garibyan and Avashia 2013) . It was named polymerase chain reaction (PCR) and has since revolutionized molecular biology. Polymerase chain reaction assays are invaluable in the field of infectious diseases and in forensic medicine. Samples can be comprised of genomic DNA or complementary DNA (cDNA) and can be used for cloning and other molecular biology techniques (Ishmael and Stellato 2008).

The process of developing a PCR assay starts with the design of primers *in silico* that have sequence complementarity to the target DNA (Lorenz 2012). This means that knowledge of the DNA sequence is crucial for primer design. A standard reaction mixture is comprised of dNTPs, DNA polymerase, buffer for the DNA polymerase, sterile water and template. Additional reagents can be added as needed depending upon reaction conditions. The PCR process involves three steps: denaturation, annealing, and extension (Lorenz 2012). During the initial denaturation step dsDNA is separated by being heated to approximately 94°C for a minute. The second step is where primers anneal to the complementary strands of DNA once the reaction temperature has been decreased. DNA synthesis occurs in the final step by incorporation of dNTPs to the strand adjacent to where the primers annealed. These steps are repeated 30-40 times resulting in a large quantity of a specific amplicon. Amplified product can be visualized on agarose gel either by staining with ethidium bromide or a similar dye or by labeling the primers or dNTPs with a fluorophore prior to amplification (Garibyan and Avashia 2013). Care must be taken when using ethidium bromide, a known mutagen, since it intercalates between the bases of the DNA strands. The inclusion of a DNA ladder within the agarose gel acts as a molecular marker from which the size of the amplified product can be determined.

Conventional PCR has several advantages such as cost-effectiveness, ease of performance, generation of copious amount of a specific product, and the ability to use the product for cloning or sequencing. However, there are limitations to this assay. Prior knowledge of the target sequence is needed for the proper design of primers. The specificity of the assay can be reduced due to nonspecific binding of the primers to areas other than the intended target. The DNA polymerase is not error proof and unintended variations in base sequences can be incorporated into the newly

synthesized strand. Also, the assay is qualitative and cannot be used to quantify the amount of target within a sample.

Polymerase chain reaction assays have been frequently used for detection of *M. ovis* infections within sheep (Hornok, Meli et al. 2009, Tagawa, Takeuchi et al. 2012, Hampel, Spath et al. 2014, Rjeibi, Darghouth et al. 2015) and goats (Hornok, Hajtos et al. 2012, Machado, Vidotto et al. 2017) throughout the world.

1.14.2 Nested PCR

Nested or semi-nested PCR allows for increased specificity and sensitivity of the assay by using a second primer set, internal to the first primer set, in addition to a second PCR assay that utilizes the template from the first reaction. Song (Song, Song et al. 2014) developed a semi-nested PCR assay for detection of *M. ovis* in goats in China with comparison to a universal PCR. Blood samples from 371 goats from seven different regions within the country were used for comparison. The semi-nested PCR assay was more sensitive than the traditional PCR in detecting infection. Wang (Wang, Cui et al. 2017) reported using a nested-PCR to detect hemoplasma infection in sheep and goats with prevalence of 45.8% and 44.1%, respectively. He also reported a greater prevalence of infection within the grazing sheep and goats vs. their house feeding counterparts.

1.14.3 Real Time-quantitative PCR

Real-time PCR (qPCR) is based on the methodology of PCR, but with the capability to monitor amplification while the reaction is occurring. Denaturation of target DNA, annealing of DNA with oligonucleotide primers, extension of the DNA strand and detection of amplification occurs within the same tube. DNA amplification occurs efficiently during the exponential phase allowing for the measurement of product (Valasek and Repa 2005). This means that the amount of target DNA within a sample can be determined based on when the fluorescent signal reaches a threshold level. Several reporter systems are available such as the DNA binding dye (SYBR® Green) and the hydrolysis probe method (TaqMan®) (Postollec, Falentin et al. 2011). The SYBR® Green dye is less expensive than TaqMan® chemistry, but is not sequence specific like the hydrolysis probe. Any double-stranded DNA that is amplified during the reaction will bind SYBR® Green (Ishmael and Stellato 2008). Therefore, dissociation curves of the amplified product must be included to ensure specificity of the amplification. An important advantage of

qPCR is the level of sensitivity the assay offers. A properly developed qPCR assay may have detection capabilities to less than 5 copies of a target sequence proving invaluable for biomedical research and diagnostic laboratories (Valasek and Repa 2005). Other advantages include minimal chance for cross contamination of samples since the reaction occurs in a closed tube or plate, and the speed of the reaction within the thermocycler. However, proper sample preparation is crucial since inhibitors within biological samples may reduce the efficiency of the PCR. Also, the cost of the real-time platform may be inhibitive in some resource limited laboratories.

The majority of research on hemoplasma infection in sheep has relied on cPCR for detection of the bacterium. However, six out of 11 sheep in Japan were found positive using qPCR (Suzuki, Sasaoka et al. 2011). A real-time qPCR assay for the detection and quantification of *M. ovis* in goats found 61% (37/61) of farms having a *M. ovis*-positive goat (Johnson, do Nascimento et al. 2016). It was found (although not statistically significant) that sheep had a protective effect against a positive result for a goat. The importance of using a sensitive assay for *M. ovis* detection was demonstrated when 8/10 research sheep (believed to have subclinical infections) were positive by qPCR, but cPCR detected only three positive sheep (Nascimento 2011).

1.14.4 Loop-mediated isothermal amplification (LAMP)

In 2000, a highly sensitive and specific assay was developed which amplified DNA under isothermal conditions, and was named loop-mediated isothermal amplification (LAMP) (Notomi, Okayama et al. 2000). This assay uses a DNA polymerase with a high strand displacement activity and requires only a water bath or heat block for the reaction to occur. Four or six primers (two inner, two outer, two loops) recognizing six to eight distinct regions of the targeted gene are used making this assay highly specific. Initially, the inner and outer primers are involved with DNA synthesis and strand displacement, respectively, but as the reaction proceeds to the cycling stage only the inner primers and loops are used. The cycling conditions are rapid and amplification of large amounts of targeted DNA within an hour have been reported (Notomi, Okayama et al. 2000) (Duan, Zhang et al. 2014) (Kim, Kim et al. 2014). Single-stranded DNA synthesis in the early steps of LAMP is initiated from one end of the target sequence by an inner primer and thereafter strand displacement DNA synthesis is begun by the outer primer at the same end. This leads to the displacement of the complementary strand, which then forms a looped out structure at one end due to its complementary sequence design. Another round of DNA synthesis occurs with the inner

primer annealing to the strand with the loop at one end, followed by subsequent strand displacement due to the outer primer. This produces a dumb-bell shaped DNA which is converted to a stem-loop DNA by self-primed DNA synthesis (Notomi, Okayama et al. 2000). The stem-loop DNA is the starting template for the next stage of the reaction. Ultimately, the final products are a combination of stem-loop DNAs of various stem lengths and structures having multiple loops and the byproduct, magnesium pyrophosphate is formed.

Modifications to the traditional LAMP assay have resulted in enhanced diagnostic capabilities. Reverse-transcriptase (RT-LAMP) and multiplex LAMP (mLAMP) are variations from the traditional LAMP, but offer the ability to detect RNA via cDNA and multiple gene targets, respectively (Wong, Othman et al. 2018).

There are various methods for the detection of LAMP products. Visual observation of the presence of a white precipitate or a color change within the tube, presence of ladder-like bands on agarose gel, and also real-time monitoring of turbidity using a turbidimeter. Dyes can be added pre and post-amplification, but cross-contamination of samples may occur when opening tubes after amplification. pH sensitive dyes, metal ion indicators, fluorescent ion indicators, and fluorescent intercalating dyes have been used successfully in various LAMP assays (Goto, Honda et al. 2009) (Fischbach, Xander et al. 2015, Tanner, Zhang et al. 2015).

Advantages of LAMP are the speed of the reaction (<1 hr), high specificity of amplified product, inexpensive equipment for maintaining the reaction temperature, evaluation of results using the naked eye, high sensitivity of the assay, and no required electrophoresis of products (once validated). LAMP assays have been frequently found to be more sensitive than PCR (Li, Yang et al. 2017, Ramos, Munoz et al. 2017, Wong, Othman et al. 2018). Although there are many advantages to LAMP, there are limitations as well. For efficient amplification the size of the product is recommended to remain less than 300 bp (Notomi, Okayama et al. 2000). Due to the large, multi-shaped structures produced by LAMP, the product is not able to be used for cloning or other molecular biological purposes. Primer design can be difficult due to the requirement of multiple primers targeting several different regions on the target sequence. However, primer design software specifically for LAMP is available. The production of millions of copies of the target sequence increases the risk for cross-contamination among samples; therefore, care must be taken to avoid aerosolizing reaction products. Lastly, determination of a positive reaction is

subjective when based on visual observation. Eyesight and perception of color vary among people, so training may be needed to properly identify positives.

Reports of isothermal amplification to detect mucosal Mycoplasmas are common in the literature. To date, only one report has been published using LAMP for detection of a hemoplasma. Song (Song, Wang et al. 2013) from China reported detection of *M. wenyonii*, a cow hemoplasma, using this method. The LAMP assay detected more positive samples then were identified by PCR. In the United States, a LAMP assay for the detection of *M. ovis* was developed and was also more sensitive, but less specific than PCR (unpublished data, Johnson).

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CHAPTER 2. DETECTION OF HEMOPLASMA INFECTION OF GOATS BY USE OF A QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY AND RISK FACTOR ANALYSIS FOR INFECTION

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2.1 Introduction

Hemotropic *Mycoplasma* spp (hemoplasmas) are epierythrocytic bacteria lacking cell walls that have been found in several mammalian species. The bacteria were previously known as *Hemobartonel- la* spp and Eperythrozooon spp, but studies[1,2] conducted by use of the 16S rRNA gene sequences led to reclassification of these bacteria as members of the *Mycoplasma* genus.

Mycoplasma ovis and *Candidatus* Mycoplasma hemovis have been found to infect sheep and goats, whereas *M ovis*–like organisms have been identified in white-tailed deer (*Odocoileus virginianus*), rein- deer (*Rangifer tarandus*), South American deer (*Blastocerus dichotomus*, *Mazama nana*, and *Mazama americana*), and Japanese serows (*Capricornis crispus*).[3-6] Comparison of the 16S rRNA gene sequences of *M ovis* and *Candidatus* Mycoplasma hemovis has revealed several nucleotide mismatches and a 17-bp deletion in the sequence of *Candidatus* Mycoplasma hemovis,[7] which results in 97% identity between these 2 organisms. However, recent evidence suggests that rather than 2 genetically distinct species, a single species infects sheep; this single species has 2 dissimilar copies of the 16S rRNA gene in its genome.[8] Information regarding hemoplasma infections in goats is limited, and the authors are aware of no published reports of hemoplasma infections of goats in the United States. On the other hand, sheep infected with *M ovis* were first described in South Africa in 1934[9] and have subsequently been found throughout the world. Prevalence of hemoplasma infections in Japan and Hungary ranged from 44% to 67%, as determined on the basis of results for a qPCR assay,[7,10] and in China from 26% to 41%,[11] as determined on the basis of results for a conventional PCR assay. Molecular evidence for infection with *Candidatus* Mycoplasma hemovis in sheep in Japan[10,12] and Hungary[7] and goats in Hungary[13] has been reported.

Transmission of infection by several blood-feeding arthropods[14,15,a] as well as by blood-contaminated equipment has been reported. Clinical signs associated with hemoplasma infections in sheep differ depending on whether the infection is acute or chronic. Acute infections are characterized by hemolytic anemia[12] and decreased exercise tolerance.[16] Chronic hemoplasma infections are characterized by a reduction in body weight and ill thrift and are believed to be the main source of infection within a herd. Stress, illness, pregnancy, or immunosuppression can cause reversion of a chronic infection to an acute infection.[17]

Before molecular assays (specifically PCR assays) were available, examination of Giemsa-stained blood films and serologic assays were used to diagnose hemoplasma infections. However, sensitivity and specificity of blood film evaluation are low, and seroconversion indicates only exposure and not necessarily active infection. It is also known that bacterial concentrations in the blood fluctuate during the course of a hemoplasma infection; at their nadir, the concentrations may not be detected by use of microscopy. This is especially true for animals that are chronically infected. Furthermore, it has been reported that the degree of bacteremia in goats detected by use of microscopy[18] is lower than that in sheep; this finding has been substantiated with molecular techniques.[13] The use of more sensitive and specific molecular techniques, such as conventional PCR assays and qPCR assays, has greatly improved the ability to diagnose hemoplasma infection in various host species. However, PCR and serological assays have been used to investigate risk factors for hemoplasma infection in dogs, cats, and sheep.[19-22]

The use of molecular diagnostic methods (specifically qPCR assays) allows for the detection and quantification of hemoplasma infections.[23] Advantages of the use of a nucleic acid dye in a qPCR assay are that it is inexpensive and easy to use and binds to any double-stranded DNA; thus, there is no requirement for design and optimization of probes. Melting curve analysis of the qPCR products also can be used to determine whether single specific products are amplified; thus, nonspecific product formation can be distinguished by use of a melting curve profile of the amplified target DNA.[23,24] However, differentiation of melting curves in a hemoplasma coinfected sample requires that the size and nucleotide sequences selected by the primers differ between the hemoplasma species.[25]

[a] Howard GW. The experimental transmission of *Eperythrozoon ovis* by mosquitoes (abstr). *Parasitology* 1975;71:xxxiii.

It is estimated that there are 41,700 goats and 55,000 sheep in Indiana[26]; however, the prevalence of hemotropic *Mycoplasma* spp–infected goats and sheep in Indiana is unknown. Therefore, the objectives of the study reported here were to develop and validate a qPCR assay for the detection and quantification of *M ovis* in goats, evaluate the prevalence of hemoplasma infection in goats located in Indiana, and determine risk factors for hemoplasma infection in infected goats.

2.2 Materials and Methods

2.2.1 Animals

Blood samples were collected via jugular venipuncture from 362 female goats located on 61 farms in Indiana. Samples were collected between June 2012 and June 2013 and were placed in tubes containing EDTA as an anticoagulant. The goats were involved in an unrelated study[27] designed to estimate the prevalence of infection with *Coxiella burnetii* in goats in Indiana. Inclusion criteria were that does be > 1 year old and not pregnant at the time of sample collection. Each producer selected up to 10 eligible does/farm to be used for sample collection. Each producer provided informed consent for use of the does in the study. All procedures involving goats were approved by the Animal Care and Use Committee at Purdue University.

Most of the farms were located in rural and suburban settings and consisted of goats raised for noncommercial purposes, as determined by one of the authors (AEB). Herd management practices differed greatly among farms, with most goats having substantial access to the outdoors.

2.2.2 Hct measurement and blood film preparation

Measurement of PCV and total solids concentration and evaluation of blood films were performed on all EDTA-anticoagulated blood samples. A plain microhematocrit capillary tube was used to obtain an anticoagulated blood sample from the blood collection tube; microhematocrit capillary tubes were centrifuged for 5 minutes at 12,100 X *g*. Blood films were prepared by use of EDTA-anticoagulated blood stained with modified Wright stain, and the presence or absence of hemoplasmas was assessed microscopically.

2.2.3 DNA extraction

Genomic DNA was extracted from 100 μL of EDTA-anticoagulated goat blood with a commercially available kit[b] used in accordance with the manufacturer's instructions. Extracted DNA was stored at –20°C. Nuclease-free water was extracted as a negative control sample.

2.2.4 Housekeeping gene for the qPCR assay

A housekeeping gene (GAPDH) was used to confirm the presence of amplifiable DNA from all samples. Each sample was assayed in a single well. Forward (5-GGG TCA TCA TCT CTG CAC CT-3) and reverse (5-GGT CAT AAG TCC CTC CAC GA-3) primers were designed[c] to amplify a 176-bp sequence of the GAPDH gene of goats. The PCR mixture contained 12.5 μL of a mastermix,[d] 0.75 μL of each primer (300nM), 6.0 μL of DNase-free water, and 5.0 μL of template DNA. Cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Melting curve analysis was performed on the PCR product by use of a step-up cycling process from 60° to 95°C.

2.2.5 Development of a standard curve

A forward (5-ATT GAA TCA GGT CAG CCA AA- 3) and reverse (5-TCC ATC AGA AAC ATC AAG CA-3) primer were designed[c] on the basis of a fragment of the dnaK gene of *M ovis* strain Michigan (GenBank accession No. CP006935.1).[8] These primers were used to amplify a 498-bp fragment of the gene in a blood sample obtained from an *M ovis*–infected sheep. The PCR product was amplified with a conventional PCR assay and purified from an agarose gel by use of a commercially available kit.[e] Cloning of the products was conducted in accordance with the manufacturer's protocols by use of a commercial vector system.[f] Plasmids were extracted from transformed strain JM109 cells by use of a commercially available kit.[g] A recombinant plasmid containing the fragment of the dnaK gene of *M ovis* (which was confirmed by Sanger sequencing) was used as a template for creating standard curves for quantification of hemoplasma DNA in

[b] Quick-gDNA Miniprep, Zymo Research Corp, Orange, Calif.
[c] Integrated DNA Technologies Inc, Coralville, Iowa.
[d] 2X SYBR Green Mastermix, Applied Biosystems Corp, Foster City, Calif.
[e] QIAquick gel extraction kit, Qiagen, Germantown, Md.
[f] pGEM-T easy vector system, Promega Corp, Madison, Wis.
[g] QIAprep spin miniprep kit, Qiagen, Germantown, Md.

unknown samples. Concentration of plasmid containing the dnaK insert was 49 ng/μL as assessed by use of a spectrophotometer.[h] The number of copies of the recombinant plasmid was determined through the use of an online calculator.[i] Values used included the following: amount of DNA = 49 ng and length of template = 3,015-bp vector + 498-bp insert = 3,513 bp, which resulted in 1.29 X 10^{10} copies. Subsequently, 10-fold serial dilutions of plasmid DNA (10^9 copies of plasmid/ reaction to 1 copy of plasmid/reaction) were prepared by adding 15.5 μL of recombinant plasmid DNA to 184.5 μL of 1X tris-EDTA buffer combined with herring sperm DNA[j] (30 μg/mL). The standard curve was created by plotting the logarithm of the initial copy number of input plasmid DNA against the C_q value.

2.2.6 Primers for real-time PCR assay for detection of *M ovis*

Forward and reverse primers were designed on the basis of a fragment of the dnaK gene of the published *M ovis* strain Michigan genome[8] by use of primer design software.[28] The forward primer was dnaK F (5-TGC TTT GCT AAT TGA GGT TCC-3), and the reverse primer was dnaK R (5-TTG TTG GAG AAA GCG CTA AGA-3).[c] Use of these primers resulted in a 194-bp product.

Optimal primer concentrations were determined by testing combinations of forward and reverse primers in triplicate at 200, 300, 500, and 700nM by use of plasmids containing a fragment of the dnaK gene of *M ovis*. The lowest concentration of a primer that did not cause dimer formation and had a low C_q value was selected. The reaction mixture consisted of 12.5 μL of a mastermix,[d] 200 to 700nM forward and reverse primers, 5.0 μL of template DNA, and sufficient DNase-free water to achieve a final volume of 25 μL. The PCR protocol used was the same as that used previously for the GAPDH gene. To determine the optimum annealing temperature of the primers dnaK F and dnaK R, the qPCR assay was performed over a range of temperatures (57° to 62°C) by use of duplicates at each of several plasmid copy numbers (1 X 10^9, 1 X 10^7, 1 X 10^5, 1 X 10^3, and 1 X 10^1).

[h] Nanodrop 1000 spectrophotometer, ThermoScientific, West Palm Beach, Fla
[i] URI Genomics and Sequencing Center, Kingston, RI.
[j] Sigma-Aldrich Corp, St Louis, Mo

2.2.7 qPCR assay

A thermocycler was used for absolute quantification assays [k] with plasmid DNA serial dilutions included in each run. A no-template sample that used DNase-free water was included in each reaction as a negative control sample, whereas DNA from the blood of an infected sheep (confirmed by qPCR assay and DNA sequencing) was used as a positive control sample. The reaction was performed in a final volume of 25 μL, and samples were assayed in duplicate. The PCR mixture contained 12.5 μL of a commercially available mastermix,[d] 0.5 μL of each primer (200nM), 6.5 μL of DNase-free water, and 5.0 μL of template DNA. Cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 61°C for 1 minute. Melting curve analysis was performed on the PCR product by use of a step-up cycling process from 61° to 95°C.

2.2.8 Validation of the qPCR assay

Efficiency of the primers for DNA detection or limit of detection was determined by use of standard curves generated from 10-fold dilutions of plasmids containing the dnaK gene insert. Interassay variability was assessed by assaying triplicates of all concentrations of the plasmid dilution on 5 days. Intra-assay variability was performed on a single qPCR assay with 5 replicates of each of the plasmid dilutions. Analyses of the results included determination of the coefficient of variation, mean coefficient of variation, and amplification efficiency. The DNA extracted from mycoplasmas known to infect goats or sheep (*Mycoplasma mycoides* subsp *capri*, *Mycoplasma arginini*, *Mycoplasma agalactiae*, *Mycoplasma ovipneumoniae*, *Mycoplasma capricolum* subsp *capripneumoniae*, and *Mycoplasma mycoides* subsp *mycoides*) and other bacteria that may be associated with septicemia in these ruminant species (*Borrelia burgdorferi*, *Pasteurella multocida*, *Escherichia coli*, *Clostridium perfringens*, *Clostridium difficile*, *Listeria monocytogenes*, *Listeria ivanovii*, *Yersinia pseudotuberculosis*, *Mannheimia haemolytica*, *Leptospira interrogans* serovar pomona, *L interrogans* serovar icterohemorrhagiae, and *Salmonella enterica* serovar Typhimurium) was used to determine specificity of the qPCR assay. Additionally, DNA extracted from hemotropic mycoplasmas (*Candidatus* Mycoplasma turicensis, *Mycoplasma hemofelis*, *Candidatus* Mycoplasma hemominutum, *Mycoplasma parvum*, *Mycoplasma suis*, *Mycoplasma*

[k] 7300 Real-time PCR system, Applied Biosystems, Foster City, Calif.

hemocanis, and a hemoplasma from a deer) was also included. Bacterial pathogens were grown in solid or liquid media, and DNA was extracted by use of a commercially available kit.[l] Conventional PCR assays with universal eubacterial primers described elsewhere[29] were used to confirm successful DNA extraction from the bacteria.

2.2.9 DNA sequencing

To confirm the target size and specificity of the products, DNA sequencing of amplicons from a subset (approx 15%) of field samples was performed; C_q values for the samples ranged from 29.2 to 34.0. The qPCR amplicons were separated by electrophoresis on a 3% agarose gel and were subsequently stained with ethidium bromide. A commercially available kit[e] was used for purification of DNA from the gel. The fragment of the dnaK gene was directly sequenced in both directions by personnel at the Purdue Genomics Core Facility using the previously described qPCR primers dnaK F and dnaK R.

2.2.10 Statistical analysis

Variables assessed as risk factors for hemoplasma infection in the goats included age, herd size, production use, farm location, lactation status, and the presence of sheep on a farm. Mixed logistic regression, with farm as a random effect, was used for the analysis of risk factors on both farm and goat levels. Age, herd size, production use of a goat, and farm location were assigned to categorical groups prior to analysis. For statistical comparisons, 3 age groups were created: < 2 years old (n = 106), 2 to < 8 years old (232), and ≥ 8 years old (22). Three groups were created for herd size: < 14 goats (n = 104), 14 to < 37 goats (151), and ≥ 37 goats (96). Two categories of production use were created: dairy (n = 267) and meat (94). Three regions for farm location in Indiana were created: Northern (n = 96), Central (160), and Southern (106). The Fisher exact test was used to assess whether anemia status was associated with a positive result for the qPCR assay. The Spearman rank correlation coefficient was used to assess the association between PCV and hemoplasma load in blood. All analyses were performed with commercially available statistical software.[m,n] The 95% CI was calculated for observed prevalence. Values of $P < 0.05$ were

[l] ZR fungal/bacterial DNA miniprep, Zymo Research Corp, Orange, Calif.
[m] IBM SPSS Statistics for Windows, version 19.0, IBM Corp, Armonk, NY.
[n] SWOG Statistical Center, Seattle, Wash.

considered significant. Samples that yielded indeterminate results for the qPCR assay were categorized as negative for analysis purposes.

2.3 Results

2.3.1 Goats

Blood samples were obtained from 362 female goats located on 61 farms in Indiana for use in a qPCR assay. Goats ranged from 1 to 14 years of age (median, 2 years); the age of 2 goats was not reported. Herd size ranged from 2 to 150 goats (median, 20 goats); herd data were not available for 11 goats. There were 267 goats used for dairy production and 94 used for meat production; production use for 1 goat was not reported.

2.3.2 Validation of the qPCR assay

Concentration for the forward and reverse primers was 200nM. Reaction efficiency of the qPCR assay ranged from 90.1% to 98.5% during testing with annealing temperatures of 57° to 62°C; therefore, the assay was subsequently performed at an annealing temperature of 61°C. Reaction efficiency of the qPCR assay was 94.45% (R_2 = 0.99; slope = –3.4623; Figure 2.1). Results for the qPCR assay were linear over 8 orders of magnitude; the qPCR assay detected 1 copy of plasmid/reaction in 45% of the assays. Thus, the limit of detection by use of the plasmid dilutions was 10 copies/reaction, which resulted in consistent detection in 100% of the assays. Intra-assay and interassay repeatability of the qPCR assay were determined (Table 2.1). The C_q value used for the cutoff for samples with positive results was 34.0. This value was determined by calculating the mean of the mean C_q values for 10_1 and 1 copy of plasmid/reaction from the interassay data. Samples from goats infected with *M ovis* were used to assess the ability of the qPCR assay to detect replicates with C_q values < 34.0 and > 34.0. Four to 6 replicates of each of 5 samples with a C_q value < 34.0 were tested. The assay detected *M ovis* in 93.3% of the replicates. However, when 6 replicates of each of 3 samples with a C_q value > 34.0 were tested, detection of *M ovis* decreased to 72.2%.

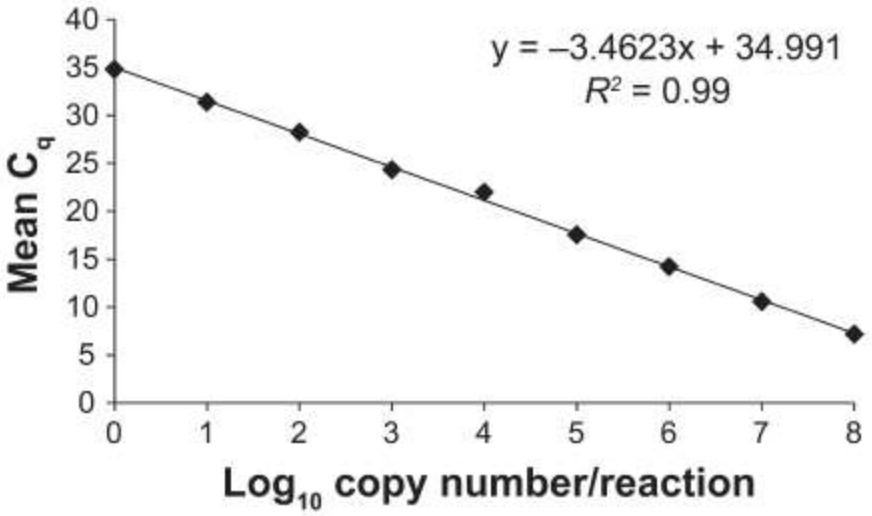

Figure 2.1. Standard curve for 10-fold serial dilutions of a plasmid containing a fragment of the dnaK gene of *Mycoplasma ovis*. The amplification efficiency was calculated as $10^{(-1/slope)} - 1$; the amplification efficiency was 94.4%.

Table 2.1. Intra-assay and interassay repeatability of a qPCR assay developed for the detection of *Mycoplasma ovis* infection in goats.

	Intra-assay repeatability*		Interassay repeatability*	
Plasmid copies	C_q†	CV (%)	C_q†	CV (%)
10^9	5.42 ± 0.35	6.47	5.81 ± 0.15	2.60
10^8	7.18 ± 0.05	0.67	7.37 ± 0.12	1.62
10^7	10.58 ± 0.06	0.58	10.89 ± 0.12	1.11
10^6	14.22 ± 0.07	0.49	14.55 ± 0.14	0.96
10^5	17.54 ± 0.13	0.72	18.02 ± 0.11	0.64
10^4	21.99 ± 1.67	7.55	21.89 ± 0.16	0.74
10^3	24.34 ± 0.08	0.33	24.89 ± 0.16	0.66
10^2	28.25 ± 0.32	1.16	28.76 ± 0.23	0.81
10^1	31.37 ± 0.63	2.01	32.21 ± 0.46	1.44
1	34.81 ± 0.80	2.29	35.94 ± 1.56	4.36

*Plasmid was diluted in 1X tris-EDTA buffer combined with herring sperm DNA (30 µg/mL). †Value reported is mean ± SD.
CV = Coefficient of variation.

2.3.3 Specificity of the qPCR assay

There was no amplification of DNA from any of the bacterial pathogens tested by use of the primers developed on the basis of a fragment of the dnaK gene of *M ovis*. When the universal

eubacterial primers were used, a band with the expected size was present for all of the tested bacteria, which indicated successful DNA extraction.

2.3.4 Results of the qPCR assay and microscopy

There was amplification of the targeted sequence for the housekeeping gene GAPDH for all DNA samples, which indicated successful DNA extraction. Mean melting temperature for the GAPDH amplicons was 82.6°C. Of the 362 samples, 65 (17.9%; 95% CI, 14.1% to 22.3%) had positive results for *M ovis* when tested by use of the qPCR assay. Mean C_q was 32.1 (range, 26.4 to 34.0), and mean melting temperature was 74.6°C. Bacterial loads in goats infected with *M ovis* ranged from 1.05 X 10^3 target copies/mL of blood to 1.85 X 10^5 target copies/mL of blood (mean, 1.31 X 10^4 target copies/mL of blood). An additional 60 samples with C_q values > 34.0 (range, 34.1 to 39.9; mean, 35.5) and mean melting temperature of 74.7°C were categorized as indeterminate. No bacteria were observed on blood films of samples collected from any of the goats.

2.3.5 DNA sequencing

Sequencing of amplicons of 11 of 65 (16.9%) samples that yielded positive results by use of the qPCR assay were confirmed to be *M ovis*. These amplicons had 98% to 100% identity with the dnaK gene of the *M ovis* strain.

Table 2.2. Risk factors for *M ovis* infection in goats in Indiana (362 goats located on 61 farms).

Variable	OR	95% CI	*P* value*
Age (y)			0.993
< 2 vs 2 to < 8	0.96	0.49–1.87	
< 2 vs ≥ 8	1.01	0.33–3.04	
2 to < 8 vs ≥ 8	1.05	0.34–3.19	
Herd size (No. of goats)			0.727
< 14 vs 14 to < 37	0.89	0.42–1.89	
< 14 vs ≥ 37	1.23	0.52–2.89	
14 to < 37 vs ≥ 37	1.38	0.60–3.18	
Farm location in Indiana			0.707
North vs Central	1.35	0.63–2.91	
North vs South	1.32	0.52–3.33	
Central vs South	0.97	0.44–2.16	
Lactation status			0.273
Yes vs no	1.5	0.71– 3.37	
Presence of sheep on farm			0.187
Yes vs no	0.51	0.18–1.41	
Production use			0.004
Dairy vs meat	3.3	1.49– 7.31	

*Values were considered significant at $P < 0.05$.

2.3.6 Prevalence of infection and risk factors for infection

Prevalence of hemoplasma infection in goats, as determined by use of the qPCR assay, was 18% (95% CI, 14% to 22%), and 61% (95% CI, 47% to 73%) of farms had at least 1 hemoplasma-infected goat. Goats used for dairy purposes had a significantly ($P = 0.004$) higher prevalence of hemoplasma infection (3.3-fold increase; 95% CI, 1.5- to 7.3-fold), compared with the prevalence in goats used for meat (Table 2.2). Frequency of hemoplasma infection did not differ significantly among age groups; it was 17% (95% CI, 10% to 25%) for the youngest age group, 18% (95% CI, 14% to 24%) for the middle age group, and 14% (95% CI, 3% to 35%) for the oldest age group. Frequency of hemoplasma infection did not differ significantly among herd sizes; it was 18% (95% CI, 11% to 27%) for the smallest herds, 19% (95% CI, 13% to 26%) for the intermediate-sized herds, and 15% (95% CI, 8% to 23%) for the largest herds. No significant correlation ($\rho_s = 0.045$; $P = 0.727$) was found between PCV and hemoplasma blood load for 64 samples with positive results for the qPCR assay. Analysis of anemia status and positive results for the qPCR assay

revealed no significant (P = 0.515) correlation. Mean ± SD PCV for goats with positive and negative results for the qPCR assay was 26.9 ± 4.6% and 27.2 ± 5.2%, respectively.

2.4 Discussion

To the authors' knowledge, the study reported here was the first in which hemoplasma infection in goats in the United States has been described. In the present study, development and validation of a qPCR assay for the detection of *M ovis* in the blood of goats were described, and risk factors for infection were analyzed. Results indicated that the qPCR assay had high sensitivity for detection of *M ovis* in goat blood samples. Ten copies of plasmid/reaction were consistently detected. The positive cutoff was set at a Cq value of 34.0 to minimize the amount of indeterminate results. However, because DNA sequencing of amplicons from samples with indeterminate results was not performed, it is difficult to know whether the positive cutoff value was selected correctly.

Reports about the prevalence of *M ovis* infection in goats determined by use of molecular methods are scarce. In the study reported here, prevalence was 18% (65/362), which is similar to results in a study[13] of the prevalence in goats in Hungary (20% [4/20]).[13] Goats in that study,[13] similar to goats in the present study, were in good physical condition and were not anemic. A study[11] in which investigators used a semi-nested PCR assay to detect *M ovis* in goats in China revealed a prevalence of 41% (151/372), but use of a PCR assay on the same samples yielded a prevalence of only 26% (97/371). Although prevalence of *M ovis* infection in sheep in the United States, as determined on the basis of PCR assays, is largely unknown, a prevalence of 14% for subclinical hemoplasma infection of sheep in Michigan was reported in a recent study.[30] In sheep in Japan with no apparent signs of infection, prevalence was 24% (as determined by use of a PCR assay).[12] On the other hand, 67% and 55% of sheep with clinical signs in Hungary[7] and Japan,[10] respectively, were infected with *M ovis*, as determined on the basis of testing with a qPCR assay.

Information is limited regarding the herd prevalence for *M ovis* in goats. A herd prevalence of 61% (37/61) in the present study was, to the authors' knowledge, the first estimated herd prevalence determined by use of an appropriate sample size. Investigators of the study[13] of goats collected samples from 20 of 310 goats in only 2 herds (150 goats of one herd and 160 goats of the other herd); therefore, an appropriate comparison of herd prevalence estimates cannot be made.

Several hematophagous vectors have been implicated in the transmission of *M ovis*[14,15,a]; however, their role in the natural spread of infection has not been established. It is believed that

the successful transfer of erythrocytes infected with *M ovis* requires high amounts of biting activity by vectors when circulating bacterial loads are low.[31] However, when bacterial loads are high, such as during an acute infection, biting activity of vectors can be low. Investigators of previous studies[18,32] on hemoplasma infection in goats and sheep have reported lower numbers of circulating bacteria as well as lower antibody titers and a lack of clinical signs in goats, compared with results for sheep. Bacterial loads of 4 goats from the 150-goat herd in the study[13] in Hungary ranged from 62 to 1,160 copy numbers/µL of blood (mean, 470.5 copy numbers/µL of blood). In the study reported here, mean bacterial load for 65 goats was 13.1 copy numbers/µL of blood (range, 1 to 185 copy numbers/µL of blood). Goats from the 150-goat herd of the Hungarian study as well as from the present study were in good physical condition. The low copy numbers per microliter of blood detected in goats of both studies, in addition to the lack of clinical signs, suggested chronic infection.

Not surprisingly, microscopy in the study reported here was found to be insensitive for detecting hemoplasma infections in goats with low bacteremia. A study[o] that involved 10 research sheep (1 was anemic and 9 were healthy) determined that half the sheep had positive results for *M ovis* infection when tested by use of a qPCR assay; however, no bacteria were detected by use of light microscopy for the 9 healthy sheep. Four healthy sheep had bacterial loads that ranged from 1.3 to 760 copies/µL, which suggested chronic infection, whereas the anemic sheep had 10^7 copies/µL, which was consistent with an acute infection.[o] The ability of a qPCR assay to detect and quantify low-level hemoplasma infections in the absence of clinical signs may be particularly useful in epidemiological studies and for reducing the rate of false-negative results.

Although the pathogenicity of *M ovis* in goats is unknown, no correlation between *M ovis* blood loads and PCV, nor between anemia and positive results for the qPCR assay, was detected in the present study. It is possible that there was too few samples with positive results (n = 65) to enable us to detect a correlation, or perhaps antimicrobials were administered to some animals, which could have caused the hemoplasma blood load to be below the detection limit of the assay. However, studies of alpacas confirmed to be chronic carriers of *Candidatus* Mycoplasma haemolamae did not detect a significant association between bacterial load and clinical signs or anemia,[33] and no correlation was detected between PCV and positive results for a qPCR assay.[34]

[o] Deschuillers PL, Santos AP, do Nascimento NC, et al. Detection of *Mycoplasma* ovis and '*Candidatus* Mycoplasma haemovis' in sheep by conventional and quantitative PCR (abstr), in *Proceedings*. 47th Annu Meet Am Coll Vet Pathol–Am Soc Vet Clin Pathol 2012;E63.

In general, chronic hemoplasma infection in an otherwise healthy animal does not lead to overt disease and anemia. Nevertheless, more subtle changes, such as production-related issues (growth rate and weight gain, susceptibility to other infections, and reproduction issues) and modulation of innate and adaptive immune responses, need to be further investigated in animals with chronic infection.

Investigators of other studies conducted to analyze risk factors for hemoplasma infection in cows and sheep have reported that farm location[22,35] and living conditions and age[35] were significantly different between infected and noninfected animals, and only the production use of a goat (dairy vs meat) was found to have a significant effect on infection status in the present study. Dairy goats had a 3.3-fold increase for hemoplasma infection, compared with that of meat goats (Table 2.2). Although it was not significant, lactating goats had a 2.5-fold increase in hemoplasma infection, compared with that of non-lactating goats. The small sample size, in particular the low number of meat goats that had positive results and that were lactating, limited our ability to test for differences in effects of lactation and positive results for the qPCR assay between meat and dairy goats. It is possible the immunosuppressive effects during birthing and the continued care of a kid (or kids) could cause a recrudescence of bacteremia in these animals during stressful periods. There is a decrease in immune function during the periparturient period in sheep.[36,37] However, a larger sample size consisting of lactating and nonlactating goats would be needed to further investigate this relationship.

A limitation of the present study was that information collected through questionnaires was geared toward identifying risk factors for *C burnetii* infection. Thus, specific questions pertinent to the transmission of hemoplasmas (eg, exposure to blood-sucking parasites, population density of the herd [population per unit area], and detailed housing aspects) were not posed. Also, the study reported here involved the use of convenience-based sampling (does at least 1 year old and not pregnant were inclusion criteria), so the estimated prevalence obtained may not have been representative of goat herds in Indiana.

The presence of sheep on goat farms was found to have a protective effect against a positive result for the qPCR assay in samples obtained from goats, although the finding was not significant (Table 2.2). This outcome raised questions as to whether confounding variables were influencing the relationship between the presence of sheep on goat farms and goats having a positive result for

the qPCR assay. Overall, the small sample size, in particular the low number of goats with positive results for the qPCR assay, limited our ability to test for interactions among risk factors of interest.

In the present study, a qPCR assay was developed; the assay was sensitive and specific for the detection and quantification of *M ovis* in infected goats. This assay may be especially useful in epidemiological studies to further characterize subclinical hemoplasma infection in goats.

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2.5 References

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CHAPTER 3. CROSS-SECTIONAL AND LONGITUDINAL STUDIES OF *MYCOPLASMA OVIS* IN PREGNANT EWES AND LAMBS

3.1 Introduction

Hemoplasma infection in sheep has been documented throughout the world (Overas 1969, Hornok, Meli et al. 2009, Nascimento 2011, Deshuillers, Santos et al. 2014, Wang, Cui et al. 2017) and acute infection with high bacterial loads may lead to severe anemia. Historically, infection was diagnosed by blood smear evaluation which revealed bacteria on red blood cells (Neitz 1934). This method was not always reliable in detecting infection and on occasion, splenectomy or inoculation of blood from a suspect-positive sheep into a naïve one was necessary to demonstrate infection (Harbutt 1969). The development of molecular-based methodologies, such as PCR and real-time qPCR, has greatly improved our ability to detect hemoplasma infection in chronically infected sheep. Animals with chronic infections have lower bacterial loads usually $<10^4$ copies /uL and are asymptomatic, whereas animal that have acute infections typically have loads in excess of 10^6 copies/uL and these animals may become profoundly anemic. Unfortunately, sheep under periods of stress may have recrudescence of a previously controlled hemoplasma infection, resulting in hemolytic anemia and possibly death. Routes of transmission are incompletely characterized, but the transfer of infected blood is required (Daddow 1979).

Sheep have been used in biomedical research for a variety of purposes (Barry and Anthony 2008, Scheerlinck, Snibson et al. 2008, Derscheid and Ackermann 2012, Oheim, Amling et al. 2012), often without concern as to their hemoplasma infection status or the effect that an infection may have on the study's outcomes. In 2014, a flock of research sheep comprised of wethers and ewes, believed to have subclinical hemoplasma infections, were tested using a PCR assay. Approximately 14% of the sheep were positive. Further, this research group showed that hemoplasma infection was associated with an increased membrane fragility of the red blood cells (Hampel, Spath et al. 2014). Even before the development of molecular assays, researchers understood and acknowledged the possible confounding effect that subclinical *M. ovis* infection of sheep might have on experimental studies. *Mycoplasma ovis* has the potential to muddle research results, and screening of sheep intended for research should be utilized (Baker, Cassell et al. 1971). Unfortunately, there is a lack of information regarding the frequency of *M. ovis* infections among sheep that are used in research studies.

The Purdue University's Animal Sciences and Research Education Center (ASREC) sheep farm is comprised mainly of breeding ewes and lambs, and supplies sheep for biomedical research. The prevalence of *M. ovis* infection in this population of potential research sheep was unknown. Therefore, the aim of the present study was to characterize *M. ovis* infection in naturally-infected ewes and lambs using cross-sectional and longitudinal studies, and to gain insight into possible transmission routes. Our primary hypotheses were that: 1) *M. ovis* is common in sheep intended for biomedical purposes; 2) the prevalence of *M. ovis* infection in mature ewes varies among pregnancy, and pre- and post-weaning of lambs; 3) bacterial loads in infected adult ewes varies among pregnancy, and pre- and post-weaning of lambs; and 4) the incidence of *M. ovis* infection in lambs is higher post-weaning versus pre-weaning. Our secondary hypotheses were that: 1) there is a negative association between PCV and bacterial loads in positive ewes; 2) PCV (2-6 days of age) will be different between lambs born from infected and uninfected ewes.

3.2 Materials and Methods

3.2.1 Study population

All studies involving sheep were approved by the Animal Care and Use Committee at Purdue University. Pregnant ewes and lambs located at Purdue's ASREC sheep farm were targeted for inclusion in the projects at the discretion of the herdsman.

3.2.2 Sampling strategy

Between December 2016 and January 2017, whole blood was collected from 87 pregnant ewes for *M. ovis* testing. This cross-sectional study was undertaken to determine the prevalence of *M. ovis* within the pregnant ewe population.

A subset of the 20 ewes that had given birth within the previous seven days in February 2017 were selected for participation in a longitudinal study which lasted from December 2016 through June 2017. Ewes had whole blood sampled five different times: once while pregnant, three times at monthly intervals while lambs were suckling, and once post-weaning of lambs. Milk was collected three times after parturition and prior to the lambs being weaned, but on the same days as blood collection. The lambs had six sampling times: three pre-weaning and three post-weaning. The first sampling was within seven days of birth while the remaining two pre-weaning

blood samples were at monthly intervals. The first post-weaning sampling was at a monthly interval followed by biweekly sampling the subsequent month.

Additionally, samples from five ewes and their lambs that were collected from July through November 2016, were also included in the longitudinal study. Ewes that had given birth within the previous 30 days were selected for inclusion in the study. The ewes and their lambs were tested for *M. ovis* over the course of six visits with three samplings occurring prior to weaning of the lambs. The initial two samplings occurred within a week of each other soon after the ewe gave birth, with subsequent samplings at monthly intervals. Ewes had whole blood, milk and feces collected at each visit, while the lambs had whole blood and feces collected. Whole blood and milk samples were tested for *M. ovis*, while feces were used to determine the presence of gastrointestinal parasites and fecal egg and oocyst counts when applicable. Anemia may develop in sheep infected with gastrointestinal nematodes (GIN), (Littlejohns 1960, Preston, Sandeman et al. 2014). Therefore, knowledge of a flock's GIN infection levels through the use of fecal egg counts is not only crucial to maintaining a healthy herd but is essential information for our understanding of potential causes of anemia.

Farm-level and animal-level questionnaires (Appendices A to E) were provided to the herdsman and included questions related to management practices, healthcare, demographic information and environmental information.

3.2.3 Sample collection

Blood was collected via jugular venipuncture into tubes containing the anticoagulant ethylenediamine tetra-acetic acid (EDTA). Up to 10 mL of milk per ewe was collected into a sterile, plastic vial using gloved hands. Dipping of the teat using iodine or a similar product prior to sampling was not recommended by the herdsman due to the possibility of the lambs then avoiding suckling. Feces were manually removed from the rectum of the sheep using a glove covered with a veterinary lubricant. A new glove was used for each animal. All samples were placed in a cooler containing icepacks for transport back to the laboratory. Blood and milk samples were stored at -80°C, while fecal samples were stored at 4°C until processed.

3.2.4 Hematocrit measurement

The measurement of packed cell volume (PCV) and total solids (TS) concentration were performed on the EDTA-anticoagulated blood samples. A plain microhematocrit capillary tube was used to obtain an anticoagulated blood sample from the blood collection tube, which was then centrifuged for 5 minutes at 12,100 X g.

3.2.5 DNA extraction

Genomic DNA was extracted from 100 µL of EDTA-anticoagulated sheep blood using the Quick-DNA™ Miniprep kit in accordance with the manufacturer's instructions. Nuclease-free water was extracted as a negative control with each batch of extractions.

Approximately 1 mL of milk was placed into a microcentrifuge tube and centrifuged at 7,400 RPM for 20 minutes then increased to 11,000 RPM for 10 minutes. A sterile transfer pipette was used to remove the top and middle layers from the pellet. Genomic DNA was extracted from 200 µL of centrifuged milk using the DNeasy® Blood and Tissue kit in accordance with the manufacturer's instructions. Nuclease-free water was extracted as a negative control with each batch of extractions.

3.2.6 Fecal exams

A modified McMaster technique (Zajac 2006) was used to determine fecal egg and oocyst counts (FEC/FOC) from samples obtained from ewes and lambs in 2016. The level of sensitivity of the procedure was 100 eggs/oocysts per gram (EPG/OPG) of feces when 1 gram of feces was used (Zajac 2006). A centrifugal fecal flotation, using a Sheather's sugar solution with a specific gravity of 1.25, was used to recover eggs and oocysts from GI nematodes, as well as protozoal parasites from lambs when sample volume was insufficient for a fecal egg/oocyst count.

3.2.7 Molecular analyses

Screening of blood and milk samples was performed for the dnaK gene of *M. ovis* using a previously reported SYBR® Green qPCR assay (Johnson, do Nascimento et al. 2016). DNA from a known *M. ovis*-positive sheep and nuclease-free water were used in all runs as positive and negative controls, respectively. A housekeeping gene (GAPDH) was used to confirm the presence of amplifiable DNA, tested as a single replicate, from all samples using the qPCR assay mentioned

previously. Milk samples that were GAPDH-negative had their DNA concentrations assessed by use of a Nanodrop 1000 spectrophotometer (ThermoScientific™, West Palm Beach, FL) and were subsequently diluted to a concentration of 16 ng/µL and then retested. DNA sequencing of amplicons from milk samples that tested positive for *M. ovis* were processed as previously described (Johnson, do Nascimento et al. 2016).

3.2.8 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows version 24.0 (Released 2016 IBM Corp., Armonk, NY) and Stata statistical software version 14 (StataCorp 2015, College Station, Tx). Descriptive statistics were performed to describe the distributions of breeds, sexes and bacterial loads in the study samples. Prevalence estimates and 95% confidence intervals (CI) were calculated using OpenEpi (Dean A.G. 2013) and Swog Statistics and Data Management Center[1]. $P < 0.05$ was considered significant. Longitudinal data from the 2016 and 2017 sheep flocks were merged for analysis purposes. Infection status of a sheep was determined by the qPCR assay. Anemia in a ewe was defined as a PCV ≤ 26 % (Jain 1986). A mixed logistic regression model was used to compare the prevalence of *M. ovis* infection in mature ewes across pregnancy, pre-weaning, and post-weaning of lambs. Mixed general linear regression with time as a fixed effect, was used to test for differences in log-transformed *M. ovis* bacterial loads in ewes during pregnancy, pre-weaning, and post-weaning of lambs. The pre-weaning phase of lambs was comprised of three sampling times, with the second pre-weaning measure used in the analysis. Life-table analysis was performed to determine the incidence rate of *M. ovis* infection in lambs pre and post-weaning. Mixed linear regression was used to model the association between PCV and log-transformed bacterial loads in infected mature ewes. A two-independent sample t-test was used to assess for a difference in mean PCV between lambs born from infected and uninfected ewes.

3.3 Results

3.3.1 Study site

In the fall of 2016, data pertaining to the farm-level survey were obtained from the ASREC herdsman. In addition to the purposes of teaching, extension work, research and meat production,

[1] SWOG Statistics Center, Seattle, WA https://statools.crab.org/Calculators/binomialConfidence.htm.

the ASREC sheep farm also supplies sheep to companies for use in biomedical research. At the time of survey, ASREC was responsible for an estimated 343 sheep. The flock was comprised of 150 mature ewes expected to lamb in spring 2017, 10 mature rams, and approximately 183 ewe and ram lambs, which were less than a year old. The flock of sheep was divided into smaller groups, some groups were housed indoors only while others were housed outdoors all summer. Other animals on the farm with access to the same areas as the sheep were stray barn cats and a guard dog.

The main building utilized by the sheep farm was divided into two parts. The first part was an open front barn with concrete floors while the second part, considered the growth pen area, had metal flooring over an open pit. Parturition took place in an enclosed pen within the open front barn. The main lambing season was spring, although in 2016 six lambs were also born in the summer. Culling of sheep was utilized and in 2016 one sheep was culled due to advanced age. That same year, nine deaths within the flock were attributed to the following: a birth defect, lack of colostrum ingestion, pneumonia, possible poison hemlock ingestion and unknown causes. At the time the survey was administered in 2016, none of the sheep within the flock had been diagnosed with anemia.

Regarding management practices, shearing of the adult sheep occurred in April. Disinfection of shears between animals or equipment used during ear-tagging was not part of the operating procedures of the farm, however, the reuse of needles among animals was never allowed. Prior to deworming, weights were obtained for a few sheep. The average weight of the heaviest sheep would then be used as the standard to base the dewormer dose. Fecal egg counts were sometimes used to indicate when to deworm, but generally convenience-based deworming was used.

Mosquitoes and flies were frequently seen bothering the sheep during the warmer months. Chemical products used to control insects were not applied to the animals; alternative methods of insect control were utilized. These included fly traps, fans, shutting lights out at night within the barn, and applying fly spray to the inside surfaces of the barn. Other external parasites such as ticks, lice and sheep keds had not been noticed within the flock, and in this management system were not problematic.

Responses to the animal-level questionnaires indicated that none of the ewes enrolled in the 2017 longitudinal study received oxytetracycline or any antibiotic at any point during the

project, but one lamb started Baytril® (enrofloxacin) and dexamethasone four days before the April collection. In 2016, prior to the September sampling, the five ewes were given a subcutaneous injection of Zactran® (gamithromycin) as a prevention for footrot.

3.3.2 Sheep

Eighty-seven pregnant ewes of differing breeds were enrolled in a cross-sectional study to estimate the prevalence of *M. ovis* within the pregnant ewe population (Figure 3.1). The ewes were bred naturally with rams on the farm. The ages of the ewes ranged from 1 to 9 years old with the median age of 3 years.

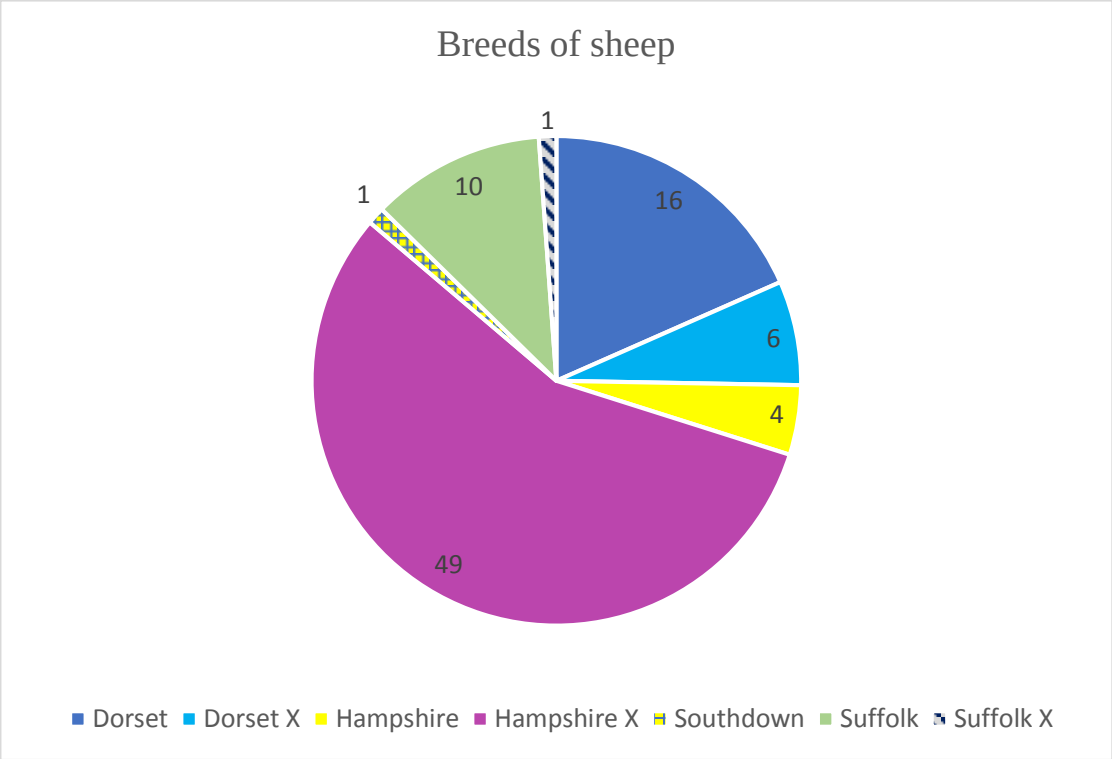

Figure 3.1. Number of ewes by breed for the cross-sectional study (n = 87).

A subset (n=20) of the pregnant ewes from the cross-sectional study were enrolled in the longitudinal study, which spanned from December 2016 through June 2017. Thirty-four live lambs and two stillborn lambs were born from the 20 ewes. There was no history of weak lambs for the ewes that had previously given birth. Four ewes had single births while 16 ewes gave birth to multiples. From the 16 ewes having multiple births, 15 were twin births and one was a triplet birth. The ages of the ewes ranged from 1 to 9 years old with the median age of 2.5 years. The breeds of

the ewes were Southdown (n=1), Hampshire (n=4), Hampshire cross (n=9), Dorset (n=4) and Dorset cross (n=2). There was a wide range of prior pregnancies among the ewes; nulliparous (n=8), primiparous (n=1), and multiparous (n=11). Of the eleven multiparous ewes, five ewes had two prior pregnancies, four ewes had four prior pregnancies, one ewe had five prior pregnancies and one had eight previous pregnancies. The ewes were housed indoors from January through March 2017, but were previously housed outside on pasture during the summer and fall of 2016. In April and May 2017, the ewes spent time in an outside lot but not in the pasture. Also, retrospective data on ewes that gave birth in June 2016 were incorporated with the 2017 data. Five, one-year old nulliparous ewes produced six live lambs in June 2016. These ewes were initially sampled between 25 to 29 days post-parturition. Four ewes were of Polypay breed while one was a Dorset cross. The ewes and lamb were housed indoors throughout the duration of the project. All six Dorset cross lambs were born alive, but one twin lamb died on the day of parturition. The sexes of the lambs were three ram lambs, two ewe lambs while the sex of the dead lamb was not recorded.

3.3.3 Fecal exams

Between July and November 2016, five ewes had 30 fecal collections with a total of 6 fecal collections per ewe. However, there were three instances that feces could not be collected: once due to the unavailability of the ewe and twice because feces were not available during the time the ewes were restrained. From the 27 successful collections, *Eimeria* spp. were identified 70.4% (19/27) of the time. All the ewes shed oocysts of this protozoan parasite at some point during the collection period. The level of sensitivity of the modified McMaster technique was 100 EPG/OPG of feces. Fecal oocysts counts ranged from less than 100 OPG to 700 OPG with a median FOC of 200. Trichostrongyle-type eggs were identified in three ewes from feces collected in October and November. One ewe had FECs less than 100 EPG, while another ewe decreased from 200 EPG to less than 100 EPG. A FEC of 2200 EPG was obtained in October for a third ewe, but decreased to less than 100 EPG in November. *Trichuris ovis* eggs were found only once from two ewes in July and September with FECs not higher than 100 EPG. *Strongyloides papillosus* was also found once from two ewes, both having FECs of less than 100 EPG with one ewe also shedding *Giardia* sp. cysts. Less than 100 EPG of *Moniezia expansa* were recovered from a single ewe in November.

Eimeria spp. oocysts, *S. papillosus* eggs and *Giardia* sp. cysts were recovered from feces from the lambs over the course of the study. One hundred percent of the lambs (5/5) had patent

Eimeria spp. infections with decreasing FOCs as time went on. Median *Eimeria* spp. FOCs from August through November were 11,133; 1,000; 200; and <100 OPG, respectively. Sixty percent (3/5) of the lambs were infected with *S. papillosus* with FECs remaining at or below 100 EPG. One lamb shed *Giardia* sp. cysts at the last collection in November.

3.3.4 Prevalence of *Mycoplasma ovis*

The prevalence of *M. ovis* in the 87 pregnant ewes from the cross-sectional study was 42.7% (37/87), (95% CI, 32.7% to 53.0%). Bacterial loads of ewes infected with *M. ovis* ranged from 1.54 X 10^4 copies/mL of blood to 2.45 X 10^9 copies/mL of blood, with a median bacterial load of 9.48 X 10^5 copies/mL of blood. Ages of infected and uninfected ewes were the same (median: 3 years, range: 1 to 9 years). Breeds of *M. ovis*-positive ewes are shown in Figure 3.2.

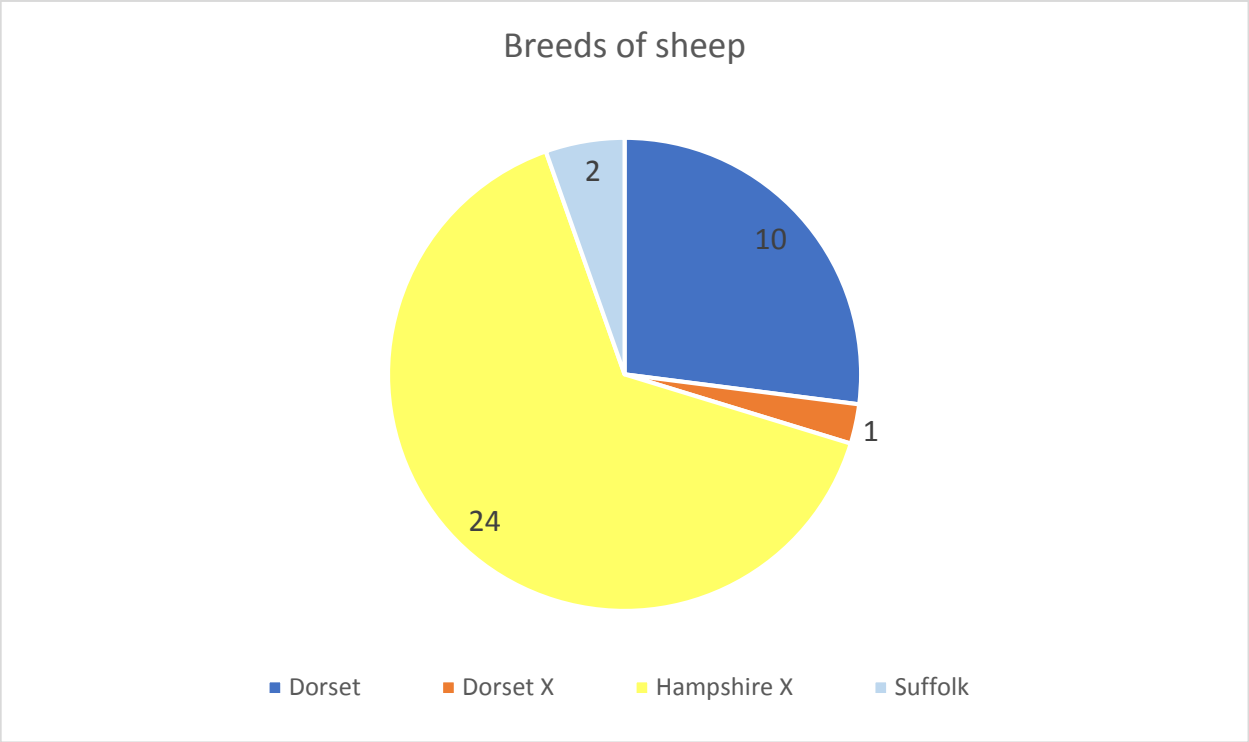

Figure 3.2. Number of positive ewes by breed for the cross-sectional study (n = 37).

3.3.5 Longitudinal study of ewes

Twenty-five ewes were enrolled in a longitudinal study with all 25 ewes completing the project. Prevalence of *M. ovis* infection in ewes during pregnancy, and before and after weaning of her lambs, was not found to be significantly different ($P = 0.518$) across these times (Table 3.1), with prevalence estimates of 45% (95% CI, 23.1 – 68.5), 36% (95% CI, 17.9 – 57.4) and 44% (95% CI, 24.4 – 65.1), respectively. Some ewes did not remain positive for all of the collections, while some were positive only once; thus, the prevalence by the end of the project was 56% (14/25) (95% CI, 34.9% to 75.6%). The breeds of the 14 positive ewes were Dorset (n = 2), Dorset cross (n = 1), Hampshire (n = 2) Hampshire cross (n =7), and Polypay (n = 2). When comparing the mean bacterial loads of *M. ovis* across samples collected during the pregnancy, pre-weaning and post-weaning phases, no statistically significant difference was found ($P = 0.193$) (Figure 3.3), with the mean bacterial loads between 10^4 and 10^6 copies/mL of blood/mL (Table 3.1).

Four ewes had *M. ovis* detected at all collection times, while ten ewes were positive between one and four times, with an additional eleven ewes remaining negative throughout the entire study. Mean ± SD of PCV for ewes that were consistently positive, intermittently positive,

and negative were 34.1% ± 3.3%, 29.7% ± 4.9%, and 29.9% ± 4.5%. No significant correlation (*P* = 0.989) was found between PCV and log-transformed *M. ovis* bacterial loads from infected mature ewes (Figure 3.4).

Table 3.1. Prevalence and bacterial loads of *M. ovis* from ewes by collection phase for the longitudinal study.

	%, (+/n)	95% CI	mean back-transformed bacterial loads per mL blood
Pregnancy	45, (9/20)	23.1 – 68.5	1.17×10^6
Pre-weaning	36, (9/25)	17.9 – 57.4	2.96×10^5
Post-weaning	44, (11/25)	24.4 – 65.1	9.46×10^4

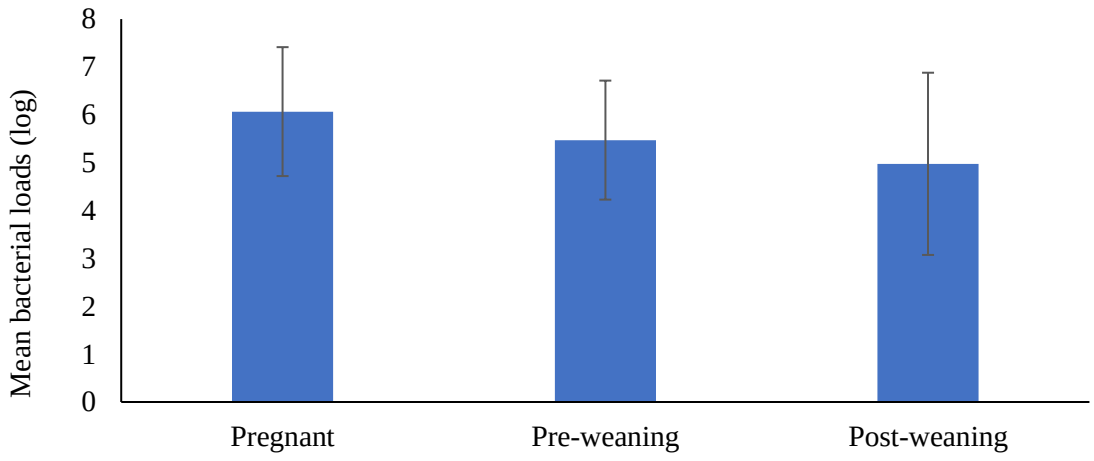

Figure 3.3. Mean bacterial loads (log-transformed) of *M. ovis* from ewes by reproductive phase. Error bars are standard deviations.

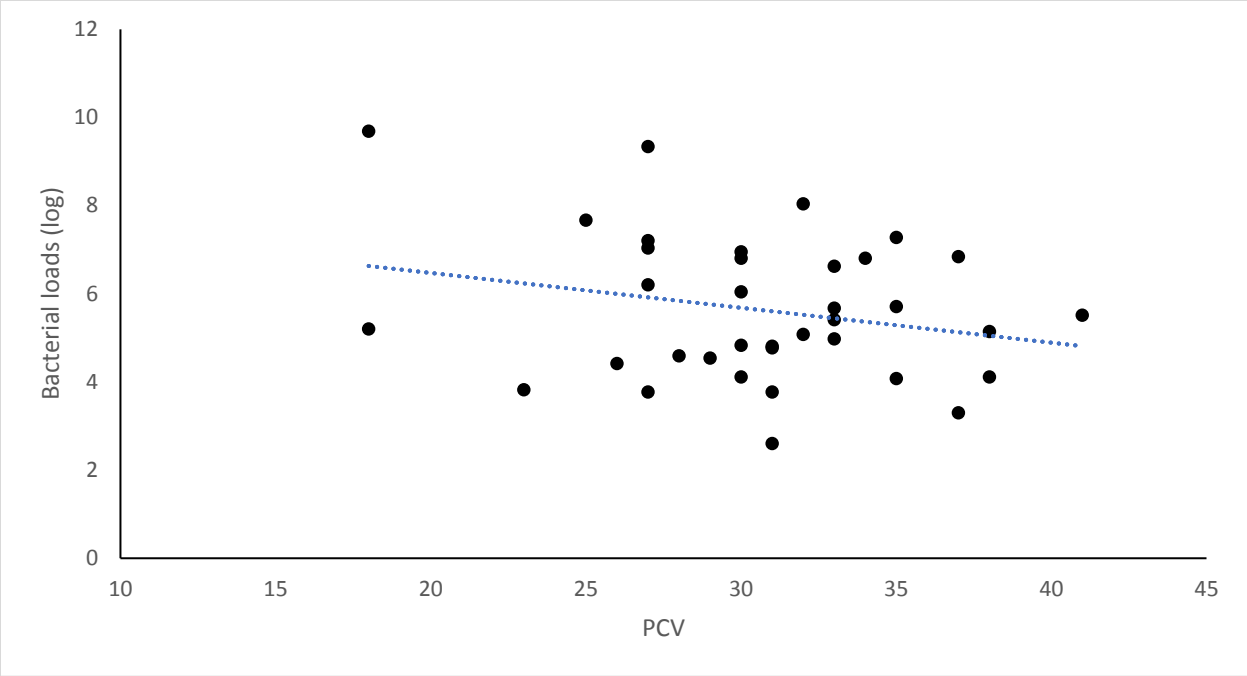

Figure 3.4. Testing for an association between PCV and log-transformed *M. ovis* bacterial loads in mature ewes. (P = 0.989).

3.3.6 Milk samples

Seventy-two milk samples were analyzed for the presence of DNA of *M. ovis* with one sample identified as positive (Figure 3.5). Gross hemolysis was not observed in any of the milk samples. The bacterial load of *M. ovis* in the milk sample was 1.01 x 10^3 copies/mL of milk from a positive ewe with a bacterial load of 6.40 x 10^6 copies/mL of blood. Sequencing of amplicons from the positive milk sample had 99% to 100% identity with the dnaK gene of *M. ovis*. The lamb suckling from this positive ewe remained negative until 117 days old.

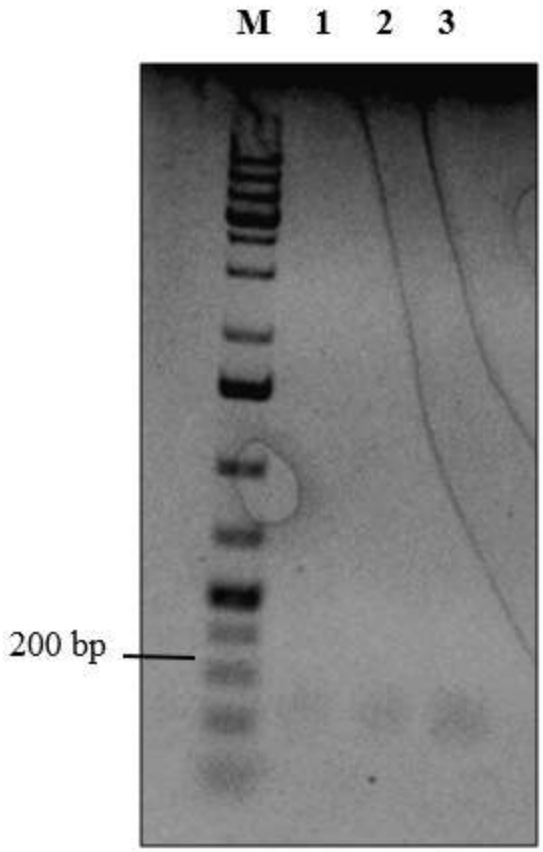

Figure 3.5. Agarose gel electrophoresis (3%) of qPCR amplified products (194 bp) using dnaK F & dnaK R primers. (Lanes 1, 2, 3: genomic DNA from a milk sample; Lane M: DNA Ladder [GeneRuler™ 1 kb Plus DNA Ladder, ThermoScientific™, West Palm Beach, FL]).

3.3.7 Longitudinal study of lambs

Ten lambs (seven ewe lambs and three ram lambs) from nine ewes were positive for *M. ovis* by the completion of the longitudinal study. The breeds of the positive lambs were Dorset, Dorset cross, and Hampshire cross, with the majority of lambs born of multiple births. The first and only lamb to be detected positive prior to weaning was a ewe lamb. This lamb had a bacterial count of 1.42×10^4 copies/mL of blood and was 61 days old at the time of the collection. Unfortunately, this ewe lamb died prior to the next collection, so subsequent information could not be obtained. However, the lamb's dam remained negative for *M. ovis* throughout the entire study duration.

The incidence rate of infection in the lambs was higher post-weaning than pre-weaning (Figure 3.6). Nine of the ten lambs were found to be positive post-weaning. Lambs were first detected positive as young as 61 days old and as old as 159 days, with a median age of 116 days.

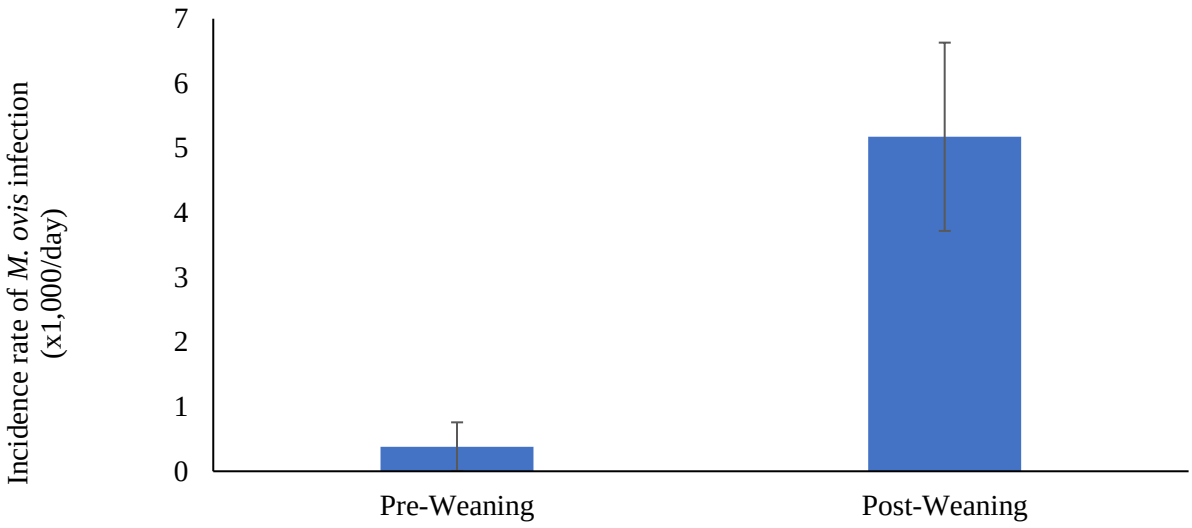

Figure 3.6. Incidence rate of *M. ovis* infection in suckling and weaned lambs. Error bars are standard errors.

Bacterial loads of the positive lambs ranged from 1.00 x 10^3 to 4.15 x 10^{10} copies/mL of blood with most lambs being detected positive only once (Table 3.2). The initial bacteremias of the lambs could be categorized into two groups. One group (n=8) had bacterial loads $\leq 10^4$ copies/mL of blood, while the other group (n=2) had 10^7 and 10^{10} copies/mL of blood. Twin lambs that were 116 days old when first detected *M. ovis* positive had dissimilar bacteremias; one lamb remaining positive at 1.4 x10^9 copies/mL of blood while the other went from 9.4 x 10^3 copies/mL of blood to negative.

Table 3.2. *Mycoplasma ovis* bacterial loads/mL of blood of lambs by age range.

(Age range in days)	(53 – 62)	(90 – 99)	(108 – 117)	(122−133)	(157−159)
1	1.42×10^4				N/A[a]
2		9.31×10^7	2.15×10^8	3.70×10^6	N/A
3			1.00×10^3		N/A
4			1.30×10^3		N/A
5			4.15×10^{10}	1.40×10^9	N/A
6			2.62×10^3		N/A
7			3.84×10^3		N/A
8			9.40×10^3		N/A
9				9.20×10^3	N/A
10					2.50×10^4

[a] Not tested

When analyzing for differences in mean PCV between lambs born of uninfected and infected mature ewes, no significant difference was found (P = 0.292) (Figure 3.7). The small sample size of *M. ovis* positive lambs (n=10) limited direct comparisons of PCV from infected and uninfected lambs at each sampling point. However, PCVs were similar across the age ranges for both negative and *M. ovis*-positive lambs, except for one positive lamb at the 157-159 age range (Table 3.3) who had a bacterial load of 2.40×10^4 copies/mL of blood.

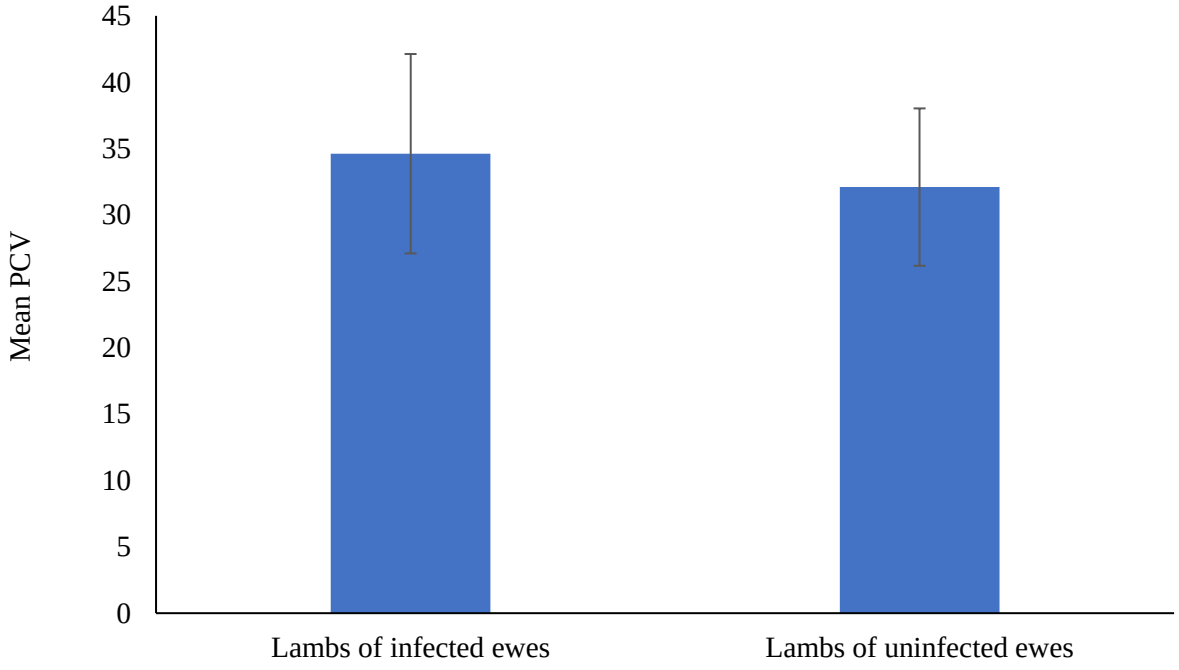

Figure 3.7. Comparison of mean PCV between lambs born of *M. ovis*-positive and negative ewes. Error bars are standard deviations.

Table 3.3. Packed cell volume (PCV%) by age for *M. ovis*-positive and negative lambs

Negative lambs, n (mean PCV%±SD)						
(Age range in days)						
(2 – 6)	(22 – 36)	(53 – 62)	(90 – 99)	(108 – 117)	(122 – 133)	(157-159)
33	32	31	32	23	31	2
(34.0 ± 3.7)	(32.4 ± 3.9)	(35.0 ± 2.1)	(34.5 ± 1.9)	(33.6 ± 3.8)	(34.7 ± 1.7)	(34.0 ± 2.8)
Positive lambs, n (mean PCV%±SD)						
(Age range in days)						
(2 – 6)	(22 – 36)	(53 – 62)	(90 – 99)	(108 – 117)	(122 – 133)	(157-159)
0	0	1	1	7	3	1
(N/A)	(N/A)	(36.0)	(30.0)	(35.1 ± 1.5)	(32.7 ± 2.9)	(44.0)

3.4 Discussion

These studies demonstrated that hemoplasma infection was common within the ASREC sheep population, and that additional work needs to be undertaken to determine the extent of hemoplasma-infected sheep used in biomedical research. The prevalence of *M. ovis* within the longitudinal study ranged from 36% to 45% for the ewes. Prevalences of 14% to 80% using PCR or qPCR assays and 43% based on blood smear detection have been reported for sheep involved in biomedical research, with sample sizes of tested animals between 10 and 99 sheep (Martin, Chrisp et al. 1988, Nascimento 2011, Deshuillers, Do Nascimento et al. 2012, Hampel, Spath et al. 2014). Prevalences of hemoplasma infection in sheep not used in biomedical research, with varying degrees of clinical signs, have been found to be between 45% to 67% (Hornok, Meli et al. 2009, Suzuki, Sasaoka et al. 2011, Wang, Cui et al. 2017). Taken together, the study herein and previous studies indicate the frequent occurrence of *M. ovis* infection in sheep populations.

Studies focusing on the course of infection in sheep have been well-documented (Littlejohns 1960, Overas 1969, Daddow 1979, Gulland, Doxey et al. 1987), but detection was based on blood smear examinations. The success of this method in correctly detecting *M. ovis* is dependent upon several factors, most importantly, the bacterial load and ability of the assessor. The current study is unique in that the progression of the infection within the pregnant ewes was followed using a highly-sensitive and specific qPCR assay (Johnson, do Nascimento et al. 2016), allowing for bacterial loads to be quantified, which has been absent from previous works. Mean bacterial loads for the ewes during pregnancy, pre-weaning, and post-weaning of lambs were not significantly different, varying between 10^4 and 10^6 copies/mL of blood. The lower bacterial loads ($\leq 10^6$ copies/mL of blood) across these three time periods, along with the mean PCV values remaining above 29.0% for the positive and negative ewes, suggest the ewes are chronically infected. Although the bacterial loads of the ewes from the cross-sectional study ranged up to 10^9 copies/mL of blood, no clinical signs were apparent in any of the animals, and the median bacterial load was 10^5 copies/mL of blood. While the pathogenicity of *M. ovis* in sheep has been reported, the current study found no correlation between PCV and *M. ovis* bacterial loads. Of course, a larger sample size of infected ewes may be needed in order to detect a relationship between these variables. However, studies of alpacas chronically infected (Meli, Kaufmann et al. 2010) with *Candidatus* Mycoplasma haemolamae and goats with *M. ovis* (Johnson, do Nascimento et al. 2016) also failed to detect a significant association between bacterial loads and anemia, and no

correlation between PCV and qPCR positivity was detected. *Mycoplasma ovis*-chronically infected sheep that are healthy and on a sufficient feeding program are typically asymptomatic (Kanabathy 2004)

Although the current studies were not designed to directly investigate whether vertical transmission of *M. ovis* occurs, it appears none of the lambs were infected *in utero* or during the birthing process. The first positive lamb was not detected until 2 months of age. The ovine placenta is characterized as synepitheliochorial with the transfer of nutrients and gases across the placenta occurring through diffusion, active transport mechanisms and carrier molecules, but the placenta limits the exchange of immunoglobulins (Sammin, Markey et al. 2009). Direct mixing of blood from the ewe and lamb does not occur, therefore transplacental transmission of *M. ovis* is unlikely. Littlejohns (Littlejohns 1960) also demonstrated the unlikeliness of transplacental and transmammary transmission in two groups of sheep after splenectomizing five yearlings and four adults (4 years or older). Further, Overas concluded that perinatal infection did not occur among lambs born from infected ewes (Overas 1969). The latter two studies relied on microscopic detection methods for establishing a diagnosis of *M. ovis* infection, which has a lower sensitivity than the PCR methods used in the study herein.

The possibility of vertical transmission has been examined in several other ruminant species. In alpacas, the likelihood of *in utero* transmission was suggested after blood samples from a 4-day old cria (Almy, Ladd et al. 2006) and another cria (prior to colostrum ingestion) (Pentecost, Marsh et al. 2012) were found to be PCR-positive for *M. haemolamae*. Additionally, transplacental transmission of bovine hemoplasmas was reported after precolostral blood samples tested positive for four calves (Hornok, Micsutka et al. 2011). However, the possibility of exposure to infected blood during the birthing process instead of *in utero* transmission could not be ruled out. These studies and the studies herein are comparable as PCR was also used to detect the hemoplasma infection. It is unknown why perinatal infection occurs in these ruminant species and not in sheep.

One milk sample out of the 72 samples collected from the ASREC ewes was positive for *M. ovis*, indicating the possibility but low probability that *M. ovis* may be transferred via colostrum or milk. None of the milk samples exhibited gross hemolysis; however, this does not completely exclude the possibility of blood contamination or hemorrhage into the milk as the reason for the positive result. Contamination of the sample is not believed to have occurred; 71 samples remained negative, and a negative-control per batch of DNA extractions was included. Daddow reported

that five lambs suckling from *M. ovis*-positive ewes failed to develop an infection even after splenectomy (Daddow 1982). In addition, Pentecost reported that colostrum samples from both infected and uninfected alpacas were PCR-negative (Pentecost, Marsh et al. 2012). The ram lamb suckling the ewe with the *M. ovis*-positive milk remained negative. Therefore, it appears the risk of developing a hemoplasma infection through colostrum and milk is negligible.

Age-related or acquired immunity to *M. ovis* appears to occur in sheep. Studies have demonstrated the susceptibility of lambs and yearlings to *M. ovis* with the presence of clinical signs, but despite infection the mature ewes remained asymptomatic with fewer positive animals detected (Littlejohns 1960, Campbell, Sloan et al. 1971). The current study and other studies (Overas 1969, Gulland, Doxey et al. 1987) have found ewes are intermittently positive for *M. ovis*. Resurgence of a latent infection, when bacterial loads are low or non-detectable, to an acute crisis may occur during times of stress. This reiterates the ineffectiveness of antibiotic therapy as well as the inability of the immune system to completely clear the infection. The mechanisms behind the hemotrophic mycoplasmas' ability to evade immune detection remain unknown.

In the current study, the majority of lambs were detected *M. ovis*-positive post-weaning. Other studies have found lambs frequently infected pre-weaning. However, management styles, weaning status, sampling frequency, and detection methods were different or unknown, which made direct comparison of these studies difficult (Jolly 1967, Harbutt 1969, Maxwell 1969, Mason, Manuel et al. 1981, Gill 1990). Only one lamb from the ASREC sheep farm was found to be *M. ovis* positive prior to weaning and it was not anemic at any of the sampling time points. The lambs bacterial load was 10^4 copies/mL of blood at the last sampling before the lamb died, but whether the lamb became anemic or an increase in the bacteremia occurred, is unknown. The median age at detection for lambs was approximately 116 days old. More frequent testing of the lambs pre- and post-weaning would have allowed a more accurate determination of initial patency. Awareness regarding initial detection in lambs would be important from a herd management perspective.

The small sample size of positive lambs (n = 10) does not allow for meaningful comparisons in detection rates among sampling age or for determining the effect weaning may have had on developing a hemoplasma infection. However, given that some of the lambs spent time outdoors, one could speculate that the increase in positive lambs post-weaning may have been attributed to increased exposure to *M. ovis* via hematophagous insects. The routes of hemoplasma

transmission have not been definitively resolved, but exposure to infected blood appears to be essential (Neimark, Hoff et al. 2004) A report from China found a higher prevalence of hemoplasma infection in grazing sheep and goats than in household sheep (Wang, Cui et al. 2017), while farms with lambs grazing adjacent to swampy areas reported deaths of suckling lambs (Campbell, Sloan et al. 1971, Gill 1990).

The bacterial loads of *M. ovis* from the ASREC lambs could be divided into two distinctive groups. The majority of the lambs (8/10) had bacterial loads $\leq 10^4$ copies/mL of blood, while two lambs had bacteremias of 10^7 and 10^{10} copies/mL of blood. To the author's knowledge, this is the first study reporting bacterial loads within a lamb population using a qPCR assay. The majority of the lambs were detected qPCR positive only for a single collection time. It is possible that if the study had continued longer more instances of positivity would have been identified. Studies examining the percentages of infected red blood cells have shown the ability of the bacterium to vary greatly both within the same animal and among herd members (Overas 1969, Campbell, Sloan et al. 1971). The ASREC lamb flock experienced a sore mouth outbreak during the last sampling in June. It would have been interesting to see the effect, if any, a concurrent infection would have on *M. ovis*-positive lambs within the flock.

Fecal samples collected from the 2016 sheep showed *Eimeria* spp. were more common than gastrointestinal nematodes (GIN) under this management system. Three of the 2016 ewes were slightly anemic (PCV % 24-26) at some point during the study period, but the small sample size limited comparisons between hemoplasma positivity, PCV%, and fecal egg and oocyst counts. Trichostrongyle-type egg counts remained low with a median count of <100 EPG, but one ewe had a FEC increase in October to 2200 EPG. The ewes were dewormed with Cydectin® five days before the September collection, so it was surprising to see such an increase as the ewes were reported to have been kept inside, and thus not exposed to infective third-stage larvae present on pasture. Therefore, this may indicate either improper dosing of the ewe with the anthelmintic, presence of dewormer resistance, activation of hypobiotic larvae, or re-infection and/or inability of the ewe's immune system to suppress the current infection. The ewes had been synchronized for estrus two days before the September collection. Induction of estrus occurred two weeks prior to the October collection with the removal of the progesterone CIDR insert and IM injection of Estrumate®. Perhaps the hormonal changes and associated stresses within the ewes were responsible, in part, for the increased trichostrongyle FECs (Brunsdon 1967, Ayalew and Gibbs

1973). By the November collection, the trichostrongyle FECs returned to <100 EPG for all the ewes. Since the ewes had not been dewormed again (according to the survey responses), the most likely cause for this decrease in FEC is suppression of the infection by the immune system (McRae, Stear et al. 2015). *Eimeria* spp. were the most frequently seen GI parasite within the lamb fecal samples, with FOCs decreasing over time as immunity built up within the lambs. Trichostrongyle-type eggs were not present in any of the fecal samples from the lambs. Since they were housed indoors and did not have prior exposure to infective larvae on pasture, this was expected. In the current study, there is no indication that GINs were a confounding factor in regards to anemia in these sheep.

3.4.1 Limitations

The main limitations of this study are the use of sheep from a single flock, the limited number of ewes and their lambs in the longitudinal studies, and the inability to compare results across different management styles. Convenience-based sampling was used, which may lead to an inaccurate representation of the population under study. A larger sample size for the longitudinal studies of ewes and lambs would allow sufficient statistical power to detect important differences and associations while account for potential confounders. Lastly, necropsies of the deceased lambs were not performed and may have provided insight into the causes of death.

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CHAPTER 4. GENETIC DIVERSITY OF *MYCOPLASMA OVIS* BASED ON SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) AND EVALUATION OF FIVE GENES FOR USE IN A MULTI-LOCUS SEQUENCE TYPING (MLST) ASSAY

4.1 Introduction

The hemotropic *Mycoplasma* spp., known by the trivial name hemoplasmas, exhibit an affinity for red blood cells and have been found in a range of mammalian species (Messick 2004). *Mycoplasma ovis* (*M. ovis*) has been found to infect both sheep and goats (Neimark, Hoff et al. 2004), but varying degrees of clinical signs have been reported both within sheep herds and between host species. Acutely infected animals may develop a life-threatening hemolytic anemia, while latent carriers may not show signs of being infected. Transmission of the bacteria is believed, in part, due to exposure to infected blood whether from fighting among herd members, medical procedures, parturition or from blood-feeding arthropods. The inability to successfully culture the bacteria has made it difficult to completely characterize the transmission cycle; thus, questions remain as to the role that *M. ovis* plays in production of farm-raised populations of sheep and goats. Typically, molecular assays such as polymerase chain reaction (PCR) (Hornok, Meli et al. 2009, Tagawa, Takeuchi et al. 2012) and real-time quantitative PCR (qPCR) (Johnson, do Nascimento et al. 2016) are used to detect infection with *M. ovis*, but if one wants to study the molecular epidemiology of this bacteria, such as unraveling its transmission cycle, then other methods must be included.

Molecular typing methods such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), automatic ribotyping, rep-PCR, multi-locus sequence typing (MLST) (Maiden, Bygraves et al. 1998) and genotyping based on single nucleotide polymorphisms (SNPs) (Touati, Blouin et al. 2015) have been useful methods for molecular characterization of isolates of pathogens. To the author's knowledge, only RFLP-PCR has been used to describe *Mycoplasma hemocanis* and '*Candidatus* Mycoplasma hematoparvum' from dogs in Iran (Sharifiyazdi, Abbaszadeh Hasiri et al. 2016). There are no published reports about typing methods for *M. ovis* or for any hemoplasma (excluding the above mentioned canine hemoplasmas).

Typically, molecular typing methods are used to study the epidemiology of local and global disease outbreaks. An added advantage of using sequence-based typing methods is that the methodology does not require cultivation of the bacteria; this is especially important since the hemoplasmas have not been successfully cultured *in vitro*. A nucleotide-based approach known as multilocus sequence typing (MLST) for characterizing bacterial isolates was first proposed by Maiden (Maiden, Bygraves et al. 1998). In MLST, typically five to ten housekeeping genes are selected for sequencing for each isolate or sample. A unique allele number is assigned to a gene when a distinct sequence or nucleotide variant has been detected. The combination of the genes and their unique, but arbitrary allele numbers, gives rise to the samples sequence type (ST) (Maiden 2006). One great advantage of using MLST, is the ability to easily compare allelic profiles of isolates to those stored in a large, centralized database that can be accessed by the Internet. This method has been applied as a tool for epidemiologic analysis to a number of different bacteria and is particularly robust for characterizing isolates and their evolution.

Multi-locus sequence typing (MLST) has been used to study global epidemiological outbreaks. This typing method utilizes genes that are known to slowly evolve or diversify (housekeeping genes) (Enright, Day et al. 2000, Arvand, Feil et al. 2007). These genes are involved in basic cellular functions and essential to the survival of the pathogen. In some instance to improve the discriminatory power for detecting sequence variations, virulence and virulence-associated genes are selected as targets for typing (Zhang, Jayarao et al. 2004); this method is known as multi-virulence-locus sequence typing (MVLST). It is believed that virulence-associated genes due to evolutionary pressures may display increased nucleotide sequence polymorphism needed for studying local outbreaks. It is unknown which genes are associated with virulence in *M. ovis*, therefore this pilot project was undertaken selecting genes previously published for mucosal mycoplasma MLST assay development.

The overall goal of the study was to characterize the genetic diversity of *M. ovis* field samples based on SNPs found within a subset of genes. The objectives included: 1) to evaluate several housekeeping genes of *M. ovis* for their potential use in a MLST assay; 2) to determine if pooled DNA amplicons processed using NGS would generate sufficient, quality reads for mapping to *M. ovis* references; and 3) to determine quality and depth of coverage for SNPs identified from pooled DNA amplicons.

4.2 Materials and Methods

4.2.1 Animal selection

A subset of 30 sheep that were involved in an unrelated study and had been previously identified as *M. ovis*-positive were selected for inclusion in this project. Two lambs and 28 ewes that were pregnant or had recently given birth at Purdue University's Animal Science and Research Education Center (ASREC) sheep unit had blood collected via jugular venipuncture. All procedures involving sheep were approved by the Animal Care and Use Committee at Purdue University.

4.2.2 DNA extraction and qPCR assay

Genomic DNA was extracted from 100 µL of EDTA-anticoagulated sheep blood with a Quick-gDNA Miniprep kit[1] used in accordance with the manufacturer's instructions. Extracted DNA was stored at –20°C. Nuclease-free water was extracted as a negative control sample. Confirmation of amplifiable DNA using a housekeeping gene (GAPDH) and quantification of positive samples were tested as previously described (Johnson, do Nascimento et al. 2016).

4.2.3 Selection of genes

Nine genes (recA, ldh, grpE, dnaK, adk, pgm, tpiA, gyrB and "hypo"-locus tag MR07-RS03555) within the *M. ovis* strain Michigan genome, NC_023062.1 were evaluated for use in the MLST assay. These genes were selected based on their prior reported use in mucosal mycoplasma MLST studies, presence of only one copy of the gene in the *M. ovis* strain Michigan genome and scattered locations of the genes across the reference genome. Negative controls (DNase-free water, and qPCR negative sheep) and positive controls at two different bacterial loads (10^8 copies/mL, and 10^5 copies/ mL) were used.

4.2.4 PCR primer design and amplification

Primer3 was used to generate primer pairs having approximate annealing temperatures of 53°C and were designed to amplify 550-650bp fragments of the genes as listed in Table (4.1). The following PCR protocol was used while testing the loci at annealing temperatures of 52°C, 56°C and 60°C. The PCR mixture contained 5 µL of a 10x STD Taq Buffer, 1 µL of 10 mM dNTPs, 1

[1] Quick-gDNA Miniprep, Zymo Research Corp, Orange, CA.

µL of $MgCl_2$ (2mM), 1.5 µL of each primer (300 nM), 34.75 µL of DNase-free water, 0.25 µL of Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 5 µl of template in a final volume of 50 µl. Cycling conditions consisted of an initial denaturation at 95°C for 30 seconds, followed by 35 cycles of 95°C for 30 seconds, an annealing phase at (52°C, 56°C, or 60°C) for 60 seconds, an extension phase at 68°C for 45 seconds, with a final extension at 68°C for five minutes. The annealing temperature of the final reaction was optimized to be 60°C. All reactions were performed using the Bio-Rad icycler (BioRad Laboratories, Hercules, CA).

Table 4.1. Selected genes for the MLST assay

Gene	Primers	Sequence (5′ − 3′)	Location of gene[b]	PCR product (bp)	MLST segment (bp)
hypo[a]	Fw	AAGTGCAACGATTGGTGTAGG	621263-622141	647	631
	Rv	TCGTTGAACCCAACAAACTT			
ldh	Fw	ACTGCTGGGAGAGCTCAAAA	158350 – 159315	628	614
	Rv	CGATTCCACATGAACCAACA			
pgM	Fw	ATTCTCCCGTCATTTCCTCA	587941 – 589467	607	590
	Rv	TCTGGTGTGTTCTTCCCAACT			
recA	Fw	GGAGCTTTCTTTCAAGTGCTTC	58918 – 59898	606	589
	Rv	AAGCTCAGGCGCAAAATAAA			
tpiA	Fw	CCATCACATTCTGGAAGACTCA	589467 – 590180	632	620
	Rv	GTCATTGGAAACCTGAAGATGA			

[a] Locus tag MR07-RS03555 ("hypothetical" gene) from the *M. ovis* strain Michigan genome, NC_023062.1.

[b] Based on the *M. ovis* strain Michigan genome, NC_023062.1.

4.2.5 DNA sequencing of amplicons

To confirm amplification of the loci under varying protocol conditions, amplicons were separated by electrophoresis on a 1.5% agarose gel that was subsequently stained with ethidium bromide. Amplicons producing a single band of the expected size specific for each loci led to five genes being retained for further analysis. To confirm the target size and specificity of the products, DNA sequencing of amplicons for each of the five loci was performed. Purification of DNA from the gel was performed using a commercially available kit. Purified DNA was sent for sequencing in both directions at the Purdue Genomics Core Facility using primers specific for each gene.

4.2.6 MLST sample submission and analyses

A composite sample of the pooled amplicons from the five loci (hypo, recA, tpiA, pgM, and ldh) per sheep were submitted for NGS, specifically WideSeq (reference ask Phillip), at the Purdue Genomics Core Facility, which used an Illumina MiSeq sequencing analyzer. Data analyses were performed by the Bioinformatics Core at Purdue University. Libraries were constructed using a Nextera XT DNA library preparation kit (Illumina®, Inc., San Diego, CA). The sequence quality of the raw reads were assessed and after quality trimming, BBMap (Bushnell 2014) was used to align the reads from each sample to the five reference genes.

The Phred-quality score (Q) was used to assess the accuracy of a base call by the sequencer. The quality score is logarithmically related to the base calling error probabilities (Nielsen, Paul et al. 2011) as indicated by the formula

$$Q = - 10 \log_{10} P(\text{error})$$

where $P = 10^{(-Q/10)}$, which is the probability that the corresponding base call is incorrect (Del Fabbro, Scalabrin et al. 2013) . A higher Q score indicates a higher probability that a base has been correctly called while a lower score indicates a higher probability of an incorrect call. While the quality score for a base gives information about the accuracy of the call at a particular nucleotide, the quality of a read takes into account the average quality scores across the entire length of that sequence. The depth of coverage conveys information regarding the number of filtered reads that supports the reported allele.

GATK HaplotypeCaller (McKenna, Hanna et al. 2010) was then used to call SNPs based on the reference aligned bam files. A consensus sequence for each sample for each gene was then generated following removal of indels using vcftools utility (Danecek, Auton et al. 2011). Custom scripts were used to assign the allelic profiles for the loci and overall ST per sample. Sequence types having similar alleles at three or more loci were grouped into a distinct clonal complex using eburst (Feil, Li et al. 2004). The discriminatory index (DI) for each locus and concatenated sequence was calculated using the method by Hunter and Gaston (Hunter and Gaston 1988) and 95% confidence intervals were generated (Grundmann, Hori et al. 2001). The discriminatory index (*D*) was calculated using the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} nj(nj-1)$$

where *N* is the total number of strains in the sample population, S is the total number of sequence types described, and n_j is the number of strains belonging to each of the sequence types. A value of 1 is highly discriminatory while a value of 0 is not discriminatory.

The ratios of nonsynonymous to synonymous substitutions (dN/dS) were calculated using a KaKs calculator based on the Nei and Gojobori method (Nei and Gojobori 1986). The index of association (IA), which tests for recombination events, was calculated using poppr (Kamvar, Tabima et al. 2014).

4.3 Results

4.3.1 Choice of MLST loci

After testing 9 genes (Table 4.2), whose reference sequence data was obtained from the only complete genome of *M. ovis* listed in GenBank, five genes (hypo, ldh, recA, pgM, tpiA) were retained for further analysis. The four genes that were excluded (grpE, dnaK, adk, pgm, gyrB) either failed to amplify the target sequence or produced nonspecific bands at the various annealing temperatures tested (Table 4.2).

Table 4.2. List of genes tested for use in a MLST assay from the *M.ovis* strain Michigan genome, NC_023062.1

Gene	Gene product	Locus tag	Genome location	Status
hypo	hypothetical protein	MR07_RS03555	621263-622141	Selected
ldh	lactate dehydrogenase	MR07_RS00945	158350-159315	Selected
pgM	2,3-biphosphoglycerate-independent phosphoglycerate mutase	MR07_RS03375	587941-589467	Selected

Table 4.2 continued

Gene	Gene product	Locus tag	Genome location	Status
recA	Recombinase	MR07_RS00380	58918-59898	Selected
tpiA	triose-phosphate isomerase	MR07_RS03380	589467-590180	Selected
GrpE	nucleotide exchange factor	MR07_RS01495	271667-272230	Nonspecific
dnaK	molecular chaperone	MR07_RS01740	314404-316251	Nonspecific
adk	adenylate kinase	MR07_RS02310	419696-420373	Nonspecific
gyrB	Type IIA topoisomerase subunit B	MRO7_RS03445	604525-606480	failed to amplify

4.3.2 Assembly of reads to reference loci

Illumina MiSeq sequencing of raw reads for the 30 samples generated 12,124 to 399,625 reads with a median value of 110,659. The median value for reads after trimming was 110,558 with only 0.09% of reads discarded due to low quality or short length. The average length of the reads for the 12 samples tested by MLST and for 18 samples not tested by MLST were 219 bp and 209 bp, respectively. The range for read length for both groups was 30 to 251 bp.

The median quality of reads which mapped to the reference loci for the MLST samples, and median mapping coverage for each of the genes were (33.87, 1066.5X), (30.87, 5694X), (17.92, 1095X), (29.95, 5130X) and (40.38, 4158.5X) for hypo, ldh, pgM, recA and tpiA, respectively. The median values for mapped reads to hypo, ldh, pgM, recA and tpiA were 3942, 23265, 6281, 22019, and 12458, respectively. Although there were sufficient amounts of trimmed reads, the majority of quality reads did not map to the reference loci. This can be seen with mapping percentages of 4.5%, 25%, 7.5%, 20% and 14% for the loci as listed above.

4.3.3 Typing results by MLST

Five loci, comprising a total of 3044 bp (Table 4.3), from 12 sheep samples were sequenced and compared to the *M. ovis* strain Michigan genome retrieved from the GenBank database (NC_023062.1). Although there were a small number of samples typed, a total of 11 STs were identified with 10 STs belonging to the 12 sheep samples and 1 ST designated to the reference (Table 4.4). Two to 3 alleles (Table 4.3) were found at each of the five loci for these samples. The

discriminatory power of each loci and concatenated sequences were compared by using Simpson's index of diversity. The indices for the loci ranged from 0.42 to 0.69, with an overall diversity index of 0.92 (Table 4.3). The majority of the 12 samples were found belonging to the same clonal complex (Table 4.4) as identified by eburst. Clonal complexes were defined as groups of two or more samples that have identical alleles at three or more of the loci. The index of association (I_A) which is a measure of the multilocus linkage disequilibrium of a population was calculated for the 12 MLST samples and was found to be (I_A= 0.13, p-value 0.24). A low I_A value indicated the presence of linkage equilibrium and the role of recombination within the population. Fourteen polymorphic sites, 12 alleles and 27 SNPs (Table 4.5) were identified from the samples tested by MLST. The quality of the base calling for the SNPs ranged from 18.59 to 222,896 with a median value of 220.2. The depth of coverage for the SNPs ranged from 1X to 5696X with a median value of 42.5X.

Table 4.3. Description of the MLST loci from nucleotide sequence data from 12 samples

	hypo	**ldh**	**pgM**	**recA**	**tpiA**	**Concatenated sequence**
Segment size (bp)	631	614	590	589	620	3044
% G + C content	26.78	34.81	36.46	34.74	32.07	32.97
Number of alleles	3	3	2	2	2	12
Polymorphic sites	4	3	3	2	2	14
% polymorphic sites	0.63	0.49	0.51	0.34	0.32	0.46
Diversity Index	0.69	0.54	0.73	0.42	0.53	0.92
(95% CI)	0.47-0.91	0.22-0.86	0.61-0.85	0.1-0.74	0.23-0.83	0.8-1.04

Table 4.4. Allele numbers, sequence types (STs) and lineages (clonal complexes) of *M. ovis*-positive samples

Sample	Allelic profile					ST	Lineage
	hypo[b]	ldh	pgM	recA	tpiA		
Reference[a]	1	1	1	1	1	1	Singleton
1612	3	3	3	2	2	2	CC1
1613	2	2	2	2	3	3	CC1
1614	2	2	3	2	2	4	CC1
1624	2	2	3	2	2	4	CC1
1625	2	2	3	2	2	4	CC1
1619	2	2	4	2	2	5	CC1
1620	4	2	3	2	2	6	CC1
1621	3	2	2	3	4	7	Singleton
1622	2	2	4	4	3	8	CC1
1627	5	2	2	2	2	9	CC1
1630	2	4	2	2	2	10	CC1
1631	3	5	2	2	2	11	CC1

[a] DNA sequences of the five loci from the *M. ovis* strain Michigan genome, NC_023062.1.

[b] Locus tag MR07-RS03555

4.3.4 Allelic diversity of samples not tested by MLST

The median quality of reads which mapped to hypo, ldh, recA, and pgM loci for the samples, and median mapping coverage for each of the genes were (33,519X), (30.7,4025X), (28.8, 1480X), and (17.3,292X), respectively. Eighteen samples were included for analysis of the genes ldh, recA and hypo, but only 7 samples were available for testing of the pgM gene. The median values for mapped reads to hypo, ldh, pgM, and recA were 2155, 16955, 2139, 6660, respectively. The genes hypo, ldh, pgM and recA had low median mapping percentages of 2%, 16.5%, 2% and 8%, respectively. The number of polymorphic sites for hypo, ldh, recA and pgM were 5, 6, 2, and 3, respectively. The quality of base calling for the SNPs ranged from 10.2 to 45704.7 with a median value of 514.3. The depth of coverage for the SNPs ranged from 2X to 392.4X with a median value of 117X. Two samples were identified as having SNPs, but were

considered false as neither sample had pooled amplicons for the pgM genes. The base calling quality and depth of coverage for those two false positives were (21.77, 2X) and (113.9, 5X).

The ratio of non-synonymous to synonymous base substitutions (d_N/d_S) was calculated for each of the 30 samples (12 MLST samples and 18 non-MLST samples) and the average for all the samples for each gene was computed. There were no statistically significant d_N/d_S ratios found for any of the five genes.

Table 4.5. Nucleotide substitutions at the polymorphic sites for the 12 samples tested by MLST

SNP locations within the genes

	hypo				ldh			pgM			recA		tpiA	
Sample[a]	621321	621327	621922	621923	158596	159209	159212	588316	588380	588416	59058	59662	590156	590165
Reference[b]	A	G	A	A	A	A	G	T	C	T	T	C	C	T
1612	.	A	.	.	C	.	.	C	T	.	.	.	.	.
1613	.	.	.	.	.	.	.	.	.	.	.	.	A	.
1614	.	.	.	.	.	.	.	C	T	.	.	.	.	.
1619	.	.	.	.	.	.	.	.	.	C	.	.	.	.
1620	.	.	C	T	.	.	.	C	T	.	.	.	.	.
1621	.	A	.	.	.	.	.	.	.	.	G	.	A	G
1622	.	.	.	.	.	.	.	.	.	C	.	G	A	.
1624	.	.	.	.	.	.	.	C	T	.	.	.	.	.
1625	.	.	.	.	.	.	.	C	T	.	.	.	.	.
1627	G	.	.	.	.	.	.	.	.	.	.	.	.	.
1630	.	.	.	.	.	.	C	.	.	.	.	.	.	.
1631	.	A	.	.	.	T	.	.	.	.	.	.	.	.

[a] Identical nucleotides at each polymorphic site to the reference are indicated by periods.

[b] DNA sequences from the five loci within the *M. ovis* strain Michigan genome, NC_023062.1.

4.3.5 Quality control of samples

Quality control generated per sample by the Illumina MiSeq platform showed the majority of samples having two different peaks relative to G + C content indicating possible contamination with another organism. Percentages of G + C were approximately 34 and 65 percent.

4.4 Discussion

Multi-locus sequence typing is an established molecular typing method for a variety of pathogens (Iredell, Blanckenberg et al. 2003, Brown, Holden et al. 2015, Olaogun, Kanci et al. 2015, Herrera, Hernandez et al. 2017, Yin, Zhang et al. 2018, Yu, Chen et al. 2018). It is considered the gold-standard for typing, since sequence data generated by laboratories can be uploaded to a common database, and results from individual laboratories can be directly compared. *Mycoplasma ovis* has not previously been described using a molecular typing method. Therefore, as proof of concept that high throughput sequencing using pooled PCR amplicons from several genes of *M. ovis* would generate quality data was tested.

WideSeq, a service offered by the Genomics Core at Purdue University, provides high throughput sequencing using an Illumina MiSeq platform, offering a cost-effective alternative to whole-genome sequencing. Polymerase chain reaction products are pooled prior to sequencing using WideSeq (AlAbdi, He et al. 2018, LaBonte, Jacobs et al. 2018), ultimately generating short reads, but with greater depth than Sanger sequencing. The use of next-generation sequencing (NGS) in genotyping and SNP detection using pooled products has been documented (Brockman, Alvarez et al. 2008, Mullen, Creevey et al. 2012). Miscall error rates of up to 1% have been reported for Illumina MiSeq (Nielsen, Paul et al. 2011), with higher error rates for other NGS platforms (Quail, Smith et al. 2012). Base miscall errors, in addition to alignment errors, reduce the accuracy of NGS data and affect downstream analyses. Therefore, a scoring system was created to assess the reliability of the read data; this is known as the phred score (Ewing and Green 1998, Ewing, Hillier et al. 1998). However, a threshold set too stringently may result in a loss of accurate information even when the bases have been correctly called, while too low of a threshold may include data with incorrect base calls. Typically, phred scores of 30 or higher are preferred (Quail, Smith et al. 2012). In addition to the phred score, the read depth of a locus must be taken into

consideration, since a low read depth may affect the accuracy of base calling and therefore correct identification of a SNP (Liao, Satten et al. 2017).

Reference-based mapping was used to identify SNPs from *M. ovis* naturally-infected sheep based on five loci (tpiA, ldh, recA, pgm and "hypo" locus tag MR07- RS03555) within the bacterium. Although sufficient numbers of reads were generated per sample, low percentages of reads (2- 25%) mapped to the reference loci. This may be a result of the reference genome not being representative of the strains present within the sheep selected for this study, poor amplification of the intended target with copious amounts of genomic DNA, and/or DNA of other organism(s) that has been amplified in addition to *M. ovis*. Although high levels of identities were found for consensus sequences of two loci (recA & tpiA) to the bacterium, *Stenotrophomonas. maltophilia*, the G + C content for the mapped reads across all loci ranged from 26 to 36%. A low G + C content is characteristic of hemoplasma genomes (do Nascimento, Dos Santos et al. 2013, Deshuillers, Santos et al. 2014), while the median G + C content for *S. maltophilia* is 66% (Crossman, Gould et al. 2008). Interestingly, when the primers that were used to amplify each of the five genes were compared for similar sequences in the Stenotrophomonas group (taxid 995085) using BLASTn®, no significant similarities were found. *S. maltophilia* is considered a nosocomial pathogen in humans with chronic respiratory disease, but in veterinary medicine it is believed to be a commensal bacterium at this site (Albini, Abril et al. 2009). However, 286 strains of *S. maltophilia* were isolated from 721 fly-strike and fleece rot samples in sheep located in New South Wales (Macdiarmid and Burrell 1986).

Overall, the quality of reads that mapped to the references had scores approximately 30 with up to 88% coverage across the genes. The pgM gene was the only gene which had a low score of approximately 17, with only 38% coverage across the gene. Several samples for the pgM and tpiA genes either failed to produce bands of the expected size (607 or 632 bp, respectively), or very faint bands were visible. Perhaps robust amplification within these two genes was not obtained due to reduced efficiency of the primers annealing to the targeted sequences because of insufficient starting template from low bacterial loads. Differences were present in the bacterial loads between the samples producing bands as expected, as well as those with faint or absent bands. The average bacterial loads for the pgM and tpiA genes which didn't produce bands were 10^5 and 10^6 copies/mL of blood, respectively, while the samples producing bands had an average of 10^8 copies/mL of blood. Also, it's possible that reanalysis of the primer concentrations, reagents,

and reaction conditions may have improved *M. ovis* amplification from template originating from low bacterial loads.

Although the sample size for the multi-locus sequence typing assay was evaluated using only 12 samples from *M. ovis*-infected sheep from a single herd, 27 SNPs were identified. The median qualities and depths of coverage of the SNPs from these 12 samples was 220 and 42X, respectively, which according to published reports is sufficient for calling SNPs accurately (Quail, Smith et al. 2012, Del Fabbro, Scalabrin et al. 2013). The two alleles that were falsely identified as SNPs had qualities of 21 and 113, and depths of coverage of 2x and 5X. This illustrates the importance of taking into consideration both values when calling SNPs.

While each of the five genes had less than 1% polymorphic sites across their segments (Table 4.3), with only slight variation between the genes, this was not surprising. Conservation of nucleotide sequences is essential for "housekeeping" genes, since significant changes to a nucleotide sequence could alter the protein product and function of the gene. *Mycoplasma* spp. are known for their small genomes (Citti and Blanchard 2013); therefore, retained genes and their products are essential for survival of the bacterium.

The diversity index for the five locus MLST assay was 0.92, which is above the suggested cut-off of 0.90 for a typing assay (Dillon, Rahman et al. 1993, Coenye, Spilker et al. 2002). Nevertheless, the addition of more genes and genes associated with virulence may help to improve the discriminatory ability of the assay and should be considered. One important consideration to be aware of is the requirement for calculation of the diversity index. The equation is based partly on knowing the number of strains within the sample population (Hunter and Gaston 1988). This is problematic since the hemoplasmas are uncultivable and the number of strains was unknown. Each sample was considered a separate strain for calculation purposes, but this assumption may have been inaccurate. Ten STs were identified from the 12 samples with most of the samples (11/12) belonging to the same clonal complex. Since the sample size and geographical locations of the *M. ovis*-positive sheep samples were limited, it was expected that the samples would belong to the same clonal complex. Interestingly, all the 12 samples had SNPs within one or more of the five loci; producing nucleotide sequences which differed from the reference strain. This suggests that variation within the *M. ovis* population may be diverse, and warrants further investigation using a larger sample size. Phylogenetic analyses based on the concatenated nucleotide sequences of the loci in MLST assays are often used to investigate the evolution of an organism. However, Tsang

and colleagues (Tsang, Lee et al. 2017) reported that phylogeny inferred from MLST did not represent the genome phylogeny of a bacterial species and resulted in less than 70% bootstrap support at most nodes with more polytomies found with the MLST trees. Phylogenetic trees were not generated for this project due to the limited number of samples.

4.5 Limitations

The inability to culture *M. ovis* directly influences the way in which this pathogen can be studied, relying solely on molecular methods for characterization of the bacterium. The greatest disadvantages of this study were the lack of reference genomes for comparison when selecting primers, and not having an established typing assay for comparison with the MLST results. Since the diversity of *M. ovis* and the existence of differing strains within the population is unknown, the assumption was made that since the genes selected were essential for metabolic functions within the *Mycoplasma* genus, they would be under stabilizing selection and not have extreme variation among nucleotide sequences. The efficiency of a primer is influenced in part by its ability to anneal with the intended target sequence. Since there was only one completely sequenced genome of *M. ovis* to use as a reference, the selected primer sequences might not be reflective of the entire *M. ovis* bacterial population within this study. Despite the high discriminatory power obtained from the five-locus MLST assay, validation of the assay using another typing method must be performed in order to adequately assess the performance of the assay. The addition of *M. ovis* samples from diverse geographical locations having nucleotide sequences determined either by whole-genome or targeted gene sequencing would help clarify strain variation within the existing population.

4.6 References

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CHAPTER 5. DEVELOPMENT AND APPLICATION OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID DETECTION OF *MYCOPLASMA OVIS* IN NATURALLY-INFECTED SHEEP.

5.1 Introduction

Mycoplasma ovis, the causative agent of hemoplasmosis in sheep, has a worldwide distribution (Neitz 1934, Hornok, Meli et al. 2009, Suzuki, Sasaoka et al. 2011, Tagawa, Ybanez et al. 2012, Deshuillers, Santos et al. 2014). Acute infection is characterized by hemolytic anemia that can be life threatening, whereas chronic infection may lead to a loss of body weight and ill-thrift (Messick 2004). Fluctuations in bacteremia often make microscopic detection of these bacteria on a peripheral blood smear problematic. Therefore, molecular methods such as conventional PCR (cPCR) [a]nd real-time quantitative PCR (qPCR) are frequently used for diagnosis of infection due to their higher sensitivities than Giemsa-stained blood smears. Unfortunately, these assays can be time-consuming and require expensive equipment and reagents, which limits their use to financially-stable laboratories.

In 2000, a highly sensitive and specific assay was developed which amplified DNA under isothermal conditions, and was named loop-mediated isothermal amplification (LAMP). (Notomi, Okayama et al. 2000) In LAMP, amplification of the target sequence is carried out at a constant temperature and does not require a thermal cycler. This method also eliminates the heat denaturation step in traditional PCR by employing a DNA polymerase having a high strand displacement activity. Since this assay requires only a stable heat source such as a water bath or heat block for the reaction to occur, it is especially useful for resource-poor laboratories. Assays developed using the LAMP method for the detection of viruses (Venkatesan, Bhanuprakash et al. 2012, Xie, Xie et al. 2014), bacteria (Yang, Guan et al. 2013, Kim, Kim et al. 2014, Liu, Du et al. 2015, Higa, Uemura et al. 2016), fungi (Duan, Zhang et al. 2014) and parasites (Mahittikorn, Mori et al. 2015, Oriero, Okebe et al. 2015) have been reported. There is only one article describing the use of this method for the detection of hemoplasmas and this was in cattle (Song, Wang et al. 2013).

The cycling conditions for LAMP are rapid and amplification of large amounts of targeted DNA can be obtained within an hour (Notomi, Okayama et al. 2000, Duan, Zhang et al. 2014,

Kim, Kim et al. 2014). In part, the high sensitivity and specificity of this method is due to the use of precisely designed inner and outer primer sets (four or six individual primers) that recognize six to eight distinct regions of the targeted gene. Single-stranded DNA synthesis in the initial steps of LAMP is initiated from one end of the target sequence by an inner primer and thereafter strand displacement DNA synthesis is begun by the outer primer at the same end. This leads to the displacement of the complementary strand, which then forms a looped out structure at one end due to its complementary sequence design. This amplification process ultimately results in a dumb-bell form DNA structure, which is converted to a stem-loop DNA by self-primed DNA synthesis (Notomi, Okayama et al. 2000). The stem-loop DNA is the starting template for the next stage of the reaction. Ultimately, the final products are a combination of stem-loop DNAs of various stem lengths and structures having multiple loops. A large amount of by-product, magnesium pyrophosphate, are also produced during the LAMP amplification process, which are in proportion to the amount of DNA synthesized. To aid in visualization of these by-products, dyes and indicators can be added pre and post-amplification (Rekha, Rana et al. 2014). However, the risk of cross-contamination increases when adding a dye post-amplification. Several methods may be employed to confirm the presence of amplified DNA by LAMP, which include measuring the turbidity of magnesium pyrophosphate (Mori, Nagamine et al. 2001), using a metal ion-binding indicator dye such as hydroxynaphthol blue (HNB) (Goto, Honda et al. 2009), and observing the color change in the reaction tube (Goto, Honda et al. 2009) or by observing a ladder-like banding pattern on agarose gel electrophoresis as well as using measuring the fluorescence of a DNA-intercalating dye (Parida, Sannarangaiah et al. 2008).

Herein, we report the development of a *M. ovis*-specific LAMP assay. The objectives of this study were to develop, optimize and validate a LAMP assay targeting the dnaK gene of *M. ovis*, and to compare the performance of the LAMP assay to cPCR using field samples from naturally-infected sheep previously tested by qPCR.

5.2 Materials and Methods

5.2.1 Sample collection

Blood samples were collected via jugular venipuncture into tubes containing EDTA as an anticoagulant from 145 female sheep. The sheep were located at the Purdue University Animal

Sciences Research and Education Center (ASREC) sheep unit. Animal selection was based on the herdsman's choice. All procedures involving sheep were approved by the Animal Care and Use Committee at Purdue University.

5.2.2 DNA extraction

Genomic DNA was extracted from 100 µL of EDTA-anticoagulated sheep blood with a Quick-gDNA Miniprep kit[a] used in accordance with the manufacturer's instructions. Nuclease-free water was extracted as a negative control with each batch of extractions. Extracted DNA was stored at −20°C. A housekeeping gene (GAPDH) was used to confirm the presence of amplifiable DNA from all samples using a previously developed qPCR assay (Johnson, do Nascimento et al. 2016).

5.2.3 Primer design and development of a standard plasmid

Six LAMP primers were designed based on a fragment of the dnaK gene of the published *M. ovis* strain Michigan genome (GenBank accession No. CP006935.1) using the Primer explorer V5 software program (http://primerexplorer.jp/e/) (Figure 5.1). The dnaK LAMP primers were comprised of two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LF and LB) (Table 5.1). The inner primers each have two unique sequences that are complementary to the sense and antisense sequences on the target DNA (Figure 5.2). This allows for priming in the first part and self-priming later in the cycle. One of the two unique sequences for each inner primer remains free (dangles), but is linked to the other sequence that binds to the target DNA. The dangling sequence (after strand displacement) makes a loop as it binds onto the complementary sequence within the DNA stand (Figure 5.3). Amplification of a fragment of the dnaK gene using cPCR was performed using the LAMP outer primers (F3 and B3) producing a 207-bp product.

[a] Quick-gDNA Miniprep, Zymo Research Corp, Orange, CA.

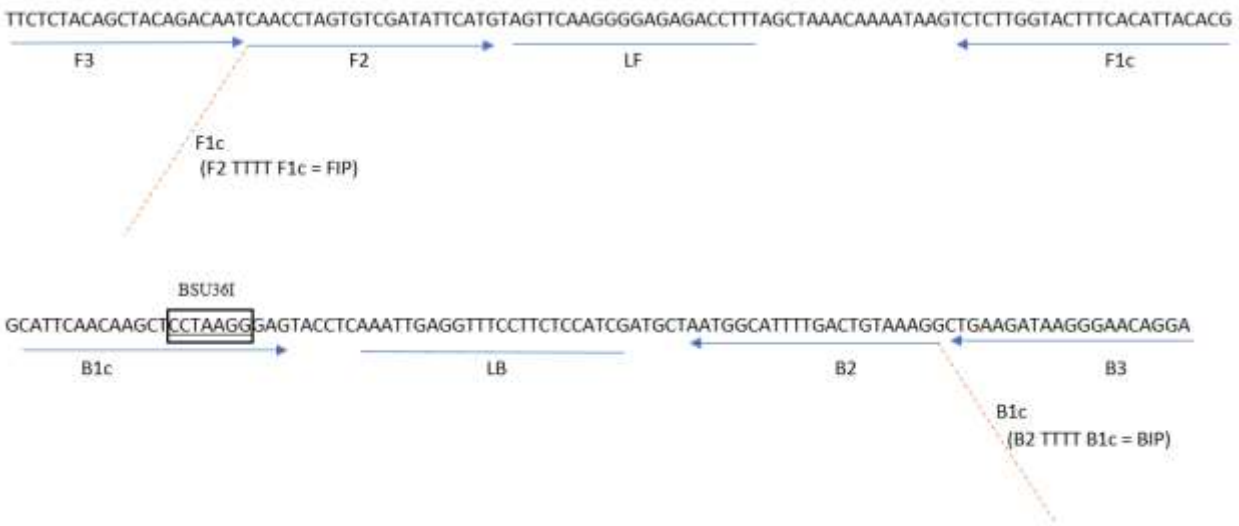

Figure 5.1. Nucleotide sequence of a fragment of the dnaK gene showing positions of LAMP primers and the BSU36I restriction site.

Table 5.1. Sequences of the primers used.

Method	Primer	Type	Sequence (5′ to 3′)
LAMP	F3	Forward outer	TTC TCT ACA GCT ACA GAC AAT
	B3	Backward outer	TCC TGT TCC CTT ATC TTC AG
	FIP	Forward internal	CGT GTA ATG TGA AAG TAC CAA GAG A TTTT CAA CCT AGT
		(F1C & F2)	GTC GAT ATT CAT G
	BIP	Backward internal	CAT TCA ACA AGC TCC TAA GGG AG TTTT CCT TTA CAG TCA
		(B1C & B2)	AAA TGC CAT T
	LF	Loop forward	AAA GGT CTC TCC CCT TGA ACT
	LB	Loop backward	AAA TTG AGG TTT CCT TCT CCA TCG A
cPCR	F3	Forward outer	TTC TCT ACA GCT ACA GAC AAT
	B3	Backward outer	TCC TGT TCC CTT ATC TTC AG

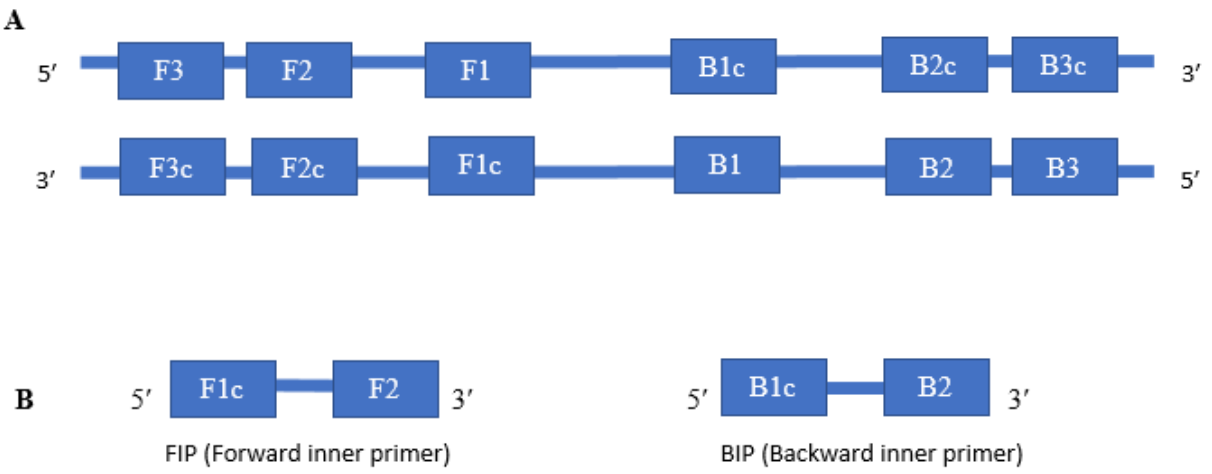

Figure 5.2. Position of LAMP primers on sense and anti-sense strands.(**A**) Positions at which each primer anneals. (**B**) Description of inner primers.

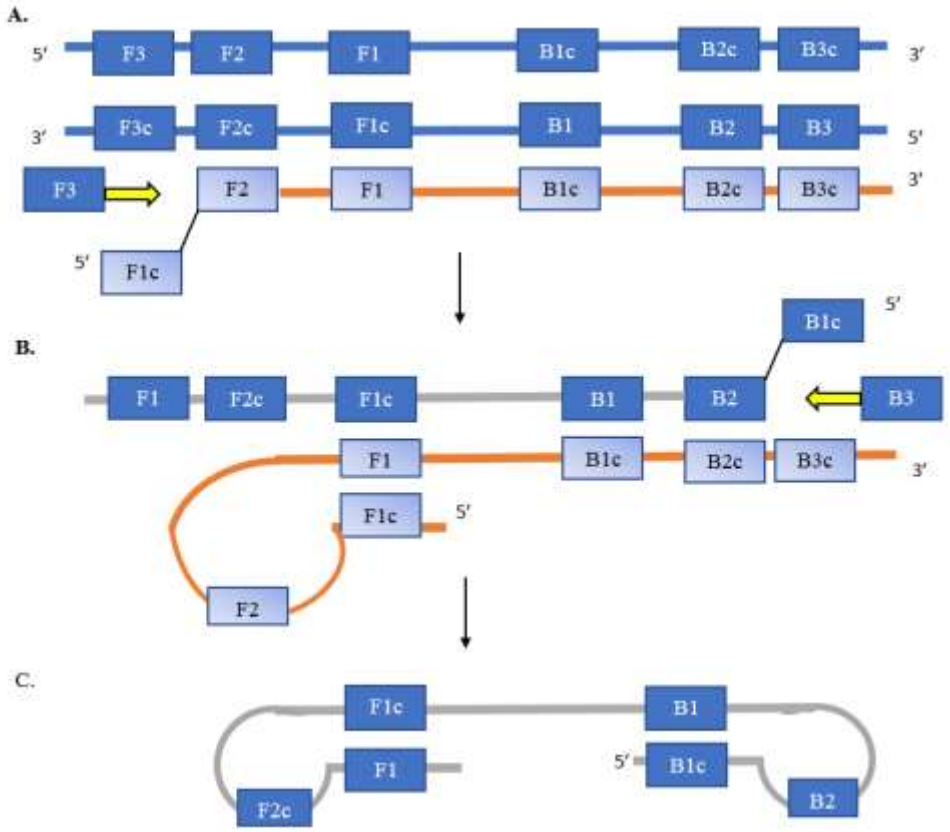

Figure 5.3. Formation of LAMP dumb-bell structure. (**A**) FIP inner primer anneals to F2c region on anti-sense strand and initiates strand synthesis followed by strand displacement via the outer primer, F3. (**B**) BIP inner primer anneals to ssDNA and initiates strand synthesis followed by strand displacement via the outer primer, B3. (**C**) Formation of a dumb-bell shape DNA structure of the displaced strand.

A recombinant plasmid containing the fragment of the dnaK gene of *M. ovis* (confirmed by Sanger sequencing) was used as a template for creating 10-fold serial dilutions of plasmid DNA (10^9 to 1 copy of plasmid/reaction) for use in development of the LAMP protocol. A 207 bp fragment (GenBank accession No. CP006935.1) was amplified and cloned into the pGEM®-T vector (Promega Corp, Madison, WI) with transformation into JM109 competent cells according to the manufacturers' instructions. A QIAprep spin miniprep kit (Qiagen, Germantown, MD) was used to purify the plasmids from the JM109 cells. A Nanodrop 1000 spectrophotometer (ThermoScientific, West Palm Beach, FL) determined the concentration of the plasmid containing the dnaK insert to be 100 ng/µL. An online calculator (URI Genomics and Sequencing Center, Kingston, RI) was used to determine the number of copies of the recombinant plasmid resulting in 2.89 x 10^{10} copies. Subsequently, 10-fold serial dilutions of plasmid DNA were then prepared using the recombinant plasmid DNA to 1X tris-EDTA buffer combined with herring sperm DNA (Sigma-Aldrich Corp, St. Louis, MO). The fragment of the dnaK gene of *M. ovis* spanned the region between the F3 and B3 primers.

5.2.4 Optimization of LAMP assay

The following reaction components were optimized individually: concentrations of inner primers to outer primers (0.8, 1.6, 2.0 µM: 0.2 µM), loop primers (0, 0.4, 0.6, 0.8, 1 µM), dNTPs (0.8, 1.0, 1.2, 1.4, 1.6 mM), $MgSO_4$ (4, 6, 8, 10 mM), Betaine (0, 0.6, 0.8, 1.0 M), hydroxynaphthol blue (HNB) (50, 100, 150, 200 µM), assay temperature (60, 62, 64, 66, 68°C), BST 2.0 DNA polymerase (0.16 U μl^{-1}, 0.32 U μL^{-1}), incubation time (30, 45, 60, 75 minutes), template amount (1, 3 µL). A non-template control (nuclease-free water) as a negative control and a positive control (genomic DNA from an *M. ovis*-positive sheep) were included in each LAMP reaction. Results were tested in triplicate using a recombinant plasmid dilution having a concentration of 10^5 copies per reaction. Results were considered positive based on HNB-visualized color change from purple to blue within the reaction tube and the presence of ladder-like bands on gel electrophoresis.

5.2.5 Optimization of cPCR assay

The outer primers (F3 and B3) designed for the LAMP assay were used in the cPCR assay. The primer concentrations (0.2, 0.3, 0.4, 0.5 µM) and assay temperature (50, 51, 52, 53, 54, 55°C) were changed one parameter at a time. Results were tested in triplicate using a recombinant

plasmid dilution having a concentration of 10^6 copies per reaction. Negative and positive controls as listed previously were used. Gel electrophoresis of amplified product using a 3% agarose gel was performed followed by staining with ethidium bromide.

5.2.6 Validation of LAMP and cPCR assays

The analytical sensitivity (limit of detection) and precision were assessed by testing triplicates of all concentrations of the 10-fold dilutions of plasmids containing the dnaK gene insert. In order to determine the precision or interassay variability, the 10-fold dilutions of the recombinant plasmid assayed in triplicate were run on three days. The specificity of the assay was determined by testing in duplicate, DNA of non-hemotrophic *Mycoplasma* spp. (*Mycoplasma mycoides* subsp *capri*, *Mycoplasma arginini*, *Mycoplasma agalactiae, Mycoplasma capricolum* subsp *capripneumoniae*, and *Mycoplasma mycoides* subsp *mycoides*) and other bacteria (*Pasteurella multocida*, *Escherichia coli*, *Clostridium perfringens, Clostridium difficile, Listeria monocytogenes, Listeria ivanovii, Yersinia pseudotuberculosis, Mannheimia haemolytica, Leptospira interrogans* serovar *pomona, L. interrogans* serovar *icterohemorrhagiae,* and *Salmonella enterica* serovar *Typhimurium).* These particular bacterial species were selected as they may be associated with septicemia and/or are known pathogens of sheep and goats. In addition to the HNB-visualized color change within the LAMP reaction tubes, the LAMP and cPCR products were separated by electrophoresis on a 3% agarose gel and subsequently stained with ethidium bromide.

5.2.7 Evaluation of LAMP and cPCR assays using naturally-infected sheep samples

Genomic DNA from 145 sheep was included in the study. Samples were evaluated for the presence of *M. ovis* by each of the following assays: LAMP, cPCR and real-time qPCR. DNA from a known *M. ovis*-positive sheep and nuclease free water were included in all runs as positive and negative controls, respectively. Samples were tested in duplicate when using LAMP and real-time qPCR and singly for the cPCR assay. To evaluate the diagnostic sensitivity and diagnostic specificity of the LAMP and cPCR assays, the samples were initially screened using a previously described real-time qPCR assay (Johnson, do Nascimento et al. 2016) as the "gold standard" for detection of *M. ovis*. Evaluation of the results for the cPCR and real-time qPCR assays was conducted by a single reviewer while two reviewers (1 blinded) interpreted the LAMP assay

results. Samples tested by the LAMP method were considered positive when at least one of the reaction tubes changed color from purple (negative) to blue (positive). When a band with the expected size of 207-bp was present after gel electrophoresis of the cPCR product, the sample was considered positive.

5.2.8 DNA sequencing

Conventional PCR assay amplicons produced from a subset (approximately 13%) of positive field samples identified were submitted for DNA sequencing. The cPCR amplicons were separated by gel electrophoresis using a 3% agarose gel followed by staining with ethidium bromide. Purification of the DNA was performed using Zymoclean Gel DNA Recovery Kit (Zymo Research Corp, Orange, CA). The fragment of the dnaK gene was directly sequenced in both directions at the Purdue Genomics Core Facility using the F3 and B3 primers listed previously.

5.2.9 Restriction endonuclease digestion of LAMP products

LAMP products from a positive sample were digested using BSU36I in a 50 µl reaction containing BSU36I (1µl) (New England Biolabs), 1X NE Buffer (5 µl), LAMP products approximately 1 µg (0.4 µl), nuclease-free water (43.59 µl), and incubated at 37°C for 1 hour. Restriction enzyme-treated and untreated LAMP products were assessed by electrophoresis using a 3% agarose gel followed by staining with ethidium bromide.

5.2.10 Statistical analyses

McNemar's tests were used to assess for differences in positivity among the LAMP, cPCR and qPCR assays. Values of $P < 0.05$ were considered significant. Cohen's kappa was used to assess the level of agreement of the results between the three assays. The real-time qPCR assay was used as the reference method when calculating the performance characteristics of the LAMP and cPCR assays. SPSS, version 23 was used for statistical analyses while 95% confidence intervals were generated for the test statistics using OpenEpi software (Dean A.G. 2013).

5.3 Results

5.3.1 Optimization of LAMP and cPCR assays

Temperature conditions and reaction time were optimized to be 64°C and 45 minutes, respectively, whereas primer concentrations were 1.6 µM, 0.2 µM, and 0.6 µM for the inner, outer and loop primers, respectively. After optimization of the reaction conditions using a recombinant plasmid, the final LAMP reaction mixture (25 µL) was comprised of 1.0 µL (1.6 µM) concentrations each of inner primers, 1.0 µL (0.2 µM) concentrations each of outer primers, 1.5 µL (0.6 µM) loop primers, 3.5 µL (1.4 mM) dNTPs (New England Biolabs, Ipswich, MA), 2.5 µL of 1X Isothermal amplification buffer (New England Biolabs), 1.5 µL (6 mM $MgSO_4$) (New England Biolabs), 2.5 µL (150 µM HNB) (Sigma-Aldrich, St. Louis, MO), 1 µL (8U) of *Bst* 2.0 DNA polymerase (New England Biolabs), 4 µL of nuclease-free water, and 3 µL of template DNA. The reaction was performed in a thermocycler set at 64°C for 45 minutes then 80°C for 5 minutes for heat inactivation of the enzyme. Positive samples were identified by a color change from purple to blue (Figure 5.4), and the presence of ladder-like bands on agarose gel (Figure 5.5).

Figure 5.4 Visualization of LAMP reaction using hydroxynaphthol blue (HNB) showing color differences between a positive and negative sample. Left tube (positive-sky blue), Right tube (negative-purple).

The final cPCR reaction mixture (25 µL) consisted of 2.5 µL of 1X Standard *Taq* reaction buffer (New England Biolabs), 0.5 µL each (0.2 µM of F3 and B3), 0.5 µL (200 µM dNTPs) (New England Biolabs), 0.125 µL (5U) of *Taq* DNA polymerase (New England Biolabs), 15.875 µL of nuclease-free water, and 5 µL of template DNA. Cycling conditions consisted of 95°C for 30 seconds, 35 cycles of (95°C for 30 seconds, 52.0°C for 30 seconds, and 68°C for 30 seconds), and 68°C for 5 minutes. Gel electrophoresis of amplified product using a 3% agarose gel and subsequently stained with ethidium bromide produced a 207-bp band (Figure 5.6).

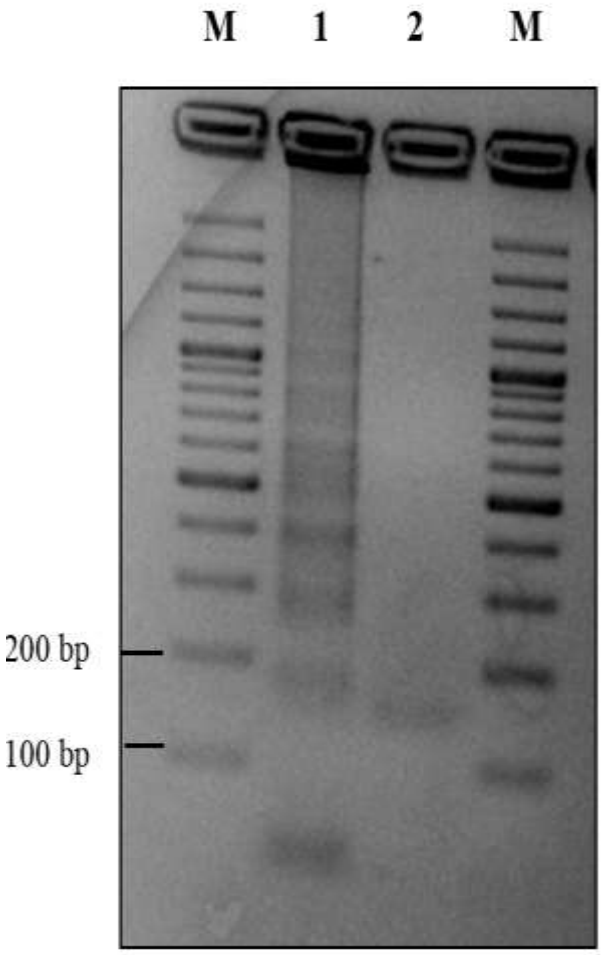

Figure 5.5. Agarose gel electrophoresis (3%) of LAMP products with and without restriction enzyme digestion using BSU36I. (Lanes M: DNA Ladder [GeneRuler 100 bp Plus DNA Ladder, ThermoScientific™, West Palm Beach, FL]; Lane 1: undigested LAMP product showing a ladder-like band pattern; Lane 2: restriction enzyme digestion of LAMP product.

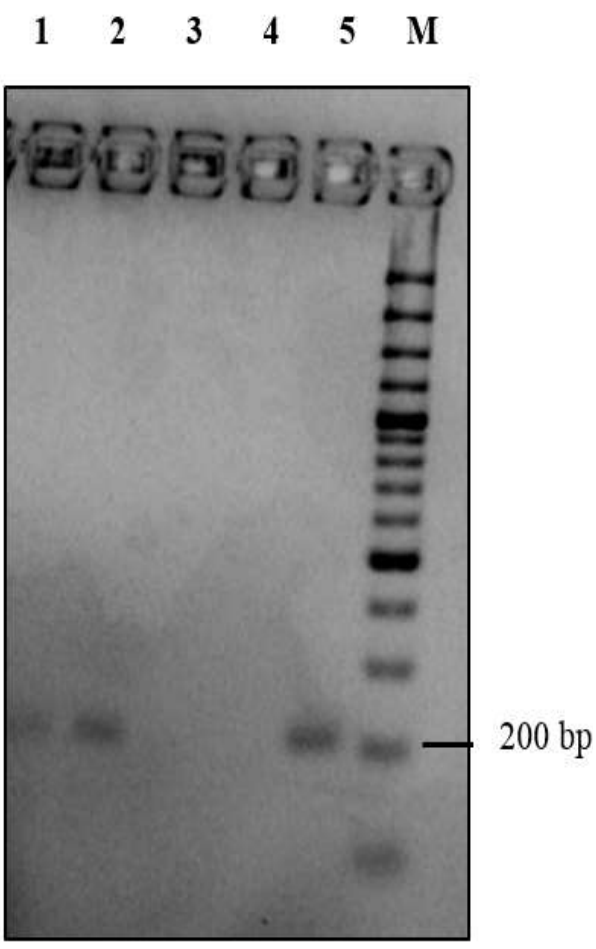

Figure 5.6. Agarose gel electrophoresis (3%) of cPCR amplified products (207 bp) using B3 & F3 outer primers. (Lanes 1, 2, 5: genomic DNA from *M. ovis* positive sheep; Lane 3: empty; Lane 4: genomic DNA from a *M. ovis* negative sheep; Lane M: DNA Ladder [GeneRuler™ 100 bp Plus DNA Ladder, ThermoScientific™, West Palm Beach, FL]).

5.3.2 Validation of LAMP and cPCR assays

To determine the sensitivities of the LAMP and cPCR assays, 10-fold serial dilutions of a plasmid (10^9 to 1 copy of plasmid/reaction) containing the dnaK gene of *M. ovis*, were used as templates and run in triplicate within the same run. The limits of detection for the LAMP and cPCR assays were determined to be 10^2 and 10^3 plasmids/ reaction, respectively. Lamp results were

considered positive based on HNB-visualized color change from purple to blue within the reaction tube and the presence of ladder-like bands on gel electrophoresis while cPCR positive results produced a single band at 207-bp. The precision or inter-assay variability was determined by testing the 10-fold serial plasmid dilutions across a total of three different runs on three separate days. No variations in the detection levels were observed across the three runs for either assay.

5.3.3 Specificity of the assays

To evaluate the specificity of the assays, DNA of non-hemotropic *Mycoplasma* spp. and bacteria associated with septicemia in sheep were tested in duplicate. No cross reactivity occurred for either assay for any of the non-target bacterial pathogens.

5.3.4 Evaluation of LAMP and cPCR assays using field samples

Blood samples were obtained from 145 female sheep from a single herd. Screening of samples for *M. ovis* using a previously reported SYBR® Green qPCR assay (Johnson, do Nascimento et al. 2016) was initially performed. The detection of *M. ovis* by qPCR, LAMP and cPCR assays was 46.2%, 36.5% and 31.0%, respectively (Table 5.2). While the LAMP assay detected more infected sheep than cPCR, both were in substantial agreement of each other with a Cohen's kappa coefficient of 0.754 (95% CI: 0.642 to 0.865) (Table 5.3). Two samples incorrectly identified as positive led to a slightly lower diagnostic specificity for the LAMP assay (97.4%) than cPCR (100%) (Table 5.2). The diagnostic sensitivity of the LAMP and cPCR assays was 76.1% and 67.2%, respectively (Table 5.2). Four samples found to be positive by cPCR, but negative by LAMP were retested with a longer reaction time of 60 minutes. Interestingly, two of the four samples then became positive. The bacterial loads for these four samples ranged from 4.24 x 10^5 to 8.25 x 10^5 copies/mL of blood. Forty-one samples identified positive by both LAMP and cPCR had a median value of 3.84 x 10^6 copies/mL of blood and ranged from 2.11 x 10^4 to 3.54 x 10^9 copies/mL of blood, while ten samples that were LAMP positive/cPCR negative ranged from 1.39 x 10^3 to 1.40 x 10^5 copies/mL of blood with a median value of 1.69 x 10^4 copies/mL of blood.

Table 5.2. Comparison of results for *M. ovis* detection for cPCR and LAMP assays.

Assay	% Positive (#pos/total)	Diagnostic Sensitivity (%), 95% CI	Diagnostic Specificity (%), 95% CI
qPCR	(67/145), **46.2**		
cPCR	(45/145), **31.0***	(67.2), 55.3-77.2	(100), 95.3-100
LAMP	(53/145), **36.5**	(76.1), 64.7-84.7	(97.4), 91.1-99.3

**P* = 0.077 for cPCR vs. LAMP *M. ovis* detection

Table 5.3. Agreement between cPCR and LAMP assays for detection of *M. ovis*.

	LAMP			
Assay	Pos	Neg	Total	kappa* (95% CI)
cPCR +	41	4	45	0.754(0.642-0.866)
cPCR −	12	88	100	
cPCR −	53	92	145	

* P <0.001

5.3.5 Confirmation of products

Sequencing of cPCR amplicons of 9 of 67 (13.4%) samples yielding positive results by qPCR, LAMP and cPCR assays were confirmed to be *M. ovis*. These amplicons had 97% to 100% identity with the dnaK gene of the *M. ovis* strain, CP006935.1. Restriction enzyme digestion with the BSU36I enzyme on a positive LAMP product resulted in the disappearance of the characteristic ladder-like pattern as expected (Figure 5.5).

5.4 Discussion

To the author's knowledge, the study reported herein was the first in which hemoplasma infection in sheep was detected using the LAMP method. This study demonstrated that the LAMP assay based on the dnaK gene provided a fast, reliable method to detect *M. ovis* using minimal equipment. A metal ion indicator, HNB, was added prior to amplification and allowed for easier visual detection of LAMP positive samples as indicated by a color change from purple to blue. The LAMP reaction produces large amounts of pyrophosphate ion byproducts that react with Mg^{2+}

ions producing magnesium pyrophosphate, an insoluble product (Figure 5.7). The color change of HNB from purple to blue is attributable to the decrease of available Mg^{2+} ions within the solution and the subsequent change in pH (Goto, Honda et al. 2009). Large amounts of amplification products produced by the reaction can easily contaminate a work area, therefore, addition of a dye prior to amplification and sterile techniques are crucial to reduce this risk. However, false positives have been reported for LAMP assays (Kim, Kim et al. 2014, Higa, Uemura et al. 2016) even when proper techniques were followed, which occurred in the current study, although minimally. Overall, the results generated from the LAMP assay were dependable and were consistent across a range of bacterial loads. This assay in comparison with cPCR offers a faster time to results, with no exposure to ethidium bromide, a known carcinogen, once it has been optimized and validated. The addition of HNB made visual detection of positives easy with 100% agreement between reviewers, one of whom was blinded to the qPCR results. During the development of the assay the results of the HNB coloration were reconfirmed at all stages by agarose gel electrophoresis, but once optimized and validated only a few of the field samples were subjected to gel electrophoresis.

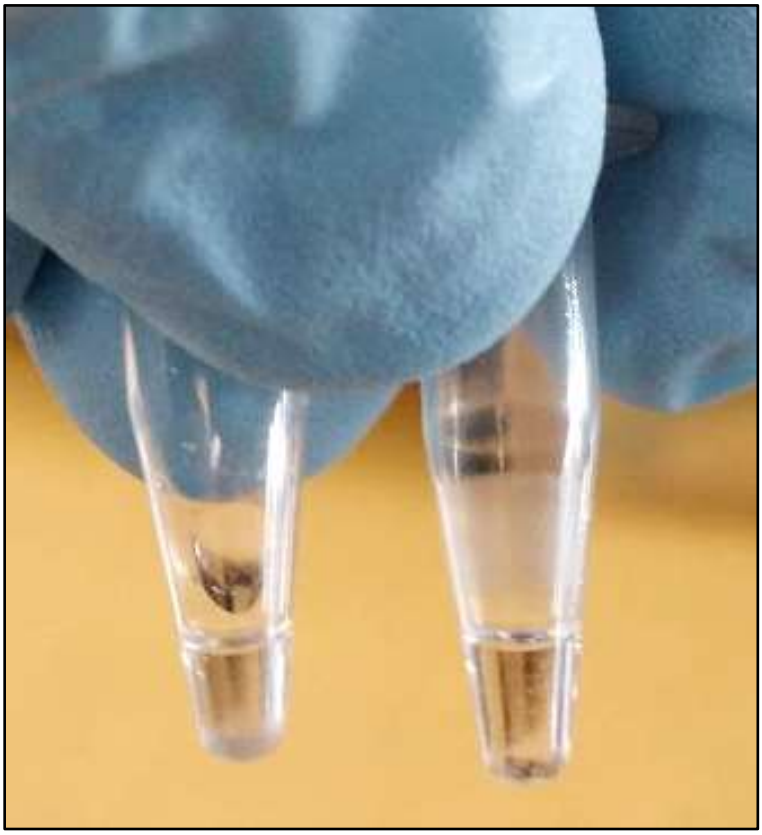

Figure 5.7. Visualization of LAMP reaction based on turbidity. Left tube (positive-white precipitate), Right tube (negative-clear).

Differences in the detection levels between LAMP and cPCR assays have been reported with LAMP displaying higher sensitivities (Venkatesan, Bhanuprakash et al. 2012, Song, Wang et al. 2013, Yang, Guan et al. 2013, Xie, Xie et al. 2014, Mahittikorn, Mori et al. 2015). The overall detection levels between LAMP and cPCR for the current study were not significantly different (P = 0.08), although more positive samples were found using LAMP. A diagnostic sensitivity of 67.2% (95% CI, 55.3 – 77.2%) for cPCR was obtained (Table 5.2). A higher analytical sensitivity for the LAMP assay than the cPCR assay was determined with limits of detection of 10^2 and 10^3 plasmids per reaction, respectively. However, a higher diagnostic specificity (100%) was obtained for the cPCR assay as two samples were incorrectly identified as positive by the LAMP assay. Sheep that were identified as positive by both LAMP and cPCR had a higher median bacterial load (3.84×10^6 copies/mL of blood) compared with sheep that were misidentified as negative by cPCR (1.69×10^4 copies/mL of blood), but positive by LAMP. This indicates the usefulness of the LAMP assay for identifying not only acutely infected sheep, but as a screening assay with the capability to identify chronically infected sheep.

To confirm the LAMP products were amplified from the dnaK gene of *M. ovis*, a positive LAMP sample was treated with the BSU36I restriction endonuclease which removed the ladder-like pattern indicating digestion of the amplified product. The expected fragment sizes were 122 bp and 85 bp, but only the 122 bp was visible after agarose gel electrophoresis. Due to the final products of the reaction having multiple shapes and sizes the fragments generated by digestion may not always be in agreement with their predicted sizes (Notomi, Okayama et al. 2000), as was the case in the present study. NEBcutter, the program used to predict the fragment sizes, is based on the assumption that the DNA is linear or circular (Vincze, Posfai et al. 2003), but LAMP products are neither. They are mixtures of stem-loop DNAs of various stem sizes with loops containing inverted repeats of the target sequence. It is possible that the 82 bp fragment was present, but was too faint for detection. If the primers had amplified the dnaK gene at a different location than intended, the size of the fragments would be different. However, the expected size of one of the fragments was visible; thus, implying the correct sequence was amplified. It would have been beneficial to sequence the purified DNA from the restriction endonuclease digested LAMP product to determine the exact nucleotide sequence amplified.

A limitation of the present study was the number of positive samples available for testing. While 67 sheep were identified positive by qPCR and 53 sheep by LAMP, a larger sample size of

positives with varying bacterial loads from multiple herds would help clarify the lower limit of the LAMP assay while testing potentially more strains of *M. ovis*. Additionally, increasing the reaction time more than 45 minutes may improve the lower limit of detection for the LAMP assay and should be considered. Restrictions regarding the size of the template to 300 bp or less (for optimal amplification) may impact the usefulness of this assay for some researchers, but was not an issue in the current study. Another consideration to be aware of is the ability of the reviewer to determine whether a color change from purple (negative) to blue (positive) has occurred. Other metal ion indicators and DNA-intercalating dyes are available and could be evaluated for improving the endpoint detection of the assay.

In conclusion, a LAMP assay was developed which was sensitive and specific for the detection of *M. ovis* in infected sheep. The frequency with which naturally-infected sheep were identified using LAMP indicates the widespread occurrence of this bacterium and the suitability of the assay for use in epidemiological studies.

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CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

The studies within this dissertation have not only expanded the ways in which *M. ovis* can be detected in goats and sheep, but have also led to a greater understanding of the infection dynamics in pregnant ewes and their lambs. The development of a *M. ovis*-specific SYBR Green qPCR assay was pivotal in demonstrating that although bacterial loads in goats with subclinical infections are $\leq 10^5$ copies/mL of blood, almost 20% of the animals were positive. The majority of studies on *M. ovis* in goats have used blood smear examination for detection of the bacterium with a few reports using cPCR. To our knowledge, this is the first assay for detection and quantification of *M. ovis* in goats which provided novel information on prevalence, bacterial loads and risk factors for goats in Indiana. It is unknown why dairy goats were found to be at a greater risk of infection in the current study than meat goats. It is possible age differences among the goats within the two production types account for the difference in risk. On the other hand, different management practices might account for a greater likelihood of exposure and infection in dairy goats. Future epidemiological studies focusing on risk factors for infection with inclusion of goats from wider geographical regions, different management practices and production uses may help to answer this question.

The second test developed for the detection of *M. ovis* was the LAMP assay. This was shown to be fast and reliable for *M. ovis* detection and had better sensitivity compared with cPCR. The ability to detect positives without the use of agarose gel electrophoresis along with a methodology not requiring a thermocycler is critical for resource-limited laboratories. In the LAMP assay, detection of a positive result (amplified DNA) is based on a color change due to the presence of a metal sensitive dye in the reaction tube. Several different types of indicator dyes are available which demonstrates the flexibility of the assay and capability to personalize the detection method based on a laboratory's need. Hydroxynapthol blue was used in the current study, allowing for fast, easy visual identification of positives. This assay has applications for use in infectious disease research and diagnostics, but does not have the ability to quantify bacterial loads. The use of different strains of *M. ovis* would help define the diagnostic capabilities and limitations of the assay, since it was developed from samples from a single sheep herd.

Massive parallel sequencing is a useful technology for rapidly sequencing whole genomes and exploring microbial diversity, however it is expensive and often exceeds the financial

resources of smaller research projects. WideSeq, a next generation sequencing service offered by the Genomics Core at Purdue University, allows full sequencing of double stranded DNA samples up to 100 kb. This cost-effective alternative to whole-genome sequencing provides high throughput sequencing using an Illumina MiSeq platform. Using this NGS platform, we successfully generated data from pooled amplicons of five housekeeping genes of *M. ovis*, having sufficient quality and depths of coverage not only for the reads, but also for detection of single nucleotide polymorphisms (SNPs). A multilocus sequence typing (MLST) assay for *M. ovis*, based on these conserved loci, was undertaken as a proof of concept project to genotype *M. ovis* from whole blood DNA extracts, with the intention of better understanding the genetic diversity among *M. ovis* isolates. The bacterium has not been successfully maintained in culture, thereby limiting the ways in which the genetic relationship among isolates can be analyzed. While the discriminatory power of the MLST for *M. ovis* was good, and the NGS methodology detected SNPs within the housekeeping genes, the assay was based only on samples from a regionally restricted area. Further development of this MLST assay must incorporate samples from geographically distant locations, as well as the inclusion of other genes, if the assay is to be useful for transmission studies. Multi-locus sequence typing is used to study global epidemiological outbreaks, utilizing genes which are known to be slowly diversified (housekeeping genes), but it has been reported that virulence-associated genes, due to evolutionary pressures, may display the increased nucleotide sequence polymorphism needed for studying local outbreaks. Currently, the genes associated with virulence in *M. ovis* are unknown.

The cross-sectional and longitudinal studies undertaken as part of this research, have expanded our knowledge and understanding of *M. ovis* infection among pregnant ewes and their lambs. Despite high bacterial loads in pregnant ewes, sometimes reaching 10^9 copies/mL of blood, the ewes did not display overt clinical signs and infection of their lambs was not detected until after weaning. These findings do not support transplacental and transmammary transmission of *M. ovis* from ewes to their lambs. It is plausible that lambs have more potential for exposure to *M. ovis* within their environment after weaning, however a protective effect during the suckling period cannot be ruled out. Both ewes and lambs were inconsistently positive throughout the longitudinal study, indicating the need for either repeat testing of sheep, or expanding the number of tested sheep within a herd when attempting to detect the bacterium. Since the transmission routes for the hemoplasmas are not well characterized, research focusing on the development of infection among

lambs is needed, particularly to determine if *M. ovis* is a vector-borne infectious organism. Finally, the prevalence of *M. ovis* within a cohort of biomedical research sheep was 42.7%. This information suggest the need to test sheep prior to inclusion in any research project to avoid confounding research results. In general, the sequelae or consequences of chronic infection of sheep with *M. ovis* are not well-defined. Prevention and treatment strategies would greatly benefit from additional epidemiological studies, particularly focused on possible transmission routes.

APPENDIX A. SHEEP FARM SURVEY

1. Date _____/_____/2016
2. Farm name _____
3. Address _____

   _____
4. County _____
5. Owner's name _____
6. Owner's phone # _____
7. Email _____
8. Number of ewe/lamb pairs sampled? _____
9. How many sheep do you own? _____
   a. Number of ewes? _____
      i. Number expected to lamb? _____
   b. Number of rams? _____
   c. Number of ram and ewe lambs? _____
10. How many different breeds do you have? _____
    a. Specify the breeds _____

       _____
11. What are the sheep used for?

a. Meat production	Yes	No
b. Milk production	Yes	No
c. Cheese production	Yes	No
d. Showing/4-H	Yes	No

e. Pets/companion Yes No

f. Other (specify) _____ Yes No

12. Do you board sheep or goats for others? Yes No

13. Do you have other animals on the farm? Yes No

a. Cattle Yes No

b. Goats Yes No

c. Alpacas Yes No

d. Llamas Yes No

e. Dogs Yes No

f. Cats Yes No

g. Horses Yes No

h. Poultry Yes No

i. Pigs Yes No

j. Other (specify) Yes No

14. Do the sheep share pastures with any of the animals listed in question 13? Yes No

a. Specify which animals _____

15. How frequently are deer seen on the farm?

a. Often Yes No

b. Sometimes Yes No

c. Never Yes No

16. Do you find deer within the enclosed pasture areas? Yes No

17. Do you see coyotes on the farm? Yes No

18. Do you know if there are farms (within 1 mile) that have sheep, goats, cattle, pigs, alpacas/llamas, or horses? Yes No Unknown

    a. Number of farms? _____
        i. Specify animals on farms _____

19. In general, does this farm have defined lambings per year? Yes No

    a. One lambing per year Yes No

        i. Specify the season _____

    b. Two lambings per year Yes No

        i. Specify the seasons _____

20. How many lambs were born in the most recent lamb crop? _____

21. In general, do the ewes have a specific area for lambing? Yes No

    a. Specify area _____

22. In general, are ewes that have recently lambed kept separated from the herd? Yes No

23. What is the average age of the lambs when weaned? _____

24. Have you ever had weak lambs born on the farm? Yes No

    a. What year and season were the most recent weak lambs born? _____

    b. How many weak lambs were born then? _____

    c. How many weak lambs survived? _____

    d. Was a diagnosis made? Yes No

        i. Specify diagnosis _____

25. In 2016, how many sheep died (exclude culled sheep)? _____

    a. Specify reason for each death _____

26. In 2016, how many sheep were culled? _____

    a. Specify reason for culling? _____

27. Have any sheep been diagnosed with anemia? Yes No

    a. Specify the month or season they were anemic _____

28. In general, what percentage of time do the sheep spend (answers must equal 100%)

    a. Indoors _____%

    b. Outdoors _____%

29. Have you noticed external parasites on the sheep? Yes No

    a. Ticks Yes No

    b. Lice Yes No

    c. Keds Yes No

30. If yes to question 29, were the sheep treated? Yes No

    a. Specify parasite treated for. _____

    b. Specify animals treated (Entire herd or infested animals) _____

    c. What product was used? _____

31. Do you apply chemical products to your sheep for controlling insects (flies, mosquitoes)? Yes No

    a. Name of product _____

    b. How often was this product used? _____

32. Do you control for flies and mosquitoes on the farm? Yes No

    a. Fly traps Yes No

    b. Fans Yes No

    c. Other (specify) _____ Yes No

33. Have you noticed insects bothering the sheep? Yes No

    a. Flies Yes No

    b. Mosquitoes Yes No

c. Other (specify) Yes No

34. What month(s) are the sheep sheared? _____

35. Do you disinfect the shears? Yes No

a. If yes, what disinfectant is used? _____

b. Do you disinfect between individual sheep? Yes No

i. If no, when do you disinfect? _____

36. Do you reuse needles on the sheep? Yes No

a. If yes, how many different sheep will the same needle be used on ? _____

37. When a procedure has the potential to cause bleeding (ear tagging, tail docking, disbudding, castration, foot trim, etc) are the instruments disinfected between animals? Yes No

a. If yes, what disinfectant is used? _____

38. Are fecal egg counts used to determine when to deworm? Yes No

39. How often are dewormers used on the herd? _____

40. Do you determine the sheep's weight prior to deworming? Yes No

APPENDIX B. ANIMAL FORM (1)-EWE

1. Date _____/_____/2016, Visit 1
2. Farm name _____
3. Animal ID _____
4. Breed _____
5. Date of birth _____
6. Most recent lambing? (Date) _____
    a. Was it a single or multiple birth? _____
    b. Were all lambs born alive? Yes No
        i. How many were born alive? _____
        ii. How many born dead? _____
7. How was the ewe bred?
    a. By artificial insemination using this farm's ram. Yes No
        i. Was fresh or frozen semen used? _____
    b. By artificial insemination using another farm's ram. Yes No
        i. Was fresh or frozen semen used? _____
    c. Naturally - ram on farm Yes No
    d. Naturally - non-owned ram Yes No
        i. Ewe brought to ram Yes No
        ii. Ram brought to ewe Yes No
8. How many times has she lambed? _____
9. Any history of weak lambs from this ewe? Yes No

10. Any history of having a blood transfusion with this ewe? Yes No

    a. What was the diagnosis? _____

11. Has this ewe been treated with an oxytetracycline antibiotic in the last month? Yes No

    a. What was the diagnosis? _____

    b. What was the dosage? _____

12. Has this ewe been treated with any antibiotic in the last month? Yes No

    a. What was the diagnosis? _____

        i. Specify antibiotic and dosage _____

13. Does this ewe spend time outdoors? Yes No

    a. Winter Yes No

    b. Spring Yes No

    c. Summer Yes No

    d. Fall Yes No

14. Does this ewe spend more time outdoors during the warmer months? Yes No

15. When is this ewe outdoors during the warmer months?

    a. Outdoors during the day only Yes No

    b. Outdoors at night only Yes No

    c. Outdoors both day and night Yes No

16. Does this ewe have direct contact with goats? Yes No

17. Have you noticed external parasites on this ewe? Yes No

    a. Ticks Yes No

    b. Lice Yes No

c. Keds Yes No

18. Date of latest deworming? _____

a. What dewormer was used? _____

b. How was the dewormer administered? _____

APPENDIX C. ANIMAL FORM (2)-EWE

1. Date _____/_____/2016, Visit # (circle) 2 3 4 5 6 7

2. Farm name _____

3. Animal ID _____

4. Has this ewe been treated with an oxytetracycline antibiotic since the last visit? Yes No

    a. What was the diagnosis? _____

    b. What was the dosage? _____

5. Has this ewe been treated with any antibiotic since the last visit? Yes No

    a. What was the diagnosis? _____

        i. Specify antibiotic and dosage _____

5. Has this ewe spent time outdoors since the last visit? Yes No

    a. Outdoors during the day only Yes No

    b. Outdoors at night only Yes No

    c. Outdoors both day and night Yes No

6. Has this ewe had direct contact with goats since the last visit? Yes No

7. Have you noticed external parasites on this ewe since the last visit? Yes No

    a. Ticks Yes No

    b. Lice Yes No

    c. Keds Yes No

8. Has this ewe been dewormed since the last visit? Yes No

    a. What dewormer was used and when? _____

b. How was the dewormer administered? _____

9. Has this ewe had a blood transfusion since the last visit? Yes No

a. What was the diagnosis? _____

10. Have there been any procedures on this ewe since the last visit? Yes No

a. Ear tagging Yes No

b. Shearing Yes No

c. Vaccinations Yes No

d. Tail docking Yes No

e. Foot trim Yes No

f. Breeding Yes No

g. Dehorning Yes No

h. Other (specify) _____ Yes No

APPENDIX D. ANIMAL FORM (1)- LAMB

1. Date _____/_____/2016, Visit 1
2. Farm name _____
3. Animal ID _____
4. Breed _____
5. Date of birth _____
6. Sex: Male Female
7. Is this lamb from a single or multiple birth? _____
   a. If multiple birth, what is this lamb's birth order? 1 2 3 Unknown
8. Is this lamb suckling solely from the birth ewe? Yes No Unknown
   a. Lamb is suckling solely from another ewe. Yes No
   b. Lamb is suckling from the birth ewe and another ewe. Yes No
   c. Lamb is being fed milk-replacer solely. Yes No
9. Has this lamb had a blood transfusion? Yes No
   a. What was the diagnosis? _____
10. Has this lamb been treated with an oxytetracycline antibiotic? Yes No
    a. What was the diagnosis? _____
    b. What was the dosage? _____
11. Has this lamb been treated with any antibiotic? Yes No
    a. What was the diagnosis? _____
       i. Specify antibiotic and dosage _____
12. Has this lamb spent time outdoors? Yes No
13. Has this lamb had direct contact with goats? Yes No

14. Have you noticed external parasites on this lamb? Yes No

    a. Ticks Yes No

    b. Lice Yes No

    c. Keds Yes No

15. Has this lamb been dewormed? Yes No

    a. What dewormer was used and when? _____

    b. How was the dewormer administered? _____

16. Have there been any procedures on this lamb? Yes No

    a. Ear tagging Yes No

    b. Shearing Yes No

    c. Vaccinations Yes No

    d. Tail docking Yes No

    e. Foot trim Yes No

    g. Debudding Yes No

    h. Other (specify) _____ Yes No

APPENDIX E. ANIMAL FORM (2)- LAMB

1. Date _____/_____/2016, Visit # (circle) 2 3 4 5 6 7
2. Farm name _____
3. Animal ID _____
4. Has this lamb been weaned? Yes No
    a. If yes, specify date of weaning. _____
5. Is this lamb suckling solely from the birth ewe? Yes No Unknown
    a. Lamb is suckling solely from another ewe. Yes No
    b. Lamb is suckling from the birth ewe and another ewe. Yes No
    c. Lamb is being fed milk-replacer solely. Yes No
6. Has this lamb had a blood transfusion since the last visit? Yes No
    a. What was the diagnosis? _____
7. Has this lamb been treated with an oxytetracycline antibiotic since the last visit? Yes No
    a. What was the diagnosis? _____
    b. What was the dosage? _____
8. Has this lamb been treated with any antibiotic since the last visit? Yes No
    a. What was the diagnosis? _____
        i. Specify antibiotic and dosage _____
9. Has this lamb spent time outdoors since the last visit? Yes No
    a. Outdoors during the day only Yes No
    b. Outdoors at night only Yes No
    c. Outdoors both day and night Yes No

10. Has this lamb had direct contact with goats since the last visit? Yes No

11. Have you noticed external parasites on this lamb since the last visit? Yes No

    a. Ticks Yes No

    b. Lice Yes No

    c. Keds Yes No

12. Has this lamb been dewormed since the last visit? Yes No

    a. What dewormer was used and when? _____

    b. How was the dewormer administered? _____

13. Have there been any procedures on this lamb since the last visit Yes No

    a. Ear tagging Yes No

    b. Shearing Yes No

    c. Vaccinations Yes No

    d. Tail docking Yes No

    e. Foot trim Yes No

    f. Debudding Yes No

    g. Castration Yes No

    h. Other (specify) _____ Yes No