A survey of Chronic Pneumonia and Polyarthritis Syndrome (CPPS) associated *Mycoplasma bovis* in western Canadian feedlots.

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Rose Whelan

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ABSTRACT

Mycoplasma bovis is generally considered the causative pathogen associated with Chronic Pneumonia and Polyarthritis Syndrome (CPPS) in feedlot cattle. However, *M. bovis* virulence may vary between strains as it is also isolated from asympytomatic cattle. The following study aims to determine the prevalence of *M. bovis* in the respiratory tract of western Canadian cattle using two sampling methods and at two time points following feedlot entry. Three study groups were sampled. In the first group nasal swabs (NS) and bronchoalveolar lavages (BAL) were taken from 36 clincally healthy cattle at the University of Saskatchewan feedlot at both 14 and 90 days on feed (DOF). In a second experiment, NS were taken from 56 animals upon arrival at a commercial feedlot and one week to three months later upon treatment for respiratory disease. Lung and joint tissue swabs were collected at necropsy from a third group of 19 animals with CPPS clinical pathology originating in 10 different western Canadian feedlots. All samples were selectively cultured for *Mycoplasma* spp. DNA was extracted from isolated putative *Mycoplasma* colonies and amplified with universal 16S rRNA gene primers for identification. Amplified Fragment Length Polymorphism (AFLP) was used to genetically differentiate *M. bovis* positive isolates. More *M. bovis* was isolated from NS than BAL and *M. bovis* prevalence increased with DOF in the feedlot in both the University of Saskatchewan and commercial feedlot trials. Three genetically distinct clusters (A, B, and C) were isolated from the necropsy group. Two of these clusters were primarily associated with isolates collected from feedlot cattle and one strain was exclusively found in CPPS-associated mortalities. No significance difference in the prevalence of *M. bovis* strains was observed between different days on feed or sampling methods. It was concluded that either the difference in disease state is a host dependent outcome, due to a multi-factorial disease complex, or the AFLP assay was not sensitive enough to differentiate strains based on virulence.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AP-PCR	arbitrarily primed polymerase chain reaction
AT	at treatment
BAL	bronchoalveolar lavage
bp	base pairs
°C	degrees Celsius
cc	cubic centimetre
cm	centimetre
CO_2	carbon dioxide
CPPS	Chronic Pneumonia and Polyarthritis Syndrome
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphates
DOF	days on feed
ELISA	Enzyme Linked Immunosorbent Assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
g	grams
G+C	guanine and cytosine
hrs	hours
IFN	interferon
IL	interleukin
kb	kilobase
kg	kilogram
MIC	minimum inhibitory concentration
min	minutes
μg	microgram
μL	microlitre
mL	millilitre
mM	micromolar
M.	*Mycoplasma*
ng	nanogram
NS	nasal swabs
OA	on arrival
PBI	Plant Biotechnology Institute
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
pmol	picomols
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
sec	seconds
TE buffer	Tris and EDTA buffer

Th1	type 1 T helper cell
Th2	type 2 T helper cell
TNF	tumour necrosis factor
U	units
UV	ultraviolet
V	volts
VNTR	variable number tandem repeat
VSP	variable surface protein

1.0 INTRODUCTION

        Mycoplasma bovis is an important pathogen in the feedlot industry from both economic and welfare concerns (Nicholas and Ayling, 2003). Previous research has shown that *M. bovis* may afflict cattle with a variety of diseases such as mastitis, otitis media, keratoconjuctivitis and chronic pneumonia and polyarthritis syndrome (Alberti et al., 2006; Gagea et al., 2006; Maeda et al., 2003). Combined, all of the clinical manifestations of this pathogen are estimated to cost $32 million dollars annually to the American cattle industry and €144 million to the European industry (Nicholas and Ayling, 2003).

        The *M. bovis* condition of particular interest to the feedlot industry is Chronic Pneumonia and Polyarthritis Syndrome (CPPS). *Mycoplasma bovis* has been shown to cause pneumonia in feedlot cattle and as the bacteria can resist many of the traditional treatments for pneumonia in the feedlot, this may develop into a CPPS, which is defined by the simultaneous presence of both persistent pneumonia and arthritis in at least one joint (Krysak, 2006; Nicholas and Ayling, 2003). CPPS can take weeks or months between onset of disease and either death, euthanasia, or recovery (Krysak, 2006; Pfützner, 1990). This long disease process confronts the producer with a great deal of labour associated costs, veterinary bills, and other disease related expenses, as well as significant welfare concerns in regards to the animal (Nicholas and Ayling, 2003).

        Mycoplasma bovis has been isolated from the respiratory tracts of healthy cattle; therefore, there has been some speculation regarding the pathogenicity of the bacteria. Further, in many cases, cattle ailing from pneumonic symptoms that test positive for *M. bovis* also test positive for many other bacteria associated with pneumonia in cattle (Blackburn et al., 2007). It is therefore, still unclear whether *M. bovis* is the primary causative agent initiating the disease or a secondary factor to one of the many other organisms associated with respiratory disease in cattle. However, pneumonic conditions have been established both in gnotobiotic and conventional cattle inoculated with *M. bovis* (Arcangioli et al., 2007; Pourmat et al., 2001; Thomas et al., 1986). Pourmat et al (2001) showed that inoculation of *M. bovis* in conventionally reared calves not only resulted in these calves showing clinical signs suggestive of pneumonia, but also increased the numbers of another bacterium, *Pasteurella multocida*, which has been associated with bovine respiratory disease, suggesting that it was indeed the primary causative agent at least in this multifactorial scenario. Another study examined not only *M. bovis*, but many other agents thought to be associated with bovine respiratory disease, and

found that in cattle afflicted with bovine respiratory disease, *M. bovis* was the most common pathogen present (Arcangioli et al., 2007). Finally, in a survey of the prevalence of *M. bovis* in stocker and backgrounding operations it was noted that cattle testing positive for *M. bovis* in nasal swabs were more likely to exhibit symptoms, such as fever and mucopurulent nasal discharge, clinical signs indicative of respiratory disease (Wiggins et al., 2007).

Members of the *Mycoplasma* genus are extremely small organisms lacking cell walls and are therefore, resistant to many antibiotics, such as the β-lactams, that target bacterial cell wall structures (Francoz et al., 2005; ter Laak et al., 1993). *M. bovis* has also shown resistance to other classes of antibiotics often used in feedlots such as many of the tetracyclines and may be developing resistance to even more antibiotics, which were at one time effective (Francoz et al., 2005; ter Laak et al., 1993). Therefore, *M. bovis* is often associated with CPPS as the pathogen survives antibiotic treatments commonly used for pneumonia in feedlots.

Successful techniques for diagnosis of *M. bovis* as the causative agent of pneumonia may; therefore, be of great importance to the treatment of pneumonia and the prevention of CPPS, as antibiotics must be chosen, which are effective for *M. bovis* in those animals testing positive for the pathogen. Research suggests that samples taken by bronchoalveolar lavages are more effective for *M. bovis* screening than nasal swabs, as *M. bovis* is thought to be a colonizer of the lower respiratory tract (Thomas et al., 2004). These samples have been tested for specific *Mycoplasma* spp. using ELISA assays or sequence analysis of PCR amplified universal target genes (Ayling et al., 1997; Butler et al., 2001; Dénes et al., 2003).

While successful identification of *M. bovis* and appropriate antibiotic administration may treat specific cattle already ailing from CPPS on a case to case basis, this treatment would be largely ineffective for elimination of *M. bovis* from a pen of feedlot animals or a herd of cows and calves. This is because *M. bovis* also colonizes healthy animals, which may shed the organism via nasal secretions to their cohorts (Butler et al., 2001). The addition of new cattle into a feedlot, as is done in western Canadian feedlots, has been shown to increase the likelihood of identifying *M. bovis* in penmates (Butler et al., 2001). Prevention of *M. bovis* induced CPPS, using effective antibiotics or other approaches is; therefore, vital to disease control.

The ineffectiveness of prophylactic antibiotics in CPPS prevention has made *M. bovis* vaccination research of great interest. However, the variability of *M. bovis* surface proteins has made successful vaccine development very difficult (Perez-Casal and Prysliak, 2007).

Development of an *M. bovis* vaccine effective against all virulent strains has also been hindered by the lack of research available regarding pathogenic strains and their virulence factors as antigenic targets (Perez-Casal and Prysliak, 2007). Vaccines are available for *M. bovis* induced mammary infections and research is ongoing for CPPS associated *M. bovis* vaccines; however, these vaccines have produced ineffective results in clinical trials (Boothby et al., 1986; Chima et al., 1981; Nicholas and Ayling, 2003; Perez-Casal and Prysliak, 2007; Maunsell and Donovan, 2009). Therefore, a better understanding of the virulent strains and virulence associated genes in *M. bovis* could greatly benefit the development of efficacious commercial vaccines.

While *M. bovis* studies have been conducted in feedlots and stocker/backgrounding operations in other areas of the world such as Europe (Arcangioli, et al., 2007; Blackburn et al., 2007), the United States (Wiggins et al., 2007) and Eastern Canada (Gagea et al., 2006), an extensive survey of western Canada has not been conducted; therefore, the prevalence of *M. bovis* as a possible causative agent of CPPS in this region is unknown. There are also many strains of *M. bovis* and these different strains may be responsible for different conditions in the animal (Blackburn et al., 2007; Miles et al., 2005). For example, the strain that causes mastitis in dairy cattle is not the same strain that causes pneumonia in feedlot cattle (Biddle et al., 2005). Conceivably the strain that causes pneumonia may be different from the strain that allows for dissemination to the joint and development of CPPS. Therefore, determining the strains of *M. bovis* associated with CPPS in western Canadian feedlots would also increase the understanding of the disease and its prevention in this area of the world.

The proposed study will aim to establish strain differentiation methods for *M.bovis*; utilize these methodologies to identify strains common to cattle with CPPS clinical pathology at necropsy; determine the prevalence of *M. bovis* strains in healthy feedlot cattle and cattle treated for respiratory disease; and compare the *Mycoplasma* spp. populations in the upper and lower respiratory tracts of healthy cattle. This will allow us to test the hypothesis that a unique strain of *M. bovis* is associated with CPPS.

2.0 LITERATURE REVIEW

2.1 *Mycoplasma bovis* taxonomy and general characteristics

The genus *Mycoplasma* belongs to the *Mollicutes* class in the phylum *Firmicutes,* which encompasses many low G+C gram positive bacteria (Sirand-Pugnet et al., 2007b; Weisburg et al., 1989; Woese et al., 1980). The *Mollicutes* are thought to have evolved from more typical Gram positive low G+C bacteria in a regressive manner involving large-scale genome reduction. The class was actually named '*Mollicutes',* Latin for 'soft skin', as their small genome size results in an inability to produce peptidoglycan cell wall. This incredibly small genome size also lacks many other genes involved in critical biochemical pathways. Therefore, *Mollicutes* are generally considered parasitic organisms, requiring their host organisms to provide essential nutrients (Sirand-Pugnet et al., 2007b; Weisburg et al., 1989; Woese et al., 1980). The inability of *Mollicutes* to synthesize essential nutrients also makes them extremely hard to culture; however, growth of many species is possible at 37°C on complex growth medias (Hannan, 2000). Replication of these *Mollicutes* occurs by binary fission like prokaryote; however, the mechanisms are not yet well understood (Miyata and Seto, 1999; Razin et al., 1998). Also recent research suggests that at least in some *Mollicutes* there is much higher horizontal gene transfer rate than thought in early studies; therefore, some members of this class may have retained or acquired sexual competence (Sirand-Pugnet et al., 2007a).

There are about 200 species of *Mollicutes*, over 100 of which belong to the *Mycoplasma* genus (Trachtenberg, 2005; Weisberg et al., 1989). The first isolation of a *Mycoplasma* was in 1896 at Pasteur institute (Nocard and Roux, 1896). The organism was cultured from cattle suffering from arthritis and pleuropneumonia (Nocard and Roux, 1896). *Mycoplasma spp.,* however, inhabit a wide array of host organisms, colonizing over 180 species in plants and animals (Weisberg et al., 1989). They were until recently thought to be exclusively extracellular pathogens, simply adhering to specific tissues and organs in the host (Weisburg et al., 1989; Woese et al., 1980). However, internalization of *Mycoplasma* spp. into host cells is now being studied and mechanisms involving adhesion induced specific changes to microtubule and microfilament structure of the host cytoskeleton have been discovered in specific species (Ueno et al., 2008; Winner et al., 2000; Yavlovich et al., 2004; Yavlovich et al., 2006). *Mycoplasma* colonization in animals is often pathogenic, causing a wide range of physiological manifestations ranging from various respiratory, cardiovascular, ocular, reproductive, urogenital, central

nervous system, skin and joint conditions (Alberti et al., 2006; Byrne et al., 1999; Gagea et al., 2006; Lu and Rosenbusch, 2004; Maeda et al., 2003). These conditions are also difficult to treat and prevent as *Mycoplasma* spp. are inherently resistance to many antibiotics and have several methods of modifying and evading the immune system (Allen et al., 1992; Behrens et al., 1996, Haines et al., 2001; Radaelli et al., 2009; Rosengarten et al., 1994; Vanden Bush and Rosenbusch, 2003). Therefore, ongoing research into *Mycoplasma* pathogens is of great importance to the livestock industries currently trying to treat and prevent *Mycoplasma*-associated disease.

2.2 *Mycoplasma bovis* and Chronic Pneumonia and Polyarthritis Syndrome (CPPS)

There is speculation regarding the relevancy of *M. bovis* as a causative agent of CPPS as *M. bovis* is also isolated from healthy animals (Allen et al., 1991; 1992; Hirose et al., 2003; Rosendal and Martin, 1986; Sachse et al., 2009; ter Laak et al., 1992b; Thomas et al., 2002; Wiggins et al., 2007). However, several studies have concluded that the presence of *M. bovis* or *M. bovis* antigens is highly associated with both the symptoms and clinical pathology of CPPS in cattle at necropsy (Booker et al., 2008; Gagea et al., 2006; Haines et al., 2001; Maeda et al., 2003; Radaelli et al., 2008; Rodríguez et al., 1996; Wiggins et al., 2007). There has also been some conjecture that *M. bovis* is an opportunistic pathogen that takes advantage of lesions caused by primary infection with other respiratory pathogens, as it is often found associated with other well established pathogens in cases of feedlot respiratory disease (Blackburn et al., 2007; Booker et al., 2008; Fulton et al., 2009; Martin et al., 1999; Shahriar et al., 2002; Thomas et al., 2002). However, cattle afflicted with CPPS have been found to carry no other respiratory pathogens but *M. bovis*. In both conventional and gnotobiotic experimental inoculations in calves, *M. bovis* was able to induce CPPS clinical symptoms (Allen et al., 1992; Arcangioli et al., 2008; Haines et al., 2001; Khodakaram-Tafti and López, 2004; Poumarat et al., 2001). This suggests that *M. bovis* may not require pre-existing respiratory disease and a multifactorial scenario to be pathogenic. Poumarat et al (2001) found that not only did *M. bovis* inoculation of calves result in an increase in clinical signs suggestive of pneumonia, but also increased the numbers of another bacteria, *Pasteurella multocida*, that has been associated with bovine respiratory disease, suggesting that *M. bovis* may even be capable of providing the primary infection for common multifactorial respiratory disease in calves.

5

2.2.1 *Mycoplasma bovis* and CPPS prevalence

   Although there have been many studies regarding the prevalence of *M. bovis* in feedlots conducted in Europe, Asia and North America, the results differ considerably from under 5% to near 100% of feedlot calves colonized with *M. bovis* (Allen et al., 1991; 1992; Angen et al., 2009; Arcangioli et al 2008; Catry et al., 2008; Haines et al., 2001; Hirose et al., 2003; Rosendal and Martin, 1986; Sachse et al., 2009; ter Laak et al., 1992a; ter Laak et al., 1992b; Thomas et al., 2002; Martin et al., 1999; Wiggins et al., 2007). The differences may be due to the large difference in geographical location; however, even prevalence studies conducted in the same area differ. Canadian studies have reported anywhere from 46-100% of animals with respiratory disease (Allen et al., 1991; 1992; Haines et al., 2001) and from 5-52% of healthy cattle colonized with *M. bovis* (Allen et al., 1991; 1992; Rosendal and Martin, 1986, Martin et al., 1999). Differences in *M. bovis* prevalence recorded in similar geographical regions could be due to differences in year or numerous other variables, which differ among the studies conducted. Enumeration methods widely vary and this may be another cause for differences between *M. bovis* prevalence studies. The Canadian studies that enumerated serum *M. bovis* antibody levels (Rosendal and Martin, 1986; Martin et al., 1999) showed much lower prevalence of *M. bovis* in both healthy animals and those with respiratory disease than the studies that used immunohistochemistry in lesions (Haines et al., 2001) or fluorescent antibody detection of culture (Allen et al., 1991; 1992) to enumerate animals with *M. bovis* at necropsy (Haines et al., 2001). Other studies outside of Canada have also used culture techniques that may be followed up by molecular, or immunoblotting typing methods to identify *M. bovis* (Angen et al., 2009; Arcangioli et al 2008; Catry et al., 2008; Hirose et al., 2003; Sachse et al., 2009; ter Laak et al., 1992a; ter Laak et al., 1992b; Thomas et al., 2002; Wiggins et al., 2007).

   The differences in sampling method have been shown to impact enumeration of *M. bovis*. For instance in several studies it was suggested that *M. bovis* prevalence, as determined by nasal swab sampling, was higher than as determined by bronchoalveolar lavage (Allen et al., 1992; Catry et al., 2008), while in one study the opposite was observed (Allen et al., 1991). In a Canadian study conducted by Haines et al in 2001, 80% of cattle with chronic pneumonia were identified as positive for *M. bovis* from lung and joint tissue samples while in a study conducted by Allen et al (1991), 46% of the animals with acute pneumonia tested *M. bovis* positive from

6

nasal swabs and 61% from bronchoalveolar lavages. The time of sampling may also affect results. The previous examples may also differ in prevalence as the Haines et al. study sampled animals upon necropsy while the Allen et al. study sampled animals at 28 days on feed with acute pneumonia. Furthermore, several groups of researchers found that *M. bovis* prevalence was increased with length of time that animals had been in the feedlot (Allen et al., 1992; Rosendal and Martin et al., 1986). In a study conducted by Allen et al (1992) *M. bovis* positive nasal swab or bronchoalveolar lavage samples in animals with chronic unresponsive respiratory disease increased from between 45-60% to 100% in a twelve day period in the feedlot.

The culturing method may also largely impact the results of *M. bovis* prevalence research. *M. bovis* is slow-growing and fastidious; therefore, the results may differ depending on conditions and time of culture (Hannan, 2000). The prevalence of *M. bovis* in studies where other respiratory pathogens are also being cultured may result in an underestimation of *M. bovis* as other bacterial pathogens are faster growing with fewer nutritional requirements; therefore, they may be cultured first (Hannan, 2000; Nicholas and Ayling, 2003).

While the exact prevalence of CPPS in feedlots is still not well understood, several studies have found that in the bovine respiratory disease complex *M. bovis* was one of the most common pathogens present (Allen et al., 1992; Arcangioli et al., 2008; Catry et al., 2008). Therefore, while quantitative data regarding *M. bovis* colonization and CPPS in feedlots may be difficult to obtain, the evidence suggests a high prevalence of *M. bovis* in feedlot respiratory disease worldwide.

2.2.2 CPPS clinical manifestations in the feedlot

The initial symptoms of CPPS are often indistinguishable from undifferentiated fever common to feedlot calves in the first months on feed (Adgeboye et al., 1995; Booker et al., 2008; Dyer et al., 2008; Krysak, 2006). Respiratory symptoms such as dyspnoea, anorexia, fever and general malaise are common clinical findings in new arrivals to the feedlot (Adgeboye et al., 1995; Booker et al., 2008; Dyer et al., 2008; Krysak, 2006). However, in cases of CPPS, common antibiotic treatment for undifferentiated respiratory disease will not improve the condition and the pneumonia often persists (Adgeboye et al., 1995; ter Laak 1992a). If signs of arthritis, including lameness, abnormal posture and reluctance to move did not exist prior to

treatment for respiratory disease, they often develop as the *M. bovis* pneumonia progresses to CPPS (Adgeboye et al., 1995; Dyer et al., 2008; Krysak, 2006). Often cattle will succumb to total recumbancy and die of severe dyspnoea, if not first euthanized (Dyer et al., 2008; Gagea et al., 2006; Haines et al., 2001; Krysak, 2006; Maeda et al., 2003).

Mycoplasma bovis pneumonia is associated with unique clinical pathology (Haines et al., 2001; Krysak, 2006; ter Laak et al., 1992a). Other pneumonia associated bacterial pathogens common to feedlots such as *Histophilus somni* and *Mannheimia haemolytica* cause fibrinosupprative bronchopneumonia characterized with fibrinous purulent liquid material and non-raised red to pink irregular shaped foci of coagulative necrosis while *M. bovis* causes chronic cranioventral bronchopneumonia characterized by white, circular friable foci with caseous necrosis, a more solid form of cheese-like purulence (Fulton et al., 2009; Gagea et al., 2006; Khodakaram-Tafti and López, 2004; Rodríguez et al., 1996). The occurence of arthritis in one or several joints in CPPS cases differentiates it from other bacterial pneumonias. The arthritis present in CPPS cases is characterized by joint distension, hyperplasic synovium, increased synovial fluid, presence of thick yellow fibrinous purulent exudates. Occasionally infection spreads to tendon sheaths, bursa and joint capsules and caseous necrosis foci develop in surrounding tissues (Dyer et al., 2008; Gagea et al., 2006; Krysak, 2006).

Studies utilizing immunohistochemistry have not only found a close association with *M. bovis* antigens and the foci of caseous necrosis in cranioventral bronchopneumonia characteristic of CPPS, but have also found more discerning characteristics between CPPS pneumonia lesions and those formed from other bacterial infections (Gagea et al., 2006; Haines et al., 2001; Rodríguez et al., 1996). The necrotic foci of *M. bovis* infections contain eosinic material with necrotic leukocytes, especially macrophages present in the periphery, while lesions from *Mannheimia haemolytica* for instance show basophilic bands around the necrotic foci and more neutrophils around the periphery (Gagea et al., 2006; Khodakaram-Tafti and López, 2004; Rodríguez et al., 1996). *Mannheimia haemolytica* associated necrotic foci also show less degradation of pulmonary architecture than those caused by *M. bovis* (Gagea et al., 2006). While the lung pathology at both visual and cellular levels does appear to be unique to the causative agent, it should be noted that often several bacterial pathogens colonize concurrently (Allen et al., 1991; Arcangioli et al., 2008; Gagea et al., 2006; Haines et al., 2001; Martin et al., 1999; Poumarat et al., 2001). Therefore, a single pneumonic feedlot calve may have an overlap of

lesion types, locations and leukocyte accumulation associated with these various pathogens (Adgeboye et al., 1995; Gagea et al., 2006, Khodakaram-Tafti and López, 2004).

2.2.3 Treatment challenges

Treatment of *M. bovis* is often complicated as *Mycoplasma* spp. are either resistant or gaining resistance to many of the antibiotics commonly used in the treatment of feedlot respiratory diseases. Therefore, the standard treatments for feedlot respiratory diseases may be changing as a result of the high prevalence of *M. bovis* being isolated from feedlot cattle with respiratory disease symptoms. The use of β-lactam group, including ampicillin, penicillin and ceftiofur, in commercial feedlots has been shown to be extremely ineffective in treating respiratory disease where *M. bovis* is a causative factor (Allen et al., 1992; Haines et al., 2001). This is due to the fact that β-lactam antibiotics inhibit cell wall synthesis of bacteria. As *Mycoplasma* spp. inherently have no cell wall the β-lactam antimicrobials have no mode of action on this genus of bacteria. Other groups of commonly utilized feedlot antibiotics such as the tetracyclines including oxytetracycline, tetracycline and chlorotetracycline and the macrolides including tilmicosin, tylosine, and erythromycin, have been found to be potentially efficacious in a few *in vivo* studies where calves were treated early (Bednarek et al., 2003; Godinho et al., 2005a; 2005b; Musser et al., 1996; ter Laak et al 1993). However, many studies indicate low susceptibility of *M. bovis in vivo* and *in vitro* to these groups of antibiotics, suggesting that perhaps *M. bovis* is capable of acquiring resistance to these antimicrobials (Cooper et al., 1993; Francoz et al., 2005; Godinho et al., 2008; Gerchman et al., 2009; Hannan et al., 1997; Hirose et al., 2003; Rosenbusch et al., 2005; ter Laak et al., 1993; Vogel et al., 2001). *Mycoplasma bovis* has been found to be highly resistant to sulfonamides group including sulfadoxine and the aminoglycosides including spectinomycin, streptomycin and gentamicin, in the majority of *in vivo* and *in vitro* studies (Allen et al., 1992; Francoz et al., 2005; Gerchman et al., 2009; Haines et al., 2001; ter Laak et al., 1993; Thomas et al., 2003b; Vogel et al., 2001).

The literature suggests that the antimicrobials with the highest potential for treatment of *M. bovis* in feedlot cattle belong to the fluoroquinolones (Francoz et al., 2005; Hannan et al., 1997; Hirose et al., 2003; Rosenbusch et al., 2005; Stipkovits et al., 2005; Thomas et al., 2003b). While Hannan et al (1997) found that *M. bovis* appears to be highly resistant to one member of this group, flumequire, the other antibiotics in this classification, such as, enrofloxacin have

shown promising antimicrobial activity in Minimum Inhibitory Concentration (MIC) studies *in vitro* and significant decreases in respiratory disease clinical symptoms and *M. bovis* colonization *in vivo* (Francoz et al., 2005; Hannan et al., 1997; Hirose et al., 2003; Rosenbusch et al., 2005; Stipkovits et al., 2005; Thomas et al., 2003b). There are also a few antibiotics not classified in any of the aforementioned groups such as tiamulin, valnemulin, lincomycin, and florfenicol, which have shown potential as effective antimicrobials against *M. bovis* associated respiratory disease (Catry et al., 2008; Hannan et al., 1997; Hirose et al., 2003; Rosenbusch et a, 2005; Stipkovits et al., 2001; 2005). Current research also indicates that treatment with either steroidal or non-steroidal anti-inflammatory drugs in conjunction with antimicrobial therapy may be more efficacious in the treatment of bronchopneumonia than antimicrobial therapy alone; therefore, these drugs should be considered for treatment of *M. bovis* associated pneumonia (Bednarek et al., 2003).

It should be noted that many of these antimicrobial susceptibility studies for *M. bovis* are conducted *in vitro* and up until the year 2000 there were no established standardized guidelines for *in vitro* antimicrobial susceptibility testing in *Mycoplasma* species (Hannan, 2000). As *Mycoplasma* spp. are very slow growing and fastidious, the differences in laboratory techniques before 2000 could have resulted in drastically different MIC values between studies of the same antimicrobial (Hannan, 2000). Additionally, *in vitro* antimicrobial susceptibilities of field isolates have been found to be much lower than susceptibilities of type strains in several studies (Hannan et al., 1997; Hirose et al., 2003; ter Laak et al., 1993). Nevertheless, *in vitro* study results for specified antimicrobials are often in poor agreement with *in vivo* studies, even if conducted by the same group (Godinho et al., 2005a; 2005b; 2008). This suggests that *in vitro* studies may be a poor reflection of antimicrobial efficacy in the field.

As for treatment recommendations, it was found in one study that the susceptibilities between field strains taken from different geographical regions may vary significantly; therefore, it was suggested that antimicrobial testing be conducted for *M. bovis* strains in specific regions before antimicrobial treatment recommendations be made (Gerchman et al., 2009). Also the studies showing promising results of antimicrobial therapy *in vivo* all treated calves within the 1st 10 days of infection; therefore, the efficacy of these antimicrobials at later disease stages is unknown and may differ greatly from results seen with early treatment (Godinho et al., 2005a; 2005b; Gourlay et al., 1989; Poumarat et al., 2001; Stipkovits et al., 2001; 2005). Therefore,

when utilizing literature results for selection of efficacious *M. bovis* antimicrobials, the experimental design, laboratory techniques, strain selection, and geographical location must be considered.

2.2.4 Prevention challenges: prophylactic antibiotics and vaccination

Antibiotic prophylaxis has been shown in many field studies to be ineffective in prevention of *M. bovis* respiratory disease (Allen et al., 1992; Booker et al., 2008; Wiggins et al 2007). However, most of the antibiotics in these cases belonged to classes like the β-lactams, macrolides or aminoglycosides, which are often ineffective in treatment of *M. bovis* diseases. However, several studies have provided evidence that antimicrobial prophylaxis with florfenicol, valnemulin or tilmicosin may reduce *M. bovis* colonization and symptoms of respiratory disease in young cattle (Catry et al., 2008; Gourlay et al., 1989; Stipkovits et al., 2001). Therefore, there may be some potential for antibiotic prophylaxis if *M. bovis* is considered when the types of antibiotics for respiratory disease prophylaxis are chosen.

Areas of the world such as the European Union are now banning the use of prophylactic antibiotics in attempts to reduce antibiotic resistance in zoonotic pathogens. Therefore, vaccination of *M. bovis* would be a preferable prevention method. While there are a few commercially available vaccines marketed in the United States of America such as Pulmo-Guard^{TM} MpB (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO), Mycomune® R (Agrilabs®, St. Joseph, MO) and MYCO-BAC® B (Texas Vet Lab, San Angelo, Tx), the efficacy has been questioned in several highly cited reviews as there is often no available literature to support the claims made by the vaccine manufacturer (Maunsell and Donovan, 2009; Nicholas et al., 2009). While vaccine production has been ongoing for 20 years with many potential vaccines having been described in the literature, the efficacy of these vaccines in the field is highly questionable. There are several reasons for the difficulties in vaccination production. Often these vaccines are either a killed or inactivated single strain of *M. bovis* or a single antigen (Calloway et al., 2008; Cho et al., 2008, Maunsell et al., 2009, Nicholas et al., 2002). However, *M. bovis* surface antigens have been shown to be highly variable; therefore, these vaccines that are designed around a single *M. bovis* strain may have very low efficacy in eliciting an immune response that would protect against other strains or even the same strain after a given amount of time. A study by Maunsell and Donovan (2009) tested a commercially

available *M. bovis* bacterin vaccine for efficacy in a field trial and found that the vaccine had no effect on serum antibody titres, respiratory disease outcome, weight gain, mortality or *M. bovis* colonization of the upper respiratory tract. While this vaccine may have tested well in the initial clinical trials conducted by the manufacturer, its efficacy after time or in field trials with diverse populations of *M. bovis* strains was low, most likely due to the high heterogeneity and variability of *M. bovis* antigens.

Recent studies have been conducted in attempts to find homologous antigens in diverse *M. bovis* populations. Behrens et al (1996) discovered a surface protein that was found to be the main antigen recognized during *M. bovis* infection and was unrelated to the common class of Variable Surface Proteins (VSP) in that it contained no repetitive structures common to these highly variable proteins, suggesting that it may be more stable. However, it was observed that this surface protein also undergoes a high rate of size and phase variation; therefore, it is an equally unstable antigen as the variable surface proteins. Perez-Casal and Prysliak et al (2007) investigated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a vaccine target as it has successfully been used in vaccine production for other pathogenic bacteria. GAPDH was suggested to have a possible involvement in adherence and low antigenic variation in *M. bovis*. Also when cattle were inoculated with *M. bovis* GAPDH they mounted a strong antibody response, suggesting this may be an effective vaccine target. Further research into potential antigen targets with low phase variation is necessary; however, if stable antigen targets are studied, efficacious vaccine preventative methods may be possible in the future. Unfortunately for now good management practices including appropriate identification, isolation and early treatment of CPPS afflicted animals may be the best prevention method.

2.3 Variable pathogenicity of *Mycoplasma bovis*

Mycoplasma related diseases are often difficult to fully define epidemiologically. Species of *Mycoplasma*-associated with disease in one animal can often also be isolated from healthy animals. Thus, colonization with *Mycoplasma* does not necessarily lead to disease in the animal. Strain differentiation studies have found that strains colonizing various infection sites can differ (Biddle et al., 2005). For instance, the strains of *M. bovis* that cause respiratory disease are not necessarily the strains that cause mastitis (Biddle et al., 2005). Also, pathogenicity can vary between strains of *M. bovis* (Adgeboye et al., 1995; Khan et al., 2005; Lu and Rosenbusch,

2004). This suggests that while there may be conditions that are more favourable to disease onset and progression, the difference in disease status between animals colonized with *M. bovis* may be attributable to variation in pathogenicity between strains of *M. bovis*. Therefore, the identification of *M. bovis* virulence mechanisms may be an important step in understanding the differences between the aetiology of *M. bovis* associated diseases and seemingly commensal colonization.

The ability of *M. bovis* to adhere to host cells appears to vary among strains and a strong link between adherence and pathogenicity in *Mycoplasmas* has been observed in several studies (Powell et al., 1976; Taylor-Robinson et al., 1981; Papazis et al., 2000). Adherence rates of *M. bovis* are significantly decreased in non-pathogenic strains when compared to strains recovered from cases of clinical mastitis (Thomas et al., 2003a). Adherence of *M. bovis* also appears to be tissue specific as adherence to endothelial cells has been observed to be significantly higher than to other cell types (Thomas et al., 2003a). It has also been observed that inhibition of adherence to one host cell type, for example by blocking membrane proteins with the addition of monoclonal antibodies, may not inhibit adherence to other tissues and cell types (Thomas et al., 2003c). Therefore, one possible explanation for the different disease outcomes associated with different strains is that there may be several mechanisms for adherence to different tissues that vary between strains or are expressed differently in a single strain under varaiable conditions (Papazis et al., 2000; Powell et al., 1976; Taylor-Robinson et al., 1981; Thomas et al., 2003c).

Pathogenic strains of *M. bovis* elicit specific immune responses in the host that not only benefit tissue degradation; therefore, nutrient acquisition and systemic infection opportunities for the pathogen, but also allow the pathogen to evade the immune system causing chronic infection (Geary et al., 1981; Lu and Rosenbusch, 2004; Thomas et al., 1986; Thomas et al., 1990; Thomas et al., 1991; Vanden Bush and Rosenbusch, 2002; 2003). Extracellular pathogens such as *M. bovis* often elicit a type 2 T-helper cell (Th2) regulated humoral based immune response characterized by the activation of antibody producing B-cells (Howard and Gourlay, 1983; Vanden Bush and Rosenbusch, 2003). The cytokine IL-4 is involved in Th2 activation of IgG_1 immunoglobulin producing B-cells and the upregulation of both IL-4 and IgG_1 has been observed in *M. bovis* infections. However, the Th2 response observed in *M. bovis* infections is only skewed not polarized, as some activation of Th1 induced cellular immune pathways has also been observed. The up-regulation of a cytokine known as interferon-gamma (IFN-ɣ) as well

as the production of IgG_2 immunoglobulins are characteristic of Th1 regulated immunity and have also been observed in the host in response to *M. bovis* infection. The immune response is both pathogen induced and host dependent. The Th2 immune response has been shown to weaken the lung cell junctions and suppress wound healing in lung tissue while the Th1 response has been shown to sensitize alveolar cells to apoptosis. A skewed response may be beneficial to the host as a polarized Th1 or Th2 response could result in severe lung damage while causing dissemination or further access of the pathogen to the host. The Th2 skewed response is most likely elicited by the host to prevent severe lung damage. However, the Th2 skewed response still provides some tissue damage, facilitating *M. bovis* nutrient acquisition and further infection, while also skewing the immunoglobulin production towards IgG_1 as opposed to the superior opsonin IgG_2. Consequently, the Th2 skewed response may benefit the longevity of *M. bovis*; therefore, the chronicity of the disease (Howard and Groulay, 1983; Van den Bush and Rosenbusch, 2003).

Up-regulation of the inflammatory response is also a common observation upon adhesion of pathogenic *M. bovis* strains to endothelial cells and some inflammatory cytokines appear to be linked with pathogenicity among *M. bovis* strains (Geary et al., 1981; Jungi et al., 1996; Lu and Rosenbusch, 2004). Chemotaxis and transmigration of mononuclear cells has been observed in epithelial cells exposed to pathogenic *M. bovis* strains. Subsequently up-regulation of the proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 that are produced by macrophages and neutrophils has also been observed in *M. bovis*. Geary et al (1981) also described an excreted toxin with pro-inflammatory effects. The inflammation is thought to be an immunomodulatory effect of *M. bovis* that increases adhesion to and permeation of endothelial cells, which is important in progression of *M. bovis*-associated diseases to systemic infection (Geary et al., 1981; Jungi et al., 1996; Lu and Rosenbusch, 2004).

While the host's immune response elicited by the immunomodulatory effects of *M. bovis* may improve access to nutrient acquisition and systemic spread, they also activate many phagocytic cells and lymphocytes that may increase the hosts chances of clearing the infection (Carroll et al., 1977; Howard and Gourlay, 1983; Howard et al., 1976; Howard et al., 1986; Lu and Rosenbusch, 2004; Vanden Bush and Rosenbusch, 2003). However, *M. bovis* has several mechanisms of evading the host immune response (Behrens et al., 1996; Le Grand et al., 1996; Lysnyansky et al., 1996; Lysnyansky et al., 1999; Sachse et al., 2000; Thomas et al., 1986;

Thomas et al., 1990; Thomas et al., 1991; Vanden Bush and Rosenbusch, 2002; 2003). While incubation of endothelial cells with *M. bovis* antigen has been observed to induce the activation of phagocytic leukocytes some mechanism in viable *M. bovis* bacteria enables it to suppress and inhibit phagocytic cells (Howard et al., 1976, Thomas et al., 1991; Vanden Bush and Rosenbusch, 2003; Vanden Bush and Rosenbusch, 2004). Induction of lymphocyte apoptosis, as well as inhibition of lymphocyte mitosis, neutrophil activation and expression of lympho-inhibitory peptides by *M. bovis* has been observed (Thomas et al., 1990; Thomas et al., 1991; Vanden Bush and Rosenbusch, 2002; Vanden Bush and Rosenbusch, 2004). The evasion mechanisms of *M. bovis* from phagocytes and lymphocytes are important to the virulence of the pathogen and the chronic disease state.

The importance of adhesion to the virulence of *M. bovis* strains has been previously described; however, the surface proteins involved in *M. bovis* adhesion to host cells serve a dual role. These surface proteins serve as antigens and serum antibodies specific to these antigens are often found in bovine serum after infection (Sachse et al., 2000). However, these antigens, including a class known as the VSP, are coded with reiterated sequences that undergo high rates of chromosomal rearrangement that cause both size and phase shift when these proteins are expressed (Behrens et al., 1996, Le Grand et al., 1996, Lysnyansky et al., 1996; Lysnyansky et al 1999; Poumarat et al., 1999; Sachse et al., 2009). This allows for a constant change in the antigenic phenotype expressed on the *M. bovis* cell surface that has been shown to effectively evade the humoral immune system proteins (Behrens et al., 1996, Le Grand et al., 1996, Lysnyansky et al., 1996; Lysnyansky et al., 1999; Poumarat et al., 1999; Sachse et al., 2009). In one study chromosomal rearrangements in the VSP coding regions were induced by the presence of bovine immunoglobulins specific to a VSP protein and a reverse of these rearrangements was observed when the antibodies were removed (Le Grand et al., 1996). This suggests that *M. bovis* has the ability to express variability in surface antigens based on the host immune response and effectively evade humoral immunity. While *M. bovis* does appear to have the ability to suppress and inhibit lymphocyte activation, an older study demonstrated that phagocytic cells did have the capacity to kill *M. bovis* if appropriate antibody opsonisation was present (Howard et al., 1976). Therefore, the evasion of both the humoral and cell mediated response by *M. bovis* is vital to the survival of this bacteria as a pathogen.

Mycoplasma bovis virulence is not yet well understood. However, studies regarding the adhesion, immunomodulatory and immune evasive behaviour of pathogenic *M. bovis* strains provide some insight into possible differences between the phenotypes of pathogenic and non-pathogenic strains. The genes responsible for the described virulence phenotypes may vary between *M. bovis* strains in either presence or expression. Further research involving virulence gene typing and molecular strain differentiation is required for a better understanding of the variable pathogenicity of *M. bovis*

2.4 Identification of *Mycoplasmas* in epidemiology studies

2.4.1 *Mycoplasma* species identification

The earliest of detection methods with any bacterial species were generally culture based. While several species of *Mycoplasma* are non-cultivatable, *M. bovis* is not one of them. *Mycoplasma bovis* is ideally cultured on Hayflick's agar or broth, at 37°C in 5% CO_2 with inclusion of a β-lactam antibiotic to inhibit contamination by other cell wall-bearing bacteria (Hannan, 2000). Unfortunately this process takes 3-10 days (Abu-amero et al., 1996; Abu-amero et al., 2000; Hannan, 2000; Hayflick, 1965). Once colonies are formed several staining and biochemical tests can be employed to determine the species represented.

There are several methods of determining that colonies are of the *Mycoplasma* genus. *Mycoplasmas* fluoresce green when stained with Acridine orange, a DNA binding flurochrome and are also stainable via the Dienes method that allows for light electron microscopy identification of the genus (Dienes, 1967; Jasper et al., 1984; Rosendal and Valdivieso-Garcia, 1981). Resistance to the antimicrobials has also been used to distinguish *Mycoplasmas* from other bacteria (Abu-Amero et al., 1996). Sterol requirements are also indicators of which genus of *Mollicutes* may be present; *Mycoplasma* being in the sterol requiring group (Miles and Nicholas, 1998). Further metabolic tests such as carbohydrate fermentation capabilities, alcohol metabolism rates, proteolytic and lipolytic activities as well as hydrogen peroxide production may then be utilized to distinguish between species (Abu-Amero et al., 2000; Khan et al., 2005; Miles and Nicholas, 1998). However, there are a limited number of biochemical and metabolic tests that can be performed with *Mollicutes* as the small genome codes for much fewer genes than other Eubacteria (Abu-Amero et al., 2000; Miles and Nicholas, 1998; Sirand-Pugnet et al., 2007b; Weisburg et al., 1989; Woese et al., 1980). Therefore, these bioactive tests may be

inconclusive as several species react very similarly (Abu-Amero et al., 1996; Abu-Amero et al., 2000; Dienes et al., 1967; Jasper et al., 1984, Khan et al., 2005; Rosendal and Valdivieso, 1981). Alternatively the efficiency at which these tests can identify *Mollicutes* genus is also affected by the variability in metabolic and biochemical behaviours observed between strains within a species (Khan et al., 2005).

To overcome the unreliability of metabolic test results, serologic methods have been developed. In the early stages of *Mycoplasma* research, tests utilizing hyperimmune serum specific to single species were utilized for species differentiation via the Growth Inhibition (GI) tests in which the inhibition of bacterial growth when exposed to hyperimmune serum for a specific species is measured (Polak-Vogelzang et al., 1978; Stanbridge and Hayflick, 1967). The indirect fluorescence test and the indirect peroxidise test in which species specific antibodies are produced that are bound with either flourochromes or peroxidase enzymes, respectively, have also been utilized to differentiate species of *Mycoplasmas* in early *M. bovis* studies (Brank et al., 1999; Cho et al., 1976; Polak-Vogelzang et al 1978). These methods, while more rapid and reliable than metabolic and biochemical testing from culture, are still often inconclusive, difficult to read, or time consuming and laborious (Brank et al., 1999; Cho et al., 1976; Polak-Vogelzang et al., 1978; Stanbridge and Hayflick, 1967).

The enzyme linked immunosorbent assay (ELISA) is an improvement on early immunological assays. It involves the absorption of immunoglobulins to a microtiter plate that are complementary to the antigen, antibody or protein in question. When the sample is added the antigen being tested will bind to the fixed complementary immunoglobulin (Engvall and Perlmann, 1971; Heller et al., 1993). Subsequent binding of free floating enzyme linked complementary immunoglobulins allows for an enzymatic determination of antigen, antibody or protein in question when substrate is added. Washing between each step ensures that color change or fluorescence produced when substrate is added is indicative of amount of antigen in the sample able to bind to the original plastic bound specific immunoglobulin (Engvall and Perlmann, 1971; Heller et al., 1993).

While ELISA is more sensitive, rapid, and easier to read than the earlier immunological methods there are still some specificity issues between closely related species, such as cross reactions between *M. bovis* and *M. agalactiae* (Boothby et al., 1981; Ghadhersohi et al., 2005; Heller et al., 1993; Thomas et al., 1987). *Mycoplasma bovis* was originally thought to be a

subspecies of *M. agalactiae*, a causative agent of caprine pneumonia, as the two organisms are so close biochemically and metabolically (Askaa and Ernø, 1976). However, *M. bovis* was reclassified as a separate species in 1976 when a DNA-DNA hybridization experiment demonstrated a much larger genetic diversity than was previously thought (Askaa and Ernø, 1976). While *M. bovis* is distinct enough to be a separate species it is still very closely related to *M. agalactiae* and shares many antigens (Boothby et al., 1981; Ghadhersohi et al., 2005; Heller et al., 1993; Mattson et al., 1994; Thomas et al., 1987). Therefore, cross-reactivity is often observed between the two species in immunological testing. As *Mycoplasma* spp. have shown specificity to particular hosts the cross-reactivity between the caprine pathogen *M. agalactiae* and the bovine pathogen *M. bovis* was originally thought to be unimportant in diagnostic assays as this would not affect the quality of diagnosis for these diseases in their respective animals (Boothby et al., 1981; Ghadhersohi et al., 2005; Heller et al., 1993; Thomas et al., 1987). However, some cross-colonization has been observed in rare cases in which *M. agalactiae* has been found to colonize cattle and *M. bovis* has been observed in goats (Bashiruddin et al., 2005; Rodríguez et al., 2000). Therefore, the importance of highly specific assays may not only be important to scientific laboratories, but may be of increasing importance to clinical laboratories as well.

There are a few published variations on the ELISA reaction that have been shown to improve specificity between closely related *Mycoplasma* species. Thomas et al (1987) observed that preincubation of bovine serum with heterologous antigens, prior to the *M. bovis* specific ELISA reaction reduced the incidence of cross-reactivity with other bovine pathogens as well as *M. agalactiae*. Another study tested a blocking-ELISA in which positive sera would bind to specific *M. bovis* antigen sites effectively blocking the enzyme bound monoclonal antibody population from binding and inducing a color reaction (Ghadhersohi et al., 2005). This modification of ELISA increased the specificity and sensitivity of the ELISA reaction to a level that was comparable with the very specific and sensitive PCR reactions available at the time. It was therefore, suggested that blocking-ELISA methods may be valuable diagnostic tools for *M. bovis* serum antibody levels in cattle (Ghadhersohi et al., 2005).

Immunoblotting assays on nitrocellulose membrane filtration paper have been developed as another serologic based method (Flores-Gutiérrez et al., 2004; Infante et al., 2002; Poumarat et al., 1991; Takahata et al., 1997). *Mycoplasma* colonies are trapped on nitrocellulose paper

with vacuum filtration before specific anti-*M. bovis* antibodies are applied. Enzyme conjugated anti-immunoglobulin antibodies are then added so that the amount of *M. bovis* can be determined qualitatively via an enzyme induced substrate color change. This method has shown to be as specific and sensitive as ELISA, which again included some cross-reactivity issues in early development between *M. bovis* and *M. agalactiae*. However, the studies have suggested that immunoblotting assays are much quicker than ELISA and as several samples can be analyzed simultaneously these assays are also thought to be high-throughput, which is desirable for clinical testing (Flores-Gutiérrez et al., 2004; Infante et al., 2002; Poumarat et al., 1991; Takahata et al., 1997).

Polymerase chain reaction (PCR) amplification of *M. bovis* specific gene elements may provide an extremely fast, sensitive, specific, reliable, and reproducible method of detecting *M. bovis* compared with both ELISA and traditional biochemical and metabolic tests from culture (Ayling et al., 1997; Bashiruddin et al., 2005; Chávez González et al., 1995; Ghadhersohi et al., 1997; Hotzel et al., 1996; Subramaniam et al., 1998; Tenk et al., 2006; Thomas et al., 2004). PCR amplification also allows for the detection of the bacterial DNA in serum or milk, as opposed to many of the immunological methods in which serum antibody levels or antigens may be detected. The first attempts to detect *M. bovis* with PCR amplification concentrated on the amplification of the 16S rRNA gene, which is universal in bacterial genomes. While this method was fast and sensitive there were again some issues with differentiating between *M. bovis* and *M. agalactiae*. After sequencing the 16S gene of these two bacteria it was found that the genes varied by only eight nucleotides and these nucleotides would have to be utilized in the 16S rRNA PCR assays for the differentiation between these closely related species. Chávez González et al (1995) developed specific primer sets for both *M. bovis* and *M. agalactiae* based on the nucleotide differences in the 16S rRNA gene that did indeed decrease cross-amplification. Other methods implemented to decrease cross-reactivity were the use of multiplex or nested secondary PCR amplifications and the addition of restriction enzyme digests post amplification to create species specific restriction fragment length polymorphism (RFLP) fingerprint patterns (Bashiruddin et al., 2005; Foddai et al., 2005; Hayman and Hirst, 2003; Johansson et al., 1996; Tenk et al., 2006). Primers have also been designed for many other conserved *M. bovis* gene targets that are more genetically distinct to improve specificity. The *uvr*C DNA repair gene, membrane protein 81 encoding gene, and *oppD* oligonulceotide permease encoding gene have all

demonstrated high specificity when used as gene targets in *M. bovis* amplification. DNA probes created from restriction fragments specific to and common in *M. bovis* have also been utilized to produce specific and sensitive PCR primers (Ghadhersohi et al., 1996; Hayman and Hirst, 2003; Hotzel et al., 1993; Hotzel et al., 1996; Tenk et al., 2006). Recently real-time PCR methods have been developed to not only detect the presence of *M. bovis* but also to quantify the abundance of *M. bovis* in samples (Cai et al., 2005; Sachse et al., 2009).

A recent study tested seven different methods of *M. bovis* and *M. agalactiae* specific PCR amplification (Bashiruddin et al., 2005). The study concluded that while some PCR methods were more specific than others, all of the systems and gene targets utilized produced useable results and were suitable for clinical use (Bashiruddin et al., 2005). In four instances the PCR amplification methods were even able to rectify incorrect identifications made by biochemical or serological methods (Bashiruddin et al., 2005). Therefore, identification by PCR may be an ideal clinical assay due to the increased speed, sensitivity, and specificity over serological and culture-based (Hotzel et al., 1996). However, it should be taken into consideration that the DNA detected with PCR may not always be from live bacteria; therefore, overestimation of active bacteria is possible.

It should also be noted that while serological and molecular identification of *M. bovis* is more sensitive, specific and rapid than early biochemical and metabolic testing of cultured isolates, bacterial culture is far from obsolete for identification of *M. bovis*. While rapidity is important for clinical testing, scientific studies often greatly benefit from culturing *Mycoplasma* samples prior to species detection. Culture stocks are not only important as a back-up but provide high concentrations of bacteria for future testing. For example high quality clean DNA is required for most modern strain differentiation techniques; therefore, while species identification may be possible from raw samples, culture of the raw samples is often necessary for future testing of *Mycoplasma* species (Butler et al., 2001; Kusiluka et al., 2000; Kusiluka et al., 2001; Mannering et al., 2009; McAuliffe et al., 2004; Mettifogo et al 2006; Parham et al., 2006; Tardy, 2007).

2.4.2 *Mycoplasma* strain differentiation

    2.4.2.1 Pulse Field Gel Electrophoresis (PFGE)

    Pulse Field Gel Electrophoresis (PFGE) was first developed by Schwartz and Cantor in 1984 in response to a need for separating large DNA fragments by size. Distinguishing between DNA fragments over 50kb by standard gel electrophoresis is not possible, as these large fragments have near indistinguishable rates of motility through an agarose gel matrix (Schwartz and Cantor, 1984). Even with very low agarose concentrations and extremely long running times resolution between bands of over 750kb via electrophoresis is not possible. Pulse Field Gel Electrophoresis (PFGE) is a variation of agarose gel electrophoresis in which the electrical field alternates between different angles from the center line. Unlike standard agarose gel electrophoresis, PFGE can separate DNA fragments up to 2000kb (Schwartz and Cantor, 1984).

    The addition of restriction enzyme digests to shorten genomic DNA into more manageable fragment lengths prior to pulse field electrophoresis has created a useful tool for strain differentiation in epidemiological studies of *Mycoplasma* species (Kusiluka et al., 2001; Mannering et al., 2009; McAuliffe et al., 2004; Mettifogo et al 2006; Parham et al., 2006; Tardy et al., 2007). This also allows the estimation of genome size based on compiling various fragment sizes (Frey et al., 1992; Hollis et al 1999, Tola et al., 1999). Restriction enzymes with very few recognition sites are desirable as they create very few large fragments for PGFE analysis and genome size estimation. Therefore, for strain differentiation in Mycoplasmas with low G+C content, restriction enzymes with recognition sites containing many G and C residues are most popular. Selection of restriction enzymes must be species specific as enzymes tend not to cut the genomes of all *Mycoplasma* species (Frey et al., 1992; Hollis et al 1999, Tola et al., 1999). With proper enzyme selection PFGE can allow for highly variable banding patterns between strains (Kusiluka et al 2001, McAuliffe et al., 2004, Tardy et al., 2007) of *Mycoplasma* spp. with improved discriminatory power over restriction fragment analysis based on the16S rRNA gene (Hollis et al., 1999). PFGE also demonstrates good reproducibility between runs, especially when enhanced software capable of normalizing runs in different conditions is utilized (Duck et al 2003; Kusiluka et al., 2001).

    PFGE has been successfully utilized in *M. bovis* associated mastitis studies (Biddle et al., 2005; Fox 2008). It has been demonstrated with PFGE patterns that while it is possible to isolate several *M. bovis* strains from one herd, or even one animal that many times the same strain will

colonize several different locations within the same animal. The observation that strains from the mammary tissue identified with PFGE were often the same as those isolated from mammary tissue in other quarters of the udder and the blood stream suggests that there may be systemic involvement in the spread of *M. bovis*. It was also observed that while strains isolated from mammary tissue also colonized several other body sites, that the heterogeneity between strains determined with PFGE was greatest between mammary tissue and respiratory tract. This indicates that the strains that cause mastitis may differ from the strains that cause *M. bovis* pneumonia (Biddle et al., 2005).

However, there are several drawbacks to PFGE when compared to other methods including lower discriminatory power and congruence with random amplified DNA (RAPD) and amplified fragment length polymorphism (AFLP) techniques in strain differentiation with some *Mycoplasma* species (Kusiluka et al., 2001; Marois et al 2001a, Marois et al 2001b, McAuliffe et al., 2004). In an *M. bovis* epidemiology study PFGE was compared with RAPD and AFLP, and found that PFGE demonstrated the least discriminatory power (McAuliffe et al., 2004). The authors elected to use primarily RAPD and AFLP as their strain differentiation tools (McAuliffe et al., 2004).

Other limitations of PFGE include the lack of universal restriction enzymes able to type all strains of *Mycoplasma* (Frey et al., 1992; Hollis et al 1999, Tola et al., 1999) and the higher labour and materials costs compared to other techniques such as RAPD (Mettifogo et al., 2006). The use of gel images also makes analysis of fingerprints difficult and advanced statistical software such as BioNumerics (Applied Maths, Austin TX) may be required (McAuliffe et al., 2004). However, despite these limitations of PFGE, in conjunction with low recognition site restriction enzyme digests the technique has proved to be a very useful tool in *Mycoplasma* epidemiological studies in several livestock animal hosts (Kusiluka et al., 2001; Mannering et al., 2009; McAuliffe et al., 2004; Mettifogo et al 2006; Parham et al., 2006; Tardy, 2007).

2.4.2.2 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA is another molecular method commonly used in *Mycoplasma* strain differentiation. The technique is a PCR amplification in which an arbitrary ten nucleotide primer anneals to several places along both strands of the genome in order to produce random fragments of varying lengths that can be visualized with agarose gel

22

electrophoresis (Geary et al 1994; Williams et al., 1990). The fragment length patterns produced vary between strains of bacteria due to polymorphisms in the genomes (Bíró et al., 2006; Charleton et al., 1999; Cherry et al., 2006; Cousin-Allery et al., 2000; Feberwee et al., 2005; Geary et al., 1994; Mannering et al., 2009; Parham et al., 2006; Stakenborg et al., 2006; Williams et al., 1990). The technique is therefore, also utilized as a method of gene mapping (Williams et al., 1990).

RAPD strain differentiation is popular as there is no need for prior sequence information, and the technique requires only a thermocycler and basic knowledge of standard PCR (Williams et al., 1990). Unfortunately, there are also several drawbacks to RAPD strain differentiation. The RAPD technique is a relatively simple variation on a standard PCR, in which very low annealing temperatures are utilized to allow the random 10mer to anneal to several locations along the genome (Williams et al., 1990). However, these low stringency conditions also result in very poor reproducibility of the fragment length patterns when analyzed on agarose gel electrophoresis, especially with the presence or absence of minor bands (Cherry et al., 2006; Cousin-Allery et al., 2000; Feberwee et al., 2005; Mettifogo et al., 2006; Stakenborg 2006). This can also lead to difficulty when analyzing results as the presence and absence of minor bands cannot be used in establishment of strain specific banding patterns, which in turn can result in decreased discriminatory power over other methods (Mettifogo et al., 2006; Stakenborg et al., 2006). While the equipment required to prepare a RAPD analysis is relatively simple, the analysis of gel photos may be very difficult and require advanced statistical software as was noted for PFGE in order to evaluate similarity and presence and absence of faint bands between isolates (McAuliffe et al., 2004).

Many strain dependent banding patterns have been produced from RAPD for several pathogenic *Mycoplasma* spp., and have proven very useful in epidemiologic studies in several host species (Bíró et al., 2006; Charleton et al., 1999; Cherry et al., 2006; Cousin-Allery et al., 2000; Feberwee et al., 2005; Mannering et al., 2009; Parham et al., 2006; Stakenborg et al., 2006). A study in the United Kingdom on 53 *M. bovis* isolates was able to group the isolates into two major clusters (McAuliffe et al., 2004). However, this is the only study to date where RAPD was utilized in the investigation of *M. bovis*; therefore, more than two main clusters may be detectable if additional strains from other geographical regions or outbreaks were analyzed with RAPD.

A variation on RAPD known as arbitrarily-primed PCR (AP-PCR) was used for strain differentiation in a *M. bovis* epidemiology case study in 2001 with greater success (Butler et al., 2001). The protocol is much the same as the RAPD protocol except instead of one random 10mer, a primer set originally used to amplify interspersed repetitive DNA elements in eubacteria was used. Within three different instances of *M. bovis* outbreaks seven different AP-PCR fingerprinting patterns were discovered. In two herd outbreaks the herds were closed and only one fingerprinting pattern was recovered from each of these operations throughout the outbreaks, signifying that there was most likely only one source of infection and one strain responsible for each of the outbreaks. The third outbreak was in a feedlot that acquired cattle from many different sources and five different fingerprinting patterns were observed throughout the outbreak, signifying several strains and sources of the infection (Butler et al., 2001). This study illustrates the potential for random PCR based methods for *M. bovis* epidemiologic studies, as several fingerprinting patterns were possible and the results provided scientific support for the epidemiologic information available about possible infection sources in these herds.

2.4.2.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism is another fingerprinting method commonly used in *Mycoplasma* epidemiology studies (Kokotovic et al., 1999; Vos et al., 1995). The technique requires four main steps. First, genomic DNA must be digested simultaneously or consecutively with two restriction enzymes. One of the restriction enzymes should have frequent cut sites the other should have relatively few cut sites. In the case of *Mycoplasma*, the rare cutter often has a high G+C content in the recognition site as *Mycoplasmas* have very low G+C content genomes. The use of both a frequent cutting and a rare cutting enzyme allows for smaller fragment sizes to be analyzed and allows for management of the number of fragments to be analyzed. The second step requires ligation of adapters to the cut sites. Two adapters with unique sequence are used; each designed to anneal to the overhang left by either the rare or frequent restriction enzyme. In the third step PCR amplification is performed with a primer set specific to the adapters, which now flank each restriction fragment. Finally, the resulting amplified fragments can be resolved on an acrylamide gel or, if one of the primers is labelled with a fluorescent marker, by automated capillary electrophoresis (Vos et al., 1995).

Like other strain differentiation techniques ALFP does not require any previous knowledge of the genome sequence. The method also has advantages over other fingerprinting methods. In particular, one study tested the reproducibility in *Mycoplasma* spp. by running all samples in duplicate and certain samples in triplicate and found near identical densitometric curves produced when analyzed via capillary electrophoresis (Kokotovic et al., 1999). As discussed above, the method was also found to have high discriminatory power between different strains within a *Mycoplasma* species (Hong et al., 2005; Kusiluka et al., 2000; McAuliffe et al., 2004). The development of AFLP databases for comparison may be more feasible than for other fingerprinting methods as the use of capillary electrophoresis makes AFLP fingerprints more consistent than other methods such as RAPD and PFGE in which agarose gel electrophoresis is utilized. The use of capillary electrophoresis in AFLP also simplifies data normalization and analysis with clustering software compared to PFGE and RAPD in which gel images must be analyzed (Hong et al., 2005; Kusiluka et al., 2000).

AFLP has been tested for species differentiation of *Mycoplasma* spp. and demonstrated high heterogeneity between species when suitable restriction enzyme pairs were utilized (Kokotovic et al., 1999). In several CPPS studies AFLP has been utilized successfully for strain differentiation of *M. bovis*. The McAuliffe study comparing PFGE, RAPD and AFLP in the differentiation of strains of *M. bovis* thought to be associated with CPPS in the United Kingdom showed that AFLP analysis of the strains resulted in similar clustering as with RAPD (McAuliffe et al., 2004). However, one of the main two clusters resulting from both of these methods could be sub-grouped with the AFLP data, suggesting possibly higher discriminatory power of AFLP over RAPD (McAuliffe et al., 2004). An early study of 42 *M. bovis* strains isolated over 17 years in various geographical regions in Denmark showed 18 different profiles; however, these profiles had very high homogeneity (Kusiluka et al., 2000). Clustering was found not to be based as much on geographical region but on epidemiological relatedness. Strains from mastitis cases were found to be near identical to each other despite length of time between the collection of the isolates, suggesting that the genomes of these strains has remained stable over long periods of time (Kusiluka et al., 2000). These studies demonstrate the usefulness of AFLP strain differentiation in *M. bovis* epidemiology research.

2.4.2.4 Other genome based strain differentiation techniques

The previously described strain differentiation methods have all involved identification of genomic diversity. However, none of these methods have attempted to determine the genomic region in which these polymorphisms occur. There are however, a few studies in which strain differentiation methods have been developed targeting the highly variable regions in the *M. bovis* genome. Beier et al (1998) investigated the genes encoding the variable surface proteins for differences between genetic structure and resultant antigen expression using Southern and Western blotting methods, respectively. The study determined that these regions were responsible for a high level of divergence between strains and subsequent antigen profiles and that these VSP profiles may be useful strain differentiation targets.

A more recent study investigated repetitive DNA elements, known as insertion sequences that vary in genome position and copy number and code only for proteins involved in their own transposition (Miles et al., 2005). As these insertion sequences are involved in chromosomal rearrangements they potentially contain the polymorphisms responsible for the genomic variation observed between strains with RAPD and AFLP strain differentiation methods. The groups that resulted from analysis of the different profiles were comparable to the groups determined via RAPD and AFLP results suggesting that the differences in length and copy number of these insertion sequences may indeed be responsible for the genome variation detected by RAPD and AFLP.

Variable number tandem repeats (VNTR) have also been studied as possible genome elements responsible for genomic variation between *M. bovis* strains in one study (McAuliffe et al., 2008). VNTRs are DNA elements that are repetitive and can vary in copy number. Databases for VNTR profiles exist for many bacteria; therefore, VNTR profiling is being used for strain typing in several species. This study utilized VNTR profiling in *M. bovis* found that like AFLP and RAPD, VNTR analysis was able to differentiate strains to a higher degree than PFGE.

Methods of strain differentiation that take into account the regions responsible for genomic variation between strains and possible differences in phenotypes may be an important step in understanding the variable pathogenicity observed in *M. bovis* epidemiology studies.

2.5 Conclusion

The literature supports a strong association between *M. bovis* and CPPS in feedlot cattle with specific pathology of the disease condition well defined and differentiated from other feedlot pneumonias. It is difficult to generalize on the prevalence of this pathogen in feedlots as differences in sample type, geography and identification methodology make comparisons of prevalence studies extremely difficult.

In addition to being associated with CPPS, *M. bovis* is also known to be a causative agent of many other bovine diseases and can be isolated from seemingly healthy animals. However, the mechanisms regulating the type of disease or in some cases the lack of disease induced after *M. bovis* colonization is unknown. The host response to infection with *M. bovis* may impact the disease outcome. In CPPS afflicted animals a Th2 skewed immune response can be observed, that may protect the host from severe immune induced lung damage associated with either Th1 or Th2 polarized response. However, this skewed response may also factor into the chronicity of CPPS. To date, there are no comparative studies examining the host immune response between different disease outcomes; therefore, host response as a factor in *M. bovis* associated diseases is not well defined.

Variability in adherence and other pathogenicity factors has been noted between different field strains of *M. bovis* indicating that different strains may be present within the species with different levels of virulence and tissue specificity. This is another plausible explanation for the variable disease states. Strain differentiation techniques based on identification of polymorphisms throughout the whole genome have been developed and utilized in strain differentiation studies. However, there is still relatively little research regarding how the different strains identified with these methods relate to disease state. Identifying these strain differences is the first step in understanding the differences between disease states in cattle upon colonization with *M. bovis*. The identification of virulent strains may also have practical benefits for detection, treatment and vaccination strategies.

3.0 A SURVEY OF *MYCOPLASMA BOVIS* STRAINS IN WESTERN CANADIAN FEEDLOT CATTLE

3.1 Introduction

Mycoplasma bovis is a bacterial pathogen associated with several different conditions in cattle including, keratoconjunctivits, otitis media, mastitis, respiratory disease and arthritis (Alberti et al., 2006; Gagea et al., 2006; Maeda et al., 2003). A lack of cell wall makes *M. bovis* inherently resistant to the β-Lactam family of antibiotics and the bacteria is gaining resistance to most of the other groups of antibiotics commonly used in cattle (Francoz et al., 2005; ter Laak et al., 1993). The surface proteins expressed by *M. bovis* are variable making an antigenic target for vaccine production difficult; therefore, no efficacious vaccine is available for most *M. bovis* associated diseases (Francoz et al., 2005; ter Laak et al., 1993). Chronic Pneumonia and Polyarthritis syndrome is an *M. bovis* associated disease in feedlot cattle that is characterized by persistent respiratory disease despite antibiotic treatment and subsequent arthritis in one or several joints (Behrens et al., 1996, Le Grand et al., 1996, Lysnyansky et al., 1996; Lysnyansky et al., 1999; Poumarat et al., 1998; Sachse et al., 2009). The difficulty in treating and preventing this condition make it of great concern to beef producers as CPPS poses significant welfare and economic concerns in the feedlot (Nicholas and Ayling, 2003).

The prevalence of *M. bovis* has been studied widely in North America, Australia and Europe (Arcangioli et al., 2008; Assié et al., 2009; Ayling et al., 2004; Bashiruddin et al., 2001; Blackburn et al., 2007; Ghadhersohi et al., 1999; Gagea et al., 2006, Le Grand et al., 2002; Wiggins et al., 2007); however, there are currently no *M. bovis* prevalence studies conducted in western Canada. The results of previously reported prevalence studies vary widely and while this may be due to the different geographical regions there are also many discrepancies in the sampling period, sampling method and identification method among these studies. Several studies have observed poor agreement between *M. bovis* isolation from nasal swabs (NS) and bronchoalveolar lavage (BAL), suggesting that sampling method may be important for consistent results and that NS may not be good indicators of *M. bovis* status in the lower respiratory tract (Allen et al., 1991; 1992; Catry, 2008; Thomas et al., 2002). Also the sampling period appears to affect the prevalence results as several studies have observed an increase in *M. bovis* isolation with an increase in the days the animal has been in the feedlot (Allen et al., 1992; Rosendal and Martin et al., 1986). The variation in results due to sampling differences and methodology in

studies from other regions make it difficult to make any inferences about the *Mycoplasma* prevalence in western Canada.

While *M. bovis* has been shown to be highly associated with CPPS and cause CPPS upon inoculation in both gnotobiotic and conventional calves, previous prevalence studies also often observed *M. bovis* colonizing the respiratory tracts of seemingly healthy cattle (Allen et al., 1991; 1992; Hirose et al., 2003; Rosendal and Martin, 1986; Sachse et al., 2009; ter Laak et al., 1992b; Thomas et al., 2002; Wiggins et al., 2007). This would suggest that there is variability of pathogenicity within the species. Molecular strain differentiation has recently been utilized to try and identify different strains in *M. bovis* epidemiology studies. Random Amplified Polymorphic DNA (RAPD) is a strain differentiation method that utilizes a random nucleotide primer in standard PCR reactions in order to reveal polymorphisms between the genomes of strains (Butler et al., 2002; McAuliffe et al., 2004). The method has been utilized in *M. bovis* epidemiology studies and has demonstrated high discriminatory power when compared to other methods. A study was conducted to compare strain differentiation methods and found that RAPD was able to separate 56 *M. bovis* isolates collected over a 6 year period in the United Kingdom into two genetically distinct clusters (McAuliffe et al., 2004). The same study also compared a strain differentiation method known as Amplified Fragment Length Polymorphism (AFLP) that utilizes a double digest with restriction enzymes followed by a PCR of the resultant fragments in order to identify genomic polymorphisms within a species. The study found that this method was consistent with RAPD and produced the same genetically distinct clusters; however, AFLP was able to identify two sub-groups within one of these clusters; therefore, may have higher discriminatory power than RAPD. While these strain differentiation methods do appear to be suitable for *M. bovis* and have provided useful information regarding *M. bovis* epidemiology, to date there are no significant relationship between strains identified with these methods and pathogenicity or disease outcome.

The aim of this study was to determine the prevalence of *M. bovis* in healthy University of Saskatchewan feedlot, commercial feedlot cattle that developed respiratory disease within 3 months of arrival at the feedlot, and in CPPS mortalities from several western Canadian feedlots upon necropsy in order to determine any differences in prevalence due to sampling method, sampling period, or disease status. In addition, AFLP was selected to genotypically differentiate *M. bovis* isolates obtained following feedlot entry and to compare these with *M. bovis* isolates

29

recovered from western Canadian feedlot that died or were euthanized following CPPS-like clinical presentations.

3.2 Materials and methods
3.2.1 Sample collection
3.2.1.1 Healthy University of Saskatchewan feedlot cattle

Thirty-six clinically healthy steers weighing between 219 – 333 kg and housed at the University of Saskatchewan feedlot were sampled at 14 and 90 days on feed (DOF) in the fall of 2008 and winter of 2009. Nasal swabs (NS) were taken with a double guarded equine uterine swab (Reproduction Resources, Walworth WI) inserted in the right nasal passage and were stored in Ames media at -80°C until culture (Mai, Ames Media, Product Number 49203, Spring Valley WI). Bronchoalveolar lavages (BAL) were collected immediately after NS by advancing a 90 cm optical endoscope (Olympus; Markham, Ontario, Canada) through the left nostril to the accessory lung lobe. Sterile plastic tubing was then advanced through the channel of the endoscope to 10 cm past the end of the scope. Approximately 120 mL of sterile buffered saline was flushed into the bronchus using a sterile 60 cc syringe. Within 10 seconds the saline was aspirated back into the syringe. In some instances, the saline gravitated into the lung field and no aspirate was collected, in which case the lung was lavaged with a 2nd 60 mL of fluid. Typically 1-5 mL was aspirated and a small aliquot (0.5 – 1.0 mL) was placed in 1.2 mL cryogenic vial (Nalgene) to be stored at -80 °C until culture. The endoscope was disinfected with alcohol between animals to prevent transfer of bacteria.

3.2.1.2 Symptomatic commercial feedlot cattle

Nasal swabs were collected on two occasions from cattle in a commercial feedlot (Pound-Maker Agventures Ltd., Lanigan, Saskatchewan) in the fall of 2007 using methods described in section 3.2.1.1. Swabs were collected from approximately 650 feedlot calves between 180 and 300 kg upon feedlot entry and again between 1 week and 3 months of arrival for 56 calves (35 from pen A and 21 from pen B) identified and treated for respiratory symptoms. Nasal swabs collected by feedlot staff at time of treatment were stored at -20 °C until transfer to the University of Saskatchewan where they were stored at -80 °C until cultures. Nasal swabs

collected from cattle sorted for treatment and from the same cattle at the time of feedlot entry were cultured for *M. bovis* identification and strain differentiation.

3.2.1.3 Necropsy samples

Sample collection kits were sent to western Canadian veterinary clinics with instructions to collect samples of CPPS lesions from lung and joint of carcasses only if both randomly distributed raised caseous micro-abscesses and evidence of septic arthritis indicative of *M. bovis* associated CPPS were evident upon necropsy. Four different veterinary clinics participated and a total of 62 cattle carcasses from 14 different feedlots in western Canada were included. Swabs were collected through sterile openings in arthritic stifle or hock joints using small swabs (Mai, Spring Valley, WI). Lung tissue samples of 3 cm by 3 cm with lesions were collected from the diaphragmatic lung lobes of the carcasses. All samples were stored at -20 °C until they could be transferred to the laboratory within two months from collection, where they could be stored at -80 °C until culture within the next six months. Due to technical difficulties, species identification was only conducted for 108 isolates (61 lung and 47 joint) from 29 animals and strain differentiation was only possible for 58 isolates (35 lung and 23 joint) from 19 animals originating from ten different feedlots.

3.2.2 Selective culture and isolation of presumptive *Mycoplasma* species

Within 6 months of collection frozen samples were thawed and streaked onto Hayflick's agar and incubated at 37 ºC in 5% CO_2 for 48 hrs. Preliminary data showed that sample storage in Ames media at -80 ºC up to 12 months did not affect the number of colonies enumerated on Hayflick's agar. Following incubation, four presumptive *Mycoplasma* spp. colonies were selected and sub-cultured onto Hayflick's agar under the same conditions for an additional 48 hrs at 37 ºC in 5% CO_2. These subcultured colonies were then inoculated into 4-10 mL of Hayflick's broth under the same temperature and atmospheric conditions for 3-7days. Stocks of 0.50 - 0.75 ml Hayflick's broth and 0.50 – 0.75 mL litmus milk were saved in 1.5 ml Eppendorf tubes at -80 ºC.

3.2.3 Species identification

One to four colonies for each sample were speciated. Fresh broth cultures (4-10 mL) were centrifuged at 3000 rpm for 1 hr and the supernatant removed. Genomic DNA was extracted from the resultant bacterial pellet using Purelink^{TM} Genomic DNA Kit (Invitrogen, Carlsbad, CA) according to the instruction manual for gram negative bacterial cell lysates. The extracted DNA was quantified and examined for purity using UV absorbance (NanoDrop® ND-1000 spectrophotometer; NanoDrop Technologies, Inc., Wilmington, USA) and stored at -80 ºC until use.

Polymerase Chain Reaction (PCR) was employed to amplify the 16s rRNA gene for nucleotide sequencing. The PCR reaction mixture included 1x PCR Buffer (Invitrogen, Burlington, ON), 1.5 mM MgCl2, 0.2 mM dNTPs, 3 mM BSA, 0.5 mM universal 16S rRNA gene forward primer (5'-GAGTTTGATCCTGGCTCAG-3'), 0.5 mM universal 16S rRNA gene reverse primer (5'-GWATTACCGCGGCKGCTG-3'), at least 10 ng of PurelinkTM extracted genomic DNA, 0.5 µL Taq polymerase (Invitrogen, Burlington, ON) and ddH20 to bring the reaction mixture volume to 50 µL. A hot start at 95 °C for 10 min in an Amplitron II thermocycler (Thermolyne, Barnstead International, Dubuque, Iowa) was utilized before Taq was added to the reaction mixture. PCR amplification continued for 40 cycles using a denaturing phase of 95 ºC for 30 sec, an annealing phase at 50 °C for 30 sec, and an extension phase at 72 ºC for 30 sec.

Successful PCR amplification was confirmed by electrophoresis of 50 µL of the completed PCR reaction with 10 µL of a 0.5% bromophenol loading buffer using a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide at 100 V for 1.5 hrs (BioRad Model 1000/500 Power Supply, BioRad, Hercules, California). PCR products of 660bp were extracted from the agarose gel with the Qiaex II Gel Extraction Kit as outlined in the DNA Extraction from Agarose Gels section of the Qiaex II handbook (Qiagen, Mississauga, Ontario). DNA in gel-purified PCR products was quantified with the NanoDrop® ND-1000 spectrophotometer and submitted to the National Research Council, Plant Biotechnology Institute (Saskatoon, SK) for DNA sequencing with the 16s universal primers previously described.

Raw nucleotide sequence data was processed with the PREGAP v1.5 and GAP4 v4.10 programs of the Staden package (Dear and Staden 1991) in order to align forward and reverse sequences, confirm sequence quality and delete primer sequences. Trimmed, quality sequences

were uploaded to the 16S Ribosomal Database Project II (Cole et al., 2207; Cole et al., 2009) website and the Sequence Match tool used to identify the nearest bacterial species match. Phylogenetic analysis and tree construction of sequences was performed using ARB phylogenetic software (Ludwig et al., 2004).

3.2.4 RAPD strain differentiation

A 50 µL RAPD reaction was prepared containing 1x PCR Buffer (Invitrogen, Burlington, ON), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.1% Triton X-100, 1 mM primer Hum4 5'-ACGGTACAT-3' (Hotzel et al., 1998), at least 50 ng of sample DNA, 5 uL Taq polymerase (Invitrogen, Burlington, ON)   and UV ddH20 to bring the reaction mixture volume to 50 µL. The RAPD reaction mixtures were subjected to 95 ºC for 3 min and then 40 cycles of 95 ºC for 30 sec, 37 °C for 30 sec, and 72 ºC for 30 sec in the Amplitron II thermocycler (Thermolyne, Barnstead International, Dubuque, Iowa). RAPD amplification products were then assessed with electrophoresis on a 2% agarose gel containing 0.5 µg/mL ethidium bromide. The voltage used was initially 140 V for 5 min followed by 90 V for 1.5 hrs (BioRad Model 1000/500 Power Supply, BioRad, Hercules, California). Gels were visualized under UV illumination.

3.2.5 AFLP strain differentiation

For isolates confirmed by 16s rRNA gene sequencing as *M. bovis*, extracted genomic DNA was amplified with the illustra GenomiPhi HY DNA Amplification Kit as described in the manual (GE Healthcare, Buckinghamshire, UK). GenomiPhi amplification products were quantified with Picogreen® as described in the manual (Picogreen® dsDNA Quantitation Kit, Molecular Probes Inc, Eugene, Oregon). Approximately 150-500 ng of DNA was digested with 10 U of MfeI and 10U of BglII restriction enzymes (New England Biolabs, Beverly, Mass.) in a buffer previously described by Kokotovic et al (1999) at 37 ºC for 6 hrs. Double stranded adapters were prepared. A ligation reaction consisting of 5 µL of restriction enzyme digest, 10 pmol of a double stranded BglII adapter, 100 pmol of a double stranded MfeI adapter, 1 U of T4 DNA ligase (New England Biolabs, Beverly, Mass.) and the previously described buffer was incubated at room temperature for 16-20 hrs.

PCR amplification from 1 uL of a 1:10 diluted ligation reaction product was performed. PCR reactions consisted of a 1x PCR Buffer (Invitrogen, Burlington, ON), 2.5mM MgCl2, 0.2

mM of each of the four dNTPs, 65 ng of MFE1-0 primer, 65ng of BGL2F-0 primer 0.3 uL Taq polymerase (Invitrogen, Burlington, ON) and UV ddH20 to bring the reaction mixture volume to 50 µL. Amplification was run in a Biorad C1000TM Thermal Cycler (Biorad, Mississagua, Ont.). An initial 3 min denaturation at 95 ºC was performed, followed by 40 cycles of denaturation at 95 ºC for 60 sec, annealing at 54 ºC for 60 sec and elongation at 72 ºC for 90 sec. Amplification completed with a final elongation step of 72 ºC for 10 min.

Amplification fragments were analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster City, CA). Mixtures of 1µl of 1:50 (v/v) dilution in ddH$_2$O of amplification product, 1 µL Genescan 600 Liz internal size standard (Applied Biosystems, Foster City, CA) and 8µl formamide were denatured at 95 ºC for 5 min and placed on ice for 2 min before detection.

Densitometric curves from the ABI 3130x Genetic Analyzer were uploaded into Genemapper v3.7 and 5 bin analysis for AFLP, in which the software searches for significant peaks every 5 nucleotides, was performed. Compared across all samples, restriction fragments within a range of 5 bp were considered a single peak and peak areas combined under a single size (bp) designation. Resulting peak area profiles were imported into Bionumerics v5.1 (Applied Maths, Austin, TX). Clusters were made based on Dice similarity coefficient similarity using the unweighted pair group method with arithmetic mean (UPGMA) algorithm with optimization and position tolerance settings at 0% and 1% respectively. A reproducibility trial was conducted in which nine samples, at least two from each AFLP plate analyzed, were run and analyzed in duplicate with the identical AFLP protocol, supplies, equipment and software.

3.2.6 Statistical analysis

Statistics were analyzed with SPSS v18.0 software. For comparison of *Mycoplasma* spp. prevalence between different sampling times and methods McNemar's Symmetry test was used. The Kappa test was used for comparing the agreement of *M. bovis* status of animals at various time points and sampling procedures. Fisher's exact two-tailed test was also used to compare the the prevalence of each strain, isolated at various sampling times and body sites as well as with different sampling methods.

3.3 Results

3.3.1 *Mycoplasma* species prevalence

3.3.1.1 Healthy University of Saskatchewan feedlot cattle

Mycoplasma bovirhinis was the most common *Mycoplasma* spp. cultured from NS and BAL samples taken from healthy University of Saskatchewan feedlot cattle (Table 3.1), followed by *M. bovis*. An increase in *M. bovis* positive animals was observed at 90 days on feed compared to 14 days on feed in NS ($P < 0.01$). Fewer animals were positive for *M. bovis* in BAL compared to NS, however, the number of *M. bovis* animals also tended to increase ($P = 0.07$) in BAL between 14 and 90 days on feed (Table 3.1). No difference was observed for animals positive for *M. bovirhinis* (NS, $P = 0.50$; BAL, $P = 0.24$) or other species (NS, $P = 0.38$; BAL, $P = 1.00$) between 14 and 90 days on feed in either NS or BAL samplings (Table 3.1). Only two *Mycoplasmas* spp., other than *M. bovis* and *M. bovirhinis,* were cultured from University of Saskatchewan feedlot cattle (Table 3.1), namely *M. alkalescens* and *M. bovoculi*. While no difference was observed between the number of animals in which no *Mycoplasma* spp. could be cultured from NS samples at 14 and 90 days on feed ($P = 0.63$), a decrease in *Mycoplasma* negative animals was observed between 14 and 90 days on feed in BAL samples ($P = 0.03$; Table 3.1). Multiple *Mycoplasma* spp. were cultured from between 6-11% of healthy University of Saskatchewan cattle tested and there was no difference between the number of animals in which multiple species could be cultured at 14 versus 90 days on feed in the either the NS ($P = 1.00$) or BAL ($P = 1.00$) samples (Table 3.1).

There was poor agreement ($K = 0.05$, $P = 0.62$) between the *M. bovis* status of an animal at 14 DOF compared to 90 DOF (Table 3.3). Fair agreement ($K = 0.25$, $P = 0.05$) was noted between the *M. bovis* status as determined by NS compared to BAL (Table 3.4). Furthermore, while not statistically significant, the prevalence of *M. bovis* was also higher in NS compared to BAL at both 14 ($P = 0.38$) and 90 ($P = 0.06$) DOF.

3.3.1.2 Symptomatic commercial feedlot cattle

Mycoplasma bovis and *M. bovirhinis* were also the most prevalent *Mycoplasma* spp. cultured from nasal swabs taken from cattle that developed respiratory disease symptoms between one week and three months after arriving at a commercial feedlot in Saskatchewan (Table 3.2). However, in these cattle, *M. bovis* was the most common. The number of animals

positive for *M. bovis* was higher upon treatment for respiratory disease than upon arrival ($P = 0.01$; Table 3.2). However, there was no difference between the numbers of animals that had positive cultures for *M. bovirhinis* when sampled at arrival compared to time of treatment ($P = 0.19$; Table 3.2). In the commercial feedlot, four other species of *Mycoplasma* were cultured including in order of prevalence *M. ovipneumoniae, M. lagogenitalium, M. alkalescens, and M. cynos*. While not significant the number of animals positive for these other species tended to be greater at the time of treatment than upon arrival ($P = 0.08$; Table 3.2). The number of animals in which multiple *Mycoplasma* spp. could be cultured also increased from the time of arrival to the time of treatment; however, this was not different ($P = 0.08$; Table 3.2). The number of animals in which no *Mycoplasma* spp. was cultured from nasal swabs was greater upon arrival than at time of treatment ($P = 0.02$; Table 3.2).

There was poor agreement ($K = 0.04$, $P = 0.78$) between the *M. bovis* status of a commercial feedlot animal on arrival at the feedlot and the status one week to three months later upon treatment for respiratory disease symptoms (Table 3.5). Consequently, for both feedlots, animals that were positive for *M. bovis* at entry occasionally became negative, and consistent with increased prevalence, many *M. bovis* negative animals at entry became positive.

3.3.1.3 Necropsied cattle

Presumptive *Mycoplasma* colonies were observed on culture of swabs on *Mycoplasma* spp. specific agar for 93% of the 61 lung samples and 77% of the 47 joint samples. However, due to technical difficulties only subcultured colonies from 29 animals were available for speciation by sequencing of 16S rRNA gene. Twenty five of the 29 animals were identified as *M. bovis* positive. Of the remaining, three animals were identified as having only *M. alkalescens* and one animal identified as *Paenibacillus chibensis*.

3.3.2 Establishment of *Mycoplasma bovis* strain differentation methodology

The RAPD strain methodology adapted from the McAuliffe et al (2004) *M. bovis* strain differentiation study failed to produce reliable and reproducible results. Success of the RAPD protocol, as determined by presence of bands when visualized with ethidium bromid under UV light, between samples run in duplicate with identical protocol, supplies and equipment was inconsistent (data not shown). The number and intensity of bands between duplicate runs was also inconsistent (data not shown); therefore, RAPD was determined to be unsuitable for this study.

Table 3.1 Classification of University of Saskatchewan feedlot cattle (percent of total in brackets) according to the bacterial species assignment of presumptive *Mycoplasma* isolates cultured from nasal swabs (NS) and bronchoalveolar lavage (BAL) samples taken from feedlot steers at 14 and 90 days on feed[a].

Sample Type	Days on Feed	Number of Animals[b]	*M. bovis*[c]	*M. bovirhinis*	Other Species	Multiple Species	No *Mycoplasma* Growth
NS	14	36	4 (11)	19 (53)	4 (11)	4 (11)	13 (36)
NS	90	36	14 (39)	15 (42)	1 (3)	4 (11)	10 (28)
P value[d]			<0.01	0.50	0.38	1.00	0.63
BAL	14	36	1 (3)	11 (31)	1 (3)	2 (6)	25 (69)
BAL	90	36	7 (19)	17 (47)	0 (0)	2 (6)	14 (39)
P value[d]			0.07	0.24	1.00	1.00	0.03

[a] Data previously reported as part of Dr. Nathan Erickson's M.Vet.Sc. at the Western College of Veterinary Medicine, University of Saskatchewan in 2009.
[b] Individual animals may be represented in more than one Mycoplasma group classification (column).
[c] Bacterial species assignment based on partial 16S rRNA gene sequence analysis
[d] *P* values determined with McNemar's test.

Table 3.2 Classification of cattle from a commercial feedlot in Saskatchewan Canada (percent of total in brackets) according to the bacterial species assignment of presumptive *Mycoplasma* isolates cultured from nasal swab samples taken from feedlot steers on arrival at the feedlot and at treatment for respiratory disease 1-3 weeks later.

Sample Time	Number of Animals[a]	*M. bovis*	*M. bovirhinis*	Other Species	Multiple Species[b]	No *Mycoplasma* Growth
On arrival	56	24 (41)	18 (32)	1 (2)	4 (7)	17 (30)
At treatment	56	38 (68)	11 (19)	14 (25)	12 (21)	5 (9)
P value[c]		0.01	0.19	<0.01	0.08	0.02

[a] Bacterial species assignment based on partial 16S rRNA gene sequence analysis
[b] Bacterial species assignment based on partial 16S rRNA gene sequence analysis Individual animals may be represented in more than one Mycoplasma group classification (column)
[c] *P* value determined using McNemar's test.

Table 3.3 Agreement between *Mycoplasma bovis* status (+ or -) of individual animals at 14 DOF compared to 90 DOF in the University of Saskatchewan feedlot[a].

	Number of Animals	*M. bovis* + 90DOF[b] # of animals (%)	*M. bovis* – 90DOF # of animals (%)
M. bovis + 14DOF[b]	3	2 (67)	1 (33)
M. bovis – 14DOF	29	15 (52)	14 (48)

[a] The *M. bovis* status of an animal at 14 DOF is in poor agreement with the status at 90 DOF ($K = 0.05$, $P = 0.62$).

[b] Defined as animals *M. bovis* positive for either a BAL or NS sample

Table 3.4 Agreement between *Mycoplasma bovis* status (+ or -) of individual asymptomatic animals with NS compared to BAL at the University of Saskatchewan feedlot[a].

	Sampling Occassion[b]	BAL +	BAL –
NS +	17	5 (29)	12 (71)
NS -	36	3 (8)	33 (92)

[a] *M. bovis* status determined in NS is in fair agreement with the status as determined by BAL ($K = 0.25$, $P = 0.05$).

[b] Represents the number of sampling occasions for which BAL and NS were available for a single animal.

Table 3.5 Agreement between *Mycoplasma bovis* status (+ or -) of individual animals OA compared to AT in a commercial feedlot (Pound-Maker Agventures Ltd., Lanigan, Saskatchewan)[a].

	Number of Animals	*M. bovis* + AT # of animals (%)	*M. bovis* – AT # of animals (%)
M. bovis + OA	21	17 (81)	4 (19)
M. bovis – OA	13	10 (77)	3 (23)

[a] *M. bovis* status on arrival at the feedlot is in poor agreement with the status one week to three months later ($K = 0.04$, $P = 0.78$).

3.3.3 AFLP Strain differentiation of *Mycoplasma bovis* isolates

The AFLP protocol resulted in much higher reliability and reproducibility of banding patterns compared to RAPD. Fingerprints consisting of between 20-60 fragments were produced for single samples with the AFLP protocol. Only minor changes in densitometric curve and band assignment was observed on analysis of nine duplicate samples. Similarity score, based on the dice coefficient, between duplicate samples was consistently above 92% (Figure 3.1).

AFLP strain differentiation and subsequent cluster analysis of *M. bovis* strains isolated from the 19 animals at necropsy revealed three main clusters, labelled A, B and C (Figure 3.2). Isolates within each cluster show a similarity score of greater than 75% based on the dice coefficient (Figure 3.2). Strain A was isolated from both the lung and joint samples of the majority of animals (Table 3.6); however, isolation of multiple strains was not uncommon from the same lung (38% of tissues) or joint sample (29% of tissues; Table 3.6). Samples for both joint and lung were available for only seven animals; therefore, this group is too small for statistical comparison. However, five of these animals were colonized by multiple strains and for three of these a unique strain was isolated from each tissue.

Only presumptive *Mycoplasma* isolates that were identified as *M. bovis* were subject to strain differentiation analysis using AFLP. Of the 67 *M. bovis* positive isolates from the University of Saskatchewan feedlot cattle, 47 could be differentiated with AFLP. These isolates represented 17 of the 21 animals that were classified as *M. bovis* positive in the study. Strain differentiation was possible on 140 of the 190 *M. bovis* positive isolates from the commercial feedlot representing 41 of 45 animals positive for *M. bovis* at either sampling point. The missing *M. bovis* positive isolates were excluded from strain differentiation analysis due to either failed culture from frozen stocks, insufficient DNA recovery or failure of AFLP to produce a fingerprint for one of the sampling periods.

The AFLP fingerprints from the commercial feedlot group isolates clustered almost entirely (99%) with necropsy group A isolates (Figure 3.3), with the exception of one isolate that clustered with necropsy group B (Figure 3.3). Of the isolates cultured from NS and BAL samples of University of Saskatchewan feedlot cattle 68% clustered with strain group B of the necropsy isolates (Figure 3.4), with 32% clustering within necropsy strain group A (Figure 3.4). None of

the isolates from either the University of Saskatchewan (Figure 3.4) or commercial feedlot animals (Figure 3.3), respectively, clustered with the necropsy group C.

There was no significant difference in the prevalence of each cluster isolated in joint and lung tissue of cattle at necropsy (Table 3.6). Similarly, no difference in the prevalence of any cluster was observed comparing 14 and 90 days on feed (Table 3.7) or comparing feedlot entry with day of treatment (Table 3.8). Finally, no significant difference between the prevalence of each cluster isolated from BAL compared to NS was observed (Table 3.9).
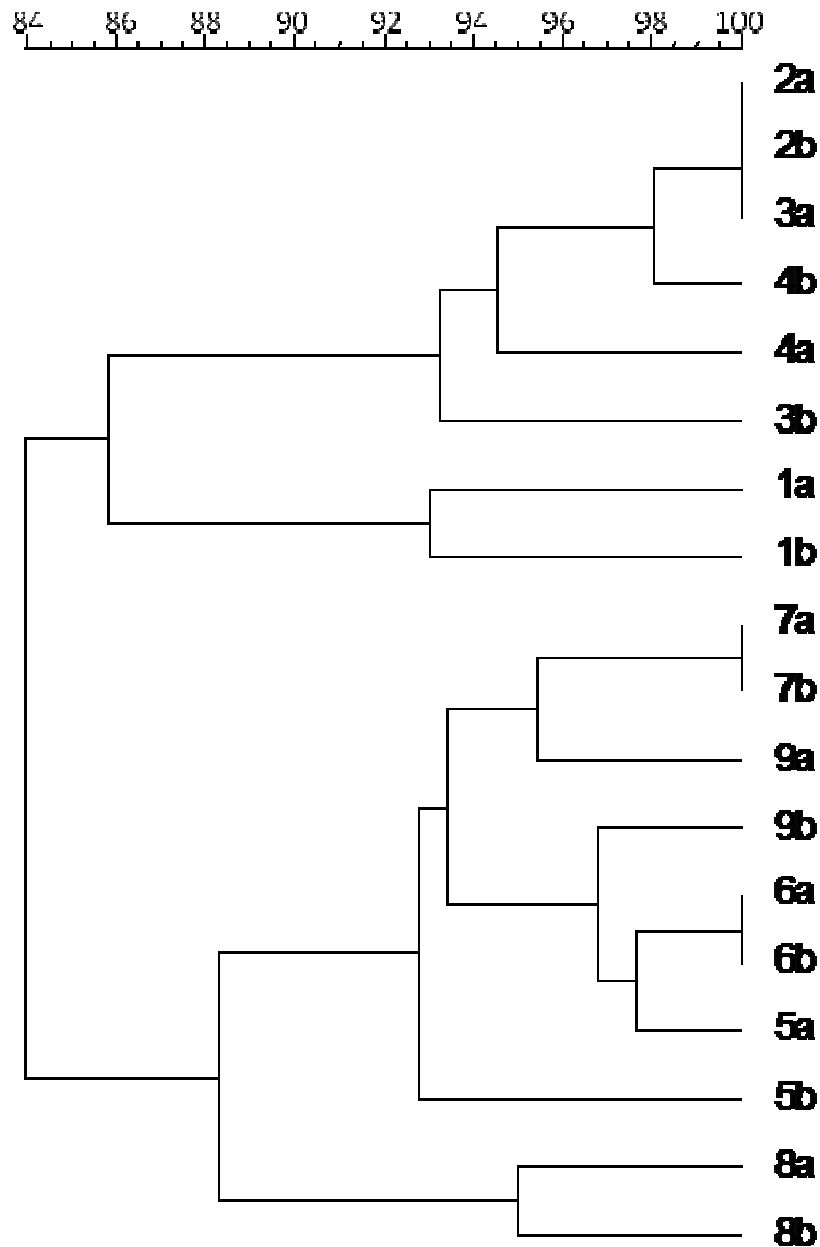
Figure 3.1 Dendogram showing the relationship between AFLP banding patterns for duplicate *Mycoplasma bovis* strains isolated from feedlot cattle. Amplified fragments were identified from the densitometric curves with Genemaper v3.7 software. The dendogram was then produced using UPGMA clustering of Dice similarity coefficient S_D (%) values (Bionumerics v.5.1). Alphanumeric characters at right include a unique numerical identifier (1-9) for each sample; duplicates are labelled a and b.
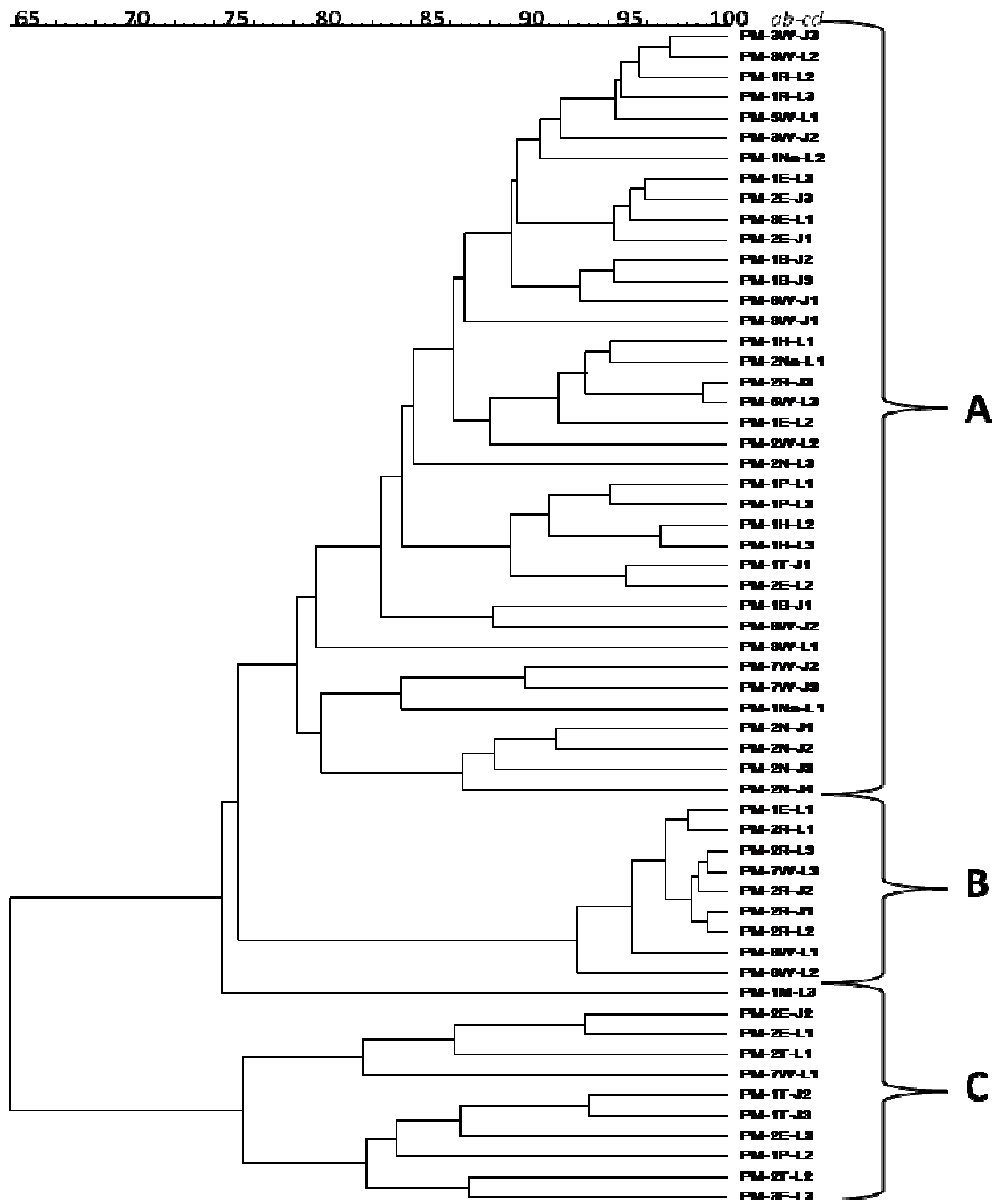
Figure 3.2 A dendogram showing relationship among AFLP banding patterns for *Mycoplasma bovis* isolates cultured from lung or joint of cattle with clinical pathology indicative of Chronic Pneumonia and Polyarthritis Syndrome. Three genetically distinct clusters are labelled A, B and C. The dendogram was produced based on UPGMA clustering of Dice similarity coefficient (S_D %) values (Bionumerics v.5.1). Unique identifiers for each isolate are given at right where PM designates post-mortem and remaining characters according to a scheme where column *a* is an animal designation (single digit numeral), *b* a feedlot designation (upper case letter), *c* indicates lung (L) or joint (J) sample site and column *d* a colony number.
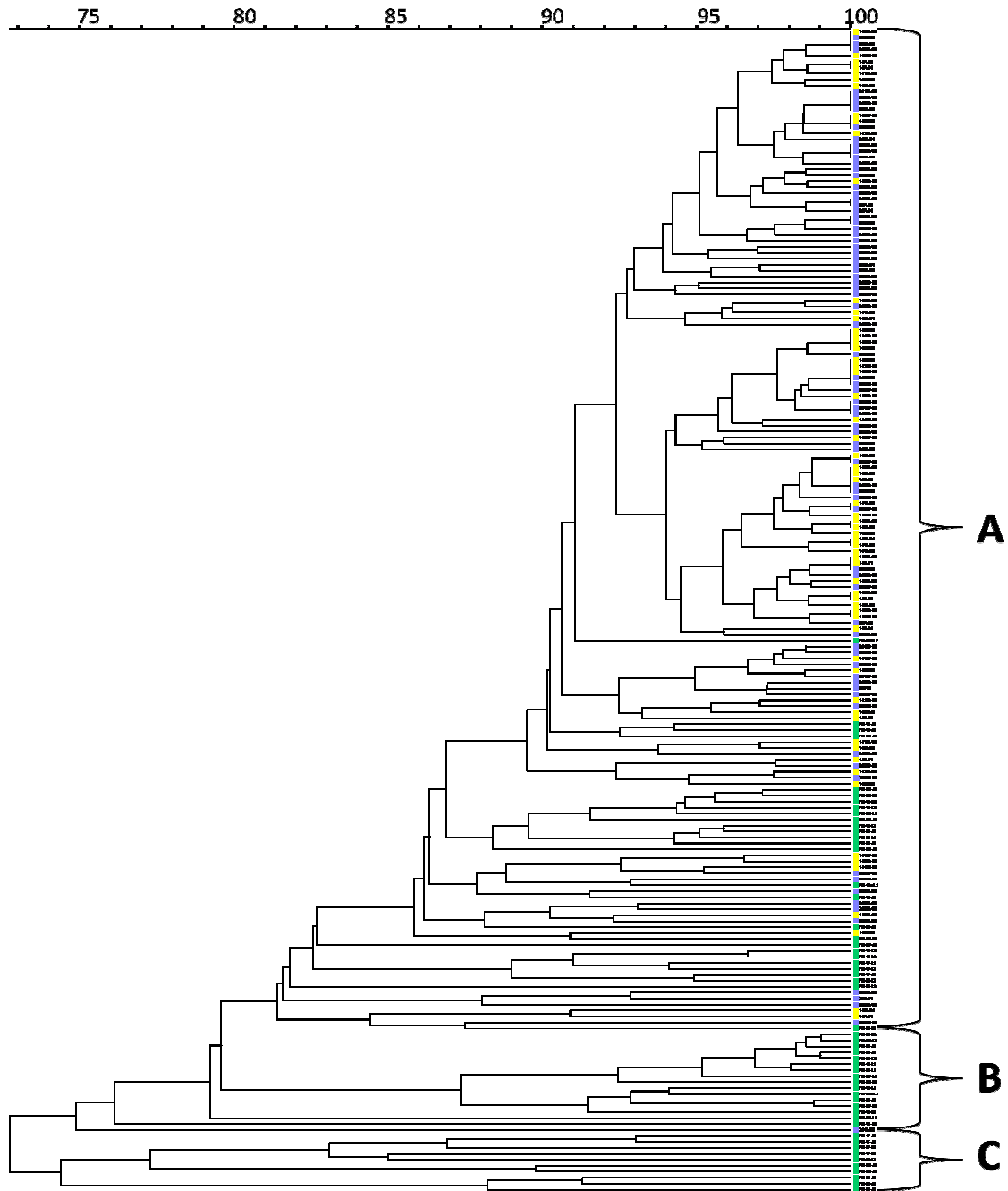
Figure 3.3 A dendogram showing relationship among AFLP banding patterns for *Mycoplasma bovis* isolates cultured from nasal swabs collected from cattle at a commercial feedlot in Saskatchewan and from joint and lung tissue of cattle with clinical pathology indicative of Chronic Pneumonia and Polyarthritis Syndrome. Three clusters are labelled A, B and C based on the clustering of necropsy samples shown in Figure 3.2. The dendogram was produced based on UPGMA clustering of Dice similarity coefficient (S_D %) values (Bionumerics v.5.1). Green represents necropsy strains, yellow represents isolates from asymptomatic calves on arrival at feedlot and blue represents isolates taken from these cattle one week to three months later upon treatment for symptoms of respiratory disease.
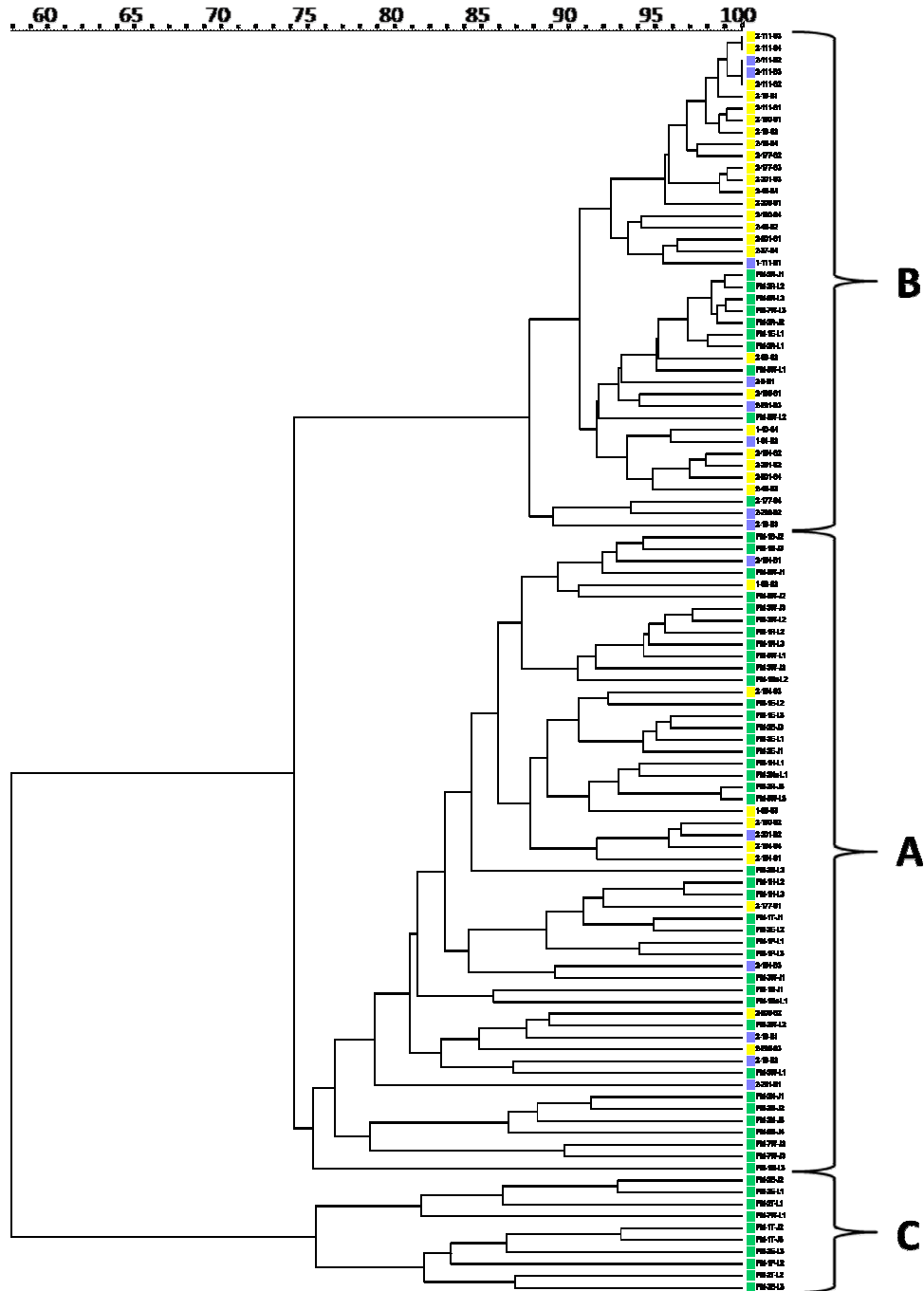
44

Figure 3.4 A dendogram showing relationship among AFLP banding patterns for *Mycoplasma bovis* strains isolated from nasal swab (NS) or bronchoalveolar lavage (BAL) samples taken from asymptomatic University of Saskatchewan feedlot cattle. Three clusters are labelled A, B and C based on the clustering of necropsy samples shown in Figure 3.2. The dendogram was produced based on UPGMA clustering of Dice similarity coefficient (S_D %) values (Bionumerics v.5.1). Green represents necropsy strains, blue represents BAL isolates, and yellow represents NS isolates.

Table 3.6 Number (and percent) of cattle in the CPPS clinical presentation necropsy group colonized in lung or joint with *Mycoplasma bovis* isolates that cluster in group A, B, and C as shown in Figure 3.2.

Sample Type	Number of Animals[a]	Cluster A	Cluster B	Cluster C	Multiple Clusters
Lung[b]	17	12 (71)	5 (29)	5 (29)	5 (29)
Joint[b]	8	8 (100)	1(13)	2 (25)	3 (38)

[a] Individual animals may be represented in more than one cluster classification (column). Cattle included in 'Multiple Strains' column not also included in 'Strain A', 'Strain B' and 'Strain C' columns.
[b] No significant difference between the prevalence of strains present in lung and joint samples (Fisher's exact test, two sided: $P = 0.54$)

Table 3.7 Number (and percent) of cattle from a commercial feedlot in Saskatchewan on arrival at the feedlot and one week to three months post arrival at treatment for respiratory disease that are colonized with *Mycoplasma bovis* strains clustering in necropsy group A, B, and C as shown in Figure 3.2.

Sample Time	Number of Animals[a]	Cluster A	Cluster B	Cluster C	Multiple Clusters
On Arrival[b]	24	24 (100)	0 (0)	0 (0)	0 (0)
At Treatment[b]	38	38 (100)	1 (3)	0 (0)	1 (3)

[a] Individual animals may be represented in more than one cluster classification (column).
[b] No significant difference in the prevalence of strains isolated from animals on arrival and at treatment for respiratory disease (Fisher's exact test, two sided: $P = 0.42$).

Table 3.8 Number (and percent) of cattle the University of Saskatchewan feedlot at 14 and 90 days on feed (DOF) that are colonized with *Mycoplasma bovis* strains clustering in necropsy group A, B, and C as shown in Figure 3.2.

Sample Time	Number of Animals[a]	Cluster A	Cluster B	Cluster C	Multiple Clusters
14DOF[b]	5	2 (40)	3 (60)	0 (0)	0 (0)
90DOF[b]	12	6 (50)	11 (92)	0 (0)	5 (42)

[a] Individual animals may be represented in more than one cluster classification (column).
[b] No significant difference in the prevalence of strains isolated from animals at 14 and 90 days on feed (Fisher's exact test, two sided: $P = 0.85$)

Table 3.9 Number (and percent) of cattle the University of Saskatchewan feedlot in which nasal swabs (NS) or bronchoalveolar lavage fluid (BAL) were colonized with *Mycoplasma bovis* strains clustering in necropsy group A, B, and C as shown in Figure 3.2.

Sample Type	Number of Animals[a]	Cluster A	Cluster B	Cluster C	Multiple Clusters
NS [b]	13	5 (38)	11 (85)	0 (0)	3 (23)
BAL[b]	7	3 (43)	6 (86)	0 (0)	2 (29)

[a] Individual animals may be represented in more than one cluster classification (column).
[b]No significant difference in proportion of each strain cluster isolated from NS and BAL (Fisher's exact test, two sided: $P = 0.91$)

3.4 Discussion

Mycoplasma bovis is associated with CPPS, a condition that is extremely difficult to treat and prevent. CPPS therefore, poses not only welfare concerns for the animals but high economic loss for the producer (Nicholas and Ayling, 2003). Prevalence studies have been conducted in Europe (Arcangioli, et al., Assié et al., 2009; Ayling et al., 2004; Bashiruddin et al., 2001; Blackburn et al., 2007; Le Grand et al., 2002;), Oceania (Ghadhersohi et al., 1999) and North American (Gagea et al., 2006, Wiggins et al., 2007); however, no studies have yet been conducted investigating the prevalence of *M. bovis* or assessing strain variation in western Canada. In this study the prevalence of *M. bovis* was examined in two different feedlots in Saskatchewan that source calves from all over western Canada. *M. bovis* prevalence varied between 41-68% of cattle tested in the commercial feedlot in the fall/winter of 2007 and between 3-39% of the cattle tested at the University of Saskatchewan in fall/winter 2008. Thus high variation in prevalence occurred within a feedlot sampled at different times as well as between feedlots. The high variability observed in this study is not uncommon among prevalence studies (Allen et al., 1991, 1992; Catry, 2008; Rosendal and Martin et al., 1986; Thomas et al., 2002) and may reflect a number of factors.

The increase in *M. bovis* prevalence in the commercial feedlot may simply have been an effect of changes in prevalence associated with the sampling year as reported previously (Ayling et al., 2004; Blackburn et al., 2007). Alternatively, while University of Saskatchewan cattle showing the lower prevalence were asymptomatic, the commercial feedlot calves were treated for respiratory disease at their second sampling. The majority of these treated animals required only one treatment with standard antibiotic therapy prior to recovery and none of the animals died of respiratory illness in the feedlot. Since *M. bovis* is resistant to most respiratory disease antibiotic therapies commonly used in feedlots and CPPS is characterized by its chronic state, the respiratory symptoms in these cattle successfully treated may be due to another bacteria, virus, or multiple pathogen complex (Francoz et al., 2005; Haines et al., 2001; Hirose et al., 2003; Rosenbusch et al., 2005; ter Laak et al., 1993). Recent studies have shown a positive relationship between *M. bovis* and other bacterial respiratory pathogens (Fulton et al., 2009; Poumarat, 2001). Therefore, while the higher prevalence of *M. bovis* recorded in the group treated for respiratory disease may not be connected to CPPS, it may be due to the presence of other respiratory pathogens.

In the current study an increase in *Mycoplasma,* specifically *M. bovis,* colonization was observed with increased days on feed for cattle in both feedlots. There are several studies that indicate an increase in *Mycoplasma* colonization with days on feed (Allen et al., 1992; Rosendal and Martin et al., 1986). This increase may be due to the length of time the calves are exposed to shedders of *Mycoplasma* in the feedlot. In North American feedlots calves are purchased from cow-calf producers and fed in large pens sorted by age or size until an optimal weight is reached for sale to slaughter (Alexander et al., 1989; Ribble et al., 1995). It is common for North American feedlots to purchase cattle from several different sources at auction and often a single shipment of 60 or more calves to a feedlot can carry no more than two calves from the same source. This high rate of commingling with cattle from many sources has been found to be associated with an increased risk of developing lower respiratory disease in these cattle within the first few months on feed (Alexander et al., 1989; Fulton et al., 2009; Healy et al., 1993; Ribble et al., 1995). Therefore, time spent commingling with many other potential pathogen sources may affect the microbial status in the respiratory tract of a calf and may increase the chances of coming into contact with and being colonized by *M. bovis,* as was observed in this study.

Although *M. bovis* prevalence increased with days on feed, there was no agreement between the *M. bovis* status of an animal early in the feedlot and at later time points. This is primarily associated and consistent with the increase in *M. bovis* prevalence with days on feed such that many *M. bovis* negative animals on feedlot entry become positive. However, we also noted that while most *M. bovis* positive animals entering the feedlot remain *M. bovis* positive, several also clear the pathogen without treatment. Alternatively, the apparent clearance of *M. bovis* from a small number of animals may have been a false negative result. Indeed, sampling method affected the number of *M. bovis* isolates identified yielding variability in prevalence. In the current study the *M. bovis* prevalence was in fair agreement between nasal swabs (NS) and bronchoalveolar lavages (BAL) and while not significant, more *M. bovis* could be isolated from NS than BAL. There are several studies that have observed a significantly higher prevalence of *M. bovis* in NS than in BAL samples (Allen et al., 1992; Catry et al., 2008); however, one study found the opposite and recorded higher *M. bovis* prevalence from BAL than NS (Allen et al., 1991). While the agreement between the two methods is fair based on the kappa statistic, further research with a greater number of animals may be needed to make significant conclusions

regarding which sampling method is a more accurate indicator of *M. bovis* prevalence and if either sampling method can be associated with disease outcome.

In the current study *M. bovis* was isolated in up to 25% of BAL samples from healthy University of Saskatchewan feedlot cattle. However, in a study in Belgium, *M. bovis* was not isolated from BAL in any of the healthy cattle sampled (Thomas et al., 2002). The investigators concluded that this indicated a small number of healthy carriers. Our results suggest a much higher number of potential carriers. However, the variability between studies may also be due to differences in *Mycoplasma* culture and identification methodology or other factors such as geography and sampling year. While Thomas et al (2002), observed different *M. bovis* prevalence from the current study they did agree with the current findings that while one or two *Mycoplasma* spp. are generally predominant in the feedlot it is not uncommon for a single animal to harbour several species. In the present study no significant increase or decrease in the numbers of animals positive for multiple *Mycoplasma* spp. was observed with days on feed even when *M. bovis* prevalence increased in these animals. Interestingly however, in NS samples there was a numerical decline in *M. bovirhinis* positive animals as *M. bovis* positive animals increased with days on feed suggesting displacement. In contrast, the number of cattle positive for *M. bovis* and *M. bovirhinis* increased (non-significantly) in BAL with days on feed. This observation suggests that while there may be some competition between closely related *Mycoplasma* spp. it is possible for them to coexist in the same animal.

The high prevalence of *M. bovis* in lung and joint samples from the necropsy animals with CPPS clinical pathology is in disagreement with Thomas et al (2002) in which relatively few necropsy animals appeared to be colonized by *M. bovis*. In the current study samples were only taken at necropsy if lung pathology associated with CPPS and septic arthritis was observed, while in the Thomas et al (2002) study samples were taken from animals at necropsy with little differentiation between *M. bovis* associated pathology and other types of pneumonias. Therefore, the mortalities in these previous reports may be more commonly due to other respiratory pathogens.

Molecular strain differentiation methods distinguish between strains based on variability in nucleotide sequence within the whole genome. Given isolation of *M. bovis* from healthy animals, one of the objectives of the current study was to identify genetic variation in *M. bovis*

by employing molecular strain differentiation to compare populations in CPPS afflicted and healthy cattle. Such a comparison could identify virulent and avirulent strains.

There are several molecular methods for *M. bovis* strain differentiation currently described in the literature with varying degrees of specificity, sensitivity, reproducibility, speed, technical and labour requirements (Beier et al., 1998; Biddle et al., 2005; Butler et al., 2001; Fox, 2008; Kusiluka et al., 2000; McAuliffe et al., 2004; McAuliffe et al., 2008; Miles et al., 2005). Originally RAPD was chosen as a seemingly ideal method of strain differentiation for this study as previous literature had found RAPD to be as sensitive and specific as other methods, but with fewer technical and time requirements (McAuliffe et al., 2004, Williams et al., 1990). However, in the current study, RAPD methodology showed extremely poor reproducibility even in the appearance of major bands visualized with ethidium bromide under UV light. Previous research did cite some reproducibility problems, but it appeared even with modifications to the original protocols the reproducibility was much lower than anticipated from this earlier research (Cherry et al., 2006; Cousin-Allery et al., 2000; Feberwee et al., 2005; Mettifogo et al., 2006; McAuliffe et al., 2004; Stakenborg et al., 2006). The low reproducibility made RAPD an unreliable method for strain differentiation in this study. AFLP methodology employed here was based on Kokotovic et al (1999) and produced reproducible banding patterns with greater than 92% similarity score based on dice coefficient values for duplicate determinations. While this reproducibility is somewhat less than the identical densitometric curves reported by Kokotovic et al (1999) when duplicate samples were analyzed, the result is not surprising given the relatively complex multi-step AFLP protocol.

Clustering of AFLP banding patterns for *M. bovis* isolates cultured from lung and joint samples at necropsy, identified three main clusters (A, B and C) for which dice coefficient values were less than 75%, well below the 92% value observed for duplicate samples. The necropsy samples came from ten different feedlots in Alberta and Saskatchewan; therefore, represent a geographically diverse group. Comparable studies in which *M. bovis* strain identification was conducted found similar results in that relatively few distinct clusters were identified in cattle from a diverse geographical sampling (Kusiluka et al., 2002; McAuliffe et al., 2004).

All necropsy samples were isolated from CPPS mortalities that occurred within a year of one another; therefore, no inferences can be made about *M. bovis* strain and sampling year in this group. Six of the ten feedlots in which CPPS mortalities were sampled had only one

51

representative necropsy animal; therefore, statistically significant conclusions cannot be made regarding the trend between geographical location and *M. bovis* strains present in this group. However, of the four feedlots in which more than one CPPS mortality was sampled, two to three *M. bovis* strains were isolated in three, suggesting that geographical location within western Canada was not a factor in *M. bovis* strains isolated. The present study indicated that the lung or joint of a single animal at necropsy could be colonized by multiple *M. bovis* strains. While only seven animals had data for both lung and joint samples, multiple strains could be identified in a single tissue for two of these animals and a different strain was isolated from the lung than from the joint in three of these animals. There is literature to support both the isolation of the same strain in multiple body sites (Fox et al., 2008)and the isolation of genetically distinct *M. bovis* strains from different body sites within a single animal (Biddle et al., 2005). Further research with strain differentiation in multiple body sites from larger groups of animals may provide a better understanding of this strain discrepancy within and between sampling locations

When the strains from cattle at both the University and commercial feedlots were compared to the strains identified in the necropsy animals no new strain clusters were identified. The strains identified in the commercial feedlot in 2006 from cattle treated for respiratory disease clustered mainly with group A while the strains identified in the University of Saskatchewan feedlot in 2007 from clinically healthy cattle clustered mainly with necropsy cluster B with some isolates clustering with strain group A. Since none of the cattle in the University of Saskatchewan or commercial feedlots presented with CPPS the A and B strains might be considered of low virulence.

The differences in strain prevalence between the two groups may be due to the years in which the samples were collected. The predominant *Mycoplasma* spp. cultured from cattle varies year to year so it is possible that the principal strains of these species also vary between year (Ayling et al., 2004; Blackburn et al., 2007). However, the tissues cultured from the animals at necropsy were all collected within a one year period between the conduct of the commercial feedlot study and the University of Saskatchewan feedlot study. Therefore, sampling year is likely not the sole cause of the strain diversity between these two feedlots. Another possible explanation for the strain discrepancy between the two feedlots is geography; however, these feedlots are both located within Saskatchewan and both purchase calves from numerous sources

so this is unlikely. Furthermore, no trends between geographical location of sampling and *M. bovis* strains isolated were observed in the necropsy group.

Mycoplasma bovis isolates clustering in strain C were not found in healthy cattle suggesting this could be a virulent strain. However, the B and C strains were of lowest prevalence in lung and joint tissue of necropsy animals compared to the A strain found in 71% of lung samples and all joint samples. Thus, the link between molecular strain differentiation and pathogenicity is still unclear. Our failure to identify a unique strain(s) associated with clinical CPPS could be due to variable host response or the presence or absence of other bacteria in a multifactorial scenario. However, studies have shown that *M. bovis* strains cultured from cattle with *M. bovis* associated disease are capable of causing disease when inoculated into both conventional and gnotobiotic calves (Allen et al., 1992; Arcangioli et al., 2007; Haines et al., 2001; Khodakaram-Tafti and López, 2001; Poumarat et al., 2001). Several recent studies have demonstrated that much of the genetic variation between *M. bovis* isolates is in repetitive non-coding regions of the genome (McAuliffe et al., 2008; Miles et al., 2005). The differentiations made by molecular strain typing techniques may not cluster the isolates by expressed genes; therefore, may not be sensitive enough to differentiate strains based on virulence. In a recent study of *Mycoplasma gallisepticum,* a respiratory pathogen in poultry, RAPD molecular strain typing was comparable with strain differentiation based on sequence polymorphisms when potential pathogen associated genes were amplified (Ferguson et al., 2005). Although the methods were comparable, RAPD demonstrated much lower discriminatory power in differentiating between vaccine strains and pathogenic field strains, than sequence analysis of expressed genes. Therefore, strain identification based on developing whole-genome fingerprints without previous knowledge of the genome may be less discriminatory and efficient in differentiating between virulent and avirulent *M. bovis* strains. Perhaps virulence factor typing and further strain differentiation within these AFLP based clusters via functional gene sequencing could further explain the differences between disease outcomes within the three identified clusters.

This study also found no significant differences between the distribution of strains isolated from NS and BAL samples. This is contrary to reported variations in adherence ability among isolates taken from different tissues (Sachse et al., 1993; Thomas et al., 2003). Furthermore, the higher prevalence of *M. bovis* in NS compared to BAL as observed here and by

others, is consistent with the presence of unique strains capable of colonizing the lower respiratory tract (Allen et al., 1992; Catry et al., 2008). The finding of all three strains in BAL in the present study suggests; however, the difference in *M. bovis* colonization between the upper and lower respiratory tract is host dependent.

In conclusion we isolated *M. bovis* from 93% of lung and 77% of joint tissues collected post mortem from cattle with clinical presentations consistent with CPPS and consistent with the identification of this organism as the causative agent. We were also able to isolate *M. bovis* from the upper and lower respiratory tract of healthy cattle. Consistent with reports from other geographical regions, the prevalence of *M. bovis* was significantly higher in the upper compared to lower respiratory tract and increased in both tract locations with days on feed. Two of the three strain clusters identified with AFLP in the CPPS clinical presentation group were also isolated from healthy animals and those with respiratory disease presentations unrelated to CPPS. No meaningful associations could be derived regarding the ability to colonize the lower respiratory tract, the development of CPPS, geographical location of samplings, year of samplings and *M. bovis* strain clusters. This could reflect a limitation of AFLP methodology to detect genetic variation associated virulence. Alternatively our results suggest that following *M. bovis* colonization of the respiratory tract, whether the animal clears this pathogen or develops CPPS is dependent on host factors and/or the presence of secondary pathogens.

4.0 GENERAL DISCUSSION AND CONCLUSIONS

The control of *M. bovis* associated disease has many challenges. Resistance to most antibiotics makes treatment difficult (Francoz et al., 2005; Gerchman et al., 2009; Hirose et al., 2003; Rosenbusch et al., 2005; ter Laak et al., 1993; Vogel et al., 2001) and the variability of the surface antigens hinders development of efficacious vaccines (Behrens et al., 1996, Le Grand et al., 1996, Lysnyansky et al., 1996). The nature of the feedlot system in North America allows rapid efficient spread of the respiratory disease pathogens including *M. bovis* (Alexander et al., 1989; Fulton et al., 2009; Healy et al., 1993; Ribble et al., 1995). Once an animal develops pneumonia the immune modulation and evasion characteristics of *M. bovis* appear to aid the chronic disease state and systemic spread to multiple joints that defines Chronic Pneumonia and Polyarthritis syndrome (Butler et al., 2001; Krysak, 2006; Pourmat et al., 2001; Arcangioli et al., 2007).

The prevalence of *M. bovis* in feedlots has been studied in most first world nations with beef production; however, due to differences in geography, sample type, sampling period and identification methods the numbers are not comparable to one another and are therefore, not applicable to western Canada (Arcangioli, et al., Assié et al., 2009; Ayling et al., 2004; 2007; Bashiruddin et al., 2001; Blackburn et al., 2007; Ghadhersohi et al., 1999; Gagea et al., 2006; Le Grand et al., 2002; Wiggins et al., 2007). The present study was the first *M. bovis* prevalence study in western Canadian feedlots and results were consistent with previous work in that prevalence varied considerably between feedlots and years and increased with days on feed. Increased prevalence was not unexpected as the more time calves are commingled with other cattle from multiple sources the more likely they are to transfer or acquire pathogens such as *M. bovis* (Alexander et al., 1989; Fulton et al., 2009; Healy et al., 1993; Ribble et al., 1995). There was also a higher prevalence in the nasal swabs than in bronchoalveolar lavages, which was also not unexpected from similar studies previously published (Allen et al., 1992; Catry et al., 2008). Therefore, nasal swabs may overestimate *M. bovis* prevalence in the lower lung. As CPPS is a condition of the lower respiratory tract BAL is presumably a more accurate predictor of CPPS. It was also observed that while animals positive for *M. bovis* upon entry did seem to be able to spread the bacteria to other animals, as seen by the increased prevalence with days on feed, some of these animals appeared to be able to clear the infection as they were found negative for *M. bovis* when sampled again later. This was also true for animals in which *M. bovis* could be

isolated from the lower respiratory tract with BAL sampling. Therefore, isolation of *M. bovis* from the lower respiratory tract is not the only predictor of CPPS development and strain variation, environmental or host factors must also play a role. It should be noted that while BAL uses alchol sterilized endoscopes and tubing some transfer of bacteria from the nasal passage to the lower lungs during the BAL sampling process is possible. Furthermore, because *Mycoplasmas* are also so slow growing and fastidious subculturing and enrichment are often utilized, as was the case in this study, and this could allow for some false positive BAL results. Future research regarding BAL and culture protocols may be of benefit to ensure the reliability of BAL prevalence recorded.

Several genomic based strain differentiation methods have been tested with *Mycoplasma* species; however, the reproducibility, sensitivity and congruence between different methods is still unclear (Beier et al., 1998; Biddle et al., 2005; Butler et al., 2001; Fox, 2008; Kusiluka et al., 2000; McAuliffe et al., 2004; McAuliffe et al., 2008; Miles et al., 2005). In the current study we attempted RAPD strain differentiation but observed undesirably low reproducibility. When duplicate samples were analyzed with identical RAPD protocols and reagents the results varied considerably in both success of the reaction and resultant banding patterns when successful reactions could be obtained. It is likely that high variability in the annealing sites of the low specificity random primer (10mer) caused this high variation in banding patterns between runs. Therefore, the RAPD protocol was deemed inappropriate for this study as statistically significant and reliable results would not have been possible.

One of the significant challenges we experienced in *M. bovis* strain differentiation was in part associated with the quantity, quality and/or stability of DNA. The DNA extracted from *M. bovis* samples did not appear to be stable even when high quality extraction kits were utilized and resultant DNA was stored at -80°C. One suggestion was that our extraction procedures produced high quantities of RNA, which might also be detected by UV spectrophotmetric quantification, but which is much more susceptible to degradation compared to DNA. However, the addition of an RNAase step did not alter the DNA yield or reduce the loss of DNA after freezing and thawing. Eventually we employed a whole genome amplification step to produce enough DNA to permit strain differentiation. Quantification of amplified DNA was then performed with picogreen to ensure that only double stranded DNA was quantified. While high quantity DNA was obtained with this method we rapidly proceeded with AFLP strain

differentiation due to concerns that the DNA quantities would still decrease over time. Therefore, the stability of this amplified DNA was never tested at a later time point and further study of the degradation of *Mycoplasma* DNA extracted with high-quality kits should be conducted.

When AFLP was utilized post genome amplification the reproducibility and reliability of the assay was far improved and duplicate results appeared consistent even when separate amplifications of the same sample were utilized. The use of specific restriction enzymes in combination with specific primers and analysis with capillary electrophoresis, which is automated and reliable, are thought to be the reasons for improved reproducibility and reliability of AFLP. However, AFLP also required some manipulation from the original protocols (Kokotovic et al., 1999) including increasing the incubation periods for the restriction enzyme digest and adapter annealing steps as well as increasing the concentration of double stranded adapters used. While the reproducibly of AFLP was adequate in our hands to differentiate clusters of *M. bovis*, we did not achieve the 100% reproducibility reported by Kokotovic et al (1999). It is possible that we could achieve 100% reproducibility with additional assay experience; however, given the complexity of the multistep protocol this would be a very high expectation.

Upon analysis with capillary electrophoresis the data often included cross-over peaks from the size standard, which were removed as they were common in all samples. This may have camouflaged peaks from the sample in the same size range and possibly decreased the sensitivity of the reaction. Large peaks visualized on the densitometric curve were also often labelled two peaks within 5 bp differences; therefore, the data needed to be manually manipulated and normalized for these peaks in all samples before being analyzed with the Bionumerics software, which proved extremely awkward in this capacity. The manual manipulation required to define bands and the numerous options for settings such as position tolerances create a high potential for over manipulation by researchers. There are to date no regulations on settings and manipulation for genomic fingerprints analyzed with Bionumerics; therefore, study comparisons at this point may be of little value. Certainly investigators should report their settings for all the parameters that can be manipulated using this software tool.

Using the successful AFLP protocols three clusters could be identified in 19 necropsy animals from ten feedlots. No relationship could be found between AFLP cluster, and disease outcome, body site from sampling, geographical sampling location or sampling period.

Therefore, it appears that our original hypothesis that one strain is responsible for CPPS in feedlot cattle is disproved. However, these clusters represent differences in the whole genome and probably differentiate based on polymorphisms in both the non-coding and functional gene regions. Therefore, whether we label these clusters as strains depends on whether a strain is defined as being morphologically, physiologically, or genetically different. In epidemiology studies such as this one, physiological differences specifically relating to virulence are of interest when defining strains. Therefore, further studies to identify and compare expressed virulence genes in these clusters would help us determine whether pathogenicity varies within or between these clusters and further test our original hypothesis when strains are defined based on physiological diversity. Koch's postulates state that a supposed pathogen must cause disease when reinfected into the host. Therefore, reinfecting cattle with representatives of the three strain clusters identified in the current study may also help determine the virulence of these clusters.

As two of the clusters identified in CPPS mortalities were also found in CPPS free cattle it could be assumed that the difference in disease outcome was at least in part due to factors other than strain. The immune response of the host may affect the outcome of *M. bovis* colonization. Immunohistochemical staining has been conducted to examine the host response in CPPS afflicted cattle; however, a comparison of expressed immune factors in asymptomatic shedders of *M. bovis* and those with *M. bovis* associated diseases has not been conducted. Monitoring immune indicators as determined by antibody titres, immunhistochemistry of lung and joint samples, and/or gene expression profiles of immune factors in animals colonized with *M. bovis* as they develop CPPS compared to animals colonized by *M. bovis* that remain asymptomatic may reveal host immune responses indicative of disease progression. The microbial population in the respiratory tract may also affect the outcome of *M. bovis* colonization. While *M. bovis* has been observed to correspond to other respiratory pathogens, studies comparing the microbial populations in cattle that develop CPPS from *M. bovis* colonization and those that remain healthy has not been conducted. Studies to investigate the role of other *Mycoplasmas* may also be of relevance as closely related species are often of the highest competition as environmental requirements are likely close. Comparative studies to determine differences in host immune status and microbial environment may further our current understanding of the factors involved in the development of CPPS.

We had originally anticipated much higher numbers of CPPS as local veterinarians had suggested that in previous years 25% of fall-placed feedlot calves developed undifferentiated fever and 5% of these animals died or were euthanized due to CPPS. We had planned a study to track the *M. bovis* strains in animals that developed CPPS in the feedlot to compare with a group of animals that remained healthy as a control based on these original estimates. When in the first year there were no animals developing pneumonias characteristic of CPPS a second sampling year was conducted at the University of Saskatchewan feedlot as a comparison and in hopes that more CPPS animals would be identified. When there was a lack of CPPS in this second sampling year it was decided that the necropsy samples would have to suffice as our CPPS group although this group was also smaller than originally anticipated due to low disease prevalence. Our inability to conduct the original study design limited our ability to determine the factors indicative of CPPS development in the feedlot. A comparison of the three AFLP clusters identified in this necropsy group with clusters in animals as they develop CPPS in the feedlot would be a beneficial future study to determine if this population changes over time and further investigate our original hypothesis that one *M. bovis* strain is responsible for CPPS in feedlot cattle.

Overall, we were unable to support or reject our original hypothesis using the current sample set and molecular methodologies. Although we could not identify specific pathogenic *M. bovis* strains, we could not rule out the possibility that AFLP was not able to detect genetic variation specifically associated with virulence. The fact that healthy animals harbouring *M. bovis* in the lower respiratory tract were able to clear this pathogen clearly suggests that host or other environmental factors (other pathogens) are key factors in disease progression.

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