AN INVESTIGATION OF ANTIMICROBIAL SUSCEPTIBILITY AND GENOTYPES OF *MYCOPLASMA BOVIS* ISOLATES DERIVED FROM WESTERN CANADIAN FEEDLOT CATTLE

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Large Animal Clinical Sciences

University of Saskatchewan

Saskatoon

By

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ABSTRACT

Mycoplasma bovis poses a significant threat to the Canadian beef industry, particularly the feedlot sector where it is associated with bovine respiratory disease (BRD) and chronic pneumonia and polyarthritis syndrome (CPPS). Furthermore, its characteristic chronic infections which are refractory to antimicrobials results in animal welfare and economic concerns. Currently, antimicrobials are the primary therapeutic option for treatment and control of *M. bovis* infections due the absence of a vaccine. This is salient given the global concern regarding antimicrobial use (AMU), antimicrobial resistance (AMR), and increased levels of AMR reported for *M. bovis* worldwide.

Due to the current reliance on antimicrobials for prevention, control, and treatment, continual surveillance of antimicrobial susceptibility is crucial for antimicrobial stewardship and therapeutic treatment of *M. bovis* related disease. Not only is there a reduced arsenal of antimicrobials for the prevention, control and treatment of *M. bovis*, but its fastidious nature and difficulties associated with culturing emphasize a need for antimicrobial susceptibility testing (AST) using rapid, accurate, molecular methods. Whole genome sequencing (WGS) provides a plethora of information that can be interrogated to investigate different aspects of *M. bovis* pathogenesis, including antimicrobial susceptibility and virulence, such as multilocus sequence typing (MLST) for classifying bacterial strains. In conclusion, an epidemiological, genotypic and phenotypic investigation into the antimicrobial susceptibility of *M. bovis* will contribute to the body of knowledge needed to assist in evidence-based decisions for the treatment and control of *M. bovis* infections in cattle and provide the rationale for the studies outlined in this thesis.

M. bovis isolates derived from western Canadian feedlot cattle sampled over a 12-year period (2006 - 2018) were used to address three objectives: 1), describe the AMU and AMR profiles of isolates derived from *M. bovis* mortalities in feedlot cattle (Chapter 2); 2), investigate the genotypic basis for macrolide resistance by assessing single nucleotide polymorphisms (SNPs) in 23S rRNA gene alleles and ribosomal proteins L4 and L22 (Chapter 3); and 3), assess the application of four genotyping methods for *M. bovis* (Chapter 4). *M. bovis* was cultured from deep nasopharyngeal swabs as well as lung and joint tissue from western Canadian feedlot cattle (cattle, $n = 134$; isolates, $n = 183$). Antimicrobial susceptibility testing (AST) was performed using a microbroth dilution assay and a customized panel of nine antimicrobials, representing

four drug classes, most commonly administered to feedlot cattle in western Canada. Furthermore, *M. bovis* isolates ($n = 129$) underwent WGS utilizing Illumina technology.

Although *M. bovis* isolates were derived from western Canadian feedlot cattle, Chapter 2 was not intended as a representative study of western Canadian feedlots but rather to provide context on AMR and AMU in feedlots and the background of the cattle and isolates represented in this thesis. In chapter 2, over 90% of cattle had received antimicrobial metaphylaxis, with tulathromycin accounting for 94.2 % of treatments. On average, cattle received three antimicrobial classes prior to dying of a mycoplasma-related pneumonia. The most commonly administered classes were macrolides (93.2%), phenicols (78.4%), and fluoroquinolones (67.6%). Isolates had the least resistance to florfenicol, with 89.9% classified as susceptible. Nearly all isolates were resistant to all five macrolides (gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin) assessed. The study described in Chapter 3 found that mutations in both domains II and V of the 23S rRNA gene alleles were found to be associated with resistance to all five macrolides. Isolates with a mutation in domain II and the L4 and L22 ribosomal proteins were also resistant to all macrolides, except tulathromycin. Lastly, in Chapter 4, four *in silico* genotyping methods were applied to *M. bovis* isolates and the Simpson's Diversity Index (D) was used to assess resolution of each method. MLST had the lowest resolution (D=0.932) but was the easiest to implement and apply; contrastingly, whole genome single nucleotide variant (wgSNV) yielded the highest resolution (D=1.000), but also involved the most complicated analysis. Application of core genome MLST (cgMLST) and core genome SNV (cgSNV) had a similar resolution of 0.987 and 0.984, respectively. No association between genotype and phenotype was resolved.

Overall, *M. bovis* isolates in western Canadian feedlot cattle were predominately susceptible to a single antimicrobial, florfenicol, and commonly administered macrolides for BRD metaphylaxis. AMR was observed to all macrolides tested, and the accumulation of SNPs in genes associated with macrolide resistance correlated to a macrolide resistant phenotype using AST. Despite being unable to associate genotype and phenotype, the typing methods yielded comparable phylogenetic relationships. Furthermore, the diversity of strain types highlighted the structure of the Canadian cattle industry and how cattle are procured for western Canadian feedlots with some strain types being dominant over geographical areas and time.

ACKNOWLEDGEMENTS

Firstly, I will forever be immensely grateful that Dr. Murray Jelinski agreed to take me on as a graduate student at the WCVM. A lot has changed over the past few years for the better, and that in large part stems from him allowing me the opportunity to complete this project. I cannot thank my supervisors Dr. Murray Jelinski and Dr. Tim McAllister enough for their guidance, patience and support throughout my graduate program. I also wish to thank my committee member, Dr. Janet Hill, and graduate chairs Dr. Cheryl Waldner, Dr. Murray Woodbury and Dr. Chris Luby.

Mycoplasma bovis is quite the bacteria to work with, and my project would not have been successful without the expertise and support of those in Dr. Jelinski's lab (Karen Gesy, Taylor Gibson, Matthew Waldner), Dr. McAllister's lab at the Lethbridge Research and Development Centre (Dr. Rahat Zaheer, Dr. Sara Andrés-Lasheras, Rodrigo Ortega-Polo), Dr. Hill's lab (Champika Fernando) and Dr. Tony Ruzzini. A big thank you to GMP, for without the right media there is no *M. bovis*.

Funding for this work was generously provided by the Saskatchewan Agricultural Development Fund, Canadian Cattlemen's Association, Beef Cattle Research Council, Agriculture and Agri-Food Canada and the University of Saskatchewan.

Finally, I would like to thank my friends and family for being the best support system I could ask for. However, the biggest thank you of all is to my husband, Mike. I know you will likely never read this, but I greatly appreciate your unconditional support as I pursued my Masters in Saskatoon – even if that meant turning our day-to-day life on its head for years on end.

TABLE OF CONTENTS

PERMISSION TO USE STATEMENT i
ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF ABBREVIATIONS xi
1 INTRODUCTION AND LITERATURE REVIEW 1
1.1 Introduction 1
1.2 Mollicutes and Mycoplasmosis 2
1.3 Overview of Canadian Beef Industry 4
1.4 *Mycoplasma bovis* in the feedlot 5
1.4.1 Bovine Respiratory Disease (BRD) 6
1.4.2 Chronic Pneumonia and Polyarthritis Syndrome (CPPS) 7
1.4.3 Antimicrobials 8
1.4.3.1 Therapeutic Use 8
1.4.3.2 Mode of Action 13
1.4.3.3 Resistance Mechanisms 13
1.4.4 Alternative and Preventative Strategies 15
1.5 *Mycoplasma bovis* 16
1.5.1 Genome 16
1.5.2 Pathogenicity and Virulence 18
1.5.3 Morphology and Growth Requirements 20
1.5.4 Diagnostics 21
1.5.4.1 Conventional Clinical Methods 21
1.5.4.2 Whole Genome Sequencing 22
1.5.5 Clinical Findings of Antimicrobial Resistance 23
1.5.6 Antimicrobial Susceptibility 24
1.5.7 Epidemiological cut-off value (ECOFF) 26
1.5.8 Strain Type Differentiation 27
1.5.9 Surveillance Programs 29

1.5.9.1 Canada 29
1.5.9.2 Europe 29
1.5.9.3 New Zealand 29
1.6 Objectives 30
2 ANTIMICROBIAL USE AND THE SUSCEPTIBILITY OF *MYCOPLASMA BOVIS* ISOLATES DERIVED FROM WESTERN CANADIAN FEEDLOT CATTLE 32
2.1 Abstract 33
2.2 Introduction 33
2.3 Materials and Methods 35
2.3.1 Sampling 35
2.3.2 Compilation of Antimicrobial Use 35
2.3.3 Culture 35
2.3.4 Identification 36
2.3.5 Antimicrobial Susceptibility Testing (AST) 36
2.3.6 Clinical breakpoints 37
2.4 Results 37
2.4.1 Cattle and *Mycoplasma bovis* isolation 37
2.4.2 Antimicrobial use 38
2.4.3 Antimicrobial susceptibility 41
2.5 Discussion and Conclusion 43
3 INVESTIGATION OF MACROLIDE RESISTANCE GENOTYPES IN *MYCOPLASMA BOVIS* ISOLATES FROM CANADIAN FEEDLOT CATTLE 46
3.1 Abstract 47
3.2 Introduction 47
3.3 Materials and Methods 49
3.3.1 Animals and Sample Collection 49
3.3.2 Mycoplasma Culture and Isolation 50
3.3.3 DNA Extraction and Identification 51
3.3.4 Whole Genome Sequencing and Assembly 51
3.3.5 Antimicrobial Susceptibility Testing 53
3.3.6 Clinical Breakpoints 54
3.3.7 Statistical Analysis 54
3.4 Results 54
3.4.1 Culture and Reference Antimicrobial Susceptibilities 54
3.4.2 Genome Sequencing and Assembly 56

3.4.3 23S rRNA gene 56
3.4.4 L4 and L22 Ribosomal proteins 61
3.5 Discussion and Conclusion 63
4 APPLICATION OF FOUR GENOTYPING METHODS TO *MYCOPLASMA BOVIS* ISOLATES DERIVED FROM WESTERN CANADIAN FEEDLOT CATTLE 68
4.1 Abstract 69
4.2 Introduction 69
4.3 Materials and Methods 71
4.3.1 Sample Collection 71
4.3.2 Culture and isolation 72
4.3.3 DNA extraction and identification 72
4.3.4 Whole genome sequencing and assembly 73
4.3.5 Genotyping methods 73
4.3.6 Data presentation 74
4.4 Results 75
4.4.1 Isolates and assembly 75
4.4.2 MLST 75
4.4.3 cgMLST and cgSNV analyses 81
4.4.4 wgSNV 86
4.5 Discussion and Conclusion 89
5 GENERAL SUMMARY AND CONCLUSION 94
5.1 Discussion 94
5.2 Future Research 96
5.3 Conclusion 96
6 REFERENCES 97
APPENDIX A – CHAPTER 2: *MYCOPLASMA BOVIS* ISOLATES 119
APPENDIX B – CHAPTER 3: *MYCOPLASMA BOVIS* ISOLATES 124
APPENDIX C – CHAPTER 4: *MYCOPLASMA BOVIS* ISOLATES 129
APPENDIX D – ANTIMICROBIAL SUSCEPTIBILITY PLATE MAP 134

LIST OF TABLES

Table 1.1 Common antimicrobials used for metaphylaxis and treatment of bovine respiratory disease (BRD) in western Canadian feedlots. 11

Table 2.1 Site of isolation of *Mycoplasma bovis* (%) across production years (*n* = 119)..... 38

Table 2.2 Antimicrobials administered to feedlot cattle upon arrival at the feedlot across five production years (*n* = 74). 38

Table 2.3 The number of feedlot cattle (*n* = 74) administered different antimicrobial classes by production year (2014 to 2018) and days on feed at the time of death. 40

Table 2.4 Use of antimicrobials in relation to the number of days on feed (DOF) at the time of death. Usage is shown as a percentage and absolute number (n) of feedlot cattle treated with each antimicrobial class for each cohort (DOF). 41

Table 2.5 Antibiotic susceptibility of *Mycoplasma bovis* isolates (*n* = 119) recovered from cattle (*n* = 74) between 2014 and 2018 42

Table 3.1 *Mycoplasma bovis* isolates (*n* = 126) by anatomical location, health status, and production year..... 55

Table 3.2 Number of *Mycoplasma bovis* isolates (*n* = 126) with a resistant (R) or susceptible (S) phenotype by health status. 56

Table 3.3 Comparison of 23S rRNA genotypes and the number (%) of *Mycoplasma bovis* isolates (*n* = 126) resistant to the five macrolides tested. 58

Table 3.4 Number and proportion of *Mycoplasma bovis* isolates (*n* = 126) resistant (R) or susceptible (S) by 23S rRNA genotype..... 61

Table 3.5 Presence of ribosomal protein mutations in different 23S genotype groups and corresponding minimum inhibitory concentrations (MIC) values..... 63

Table 4.1 Number of MLST sequence types (STs) of *Mycoplasma bovis* isolates (*n* = 125) by production year (2006 – 2018) and health status (healthy, diseased and dead)..... 77

LIST OF FIGURES

Figure 1.1 Schematic of the cell wall structure of gram negative and gram positive bacteria. Mollicutes, such as *Mycoplasma bovis*, lack a cell wall and are characterized by a single cell membrane 3

Figure 1.2 Tylosin (TYLT), and its two derivatives tilmicosin (TIL) and tildipirosin (TIP) have a common 16-membered macrolactone ring structure 12

Figure 1.3 *Mycoplasma bovis* PG45 complete genome. *rRNA* genes are depicted in red 17

Figure 1.4 The "fried-egg" morphology of *Mycoplasma bovis* on solid media [99]..... 21

Figure 1.5 Sensititre plate with alamarBlue following 48 h incubation. The *Mycoplasma bovis* isolate was derived from lung tissue collected during a postmortem examination..... 25

Figure 3.1 Minimum inhibitory concentrations (MIC) of *Mycoplasma bovis* isolates ($n = 126$) for tylosin (TYLT), tilmicosin (TIL), tildipirosin (TIP), gamithromycin (GAM), and tulathromycin (TUL), and the corresponding 23S rRNA genotype: a) wildtype (wt) or mutations in domain II only (Δ748); b) mutations in domain II and V (Δ748Δ2059, Δ748Δ2060)..... 59

Figure 4.1 MLST neighbor joining tree of 126 *Mycoplasma bovis* isolates typed by MLST..... 79

Figure 4.2 Minimum spanning tree of 126 *Mycoplasma bovis* genomes (125 field isolates plus PG45) typed by MLST. Clonal complexes 1 and 2 are surrounded by red and blue lines, respectively 80

Figure 4.3 cgMLST neighbor joining tree of 102 *Mycoplasma bovis* genomes based on alleles at 296 core genome loci..... 82

Figure 4.4 Minimum spanning tree of 102 *Mycoplasma bovis* isolates (101 field isolates plus PG45) created with cgMLST, based on alleles at 296 core genome loci. Clades 1 - 3 are denoted by a surrounding circle 83

Figure 4.5 cgSNV neighbor joining tree of 102 *Mycoplasma bovis* isolates based on 283 core genome loci (3,925 SNVs)..... 84

Figure 4.6 Minimum spanning tree of 102 *Mycoplasma bovis* isolates (101 field isolates plus PG45) based on 283 core genome loci, using cgSNV, based on 3,925 SNVs. The clades are indicated by an encompassing circle..... 85

Figure 4.7 wgSNV maximum-likelihood tree of 130 *Mycoplasma bovis* genomes based on 14383 SNVs in the core and accessory genomes..... 87

Figure 4.8 Maximum-likelihood tree of 130 *Mycoplasma bovis* isolates (129 field isolates plus PG45) based on 14383 SNVs in the core and accessory genomes typed by wgSNV. Clades 1 and 2 are indicated by a surrounding line in red and blue, respectively..... 88

LIST OF ABBREVIATIONS

ADME	Absorption, distribution, metabolism, and excretion
AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
AMU	Antimicrobial use
AST	Antimicrobial susceptibility testing
ATP	Adenosine triphosphate
BAM	Binary alignment map
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
C	Clade
CBPP	Contagious bovine pleuropneumonia
CC	Clonal complex
CEPH	Cephalosporin
cgMLST	core genome multilocus sequence typing
cgSNP	core genome single nucleotide polymorphism
cgSNV	core genome single nucleotide variant
CI	Confidence interval
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standards Institute
CPPS	Chronic pneumonia and polyarthritis syndrome

CTET	Chlortetracycline
D	Simpson's Diversity Index
DNA	Deoxyribonucleic acid
DNP	Deep nasopharyngeal
DOF	Days on feed
ECOFF	Epidemiological cut-off value
ELDU	Extra-label drug use
ELISA	Enzyme linked immunosorbent assay
ENRO	Enrofloxacin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FFN	Florfenicol
FQ	Fluoroquinolone
GAM	Gamithromycin
HTS	High-throughput sequencing
IBR	Infectious bovine rhinotracheitis
ICE	Integrative conjunctive elements
NGS	Next generation sequencing
MAC	Macrolide
Mbp	Mega base pair
MIA	Medically important antimicrobial
MIC	Minimum inhibitory concentration
MLSB	Macrolide-lincosamide-streptogramin B
MLST	Multilocus sequence typing

MLVA	Multiple loci variable number tandem repeat analysis
MST	Minimum spanning tree
NJ	Neighbour joining
OD	Optical density
OIE	World Organization for Animal Health
OXY	Oxytetracycline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PHAC	Public Health Agency of Canada
PHEN	Phenicol
PI-3	Parainfluenza 3
PPLO	Pleuropneumonia like organism
QRDR	Quinolone resistance determining regions
RAPD	random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
rRNA	ribsosomal ribonucleic acid
ROS	Reactive oxygen species
SAM	Sequence alignment map
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
ST	Strain type

SULF	Sulfonamide
TET	Tetracycline
TIL	Tilmicosin
TIP	Tildipirosin
TMS	Trimethoprim-sulfonamides
tRNA	Transfer ribonucleic acid
TUL	Tulathromycin
TYLT	Tylosin tartrate
UF	Undifferentiated fever
VCPR	Veterinary-client-patient relationship
VSP	Variable surface protein
WGS	Whole genome sequencing
wgSNV	whole genome single nucleotide variant
WHO	World Health Organization

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Mycoplasma bovis is considered to be one of the smallest self-replicating organisms, with its gene content bordering on the minimum number to sustain bacterial life [1]. *M. bovis* is associated with bovine respiratory disease (BRD) and chronic pneumonia and polyarthritis syndrome (CPPS) in feedlot cattle [2-4]. In a feedlot, death within days to weeks post-arrival is commonly attributed to BRD; whereas, death after a month is attributed to CPPS [3]. The time cattle spend in a feedlot being fed is referred to as days on feed (DOF) [5].

Mycoplasmosis is both an economic and animal welfare concern for Canadian feedlot producers. The economic impact of *M. bovis* results from reduced weight gain, increased treatment costs, and death losses [6,7]. Concerns for welfare arise because *M. bovis* is associated with chronic infections, such as CPPS, that respond poorly to antimicrobial treatment [6]. Furthermore, polyarthritis is particularly debilitating and painful manifestation of mycoplasmosis resulting in lameness, leading to decreased feed consumption and weight loss [4,8]. Poor response to treatment can also lead to antimicrobial resistance in other BRD pathogens [7]. This is of particular importance as BRD is a multifactorial infection associated with other bacterial pathogens, particularly *Manheimmia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* [9]. Further compounding the impact of *M. bovis*, is the fact that antimicrobial resistance (AMR) within Canada [10-13] and worldwide [14-22] is increasing.

AMR is a global concern, spanning both human and animal microbial species. The Public Health Agency of Canada (PHAC) has outlined three areas of focus to respond to AMR in Canada: surveillance, stewardship and innovation [23]. In animal health, antimicrobial stewardship is a primary responsibility of veterinary professionals [24]. Antimicrobial stewardship is predicated on prudent use guidelines for antimicrobial use (AMU) [25]. This thesis focuses on surveillance and stewardship of antimicrobials through antimicrobial susceptibility testing (AST), AMU, genotypic correlation to AMR and population structure of *M. bovis* in western Canadian feedlot cattle. Furthermore, investigations on AMR in *M. bovis* are crucial to reducing the welfare and economic impact of BRD and CPPS on feedlot cattle.

The literature review in Chapter 1 is intended to introduce myoplasmosis, specifically the impact of *M. bovis* related disease in cattle in the context of the Canadian Beef Industry and the

practices associated with finishing cattle in a western Canadian feedlot. A more in-depth description of *M. bovis* is presented focusing on the details relevant to the epidemiological, phenotypic and genotypic studies undertaken in this thesis.

1.2 Mollicutes and Mycoplasmosis

Mycoplasmas belong to the class Mollicutes and are characterized by not having a cell wall (Figure 1.1), making them a gram negative bacterium. However, they evolved from a gram positive ancestor, and have highly reduced genomes that possess nearly the minimum gene content needed to sustain independent bacterial life [26]. A consequence of a reduced genome is the absence of genes for some biosynthetic pathways [27]. The absence of these complete biosynthetic pathways makes *Mycoplasma* spp. reliant on exogenous sources of biosynthetic products and suggests a close association with their host. These exogenous dependencies make *Mycoplasma* spp. difficult to culture, with different *Mycoplasma* spp. having different nutrient requirements [28]. The absence of a cell wall and some biosynthetic pathways make *Mycoplasma* spp. intrinsically resistant to antimicrobials such as β-lactams and trimethoprim that target peptidoglycan and folic acid synthesis, respectively [29]. Additionally, mycoplasmas are considered to be fast evolving, due to their exploitation of spontaneous mutagenesis [26]. *Mycoplasma* spp. include both human and veterinary agents, with a predilection for colonizing mucosal surfaces in the respiratory and reproductive tracts [30], and are capable of intracellular localization which may promote the chronicity associated with infections [31].

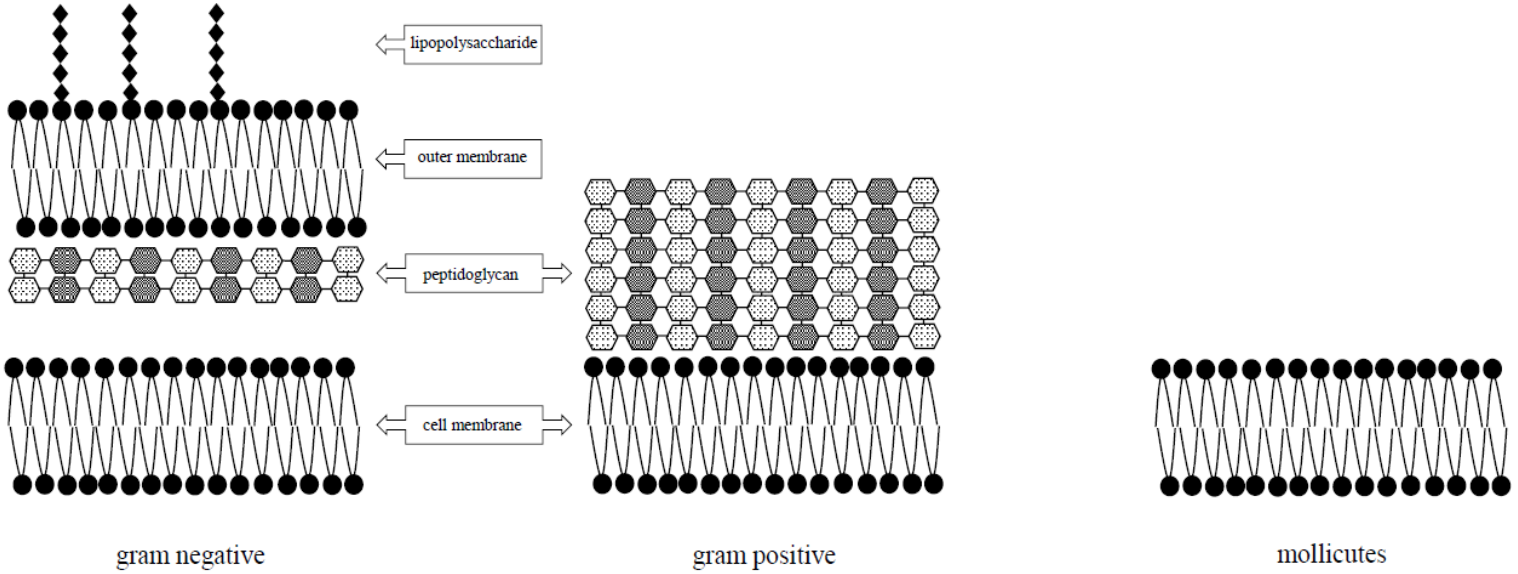

Figure 1.1 Schematic of the cell wall structure of gram negative and gram positive bacteria. Mollicutes, such as *Mycoplasma bovis*, lack a cell wall and are characterized by a single cell membrane. Graphic was prepared using Microsoft powerpoint.

Mycoplasmosis is a common disease of people and food production animals such as chickens, turkeys, pigs, and cattle. There are multiple *Mycoplasma* spp. that affect different animals, but regardless of the host infections within these different species, mycoplasma infections commonly manifest as clinical manifestations involving the mucosal surfaces within joints, respiratory or reproductive tracts [30]. Mycoplasma infections in veterinary medicine can be particularly detrimental due to their welfare and economic impact. In poultry, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are associated with infections of the respiratory tract and joints, respectively [29]. In piglets, *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* are associated with polyserositis and arthritis, and *Mycoplasma hyopneumoniae* is associated with chronic pneumonia [29].

In cattle, *Mycoplasma* spp. are associated with various ailments such as: mastitis (*Mycoplasma bovis*, *Mycoplasma bovigenitalium, Mycoplasma californicum*); arthritis (*M. bovis*); pneumonia (*Mycoplasma mycoides* subsp. *mycoides* (small colony), *M. bovis*, *Mycoplasma dispar, Mycoplasma canis, M. californicum*); and reproductive infections (*M. bovis*, *M. bovigenitalium*) [8]. *M. mycoides* subsp. *mycoides* is highly virulent and the causative agent of contagious bovine pleuropneumonia (CBPP) [32]. CBPP is a reportable disease to the World Organization for Animal Health (OIE) and was eradicated in Canada in 1876 [33]. However, CBPP is still endemic in sub-saharan Africa [34]. This organism is of concern in North America because infections caused by *M. bovis* and *M. mycoides* subsp. *mycoides* are difficult to differentiate on gross postmortem examination. *M. bovis* is not specific to cattle, as it is also associated with pneumonia in bison [35,36]. Generally, *M. bovis* infections in all species tend to be chronic and frequently refractory to antimicrobial therapy [6], which compounds the welfare and economic impacts of this bacterium on the beef industry.

1.3 Overview of Canadian Beef Industry

In Canada, as well as in the United States, the beef industry consists of three main sectors: cow-calf, stocker (also known as backgrounders), and feedlot [37]. Cow-calf operations comprise the bulk of the beef sector, providing a supply of stocker and feedlot cattle. Cows or heifers are typically bred in the summer and following a nine month gestation period have their calves the following spring. After weaning in the fall, the calves go either directly into feedlots, or they may be backgrounded.

Cattle can arrive in a feedlot via two different ways: ranch direct or auction mart. Ranch direct means that a group of cattle are purchased and transported directly from a cow-calf operation to a backgrounder. For cattle that arrived via an auction mart, cattle are transported from the home ranch to the auction mart, commingled with cattle from other ranches to form uniform groups, sold and then shipped to a feedlot. Cattle can also arrive at the same feedlot, via different auction marts or ranches. Western Canadian feedlots also source cattle from the United States.

Calves can enter the feedlot in three general ways. Firstly, calves can enter the feedlot directly following weaning in the fall. Fall-weaned calves are born in the spring, spend the summer on pasture with their dam and are shipped to feedlots for backgrounding using a forage-based diet and finishing using a grain-based diet. The next two cohorts of calves utilize backgrounding prior to entry. Calves weaned in the fall can be maintained, often by cow calf-producers, until the following spring when they enter into the feedlot. Lastly, calves can be maintained over winter and then grazed on pasture over the summer before entering the feedlot the following year as a yearling for a shorter finishing period. These stocking operations help to maintain a year-round supply of cattle for finishing outside of the peak fall entry. Depending if they enter as calves, backgrounded animals, or yearlings, cattle spend approximately 60-200 days in a feedlot [38].

Feedlots are considered an intensive method of finishing cattle. While at the feedlot, cattle are sorted into pens of animals of uniform size, sometimes more than once and fed rations with increasing proportions of grain until the animal reaches its finished weight of approximately 600-700kg [39]. Once a group of animals has reached a uniform target weight in the feedlot they are sold and transported to a packer. At the packer the animals are inspected during slaughter and processing before any meat products enter the food chain.

1.4 *Mycoplasma bovis* in the feedlot

Globally, *M. bovis* is regarded as the most ubiquitous mycoplasma of cattle [40]. *M. bovis* was first isolated in North America the 1960's in the United States from a case of mastitis [41], and has since spread worldwide through animal movement [40]. It is associated with chronic infections and lameness [3,6] causing pain and suffering and hence these infections are

considered an animal welfare issue. Additionally, economic impacts arise due to reduced weight gain, increased treatment costs, and death [6,7,40].

BRD is the most common morbidity and mortality in feedlots [42]. Clinical signs of undifferentiated bovine respiratory disease can include difficulty breathing, depression, anorexia, fever, and evidence of pneumonia [32]. Various differential diagnoses of bovine respiratory diseases exist, with differences identified based on their underlying etiology.

1.4.1 Bovine Respiratory Disease (BRD)

BRD is caused by multiple microbial agents and is attributed to causing 70-80% of morbidities in feedlot cattle and 40-50% of mortalities in the US cattle industry [43]. Focusing on western Canadian feedlot cattle, Brault et al. [44] reported that 39% of approximately 2.6 million cattle from 2008 to 2012 were classified as 'high risk' for BRD. In addition to *M. bovis*, BRD is a polymicrobial disease with various bacterial and viral etiological agents. These bacteria include *M. haemolytica*, *P. multocida*, and *H. somni*; whereas, viral components include bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis (IBR), parainfluenza 3 (PI-3) and bovine respiratory syncytial virus (BRSV) [9]. *M. haemolytica* causes an acute fibrinous pneumonia or acute fatal pneumonia, which is synonymous with classical 'shipping fever', whereas *M. bovis* is associated with chronic caseonecrotic pneumonia. BRD typically occurs within 14 DOF [32]. Cattle entering the feedlot are classified as high or low risk for BRD, based on algorithms taking into account age, source and a range of management factors that can influence stress. Age is generally used as a proxy for immune status, with younger animals being considered to be less immunocompetent [32]. High risk features include fall-weaned calves that arrive at the feedlot via auction, where they have commingled with other cattle. Low risk cattle may be backgrounded or preconditioned and/or arrive at the feedlot following a ranch-direct sale. Preconditioning is a management practice that encompasses completion of stressful events such as weaning, castration, dehorning, and the administration of vaccines, particularly those for BRD, in advance of the animals leaving the cow-calf operations [32,43]. The intent of preconditioning is to prime the animal's immune system and reduce stress, which improves the overall immune response and decreases the risk of BRD at the time of shipping and commingling in auctions and/or feedlots [32]. However, unless ownership of the calves is retained upon entry into the feedlot, cow-calf producers are not often compensated for the cost and labour associated

with preconditioning, thus reducing the adoption and economic attractiveness of this approach. Calves that fall into the high risk category typically receive tulathromycin (i.e., Draxxin®), a macrolide, upon arrival; whereas low risk animals often receive no antimicrobial or a lower category antimicrobial such as long-acting oxytetracycline [44].

Historically, studies on BRD have focused on the bacterial agents *M. haemolytica*, *P. multocida*, and *H. somni*. The role of *M. bovis* in BRD is less clearly defined and it has been considered to be opportunistic, a secondary contributor, and a causative agent [3,6,45]. Since all BRD bacteria have been isolated from the same lesion, this suggests synergism among these bacterial species [3]. The polymicrobial nature of BRD is highlighted in a study by Klima et al. [46], where they examined 68 BRD mortalities. Co-infections were observed in 97% of cases, with *Mannheimia* spp., BVDV, and *M. bovis* present with or without *H. somni*. This is likely due to challenges associated with culturing *M. bovis,* resulting in it being the least characterized of the bacterial members of the BRD complex.

It is hypothesized that BRD is associated with altering the microbiome of the respiratory tract [47]. A recent study by Holman et al. [48], looked at the effect of a single metaphylactic treatment with oxytetracycline or tulathromycin on the nasopharyngeal microbiome of beef cattle transported to the feedlot. Within days of administration both metaphylactic treatments were found to reduce the abundance of BRD-associated agents, including *Mycoplasma* spp. However, at 34 d post-administration of oxytetracycline, *Mycoplasma* spp. were the predominant BRD agents in the nasopharynx. Contrastingly, following tulathromycin metaphylaxis, the nasopharyngeal microbiome recovered to resemble the microbiota composition of the control animals after 12 d. In an earlier study by Hendrick et al. [10], they observed that calves receiving oxytetracycline metaphylactically had a reduced risk of BRD, but an increased risk of arthritis. However, the relationship between oxytetracycline and arthritis requires further investigation.

1.4.2 Chronic Pneumonia and Polyarthritis Syndrome (CPPS)

CPPS is commonly attributable to a persistent *M. bovis* infection and characterized by the combined afflictions of pneumonia and arthritis that have responded poorly to treatment [49]. Arthritis invariably leads to lameness, which further impacts the welfare of cattle by reducing their mobility and ability to obtain feed and water [4,8]. These arthritides are generally nonresponsive to antimicrobial therapy, but not necessarily due to antimicrobial resistance. Since

caseonecrotic lesions form in the lungs and fibrin forms in the joints following *M. bovis* infections [12,50], these pathologies could reduce antimicrobial efficacy due to decreased antimicrobial perfusion. Therefore, it can be difficult to control *M. bovis* infections even if *M. bovis* is susceptible to a given antimicrobial.

Complicating matters, cattle colonized with *M. bovis* do not necessarily develop BRD or CPPS, rather they can be asymptomatic carriers. Individuals can be intermittent shedders of *M. bovis* without clinical signs of disease [4], making it difficult if not impossible to eradicate this pathogen from feedlots. This is exacerbated by cattle arriving at the feedlot from different sources and being commingled. In western Canada, Brault et al. [44] reported that nearly 92.9% of antimicrobials administered to cattle were for the treatment or prevention of BRD, with nearly 90% of these through metaphylaxis. The remainder of antimicrobials administered were due to acute respiratory disease, lameness, implants or other. Therefore, given the reliance on antimicrobials for treatment and prevention of BRD the development of alternatives such as vaccines or probiotic supplementation to prevent or treat this disease are of high interest.

1.4.3 Antimicrobials

1.4.3.1 Therapeutic Use

For the purpose of this thesis, the classes of antimicrobials discussed are not exhaustive, but rather focus on classes commonly used in western Canadian feedlots to treat BRD and are presented in Table 1.1. Feedlot consulting veterinarians are responsible for developing treatment protocols specifically for individual feedlots, taking into account risk factors for disease. These treatment protocols outline the use of vaccines and antimicrobials, and aid in facilitating antimicrobial stewardship. The principles of antimicrobial stewardship include the implementation of preventative and management strategies to prevent disease, evidence-based selection of antimicrobials, evaluation of antimicrobial efficacy, and judicious antimicrobial use to maintain animal health and welfare [51]. Antimicrobials can be administered prophylactically, metaphylactically, or therapeutically for the prevention, control, and treatment of diseases, respectively [52]. Prophylactic administration of antimicrobials is done as a means of disease prevention, based on the anticipation of disease occurring based on historical, clinical or epidemiological information [52]. Metaphylaxis is the administration of antimicrobials to reduce the incidence, progression, or transmission of an infectious disease that is evident [52], such as

when an individual in a cohort of cattle (e.g. pen) shows signs of disease and as a result all cattle in the cohort are administered antimicrobials. Therapeutic administration occurs following evidence of infectious disease [52]. A common management practice for controlling BRD is for cattle to receive metaphylactic antimicrobial therapy upon arrival at the feedlot to reduce the risk of respiratory infections [53]. In western Canada, tetracyclines and macrolides are the predominant metaphylactically administered antimicrobial classes [44].

As of December 1, 2018, a veterinary-client-patient relationship (VCPR) is required for the prescription and administration of all antimicrobials to livestock in Canada. Additionally, label claims for growth promotion have been removed for antimicrobials. The classes of antimicrobials commonly used in western Canadian feedlot cattle are macrolides, fluoroquinolones, phenicols, and tetracyclines [11,44].

The World Health Organization (WHO) has categorized medically important antimicrobials (MIA) based on two criteria: the limited availability of therapies to treat bacterial infections in humans; and their use to treat infections caused by bacteria that could have acquired resistance genes from non-human sources [54]. Critically important antimicrobials (CIAs) meet both of these criteria; whereas highly important antimicrobials meet either criteria. Important antimicrobials do not meet either of these criteria, but are used in human medicine. On a global scale, the WHO lists quinolones and macrolides as critically important, and phenicols and tetracyclines as highly important antimicrobials [54].

Health Canada has also classified antimicrobials based on their importance in human medicine and availability of alternative therapies [55]. Category I antimicrobials, are considered to be of very high importance with category II, III, and IV being of high, medium, and low importance, respectively. In Canada, quinolones and macrolides differ in their classification, belonging to category I (very high importance) and II (high importance), respectively [55]. Category I and II antimicrobials are the preferred treatment of serious infections in humans, with a category I antimicrobial having no or limited alternative antimicrobials available for treatment [55]. Phenicols and tetracyclines are both classified as category III (medium importance). Category IV antimicrobials, such as ionophores, are not used in human medicine.

Label claims specific to one or more of the BRD bacterial agents exist for all antimicrobials licensed for treatment (Table 1.1). However, tulathromycin (i.e., Draxxin®) and

gamithromycin (i.e., Zactran®) have specific label claims for *M. bovis* in cattle along with the other three BRD bacterial agents (i.e., *M. haemolytica*, *P. multocida*, and *H. somni*) [56]. As BRD is a polymicrobial disease, *M. bovis* can be exposed to antimicrobials that are being administered to treat infections caused by other bacterial BRD pathogens. Given that no vaccines exist for *M. bovis*, antimicrobials remain the primary therapeutic option for prevention and treatment of infections. In instances of chronic *M. bovis* infections, these bacteria are often refractory to antimicrobial therapy [6].

Table 1.1 Common antimicrobials used for metaphylaxis and treatment of bovine respiratory disease (BRD) in western Canadian feedlots.

Antimicrobial	Year to Canadian Market†	Class	Health Canada Categorization	WHO Classification	Indication in Cattle††
Enrofloxacin (eg. Baytril® 100; DIN: 02249243)	2004	Fluoroquinolone	I, very high importance	Critically Important	BRD (*M. haemolytica* and *P. multocida*)
Tulathromycin (eg. Draxxin®; DIN: 02285452)	2006	Macrolide	II, high importance	Critically Important	BRD (*M. haemolytica*, *P. multocida*, *H. somni*, and *M. bovis*)
Gamithromycin (eg. Zactran®; DIN: 02347407)	2010	Macrolide	II, high importance	Critically Important	BRD (*M. haemolytica*, *P. multocida*, *H. somni*, and *M. bovis*)
Tilmicosin (eg. Micotil™; DIN: 00857602)	1990	Macrolide	II, high importance	Critically Important	BRD (*M. haemolytica* and *P. multocida*)
Tildipirosin (eg Zuprevo®; DIN: 02387719)	2012	Macrolide	II, high importance	Critically Important	BRD (*M. haemolytica*, *P. multocida*, and *H. somni*)
Tylosin (eg. Tylan™ 200; DIN: 00103594	1968	Macrolide	II, high importance	Critically Important	Pneumonia (*P. multocida* and *Actinomyces pyogenes),* and contagious calf pneumonia and metritis (*Actinomyces pyogenes*)
Florfenicol (eg. Nuflor®; DIN: 02216558)	1996	Phenicol	III, medium importance	Highly important	BRD (*M. haemolytica*, *P. multocida*, *H. somni*), bovine interdigital phlegmon, and infectious keratoconjunctivitis
Oxytetracycline (eg. Oxyvet® 200 LA; DIN: 02184575)	1996	Tetracycline	III, medium importance	Highly important	Bacterial pneumonia, black leg, calf diphtheria, calf scours, foot rot, joint ill, leptospirosis, mastitis, metritis, navel ill, pasteurellosis, and peritonitis.
Chlortetracycline* (eg. Chlor 50; DIN: 00641804)	1985	Tetracycline	III, medium importance	Highly important	Bacterial diarrhea and foot rot

*Administered orally. †Year to market for the drug indicated, based in their drug identification number (DIN) in the Health Canada Drug Product Database [57]. †† indications were obtained from the label insert associated with the product name and DIN [56]

One of the issues of AMR has to do with the limited development of new antimicrobials. Since the golden age of antimicrobial discovery from the 1950s to 1970s, more recent antimicrobial drug registrations are the result of structural modifications of previously discovered antimicrobials [58]. For instance, tildipirosin and tilmicosin are derivatives of tylosin [59] (Figure 1.2). It is estimated that it can cost in excess of 1.5 billion (US) dollars to develop and bring a new antimicrobial product to market [60]. In order to bring a product to market, it must undergo rigorous testing and be licensed for a specific use. This can be problematic for veterinary medicine, as it is cost prohibitive to test a new antimicrobial for safety in all livestock species prior to licensing. As a strategy to provide effective treatment of animals, extra-label drug use (ELDU) can be used at the discretion of the veterinarian with a valid veterinary-client-patient relationship [61]. ELDU refers to the use of a drug in an animal that is not in accordance with the approved label, insert, or registration, as licensed by Health Canada [62].

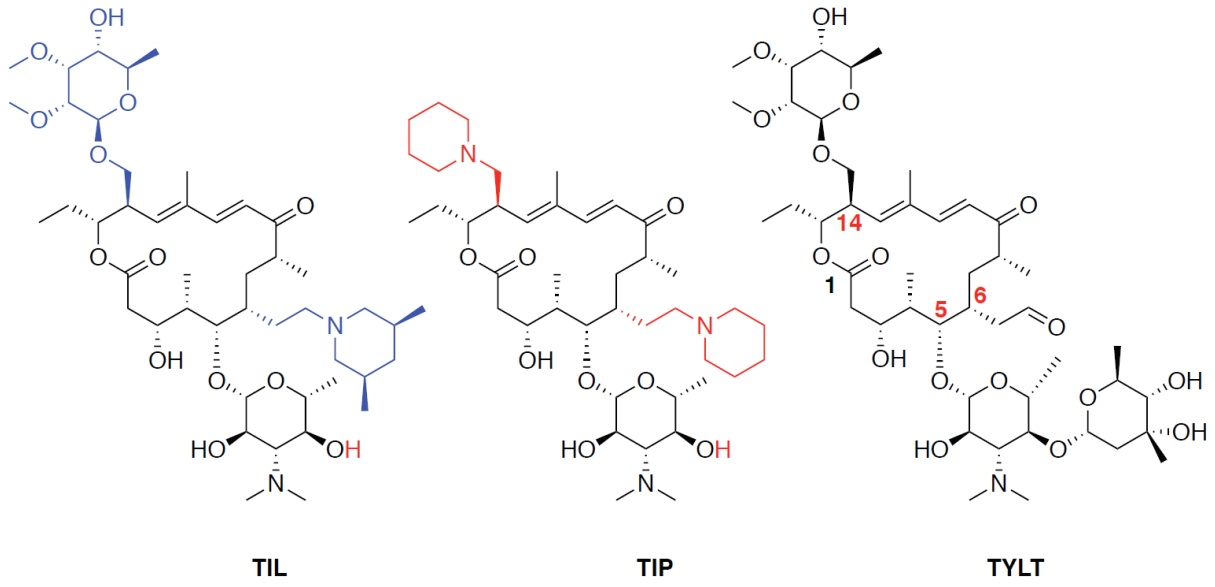

Figure 1.2 Tylosin (TYLT), and its two derivatives tilmicosin (TIL) and tildipirosin (TIP) have a common 16-membered macrolactone ring structure. Courtesy of Dr. Antonio Ruzzini (University of Saskatchewan)

1.4.3.2 Mode of Action

As *M. bovis* does not have a cell wall nor do they synthesize folate, it is intrinsically resistant to antimicrobials that target the cell wall (β-lactams) or folic acid synthesis (sulfonamides) [6,20]. Therefore, the antimicrobials that target *M. bovis* focus on protein synthesis and deoxyribonucleic (DNA) replication. Macrolides and florfenicol bind to the 50S ribosomal subunit; whereas tetracyclines bind to the 30S ribosomal subunit [29,63]. As these antimicrobials target parts of the small (30S) and large (50S) ribosomal subunits, they exert their effect by interfering with protein synthesis. The small ribosomal subunit normally binds mRNA and facilitates the interaction between mRNA and tRNA, whereas the large ribosomal subunit facilitates peptide bond formation as it contains the peptidyl transferase site [63]. Interference with protein synthesis prevents *M. bovis* growth, allowing the immune system to clear the infection. For this reason, antimicrobials that interfere with protein synthesis are considered mycoplasmostatic. Whereas, fluoroquinolones exert their effect on components of DNA replication, through interactions with DNA gyrase and topoisomerase IV [29], which leads to cell death and hence these antimicrobials are considered to be mycoplasmacidal.

1.4.3.3 Resistance Mechanisms

Unlike other bacterial agents associated with BRD, antimicrobial resistance genes have not been identified in *M. bovis*. However, genes conferring antimicrobial resistance, such as *tet(M)* encoding for a ribosomal protection protein, have been found in *Mycoplasma hominis* [64], a mycoplasma associated with humans. Ribosomal protection proteins exert their effect by interfering with the binding of tetracyclines [65]. Therefore, while it is possible that *M. bovis* could have antimicrobial resistance genes, to date they have not been reported. However, even in the absence of resistance genes, reduced susceptibility of *M. bovis* to antimicrobials has been reported in Canada [10-13] and worldwide [14-22].

There are four resistance mechanisms employed by bacteria: altered cell wall or membrane permeability, antibiotic inactivation, use of alternate metabolic pathways, and target modification [66]. In addition to intrinsic antibiotic resistance, the main mechanism of antimicrobial resistance described for *M. bovis* is through target modification as a result of single nucleotide polymorphisms (SNPs) [4,67]. A SNP is a single nucleotide change at a given position in the genome, and can be deleterious in that it can result in cell death or confer

advantages such as resistance to antimicrobials. For the purposes of this thesis SNP is used interchangeably with single nucleotide variant (SNV). *Mycoplasma* spp. are believed to undergo a comparatively high rate of mutation, due to deficiencies in DNA repair mechanisms [31], and this is thought to be one of the reasons why mycoplasmas can rapidly become resistant to antimicrobials.

Target modifications, such as SNPs in the 16S and 23S rRNA interfere with the binding of antimicrobials to these ribosomal components. Tetracycline antimicrobials interact with the 16S rRNA at the Tet-1 binding site [68]. The Tet-1 biding site forms a clamp-like structure around the A-site for binding of tRNA and possesses two main domains. These domains consist of helix 34 (1054-1056 and 1196-1200; *E. coli* numbering) and the loop next to helix 31 (964-967) [68]. Mutations at 965 (A965T) and 967 (A967T/C) in *M. bovis* increase resistance to tetracyclines [22,67-69]. Changes at position 1058 (G1058A/C) have also been shown to confer resistance to tetracyclines [22,68].

Mutations that preclude the binding of macrolides to 23S rRNA have also been observed in *M. bovis* and other *Mycoplasma* spp. [67], with mutation hot spots occurring in domains II and V [69]. Increased resistance to different 50S subunit inhibitors (i.e., tilmicosin, lincomycin, tylosin) has also been observed through the accumulation of SNPs in domain II and V [67]. The main interaction within the ribosomal tunnel occurs at A2058 (domain V), with additional interactions occurring at G745, G748, or A752 (domain II) [59]. Methylation in G748 and A2058 have also been shown to be synergistic in reducing the susceptibility of *M. bovis* to tylosin [70]. High minimum inhibitory concentrations (MICs) to macrolides and florfenicols at position 534 has also been associated with increased resistance in *M. bovis* [67]. In general, single mutations appear to reduce susceptibility, but mutations in both copies of the *rrl* gene within 23S rRNA resulted in higher MICs (>128 μg/mL) to macrolides [71]. It has been shown that different sugar residues on a macrolide interact uniquely with the ribosome [71]. For example, in a static model, tulathromycin was too small to extend across the ribosome tunnel to interact with G748, unlike tylosin, tilmicosin, or tildipirosin [59]. Mutations in ribosomal proteins L4 and L22, encoded by *rplD* and *rplV*, have also been shown to impart macrolide resistance, due to their proximity to the macrolide binding site [63,72]. Although mutations in

these proteins have been reported for macrolide resistant *M. bovis*, their role in resistance remains elusive [20].

Genes that encode for proteins that make up DNA gyrase (*gyrA* and *gyr B*) and topoisomerase IV (*parC* and *parE*), known as the quinolone resistance determining regions (QRDR) have been observed to have non-synonymous mutations that confer resistance to fluroquinolones, such as enrofloxacin [73]. Amino acid substitutions have been observed in gyrA, gyrB, and parC [67,73,74] in *M. bovis* isolates with reduced susceptibility to fluoroquiniolones. A Ser83Phe mutations in gyrA has been observed in intermediate (0.5-1μg/ml) and resistant (≥ 2 μg/mL) *M. bovis* isolates; whereas Asp84Asn in parC was observed in resistant isolates [73]. Similarly, mutations in gyrA (Ser83Phe), gyrB (Val320Ala), and parC (Ser80Ile) were reported to occur in isolates with fluoroquinolone MICs > 10 μg/mL [67]. At this point, no mutations that confer antimicrobial resistance have been identified in parE [67].

The trio of other bacterial species associated with BRD (*M. haemolytica, P. multocida*, and *H. somni),* have known genes and integrative conjunctive elements (ICE) associated with their resistance profiles [46]. ICE are associated with horizontal gene transfer, allowing for genome plasticity in both gram positive and gram negative bacteria. All ICE have three conserved modules involved in integration, excision, conjugation, and regulation [75]. However, the specific genes and mechanisms involved in these conserved modules can vary among ICE [75]. Two ICE have been detected in *M. bovis* PG45, ICEB-1 and ICEB-2 [76]. The majority of the differences between the primary reference genome of Hubei-1, a strain isolated in 2008 from a pneumonic calf in China [77], and PG45 is due to the absence of ICEB-1 which contains genes encoding hypothetical proteins and variable surface lipoproteins [77]. At present, it is unknown if this region has evolved to acquire antimicrobial genes as is the case with ICE in other BRD bacteria.

1.4.4 Alternative and Preventative Strategies

There are currently no effective commercialized vaccines for *M. bovis* [6,20], but vaccines do exist for other BRD agents, such as *M. haemolytica*. Vaccines for the other BRD bacterial and viral agents are routinely administered upon arrival at the feedlot. Vaccines are currently not a viable therapeutic alternative to antimicrobials for treating *M. bovis*, owing to its ability to evade the immune system by generating variable surface proteins (VSPs) [7].

Furthermore, in order to develop a vaccine, tissue culture models need to be developed. However, the clinical presentation of *M. bovis* is difficult to replicate in the lab, due to confounding factors associated with the host's immune system such as stress exposure, age, breed, and the polymicrobial and chronic nature of diseases associated with *M. bovis*. Thus, there are no suitable models that are representative of a natural *M. bovis* infection [78]. Therefore, the combination of varied immunogenic targets and the lack of a viable infection model has hindered the development of an effective vaccine against *M. bovis*.

1.5 *Mycoplasma bovis*

1.5.1 Genome

Until 1975, *M. bovis* was misidentified as a subspecies of *Mycoplasma agalactiae*, which is the causative agent of contagious agalactiae [79]. *M. bovis* is very small bacterium (0.2-0.5 µm), approximately 20% smaller than other bacterial species, such as *Escherichia coli* [80]. *M. bovis* is considered to be one of the smallest self-replicating organisms, with its economized genome bordering on containing the minimum number of genes to sustain bacterial life [1]. *M. bovis* has a circular genome, approximately 1 Mbp, with the first sequenced genome published in 2011 [*M. bovis* PG45, 76]. The PG45 genome was derived from an isolate taken from a mastitic milk sample on a dairy farm in the United States in the 1960's [41]. This isolate has 826 open reading frames with an 89% coding density, 61 of which are pseudogenes. The PG45 genome has a reduced guanosine/cytosine (G/C) content of 29.3%. However, the G/C content is not consistent among mycoplasmal genomes, with areas encoding for rRNA and tRNA having comparatively higher G/C content, likely due to the conserved nature of these genes in prokaryotes [31].

M. bovis has a reduced number of ribosomal RNA (rRNA) genes, with two *rrn* loci in the PG45 reference genome encoding for rRNA [76] compared to seven in *E. coli* K12 substrain MG1655 [81]. A *rrn* operon, which contains several rRNA genes under the control of a single promoter, is typically in a *rrs-rrl-rrf* configuration encoding for 16S, 23S, and 5S rRNA, respectively [82]. In *M. bovis*, the *rrn* loci consist of tandem *rrs-rrl* alleles, with *rrf* present at a distant site, as depicted in Figure 1.3. Having multiple copies of rRNA genes allows for redundancy in case of deleterious mutations. However, reduced copies of these genes results in mutations causing more frequent changes in phenotype. This is important in the context of

antimicrobial susceptibility as mutations in rRNA are associated with reduced susceptibility to the tetracyclines, macrolides, and phenicols[67].

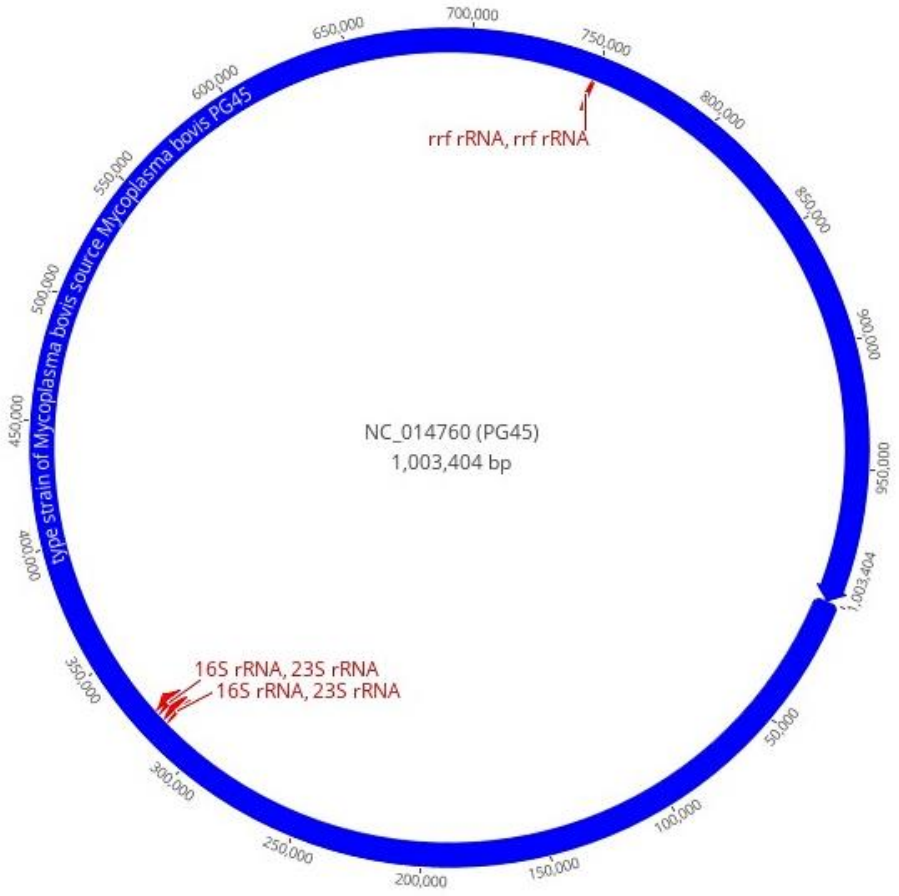

Figure 1.3 *Mycoplasma bovis* PG45 complete genome. *rRNA* genes are depicted in red. Graphic prepared using Geneious version 2020.0 [83].

A caveat to economization of the genome, is the absence of complete pathways involved in vital cellular mechanisms such as biosynthesis and DNA repair mechanisms; therefore, *Mycoplasma* spp. are reliant on nutrients from the environment that cannot be synthesized by the

cell. Based on complete sequencing of the *M. bovis* genome, deficits in both of these mechanisms have been reported [77]. The primary mechanism of maintaining the integrity of DNA in *Mycoplasma* spp. is through nucleotide excision repair, as it was the only complete repair pathway identified based on the comparative analysis of nine *Mycoplasma* spp. [84].

A number of genes encoding products in various biosynthetic pathways are missing in *M. bovis*, such as genes involved in the tricarboxylic acid cycle [77]. Therefore, it relies on glycolysis to generate cellular energy (adenosine triphosphate, ATP). However, it uses glycerol, rather than glucose to synthesize ATP through glycolysis as the 6-phosphofructokinase gene encoding phosphofructokinase is absent [77]. Pyruvate can also be used to generate ATP, following transformation into acetate via acetyl-CoA [77]. Therefore, growth media can be further supplemented with sodium pyruvate to stimulate growth. Additionally, *M. bovis* is largely reliant on external sources of amino acids, purines, pyrimidines, and lipids [4]. In *M. bovis*, genes coding for the components of the tricarboxylic acid cycle and pentose phosphate pathways are also absent [77].

1.5.2 Pathogenicity and Virulence

The ability of *M. bovis* to bind to epithelial cells is the first step in pathogenicity, where it adheres to epithelial cells through adhesins such as α-enolase and NADH oxidase that interact with plasminogen and fibronectin, respectively [85,86]. However, these proteins are not limited to a single function. In addition to being an adhesin, α-enolase is a glycolytic enzyme involved in the production of cellular energy [85] and NADH oxidase is involved in the production of hydrogen peroxide (H_20_2) [86]. Once in contact with host cells, *M. bovis* produces various virulence factors, which aid in the establishment of chronic infections. Proteins such as α-enolase and NADH oxidase that exhibit multiple functions are common in *M. bovis*. For example, VSPs play a role in both adhesion and evasion of host immunity.

Antigenic variations in *M. bovis* is generally associated VSPs [87]. There are 13 *vsp* genes at a single locus in *M. bovis* PG45: *vspA*, *vspB*, *vspE*, *vspF*, *vspG*, *vspH*, *vspI*, *vspJ*, *vspK*, *vspL*, *vspM*, *vspN* and *vspO* [87]. Tandem repeats within this region allows for rapid changes in their composition and expression, allowing VSPs to be both size and phase variable. Phase variation is due to an ON-OFF molecular switch, which is controlled within the operon and changes the composition of surface proteins through recombination. Intergenic recombination

between *vspA* and *vspO*, results in *vspC* [88]. These mechanisms create antigenic heterogeneity so as to evade the host's immune system. This rapid alteration of immunogens likely accounts for the failure to develop a suitable field vaccine against *M. bovis*, despite considerable effort by the mycoplasma community [78]. This trait could also account for the chronic nature of *M. bovis* infections.

Slow growth rate, intracellular localization, modulation of the immune system, biofilm formation, and the production of secondary metabolites have all been investigated as factors that contribute to the chronic nature of *M. bovis* infections. Their slow growth rate enables them to gradually increase in the presence of the immune system, allowing time for the host to adapt rather than illicit an immediate immune response [80]. Studies have demonstrated the *M. bovis* is able to invade and replicate in primary embryonic calf turbinate cells [89] and bovine embryonic tracheal cells [90]. Additionally, *M. bovis* has been shown to enter various types of peripheripheral blood mononuclear cells (T cells, T helper cells, B cells, monocytes, γδ T cells, cytotoxic T cells, natural killer (NK) cells and dendritic cells) and erythrocytes [91]. In addition to invading erythrocytes, *M. bovis* possesses a gene encoding a hemolysin-related protein, which can lyse erythrocytes [77].

In addition to evading the immune system, intracellular localization also reduces exposure to antimicrobials. Furthermore, *M. bovis* is able to interact with immune cells such as neutrophils, macrophages and lymphocytes, and modulate their function. Specifically, delaying apoptosis in monocytes and alveolar macrophages [92], and further impeding the immune response by altering phagocytosis and antigen presentation [93]. Increases in anti-inflammatory cytokines, and a reduction of pro-inflammatory cytokines are additional mechanisms of immune modulation [94,95].

M. bovis also forms biofilms, which form a barrier to protect *M. bovis* from immune defenses such as opsonization and complement mediated-lysis [93] and reduce the effectiveness of antimicrobials [26]. Biofilms not only contribute to the persistence of *M. bovis*, potentiating chronic infections, but also can damage host cells. As phagocytes are recruited to the biofilm, they release secondary metabolites such as lysozymes, reactive oxygen species (ROS) and reactive nitrogen species (RNS) that compromise host cells. Tissue damage can also occur by

extracellular cysteine proteases, which degrade extracellular matrix proteins directly (fibronectin and vitronectin) or indirectly (activating host metalloproteases) [77].

Mycoplasmas are also capable of producing hydrogen peroxide [86]. However, levels of hydrogen peroxide were found to not be statistically different between *M. bovis* associated caseonecrotic bronchopneumonia, acute bronchopneumonia, chronic bronchopneumonia or non-inflamed lung samples [96]. It has been suggested that hydrogen peroxide in combination with other ROS and RNS production results in the caseonecrotic lesions characteristic of *M. bovis* pneumonia [87,96]. Not only is hydrogen peroxide cytotoxic, but has been speculated that ROS produced by *M. bovis* modulates the immune response enabling persistent infections through promotion of a T helper 2 (Th2) versus a T helper 1 (Th1) response [96].

1.5.3 Morphology and Growth Requirements

On agar, *M. bovis* presents a characteristic "fried-egg" colony, with the white center being due to concentrated growth embedded in the agar (Figure 1.4). *Mycoplasma* spp. are fastidious with complicated nutritional requirements for growth *in vitro*. These characteristics, despite their significant impact on human and animal health, are largely why information on *Mycoplasma* spp. is limited. A pleuropneumonia like organism (PPLO) media base (broth or agar) is commonly used and is further supplemented with serum and yeast extract [97]. Serum is added, as a source of fatty acids, cholesterol and nucleotides [31]. Yeast extract is added as a source of micronutrients (vitamins and carbohydrates) and biosynthetic precursors (nucleosides, amino acids and peptides) [98].

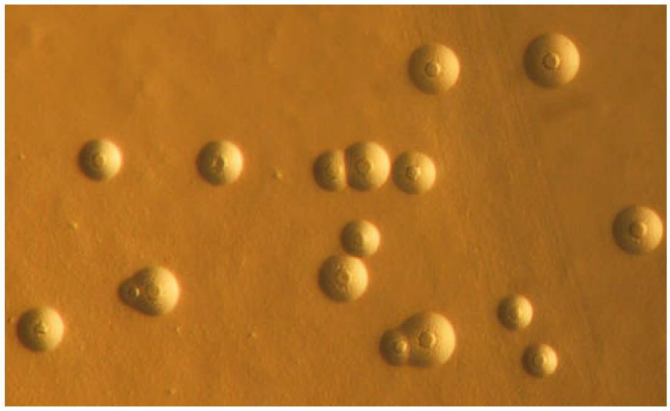

Figure 1.4 The "fried-egg" morphology of *Mycoplasma bovis* on solid media [99].

1.5.4 Diagnostics

1.5.4.1 Conventional Clinical Methods

Multiple diagnostic methods exist for *M. bovis* and each provide different information pertaining to clinical ailments or manifestations. The gold standard of bacteriology is still culturing, but given the fastidious nature of *M. bovis* this method is not ideal when results are required expeditiously. Additionally, culture-based methods are dependent on recovery of a viable organism and are sensitive to previous antimicrobial exposure [100]. Serological testing, such as enzyme-linked immunosorbent assay (ELISA) is a rapid method to detect *M. bovis* specific antibodies in serum [29], indicative of an immune response as a result of prior exposure to *M. bovis*. An additional advantage of ELISA over culturing is that it is not impacted by recent antimicrobial therapy [40]. However, it is not able to indicate whether the host is currently infected or shedding the bacterium in a transient manner.

To expedite the identification process, DNA sequence based methods are widely used due to their sensitivity, specificity, and high-throughput capabilities [4]. DNA amplification can utilize the polymerase chain reaction (PCR) in both conventional and real-time platforms. As PCR amplifies a specific target sequence, only a small amount of purified DNA is required. Often a target of interest is the *uvrC* gene, encoding for excinuclease ABC subunit C [101], a protein involved in DNA excision and repair [35]. This gene is highly conserved among *Mycoplasma* spp. and has been shown to be an effective target to screen for *M. bovis* [102]. Not only do PCR methods provide a rapid method of identification, but they can be designed for

single or multiple targets. As cattle can be colonized by multiple *Mycoplasma* spp., multiplex PCR assays have been developed to identify multiple species in a single assay. For example, Parker et al. [103], developed a multiplex PCR that simultaneously targeted *M. californicum, M. bovigenitalium,* and *M. bovis*.

As different diagnostic methods provide different information they can be combined to provide further insight into *M. bovis* infections. For instance, when *M. bovis* was confirmed to be in New Zealand's cattle herd in 2017, a surveillance program to eradicate it from their national herd was implemented. This surveillance program utilizes two identification methods designed to address *M. bovis* infections at the individual and herd level, respectively. PCR is used to detect animals that are shedding *M. bovis* and an ELISA to detect animals that have been exposed to the organism [104]. All herds found to be positive are culled in an effort to remove the *M. bovis* reservoir from the island [105].

1.5.4.2 Whole Genome Sequencing

Whole genome sequencing (WGS) is a version of high-throughput sequencing (HTS), where the input nucleic acid is DNA. In HTS, there are four general stages: data generation, primary analysis, secondary analysis, and tertiary analysis [106]. Data generation consists of bacterial isolation and nucleic acid extraction. The quality of sequencing information generated is highly dependent on the extraction method, yield, purity, and integrity of the DNA [106]. Following a quality control check of the extracted DNA, the next stages of data generation include library sequencing and computational analysis [106]. In the primary analysis, quality assurance and quality control procedures are performed on the raw read information [106]. Secondary analysis involves assembly of the raw reads, which can be done *de novo* or through alignment against a reference sequence, while the tertiary analysis is the interpretation of the sequence information [106].

HTS can be done utilizing next-generation sequencing (NGS) technologies. Regardless of the different procedures for DNA extraction, there are three main steps: lysis, precipitation, and purification. In the lysis step, cells are lysed chemically or mechanically and enzymes such as proteinase K and RNAse are added to digest contaminating proteins and RNA. Next, the DNA is precipitated and purified by separating out the DNA from contaminants via a solid (column-based) or liquid (solvent-based) phase extraction. The principle requirements of DNA for NGS is

purity, concentration, and integrity. Purity can be assessed quantitatively, using spectrophotometric (A260/A280) readings, or qualitatively, using agarose gel electrophoresis. Furthermore, concentration can be determined using fluorometric readings, such as Qubit (Thermo Fisher) and integrity can be assessed using gel electrophoresis.

Built upon the technology used to sequence individual amplicons, some NGS methodologies rely on amplification. Amplification, and subsequent sequencing, allows for reconstruction of the sequence of interest multiple times over in a high-throughput manner [107]. When utilizing Illumina sequencing technologies, DNA is fragmented into 250-1000 bp and indexed to generate a genomic library for each sample [108]. The final pooled library is loaded onto the sequencer, where the adapter sequences bind to their complementary oligonucleotide in a flow cell [109]. DNA fragments are then clonally amplified through bridge amplification to generate clusters. The reverse template is removed, and the remaining forward strand is sequenced using fluorescently labelled nucleotides in a process commonly referred to as 'sequencing by synthesis' to produce read 1. In paired-end sequencing, read 2 is generated in a similar manner by using the reverse strand as a template. Following completion of sequencing, samples can be separated based on their unique indices [109]. The cost of NGS has fallen rapidly and hence its use is growing in popularity for investigating antimicrobial resistance and in epidemiological studies [103,107,110,111]. Important for the context of this thesis, NGS can also be utilized for WGS [107].

1.5.5 Clinical Findings of Antimicrobial Resistance

For antimicrobial stewardship, it is important to know the susceptibility of pathogens to specific antimicrobials, so that the correct antimicrobial can be administered. Global efforts to monitor changes to phenotypic and genotypic traits of *M. bovis* have been undertaken. As there is currently no established methodology for *M. bovis* AST, it is challenging to compare minimum inhibitory concentration (MIC) values among studies. However, two studies have looked at *M. bovis* isolates spanning three decades, allowing for comparison of isolates overtime. These two studies have been undertaken using field isolates from cattle in France [17] and Eastern Canada [13] using agar and broth dilution methods, respectively. The French isolates were from calves with BRD acquired through the national surveillance network of ruminant mycoplasmoses (VIGIMYC). The Canadian isolates were derived from respiratory, milk and joint samples from

beef and dairy animals submitted to a clinical laboratory. In these studies, a general increase in the MIC for 50% of the isolates (MIC_{50}) over three decades (1978 to 2012) was observed, indicating reduced antimicrobial susceptibility. The MIC_{50} increased for tulathromycin, tilmicosin, and tylosin [13,17], but remained constant at 128 µg/mL for gamithromycin and tildipirosin [17]. Fluoroquinolone resistance, specifically enrofloxacin, was consistently low (MIC_{50} = 0.25 µg/mL) across the three decades. Within the tetracyclines, the MIC_{50} for oxytetracycline and chlortetracycline in Canadian isolates increased by a single dilution from 2 to 4 µg/mL; whereas, in France the MIC_{50} for oxytetracycline remained elevated at ≥ 32 µg/mL over the 30 year period. There was no change observed in the susceptibility to florfenicol over this time period. Overall, there has been a reduction in the susceptibility of *M. bovis* to macrolides, whereas the susceptibility to florfenicol, oxytetracycline, and chlortetracycline has remained largely unchanged. Fluoroquinolones remain the most effective antimicrobial, but MIC values have increased overtime [67].

In general, these studies provide evidence that over time there has been a reduction in the number of suitable antimicrobials to treat *M. bovis* infections. This reduction in susceptibility could be due to increased antimicrobial use and thus exposure and selection for resistant strains. Antimicrobials administered to cattle could be effective against other BRD bacterial agents, but not against *M. bovis*. Therefore, it is possible that *M. bovis* still undergoes antimicrobial exposure and selection indirectly as antimicrobial treatment is primarily targeted at other BRD agents. Measuring AST is time consuming and expensive, making it desirable to have a genotypic method to select suitable antimicrobial therapies. Although this approach has been explored [22,67], assessment of causative versus predictive genotypes is still ongoing and requires concordance studies with paired phenotypic (AST) and genotypic (SNP) data.

1.5.6 Antimicrobial Susceptibility

Antimicrobial resistance of *M. bovis* is of global concern as it has been increasing [12,13,17,40]. Susceptibility of *M. bovis* to tetracyclines, macrolides, phenicols, and fluoroquinolones has been studied. A number of methods including E-test, agar dilution and broth dilution can be used to estimate antimicrobial susceptibility. Broth and agar dilutions are performed by doing 2-fold serial dilutions of an antimicrobial in broth or agar [112]; whereas the E-test assesses the zone of inhibition on agar using a continuous gradient of antimicrobial on a

plastic strip [113]. Each method utilizes a standardized bacterial suspension when determining the MIC, which is defined as the minimum concentration of an antimicrobial that prevents visible growth of a microorganism [112]. When AST is done in broth for *M. bovis*, coloured indicators like alamarBlue (resazurin) [13,15,19,21,36,] or phenol red [16,18,114] can be used. AlamarBlue detects redox changes using resazurin [15] (Figure 1.5); whereas, phenol red detects a reduction in pH [115].

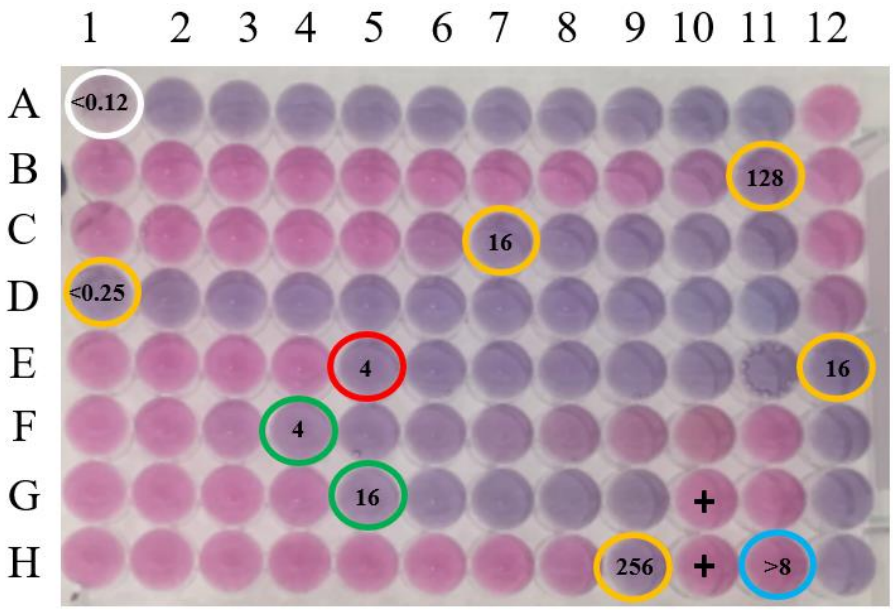

Figure 1.5 Sensititre plate with alamarBlue following 48 h incubation. The *Mycoplasma bovis* isolate was derived from lung tissue collected during a postmortem examination. Minimum inhibitory concentrations are indicated in black text with antimicrobials coded by class using coloured circles: white, fluoroquinolone; orange, macrolide; red, phenicol; green, tetracycline; and blue, β-lactam. A1-A11, enrofloxacin 0.12-128 µg/mL; B1-B11, tildipirosin 0.12-128 µg/mL; C1-C11, gamithromycin 0.25-256 µg/mL; D1-D11, tulathromycin, 0.25-256 µg/mL; E1-E11, florfenicol 0.25-256 µg/mL; F1-F10, oxytetracycline 0.5-256 µg/mL; G1-G9, chlortetracycline 1-256 µg/mL; H1-H9, tilmicosin 1-256 µg/mL; 12A-12H, tylosin tartrate 1-128 µg/mL; 11F-11H, penicillin 2-8 µg/mL; 10G/10H, positive control (+, no antimicrobial). Growth assessed based on a blue-to-pink colour change. Colour change due to interference between PPLO broth and alamarBlue in wells occurred where oxytetracycline ≥ 32 µg/mL.

One of the greatest limitations of AST for *M. bovis* is that there are no standardized breakpoints or protocols from international organizations such as Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST). Therefore, this makes comparisons of absolute MIC determinations from different publications challenging, if not impossible. Antimicrobial sensitivity testing assesses *in vitro* susceptibility to antimicrobials, but does not necessarily reflect *in vivo* sensitivity [21]. Clinical breakpoints do exist for human mycoplasmas, such as *M. hominis* and *Mycoplasma pneumoniae* [28] and for the BRD pathogens *M. haemolytica*, *P. multocida*, and *H. somni* [116]. Clinical breakpoints are used to establish ranges to determine whether a particular bacterial isolate is sensitive, intermediate or resistant to a specific antimicrobial [117]. These classifications are used to guide therapeutic choices, as they are associated with successful, uncertain, or failed antimicrobial therapy [117].

Clinical breakpoints are used to predict the probability of treatment success [112]. These breakpoints are specific to a susceptibility protocol, and take into account the pharmacokinetic and pharmacodynamic properties of a specific antimicrobial. Pharmacokinetics relates to the absorption, distribution, metabolism, and excretion (ADME) of a drug within an individual [118,119]. Pharmacodynamics focuses on how a drug works based on its unbound drug concentration and antimicrobial effect [119]. Therefore, without established clinical breakpoints for *M. bovis,* it is not possible to correlate *in vitro* MIC to clinical efficacy of an antimicrobial *in vivo*. However, MICs for human *Mycoplasma* spp. [28] and other respiratory pathogens in cattle [116] have been used as a guide for interpretation of resistance in *M. bovis* [11,12,15,16,29].

1.5.7 Epidemiological cut-off value (ECOFF)

The ECOFF is an alternative method to describe isolates in a population on the basis of antimicrobial susceptibility and was established in an effort to detect biological phenotypic resistance [117], as opposed to success of treatment indicated by clinical breakpoints [112]. In an effort to improve the basis of comparison for genotype-phenotype concordance studies, the use of an ECOFF as opposed to a breakpoint has been proposed [110]. An ECOFF is defined as the highest MIC where acquired resistance mechanisms are not phenotypically detectable [110], and is determined based on the distribution of MIC values for a specific antimicrobial in a given bacterial species [112]. Instead of the classical approach of classifying an organism as sensitive,

intermediate, or resistant to a specific antimicrobial using clinical breakpoints, isolates are classified as wildtype or non-wild type. Wildtype (WT) organisms do not have any known phenotypically detectable resistance; whereas a non-wildtype (NWT) organism exhibits phenotypic resistance. Additionally, WT organisms do not exhibit resistance regardless of year, geographic or anatomical location of sampling [110]. An ECOFF can provide complementary information to clinical breakpoints, but clinical breakpoints do not differentiate WT from NWT organisms. For each ECOFF group, isolates can be clinically categorized as susceptible, intermediate or resistant phenotypes.

1.5.8 Strain Type Differentiation

To resolve differences in bacterial isolates, bacteria can be categorized based on a set of characteristics. This can be done using phenotyping, genotyping, or a combination of both. Categorizing isolates based on these characteristics is known as typing. The ability to differentiate isolates is the cornerstone of epidemiological surveillance [120]. There have been multiple strategies employed to genotype *M. bovis*, including restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and SNP analysis [121]. Both RFLP and PFGE are examples of fingerprinting methods that use different sizes of genomic fragments to infer sequence differences. Both MLST and SNP are sequencing based methods [121] and as technological advances have been made these procedures have been more widely adopted. Sequence methods employed in this thesis include *in silico* MLST, core genome MLST (cgMLST), core genome single nucleotide variant (cgSNV) and whole genome SNV (wgSNV) analysis.

MLST is traditionally done using PCR, targeting a small number of housekeeping genes with specific primer pairs, followed by sequencing the individual amplicons [97]. However, MLST can also be done *in silico* from WGS information. There are two published MLST schemes for *M. bovis*, both consisting of seven housekeeping genes [97,122]. The scheme originally proposed by Register et al. [97] included alcohol dehydrogenase-1 (*adh-1*), glutamate tRNA ligase (*gltX*), glycerol-3-phosphate dehydrogenase (*gpsA*), DNA gyrase subunit B (*gyrB*), phosphate acetyltransferase-2 (*pta2*), thymidine kinase (*tdk*), and transketolase (*tkt*). The scheme by Rosales et al. [122] also used *tdk*, in addition to the chromosomal replication initiator protein (*dnaA*), methionine tRNA ligase (*metS*), DNA recombination and repair protein (*recA*),

elongation factor Tu1 (*tufa*), ATP synthase subunit alpha (*atpA*), and RNA polymerase sigma factor (*rpoD*). In some *M. bovis* isolates, the *adh-1* gene has not been detected using the Register et al. [97] scheme, making these isolates untypeable. However, the scheme has recently been revised, by replacing *adh-1* with *dnaA* [123]. Both of the schemes are curated and hosted on PubMLST (https://pubmlst.org/mbovis/).

Core genome MLST is built upon the same molecular principals as MLST, but instead of looking at a few genes, cgMLST uses hundreds of genes. This allows cgMLST to discriminate genotypic differences using a larger portion of the genome. cgMLST has been utilized for epidemiological studies for other veterinary *Mycoplasma* spp. [124,125], as well as in a recent epidemiological investigation of *M. bovis* associated with mastitis in two dairy herds in Finland [111].

cgSNV analysis involves determining which genomic regions are similar in all isolates of interest, then looking for SNPs that occur in these common to "core" areas; whereas wgSNV queries across the entire genome. A SNP analysis approach has been performed previously using 75 Australian *M. bovis* isolates from clinical and non-clinical dairy cattle spanning 2006 to 2015, and found these to be genetically similar with a maximum of 50 SNPs observed among isolates [103].

In silico MLST, cgMLST, cgSNV and wgSNV analysis are capable of different levels of resolution, and are compatible with WGS information. The trade-off for resolution is in the complexity of analysis for each typing approach. *In silico* MLST, cgMLST, cgSNV and wgSNV analysis have increasing levels of resolution as well as increasing analytical complexity and computational requirements. One of the benefits of a genomics based approach utilizing WGS is that as new questions arise, the genomic information can be interrogated at a later date without having to perform additional lab processes. The phylogenetic relationship of isolates constructed using the different typing method can allow the relationship between the isolates, and the possible evolutionary history to be determined. This can allow for phenotypic prediction, which can have clinical relevance with regard to antimicrobial susceptibility.

1.5.9 Surveillance Programs

1.5.9.1 Canada

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) was started in 2002 and is a One Health initiative coordinated by the PHAC [126]. The program assesses trends in AMU and AMR concerning both humans and animals [127]. One Health is a multimodal approach that considers human health, animal health, and the environment. One Health follows the tenet that these entities do not act as discrete units, but rather act in concert with one another. Therefore, the most effective way to address AMR requires collaboration of government, private industry, and academic stakeholders within these entities. The focus of CIPARS is primarily on food-borne pathogens such as *Salmonella*, *Campylobacter*, and *E.coli*. Recently, *M. haemolytica* a member of the bacterial complex associated with BRD, is a bacteria of interest for CIPARS.

1.5.9.2 Europe

In Europe, there is the MycoPath pan-European antimicrobial susceptibility monitoring program that was developed specifically to focus on mycoplasmal species in agriculture [114]. Of particular interest to the program is *M. hyopneumonia* in pigs and *M. bovis* in cattle [21]. The isolates collected in the program are from clinical cases in livestock that have not been recently exposed to antimicrobials [114]. Through continually monitoring the susceptibility of isolates, the aim of the program is to monitor resistance and ensure that effective therapies are administered.

1.5.9.3 New Zealand

In 2017, *M. bovis* was found in cattle at a dairy farm in New Zealand. This led to a trace back program to determine the source of *M. bovis*, and ultimately the decision in May 2018, to attempt to eradicate *M. bovis* from New Zealand [128]. The decision to eradicate was due to the welfare and economic impact of clinical conditions (pneumonia, arthritis, mastitis, otitis media, conjunctivitis), the additional cost of production due to treatment and disease management, and its poor response to available therapies [128]. As the number of herds impacted were limited, eradication through depopulation was chosen to limit the impact of *M. bovis* on the dairy and beef industries in New Zealand. It is estimated that full eradication will take 10 years and cost $886 million, at a cost of $16 million in lost production [105].

1.6 Objectives

1. Describe the usage of antimicrobials and the presence of antimicrobial resistant isolates in western Canadian feedlot cattle mortalities due to *Mycoplasma bovis*.
2. Investigate the genotypic basis for macrolide resistance in *Mycoplasma bovis* isolates using whole-genome sequencing to assess single nucleotide polymorphisms in the 23S rRNA gene alleles and ribosomal proteins L4 and L22.
3. Assess the concordance of four genotyping methods to *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle and the ability of each method to resolve phenotypic differences based on genotype.

The primary objectives of this thesis were to describe the usage of antimicrobials and the presence of AMR in relation to *M. bovis* related disease, investigate the genotypic basis for macrolide resistance, and assess the suitability of four different *in silico* genotyping methods for the phylogenetic analyses of *M. bovis*. In order to achieve these objectives, a series of three related and interdependent studies were conducted and described in Chapters 2, 3 and 4 of this thesis.

In general, AMU and AMR are a global concern. In western Canadian feedlot cattle, antimicrobials are central to the prevention and treatment of *M. bovis* infections. Chapter 2 describes the AMU and AMR of *M. bovis* isolates of western Canadian feedlot cattle. Although Chapter 2 describes AMR and AMU based on data obtained from western Canadian feedlot cattle, this chapter was not intended to be representative of all western Canadian feedlots but rather provide context on AMR and AMU in feedlots and the background of the cattle and isolates represented in this thesis.

AST of *M. bovis* traditionally requires culture-based methods which are time consuming and not amenable to rapid and routine assessment, unlike molecular methods which directly query the genome. In order to assess the relationship between genotype and phenotype for AMR, *M. bovis* isolates underwent both WGS and AST. Due to the elevated AMR and reliance on macrolides for treatment and prevention of *M. bovis* disease in feedlots, the study in Chapter 3 focuses on macrolides to investigate the relationship between genotype (SNPs in genes associated with macrolide resistance) and phenotype (macrolide resistance, as determined using AST).

Throughout this thesis, isolates were derived from different anatomical locations (nasopharynx, lung, joint) of cattle with varying health status (healthy, diseased, dead) in different feedlots over a 12-year period (2006 - 2018). As the ability to differentiate bacterial isolates is the cornerstone of epidemiological surveillance, the study outlined in Chapter 4 utilizes four *in silico* genotyping methods (MLST, cgMLST, cgSNV, wgSNV) to assess the phylogenetic relationship of *M. bovis* isolates derived from western Canadian feedlot cattle and the ability to resolve phenotypic differences based on genotype. Lastly, Chapter 5 provides a general discussion, future research and conclusion for the studies undertaken within the thesis.

2 ANTIMICROBIAL USE AND THE SUSCEPTIBILITY OF *MYCOPLASMA BOVIS* ISOLATES DERIVED FROM WESTERN CANADIAN FEEDLOT CATTLE

2.1 Abstract

Mycoplasma bovis has significant animal welfare and economic concerns given its association with BRD and CPPS in feedlot cattle. Antimicrobials are currently the only therapeutic option for treatment and control of *M. bovis*, making surveilling their administration and susceptibility crucial to their utility and judicious use. *M. bovis* isolates ($n = 119$) were cultured from the nasopharynx, lung, and/or joint of 74 western Canadian feedlot cattle that succumbed to *M. bovis* related disease over five production years (2014-2018). AMU was determined from feedlot treatment records and 93.2% of cattle received an antimicrobial metaphylactically upon arrival at the feedlot. An average of three antimicrobial classes was administered to an individual animal with more cattle having received additional antimicrobial classes later in the feeding period (> 60 DOF). Antimicrobial susceptibility was assessed for nine antimicrobials commonly administered to feedlot cattle using a microbroth dilution assay. Most isolates (> 92 %) were resistant to each of the five macrolides tested and 50.4% were resistant to enrofloxacin. The greatest susceptibility (89.9%) was observed to florfenicol, despite 78.4% of animals having been administered it. Overall, AMR was present in western Canadian *M. bovis* isolates.

2.2 Introduction

M. bovis infections pose both economic and animal welfare concerns for Canadian feedlot operators, through its association with BRD and CPPS in cattle [2,3]. BRD typically occurs one to two weeks after arrival at the feedlot and is associated with multiple bacterial and viral etiological agents [32]. Other bacterial species associated with BRD include *M. haemolytica*, *P. multocida*, and *H. somni* [9]. Unlike BRD, CPPS is attributed to chronic *M. bovis* infections [49]. Economic losses occur due to reduced weight gain, increased associated treatment costs, and death [6,7].

Typically, most AMU in feedlot cattle is targeted towards BRD, but the role of *M. bovis* in this condition remains nebulous [3,6,45]. More frequently, AMU is more specifically directed at *M. bovis* for chronic pneumonia and CPPS, where clinical failure is common [6]. Prolonged antimicrobial therapy against these chronic infections selects for antimicrobial resistance in other members of the BRD bacterial complex [7].

Antimicrobials exert bacteriostatic and bactericidal effects through various mechanisms, such as interfering with cell wall, DNA, and protein synthesis. As *M. bovis* does not have a cell wall nor does it synthesize folic acid, it is intrinsically resistant to β-lactams and sulfonamides, respectively [6]. Therefore, antimicrobials used to treatment mycoplasmosis target DNA (i.e., fluoroquinonlones) and protein synthesis (i,e., macrolides, tetracyclines, and phenicols) [29,63]. Over time, *M. bovis* has tended to exhibit reduced susceptibility to these antimicrobials [13,15,17,20,22]. This is problematic as there are currently no effective vaccines for *M. bovis* [6,20], and as a result, antimicrobials remain the primary tool to control mycoplasmosis. Feedlots generally administer in-feed antimicrobials for the prevention and control of liver abscesses; whereas, individual parenteral metaphylaxis of antimicrobials is more typical for the control of BRD [44].

Not only do antimicrobials have different modes of action, but they also differ in their utility to combat bacterial infections in humans. The classes of antimicrobials commonly used in western Canadian feedlot cattle to treat BRD include fluoroquinolones, macrolides, phenicols and tetracyclines [11,44]. Health Canada has classified antimicrobials based on their importance in human medicine and availability of alternative therapies [55]. Category I antimicrobials, including fluroquinolones such as enrofloxacin, are the preferred treatment of serious infections in humans for which there are no or limited alternatives [55]. Macrolides are a category II antimicrobial (high importance); whereas, phenicols and tetracyclines are both classified as category III (medium importance). A broader understanding of the sensitivity of *M. bovis* to these antimicrobials may enable veterinarians to select effective antimicrobial therapies without relying on category I or II antimicrobials. The objective of this study was to describe antimicrobial use and the subsequent antimicrobial susceptibility of *M. bovis* isolates derived from the nasopharynx, lung and joint in cattle. However, it should be noted that this chapter was not intended as a survey of AMU and AMR for all western Canadian feedlot cattle, but rather to provide general context and background for the cattle and *M. bovis* isolates used and discussed throughout this thesis.

2.3 Materials and Methods

2.3.1 Sampling

M. bovis isolates (n = 119) were sampled from the nasopharynx, lung, and/or joint of 74 feedlot cattle, spanning 5 production years (2014 - 2018) as described by Jelinski et al. [12]. Cattle were sampled from 10 feedlots. All samples were taken postmortem from cattle with pathological lesions consistent with BRD, chronic pneumonia, or CPPS. Production years were used to define the sampling cohort, as cattle often enter the feedlot in the fall and remain there until the following calendar year. Thus, the 2018 production year included samples obtained between November 1, 2018, and June 30, 2019.

2.3.2 Compilation of Antimicrobial Use

Feedlot treatment records were used to compile a history of antimicrobial treatments and included the antimicrobial, date of treatment and number of treatments. These treatment histories were compiled in spreadsheet format (Microsoft Excel, Microsoft Office, v. 15, Microsoft Corporation, Redmond, WA, USA). Although administration of antimicrobials in feed was not recorded for each animal, chlorotetracycline is frequently included in diet for 20-40 d post arrival to prevent BRD, and tylosin is administered for the remainder of the feeding period to reduce the incidence of liver abscesses [11].

Metaphylactic administration of antimicrobials was classified as those that were administered upon arrival at the feedlot. The active ingredient of each antimicrobial was determined by searching the product name in the Canadian edition of the Compendium of Veterinary Products [56]. Antimicrobials administered were then grouped into their respective classes based on active ingredients. Active ingredients were classified as follows (active ingredient, class): enrofloxacin, fluoroquinolone (FQ); ceftiofur, cephalosporin (CEPH); tulathromycin/tilmicosin, macrolide (MAC); oxytetracycline, tetracycline (TET); florfenicol, phenicol (PHEN); trimethoprim and sulfadoxine, trimethoprim-sulfonamides (TMS); and sulfanilamide/sulfathiazole/sulfamethazine, sulfonamide (SULF).

2.3.3 Culture

M. bovis was isolated from deep nasopharyngeal swabs and swabs from lung and joint tissue as described by Jelinski et al. [12]. Samples were cultured using pleuropneumonia-like organism (PPLO) broth and agar (BD Difco, Fisher Scientific, Waltham, MA, USA),

supplemented with 10 g/L yeast extract (BD Diagnostic Systems, Fisher Scientific, Waltham, MA, USA), and 20% horse serum (Invitrogen, Fisher Scientific) [12,97]. Where specified, the media was supplemented with 0.05% thallium (I) acetate, 500 U/mL penicillin G, and/or 0.5% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA).

Cultures derived from samples were filtered through 0.45 and 0.20 µm filters (Basix, VWR International, Radnor, PA, USA), and were used to inoculate PPLO broth with 0.05% thallium (I) acetate, 500 U/mL penicillin G, and 0.5% sodium pyruvate, and grown in a 5% CO_2 atmosphere with 75% humidity at 37 °C. Cultures with visible growth were subcultured onto PPLO agar and incubated for 3-6 days. A single colony with characteristic "fried-egg" morphology was subcultured onto PPLO agar, and incubated for 72 h. Up to three individual colonies per sample were used to inoculate separate aliquots of PPLO broth. After 48 h, each culture was transferred to PPLO with 20% glycerol, and stored at –80 °C. A single stock culture was selected and used to inoculate PPLO to grow cells for antimicrobial susceptibility testing and DNA extraction.

2.3.4 Identification

M. bovis isolates were grown in PPLO media for 48 h and genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's instructions with the exception that the final elution buffer was replaced with 10 mM Tris (pH 8.5). Extracted genomic DNA was assessed for quality using gel electrophoresis and quantified fluorometrically using a Qubit analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Cultures were confirmed as *M. bovis*, based on the presence of the *uvrC* gene [35] and sequencing of the 16S rRNA gene [129]. The 16S rRNA amplicon was purified using a QIAquick PCR kit (Qiagen, Nevlo, Netherlands) and Sanger sequencing at Macrogen (Seoul, South Korea). Forward and reverse sequences were assembled and edited using the Staden Package (version 1.6-r, http://staden.sourceforge.net/). The resulting sequences were compared to the National Center for Biotechnology Information (NCBI) nonredundant nucleotide database (nr) using BLASTn.

2.3.5 Antimicrobial Susceptibility Testing (AST)

AST was determined using a microdilution assay, in a customized Sensititre™ (Trek Diagnostics, Oakwood, GA, USA) plate designed to assess the common antimicrobials used

against BRD in North American feedlots [12]. AlamarBlue (ThermoFisher Scientific, DAL1100), a color redox indicator was used to assess growth in each well based on a blue to pink color transition.

The AST procedure began by inoculating an *M. bovis* isolate previously stored at –80 °C in 20% glycerol into PPLO broth with 0.5% pyruvate and incubating for 72 h at 5% CO_2 with 75% humidity at 37 °C. *M. bovis* isolates were subcultured in PPLO for 24 h. Following incubation, the optical density (OD) at 450 nm was determined using a NanoDrop One Spectrophotometer (Fisher Scientific, Waltham, MA, USA) and the culture adjusted to an OD_{450} = 0.1. The adjusted culture was diluted up to 100X, and 120 µL of the diluted culture was inoculated into 6 mL of PPLO in 2X AlamarBlue. Each well of a Sensititre™ plate was then inoculated (50 µL) into wells containing 50 µL of media with the appropriate concentration of each antimicrobial within a dilution series so as to achieve 1 x 10^3 to 5 × 10^5 CFU/mL in each well. Plates were sealed with a CO_2 permeable film, and incubated for 48–72 h. Minimum inhibitory concentrations (MICs) were determined by visual assessment of growth in plates after 48 to 72 h incubation based on a blue to pink colour change. *Mycoplasma bovis* ATCC® 25523™ was included as a positive control. Susceptibility of the isolates to penicillin was also used as a control, as all *M. bovis* isolates should be intrinsically resistant to this antimicrobial.

2.3.6 Clinical breakpoints

As there are no established antimicrobial breakpoints for *M. bovis*, breakpoints were extrapolated from other members of the bacterial BRD complex (i.e., *M. haemolytica*, *P. multocida*, *H. somni*) and human *Mycoplasma* spp., as described by Jelinski et al. [12]. The resistance breakpoints used were ≥ 0.50 µg/mL for enrofloxacin; ≥ 4 µg/mL for florfenicol, oxytetracycline, and chlortetracycline, ≥8 µg/mL for tildipirosin, gamithromycin, tilmicosin, tylosin; and ≥32 µg/mL for tulathromycin.

2.4 Results

2.4.1 Cattle and *Mycoplasma bovis* isolation

There were 119 isolates cultured from the nasopharynx (n = 12), lung (n = 66) and joint (n = 41) of 74 cattle at the time of postmortem examination (Table 2.1). Over half of the cattle (n = 42) came from a single feedlot (2016-2018), representing 62.2% of all isolates (n = 74). The remaining 30 cattle came from nine feedlots (2014-2016) and represented 37.8% (n = 45) of

isolates. There were 33 cattle that had a single isolate from the nasopharynx ($n = 2$), lung ($n = 25$) or joint ($n = 6$), 37 had isolates from two anatomical locations (6 nasopharynx/lung pairs, 31 lung/joint pairs) and 4 had isolates from all three anatomical locations. Nasopharynx samples were only collected in 2018; whereas lung and joint samples were collected across all years.

Table 2.1 Site of isolation of *Mycoplasma bovis* (%) across production years ($n = 119$).

Anatomical Location	Production Year					
	2014	**2015**	**2016**	**2017**	**2018**	**Total**
Nasopharynx					12 (10.1)	12 (10.1)
Lung	7 (5.9)	10 (8.4)	16 (13.4)	16 (13.4)	17 (14.3)	66 (55.5)
Joint	2 (1.7)	2 (1.7)	14 (11.8)	17 (14.3)	6 (5.0)	41 (34.5)
Total	9 (7.6)	12 (10.1)	30 (25.2)	33 (27.7)	35 (29.4)	119 (100)

2.4.2 Antimicrobial use

Treatment records and AST results were compiled for 74 cattle from ten feedlots over five production years. Most (93.2%) cattle received metaphylaxis with 94.2% of this being tulathromycin (Draxxin®) (Table 2.2).

Table 2.2 Antimicrobials administered to feedlot cattle upon arrival at the feedlot across five production years ($n = 74$). The number of cattle (%) that received each antimicrobial is indicated.

Antimicrobial	Production Year					
	2014	**2015**	**2016**	**2017**	**2018**	**Total**
Tulathromycin	4 (5.4)	9 (12.2)	16 (21.6)	17 (23.0)	19 (25.7)	65 (87.8)
Oxytetracycline	1 (1.4)	2 (2.7)				3 (4.1)
Tilmicosin				1 (1.4)		1 (1.4)
None	3 (4.1)			2 (2.7)		5 (6.8)
Total	8 (10.8)	11 (14.9)	16 (21.6)	20 (27.0)	19 (25.7)	74 (100)

An average of three classes of antimicrobials were administered to cattle. This was consistent over the 5 sampling years, with the exception of 2015 where an average of four classes were administered. Overall, 66.2% of cattle received ≤ 3 classes of antimicrobials (Table 2.3). The number of antimicrobials administered ranged from one to six, with 9.5% receiving 5 or 6 different classes. At the time of death, cattle had spent from 9-217 DOF with 81.1% being ≤ 60 DOF (Table 2.3). Of the cattle that received five or six classes, 57.1% were > 60 DOF at the time of death.

Table 2.3 The number of feedlot cattle ($n = 74$) administered different antimicrobial classes by production year (2014 to 2018) and days on feed at the time of death.

	Number of antimicrobial drug classes*						Average # antimicrobial drug classes†	Total # cattle (%)
	1	**2**	**3**	**4**	**5**	**6**		
Production Year								
2014	2	2	2	2			3	8 (10.8)
2015	1	1		3	4	2	4	11 (14.9)
2016	5	2	5	3	1		3	16 (21.6)
2017		7	11	2			3	20 (27.0)
2018	1	2	8	8			3	19 (25.7)
Days on Feed								
0-14	2						1	2 (2.7)
15-30	3	4	6	2	1		3	16 (21.6)
31-60	3	10	18	9	2		3	42 (56.8)
>60	1		2	7	2	2	4	14 (18.9)
Total	9 (12.2)	14 (18.9)	26 (35.1)	18 (24.3)	5 (6.8)	2 (1.7)	3	74 (100)

*Antimicrobial classes assessed : FQ - Fluoroquinolone, CEPH - cephalosporin, MAC - macrolide, TET - tetracycline, PHEN - phenicol, TMS - trimethoprim-sulfonamides, and SULF – sulfonamide. † Average number of classes administered is rounded to the nearest whole number.

Of the seven possible classes of antimicrobials administered (Table 2.4), macrolides were administered to 93.2% ($n = 69$) of the cattle, primarily for metaphylaxis. Phenicols, fluoroquinolones, and tetracyclines were administered to 78.4 ($n = 58$), 67.6 ($n = 50$), and 18.9 % ($n = 14$) of cattle, respectively. Cephalosporins and trimethoprim-sulfonamides, were administered to 36.5 ($n = 27$) and 8.1 % ($n = 6$) of animals, respectively.

Table 2.4 Use of antimicrobials in relation to the number of days on feed (DOF) at the time of death. Usage is shown as a percentage and absolute number (n) of feedlot cattle treated with each antimicrobial class for each cohort (DOF).

DOF	% (n) of Animals	Antimicrobial Classes						
		FQ	CEPH	MAC	TET	PHEN	TMS	SULF
0-14	2.7 (2)	0 (0)	0 (0)	100 (2)	0 (0)	0 (0)	0 (0)	0 (0)
15-30	21.6 (16)	68.8 (11)	31.3 (5)	93.8 (15)	12.5 (2)	56.3 (9)	0 (0)	0 (0)
31-60	56.8 (42)	64.3 (27)	31.0 (13)	90.5 (38)	14.3 (6)	85.7 (36)	7.1 (3)	2.4 (1)
>60	18.9 (14)	85.7 (12)	64.3 (9)	100 (14)	42.9 (6)	92.9 (13)	21.4 (3)	0 (0)
Total	100 (74)	67.6 (50)	36.5 (27)	93.2 (69)	18.9 (14)	78.4 (58)	8.1 (6)	1.4 (1)

FQ - Fluoroquinolone, CEPH - cephalosporin, MAC - macrolide, TET - tetracycline, PHEN - phenicol, TMS - trimethoprim-sulfonamides, and SULF – sulfonamides.

2.4.3 Antimicrobial susceptibility

Most (92.4 %; $n = 110$) isolates were resistant to all five macrolides tested with a MIC_{50} ≥ 128 µg/mL across all macrolide and approximately half (50.4 %) of all isolates were resistant to enrofloxacin (Table 2.5). Within the tetracycline class the proportion of resistant isolates ranged from 37.8 to 76.5% for oxytetracycline and chlortetracycline, respectively. Only 10.1% of the isolates tested were resistant to florfenicol.

Table 2.5 Antibiotic susceptibility of *Mycoplasma bovis* isolates ($n = 119$) recovered from cattle ($n = 74$) between 2014 and 2018. The dark vertical lines denote the resistance breakpoint, whereas the shaded cells are antimicrobial concentrations that were not tested.

Antibiotic	Class	≤0.12	0.25	0.50	1	2	4	8	16	32	64	128	≥256	%R	MIC Range (µg/mL)	MIC_{50}‡ (µg/mL)	MIC_{90}† (µg/mL)
Tulathromycin	Macrolide		6			1		2		1	3	20	86	92.4	0.25	256	256
Gamithromycin	Macrolide					1	2	2	1			3	110	97.5	2-128	256	256
Tilmicosin	Macrolide												119	100	256	256	256
Tildipirosin	Macrolide								1	1	3	114		100	16-128	128	128
Tylosin	Macrolide						2	1	2	5	4	105		98.3	4-128	128	128
Enrofloxacin	Fluoroquinolone	47	12			20	27	11	2					50.4	2-16	2	8
Florfenicol	Phenicol		2	16	45	44	11	1						10.1	0.25	1	2
Oxytetracycline	Tetracycline			2	26	45	36	9	1					37.8	0.5-8	2	4
Chlortetracycline	Tetracycline				3	25	36	30	22	3				76.5	1-32	4	16

‡MIC_{50}, 60th isolate

†MIC_{90} 107th isolate

2.5 Discussion and Conclusion

Antimicrobials are used extensively for the prevention and treatment of *M. bovis* infections in feedlot cattle in western Canada, given the absence of alternatives such as vaccines. From the 74 feedlot cattle sampled from 2014 to 2018, on average each animal was administered 3 different antimicrobial classes and 93.2% of cattle were administered an antimicrobial metaphylactically upon arrival at the feedlot. Of the 119 *M. bovis* isolates recovered, AMR was present in >50% of isolates for 7 of the 9 antimicrobials assessed. However, 55.4 % of cattle (n = 41) contributed more than one isolate, so these are not independent observations. These findings highlight the ubiquitous use of antimicrobials in western Canada and the elevated level of resistance present in *M. bovis* infected feedlot cattle.

Over the five production years, *M. bovis* isolates exhibited resistance to an average of three antimicrobial classes, one more than that observed by Anholt et al. [11] for *M. bovis* isolates predominately collected from cattle that had died of BRD (528 of 618 animals) in 2014 and 2015 in southern Alberta. This discrepancy could be due to the timing of sampling as cattle in their study were predominately sampled earlier in the feeding period (0-30 DOF) and included morbid cases; whereas, 75.7% of cattle in the current study were sampled at > 30 DOF during postmortem examination.

The percentage of resistant isolates in this study overestimates the level of AMR in the general *M. bovis* population in western Canada, as all isolates were obtained from dead cattle that had received multiple antimicrobial treatments. As approximately half of the isolates were derived from lung/joint pairs from a single animal, with a third of all isolates derived from the joints, the sample set was largely comprised of isolates derived from animals chronically infected with *M. bovis*. Furthermore, overestimation could be due to culturing more than one isolate from an individual animal, which occurred in 55.4 % of cattle (n = 41). As death attributed to chronic *M. bovis* infections are difficult to treat [6], it is no surprise that a greater number of antimicrobial classes were administered to cattle later in the feeding period in an effort to control mycoplasmosis.

Consistent with other reports, macrolides were the most common antimicrobial class administered upon arrival [11,44]. Nearly all cattle received metaphylaxis, with macrolides accounting for 95.7% AMU. Florfenicol was also commonly administered with 78.4% of cattle

being treated, but only 10.1% of isolates exhibited resistance. The minimal resistance to florfenicol in *M. bovis* is encouraging since it is a lower category III antimicrobial and could serve as an alternative to category II macrolides. In a 2007 field trial in Alberta, calves treated with florfenicol and flunixin meglumine for undifferentiated fever (UF) had a significantly lower mortality rate than those that were treated with a macrolide or cephalosporin [130]. The fact that the frequent use of florfenicol in the current study did not promote resistance could be related to the timing of administration where florfenicol administration increased later in the feeding period. As chronic *M. bovis* infections are associated with caseonecrotic pneumonia and arthritis, reduced perfusion of the infection site could render the treatment ineffective even if *M. bovis* was susceptible. Additionally, the formation of biofilms could further limit the effectiveness of antimicrobials by decreasing access. However, an elevated level of florfenicol resistance (71.7) in *M. bovis* was observed in Anholt et al. [11] when resistance was assigned at a MIC $\geq$ 4 ug/mL. This seems disproportionately higher, given that florfenicol accounted for 35.4% of treatments. However, only 38% of animals had treatment records available. Overall, the antimicrobial susceptibility results indicate that populations of *M.bovis* are largely susceptible to florfenicol. As antimicrobials remain the only therapeutic option for control and treatment of mycoplasmosis, it is crucial that an appropriate antimicrobial is given at the correct dose and time for the right disease.

Given that there was a single antimicrobial (florfenicol) to which 89.9% of *M. bovis* isolates were susceptible, the question should not be how best to use this antimicrobial but rather what other management practices could reduce *M. bovis* infections in feedlot cattle. Vaccines remain an ideal alternative to antimicrobials; however, the variability in the immunogenic surface proteins and lack of a suitable *in vitro* model have hindered the development of vaccines against *M. bovis* [7,78]. This leaves alternative management practices, such as ranch direct purchases and preconditioning of cattle as alternative mitigation strategies. Ranch-direct purchases are when cattle by-pass an auction mart and enter backgrounding operations directly from the cow-calf operation. Preconditioning is when stressful events and procedures are performed at the cow-calf operation to promote immunocompetency and to decrease the risk of respiratory disease [32,131]. Ranch direct purchases would reduce the commingling of cattle prior to arrival at the feedlot ultimately reducing the potential for transmission of *M. bovis*. Additionally, preconditioned cattle are more immunocompetent, reducing the occurrence of

disease from viral and/or bacterial infections. As preconditioning would occur in the cow-calf sector, the current lack of monetary incentive for producers to go this route unless ownership of the cattle is retained makes it less likely to be implemented.

Despite the levels of antimicrobial resistance in *M. bovis*, banning the use of antimicrobials altogether is not a reasonable alternative as they provide a crucial therapeutic tool that directly impacts the welfare of animals. Given the use of multiple classes of antimicrobials, without the benefit of recovery from a *M. bovis* infection, studies to compare the timing of therapeutic intervention could be beneficial to assess the risks and rewards to their use against *M. bovis*. This could possibly lead to the development of earlier endpoints for euthanasia when attempting to treat a mycoplasmosis. Having earlier endpoints would be beneficial in two regards. It could reduce the overall administration of antimicrobials and would positively influence animal welfare by reducing the time cattle remain in the feedlot with pneumonia and/or arthritis that is not going to respond to antimicrobial intervention.

The time consuming and labour intensive process of performing an *in vitro* based technique such as AST on *M. bovis* are caveats that limit the implementation of this method to make timely decisions on AMU. As an alternative, direct detection of the genotype is amenable to rapid molecular techniques such as PCR. Given the increased resistance and importance of macrolides in treatment and prevention of *M. bovis* related disease, Chapter 3 investigates the relationship between genotype and phenotype of macrolide resistance.

3 INVESTIGATION OF MACROLIDE RESISTANCE GENOTYPES IN *MYCOPLASMA BOVIS* ISOLATES FROM CANADIAN FEEDLOT CATTLE

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Author Contributions: Conceptualization, M.D.J., T.A.M.; Methodology, M.D.J., T.A.M., A.K., M.W., R.Z, S.A.L., J.E.M., A.C.R.; Formal Analysis, A.K., M.W., R.Z., S.P.; Investigation, M.D.J., A.K., R.Z.; Resources, M.D.J., T.A.M., J.E.M.; Data Curation, M.D.J., A.K., M.W.; Writing—Original Draft Preparation, A.K., M.DJ., T.A.M.; Writing—Review and Editing, A.K., M.J., T.A.M., M.W., R.Z., S.A.L., S.P., J.E.M., A.C.R.; Supervision, M.D.J., T.A.M., J.E.M. Funding Acquisition, M.D.J., T.A.M.

This chapter has been previously published in an open access peer-reviewed journal, *Pathogens*. The manuscript is included in its entirety, but has been reformatted for the purposes of this thesis.

Citation: **Kinnear, A**.; McAllister, T.A.; Zaheer, R.; Waldner, M.; Ruzzini, A.C.; Andrés-Lasheras, S.; Parker, S.; Hill, J.E.; Jelinski, M.D. Investigation of Macrolide Resistance Genotypes in *Mycoplasma bovis* Isolates from Canadian Feedlot Cattle. *Pathogens* **2020**, 9, doi:10.3390/pathogens9080622.

3.1 Abstract

Mycoplasma bovis is associated with bovine respiratory disease (BRD) and chronic pneumonia and polyarthritis syndrome (CPPS) in feedlot cattle. No efficacious vaccines for *M. bovis* exist; hence, macrolides are commonly used to control mycoplasmosis. Whole genome sequences of 126 *M. bovis* isolates, derived from 96 feedlot cattle over 12 production years were determined. Antimicrobial susceptibility testing (AST) of five macrolides (gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin) was conducted using a microbroth dilution method. The AST phenotypes were compared to the genotypes generated for 23S rRNA and the L4 and L22 ribosomal proteins. Mutations in domains II (nucleotide 748; *E. coli* numbering) and V (nucleotide 2059 and 2060) of the 23S rRNA (*rrl*) gene alleles were associated with resistance. All isolates with a single mutation at Δ748 were susceptible to tulathromycin, but resistant to tilmicosin and tildipirosin. Isolates with mutations in both domain II and V (Δ748Δ2059 or Δ748Δ2060) were resistant to all five macrolides. However, >99% of isolates were resistant to tildipirosin and tilmicosin, regardless of the number and positions of the mutations. Isolates with a Δ748 mutation in the 23S rRNA gene and mutations in L4 and L22 were resistant to all macrolides except for tulathromycin.

3.2 Introduction

Mycoplasma bovis is associated with various diseases of cattle such as pneumonia, mastitis, arthritis, otitis media, conjunctivitis, and reproductive disorders [6,45]. In feedlot cattle, *M. bovis* infections commonly manifest as bovine respiratory disease (BRD) and chronic pneumonia and polyarthritis syndrome (CPPS) [2,3]. Furthermore, *M. bovis* infections often respond poorly to antimicrobial therapy, resulting in a chronic infection [49]. This lack of a response frequently results in prolonged antimicrobial therapy, which indiscriminately selects for antimicrobial resistance in other pathogens that comprise the BRD complex [7]. Mycoplasmosis in the feedlot results in economic losses due to reduced production performance, increased treatment costs, and death loss [6,7]. In addition, feedlot cattle with polyarthritis may become severely lame, which is a significant animal welfare issue.

As there are currently no effective vaccines for *M. bovis*, antimicrobials remain the primary option for prevention and control of mycoplasmosis [6,20]. This has led to a number of *M. bovis* antimicrobial susceptibility studies in Canada [9-13], United States [15], Japan [22] and

throughout Europe [14,16-21]. These studies suggest that *M. bovis* will continue to become increasingly resistant to antimicrobials. This situation is exacerbated by the limited number of antimicrobials available for treating mycoplasma infections. *Mycoplasma* spp. lack a cell wall and the ability to synthesize folate, rendering them intrinsically resistant to all β-lactams and sulfonamides [6]. In addition, most aminoglycosides either lack label claims for BRD, or the formulations are not amenable to use in feedlot cattle. This narrows the selection of antimicrobials to those that target protein synthesis or DNA replication, and that have been formulated to maintain therapeutic blood levels for several days. The main class of antimicrobials that meet these criteria is the macrolides.

Macrolides have been formulated to be administered parenterally or in-feed; however, only one macrolide, tylosin tartrate (TYLT), is registered in Canada for in-feed use. Tylosin is typically administered throughout the feeding period, and is used to control liver abscesses [11]. The other four main macrolides used in the feedlot are: tilmicosin (TIL), tildipirosin (TIP), tulathromycin (TUL), and gamithromycin (GAM). All of which are formulated as long-acting injectable antimicrobials, and depending on the drug, may have label claims for the control (metaphylaxis) and treatment of BRD. A distinctive pharmacological characteristic of macrolides that makes them ideally suited for use in feedlot cattle is their predilection to concentrate in the pulmonary epithelial fluid [132]. This is notable because BRD is the most prevalent and costly disease of feedlot cattle [133]. Thus, the macrolides' pharmacokinetic and pharmacodynamic profiles are particularly well suited for metaphylaxis therapy for BRD in feedlots [53]. In western Canada, cattle deemed to be a high risk for developing BRD often receive TUL at the time of arrival to the feedlot; whereas, low risk cattle may receive either no antimicrobials or a long-acting oxytetracycline [44]. Lastly, unlike other BRD pathogens, antimicrobial resistance in *M. bovis* is not associated with antimicrobial resistance genes [134], but rather resistance arises from mutations in ribosomal RNAs [4].

Macrolides are a member of the macrolide–lincosamide–streptogramin B (MLSB) superfamily, all of which exert a bacteriostatic effect by disrupting protein synthesis [135]. Specifically, they bind with domains II and V of 23S rRNA, which is a component of 50S ribosomal subunit [63,136]. Ribosomal proteins L4 and L22 are positioned in close proximity to these macrolide binding sites [63,72]. Mutations within 23S rRNA and the L4 and L22 ribosomal

proteins are associated with macrolide resistance [4,137]. This mechanism of resistance is not unique to *M. bovis* [22,67,71], having been reported in a variety of bacterial species, including other *Mycoplasma* spp. [138,139], *Neisseria gonorrhoeae* [137], *Streptococcus* spp. [140,141], *Francisella tularensis* [142], *Escherichia coli* [143], *Chlamydia trachomatis* [144], and *Haemophilus influenzae* [145].

A limitation of antimicrobial susceptibility testing (AST) for *M. bovis* is the lack of established clinical breakpoints from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). As a result, researchers have extrapolated *M. bovis* clinical breakpoints from human *Mycoplasma* spp. and other bovine respiratory pathogens for which clinical breakpoints have been established [11,12,15,16,28,29,116]. Another challenge with performing AST on *M. bovis* is its very fastidious culture requirements, which is related to its reduced genome and limited biosynthetic capacity [27]. These requirements, coupled with relatively slow nonprolific growth, have encouraged the development of rapid molecular testing techniques for predicting antimicrobial susceptibility for *M. bovis* [22,146]. Utilization of a genotypic approach to assess antimicrobial susceptibility of *M. bovis* could allow for more expeditious evaluation of antimicrobial efficacy and evidence-based selection of antimicrobials to enable judicious use of antimicrobials, which are all principles of antimicrobial stewardship. Additionally, a genotypic approach could be more amenable as a standardized approach to assess antimicrobial susceptibility in *M. bovis* than culture-based techniques, as it would not be susceptible to variable results due to growth conditions. To support these efforts, this study assessed the concordance between genotypes known to confer macrolide resistance to AST phenotypes. Specifically, the study compared the 23S rRNA, L4, and L22 genotypes of *M. bovis* isolates to the AST results of five macrolides commonly used in western Canadian feedlot cattle to control and treat BRD.

3.3 Materials and Methods

3.3.1 Animals and Sample Collection

Mycoplasma bovis isolates were cultured from a cross-section of clinical samples derived from different anatomical regions (nasopharynx, lung, and joint) of western Canadian feedlot cattle over 12 production years (2006–2018). Deep nasopharyngeal swabs from live cattle were taken in accordance with Animal Use Protocols (#20070023; #20170021) approved by the

University of Saskatchewan's Animal Research Ethics Board and Lethbridge Research Center's Animal Care Committee (#1641).

Sampling was performed as described in Jelinski et al. [12]. Briefly, doubled-guarded uterine swabs (Reproduction Resources, Walworth, WI, USA) were used to obtain deep nasopharyngeal (DNP) samples from healthy and diseased cattle. The diseased cattle were identified by feedlot personnel trained in recognizing the clinical signs of BRD (dyspnea, depression, nasal discharge, anorexia, and fever). Swabs were immediately placed in Ames media (Mai, Ames Media, Product 49203, Spring Valley, WI, USA).

All other swabs or tissues were collected from animals purposively sampled by feedlot veterinarians recruited to provide clinical case material for the study. Samples were obtained by the veterinarians from animals that on postmortem examination were found to have pathological lesions consistent with *M. bovis* pneumonia or chronic pneumonia and polyarthritis syndrome (CPPS). Specifically, the lungs had gross pathology consistent with caseonecrotic pneumonia and/or chronic bronchopneumonia. A minimum 3 × 3 cm lung sample was excised and if septic arthritis was concurrently observed, then the diseased joints were sampled by swabbing, aspirating synovial fluid, or excising synovial tissue.

Tissue and fluid specimens were stored at –20 °C, and batch shipped by courier. Upon receipt, samples were stored at –80 °C until culturing. For each sample, the following metadata were recorded: date of sampling, type of sample (swab, tissue, joint fluid), anatomical location (nasopharynx, lung, joint), and disease status (healthy, diseased, dead).

3.3.2 Mycoplasma Culture and Isolation

Selective culture was performed on the DNP swabs and on swabs of the cut tissue surfaces as previously described by Jelinski et al. [12]. Due to the extended time span of sample collection, there were slight differences in isolation methods and media over the course of the study. Samples collected between 2006 to 2008 were cultured using Hayflick's media (made in-house), whereas in subsequent years samples were cultured using pleuropneumonia-like organism (PPLO) broth and agar (BD Difco, Fisher Scientific, Waltham, MA, USA) , supplemented with 10 g/L yeast extract (BD Diagnostic Systems, Fisher Scientific, Waltham, MA, USA), and 20% horse serum (Invitrogen, Fisher Scientific) [12,97]. Where specified, the

media was supplemented with 0.05% thallium (I) acetate, 500 U/mL penicillin G, and/or 0.5% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA).

Cultures derived from samples were filtered through 0.45 and 0.20 µm filters (Basix, VWR International, Radnor, PA, USA), and were used to inoculate PPLO broth with 0.05% thallium (I) acetate, 500 U/mL penicillin G, and 0.5% sodium pyruvate, and grown in a 5% CO_2 atmosphere with 75% humidity at 37 °C. Cultures with visible growth were streaked onto PPLO agar with 0.05% thallium (I) acetate and 500 U/mL penicillin G and incubated for 3-6 days. An isolated colony with characteristic "fried-egg" morphology was picked, replated on PPLO agar, and incubated for 72 h. Up to three individual colonies per sample were used to inoculate separate aliquots of PPLO broth with 0.05% thallium (I) acetate and 500 U/mL penicillin G. After 48 h of growth, each culture was separately stored in PPLO with glycerol (20%, v/v) at –80 °C. From the three possible cultures, a single culture was chosen to inoculate PPLO media for DNA extraction and antimicrobial susceptibility testing.

3.3.3 DNA Extraction and Identification

Mycoplasma bovis isolates were grown in PPLO media for 48 h and the genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The DNA was extracted following manufacturer's instructions for Gram negative bacteria with the final elution buffer replaced with 10 mM Tris (pH 8.5). Extracted genomic DNA was assessed for quality using gel electrophoresis and quantified fluorometrically using Qubit (Thermo Fisher Scientific, Waltham, MA, USA). Cultures were confirmed as *M. bovis*, based on confirmation of the presence of *uvrC* [35] and sequencing of the 16S rRNA gene [129]. The 16S rRNA amplicon was purified using a QIAquick PCR purification kit (Qiagen, Nevlo, Netherlands) and sent to Macrogen (Seoul, South Korea) for Sanger sequencing with the amplification primers. Forward and reverse sequences were assembled and edited using the Staden Package (version 1.6-r, http://staden.sourceforge.net/). The resulting sequences were compared to the National Center for Biotechnology Information (NCBI) nonredundant nucleotide database (nr) using BLASTn.

3.3.4 Whole Genome Sequencing and Assembly

Genomic DNA was prepared for sequencing using Illumina Nextera XT DNA Library Preparation (Illumina Inc., San Diego, CA, USA) and sequenced on a Illumina MiSeq platform

using the MiSeq v2 Reagent Kit to generate 250 bp paired-end reads. Illumina reads were trimmed using Trimmomatic v0.38 [147] with settings slidingwindow:5:15 leading:5 trailing:5 and minlen:50. Genomes were assembled with *M. bovis* PG45 as the reference genome (CP002188) using BWA-MEM v0.7.10-r789 [148] with default settings, producing SAM formatted assemblies. SAMtools [149] was used to convert the assemblies to BAM files and then sort and index for further processing. The Picard v2.18.4-SNAPSHOT [150] marked and removed duplicate reads from the BAM file. The Genome Analysis ToolKit v3.4-46-gbc02625 was used to perform local indel realignment and base quality score recalibration to improve the alignment quality, according to GATK best practices pipeline [151]. Consensus sequences for each assembly were created from each BAM file using bcftools [149]. This was performed by piping results from bcftools mpileup to bcftools call to create a vcf file for each BAM file. Each vcf file was used as input for vcfutils vcf2fq to generate a consensus fastq file. The fastq files were converted to fasta files using a bash script.

Genes encoding for 23S rRNA (*rrl3* and *rrl4*), L4 (*rplD*), and L22 (*rplV*) ribosomal proteins were identified using BLASTn [152] to compare *M. bovis* strain PG45 genes to the assembled genomes. For *rrl3* and *rrl4*, the closest matching sequence to the start of the genome being analyzed was identified as *rrl3*, the furthest as *rrl4*. As two start sites have been proposed for ribosomal protein L4, for the purposes of this study the position of *rplD* and overall numbering was based on locus ID MBOVPG45_0263. Extraction of gene sequences was performed using the start and ends of the match as reported by BLASTn for input to SAMtools faidx [149]. Genes of interest extracted from each isolate were aligned with the equivalent region in the *M. bovis* PG45 reference genome (CP002188.1) in Geneious Prime 2020.0.5 (https://www.geneious.com) using MUSCLE to identify SNPs with a minimum variant frequency of 0.01. For L4 and L22 ribosomal protein gene alignments, they were translated using the *Mycoplasma* spp. genetic code. To verify the nucleotide composition in *rrl3* and *rrl4* at positions within hairpin 35 in domain II and the peptidyl transferase loop in domain V within the MLS_B binding pocket [153], the SAM files were queried for ambiguity to determine the representative nucleotide(s). In cases of ambiguity, the percent of reads for a given allele was >20%. The raw paired reads for the isolates used in this study are available at NCBI SRA (www.ncbi.nlm.nih.gov/sra) with BioProject ID PRJNA642970.

The *M. bovis* sequences were aligned to their respective 23S rRNA (*rrlA*), L4 (*rplD*), or L22 ribosomal protein (*rplV*) genes isolated from the *E. coli* K12 substrain MG1655 genome to determine equivalent positioning to generate numbering to allow for comparison between different studies and bacterial species.

3.3.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility (AST) was determined using a microdilution assay, in a Sensititre™ (Trek Diagnostics, Oakwood, GA, USA) plate format and a customized panel designed to assess the antimicrobials most commonly used in North American feedlots for the treatment and control of BRD. The panel consisted of ten antimicrobials as described by Jelinski et al. [12], five of which were macrolides: tildipirosin (TIP; 0.12–128 µg/mL), gamithromycin (GAM; 0.25–256 µg/mL), tulathromycin (TUL; 0.25–256 µg/mL), tilmicosin (TIL; 1–256 µg/mL), and tylosin tartrate (TYLT; 1–128 µg/mL). AlamarBlue (ThermoFisher Scientific, DAL1100), a color redox indicator, was used to assess growth in each well based on a blue to pink color transition.

The AST procedure began by inoculating an *M. bovis* isolate previously stored at –80 °C in 20% glycerol into PPLO broth with 0.5% pyruvate and incubating for 72 h at 5% CO_2 with 75% humidity at 37 °C. Broth cultures were then subcultured into neat PPLO (without antibiotics) and incubated for 24 h. Following incubation, the optical density (OD) at 450 nm was determined using a NanoDrop One Spectrophotometer (Fisher Scientific, Waltham, MA, USA) and the culture adjusted to an $OD_{450} = 0.1$. The adjusted culture was diluted up to 100X, and 120 µL of the diluted culture used to inoculate 6 mL of PPLO in 2X alamarBlue. Each well of a Sensititre™ plate was inoculated to a final concentration of 10^3 to 5×10^5 CFU/mL with 50 µL of culture in 2X alamarBlue in 50 µL of media with each of antimicrobials within a series of Sensititre wells (final working concentration of alamarBlue: 1X; final well volume: 100 µL). Plates were sealed with a CO_2 permeable film, and incubated for 48–72 h. Minimum inhibitory concentrations (MICs) were determined by visual assessment of plates at 48 and 72 h, based on a blue to pink colour change. The *M. bovis* reference strain (*Mycoplasma bovis* ATCC® 25523™) was tested five times for quality control.

3.3.6 Clinical Breakpoints

As there are no established macrolide breakpoints for *M. bovis,* they were extrapolated from other members of the bacterial BRD complex (i.e., *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*) and human *Mycoplasma* spp., as described previously in Jelinski et al. [12]. The resistance breakpoints were ≥ 8 µg/mL for TIP, GAM, TIL, and TYLT, and ≥32 µg/mL for TUL.

3.3.7 Statistical Analysis

As *rrl3* and *rrl4* genes in the reference sequence for *M. bovis* PG45 differ by only a single nucleotide, alleles in each isolate could not be assigned to a specific locus. Instead, allele(s) at a given position were reported and isolates were grouped into genotypes according to the presence of mutation(s) in domain II and V. This created four 23S rRNA genotype groups: wildtype, Δ748 only, Δ748Δ2059, and Δ748Δ2060.

As phenotypically resistant and susceptible isolates were derived from cattle in each health status cohort (healthy, diseased, and dead), all isolates were analyzed together regardless of their source. Confidence intervals were calculated using the Wilson score interval method for estimating intervals for proportions. The confidence intervals were used to represent the antimicrobial resistance for a given 23S rRNA genotype using Epitools [154]. To assess the agreement in classification of resistance between the presence of a mutation in domain V of the 23S rRNA genotype and phenotype (MIC value), the Cohen's kappa statistic interpretation criteria (value, level of agreement): 0–0.20, none; 0.21–0.39, minimal; 0.40–0.59, weak; 0.60–0.79, moderate; 0.80–0.90, strong; > 0.90, almost perfect [155] were calculated using a commercial statistical program (SPSS 26, IBM SPSS Statistics version 26, IBM Corporation, Armonk, NY, USA). All descriptive statistics were calculated using a commercial spreadsheet software (Microsoft Excel version 15; Microsoft Corporation, Redmond, Washington, WA, USA).

3.4 Results

3.4.1 Culture and Reference Antimicrobial Susceptibilities

A total of 126 *Mycoplasma bovis* isolates were derived from 96 head of feedlot cattle from 21 feedlots over 12 production years, 2006 to 2018 (Table 3.1). Thirty head of cattle provided paired lung/joint isolates ($n = 60$), 14 provided a lung sample, 5 provided a joint isolate,

and 47 isolates came from the nasopharynx. Nasopharyngeal isolates were derived from healthy ($n = 30$), diseased ($n = 15$), and dead ($n = 2$) cattle. Phenotypically resistant isolates to the macrolides tested were derived from samples taken from healthy, diseased, and dead cattle (Table 3.2). Production years were used to define the sampling cohort, as animals often enter the feedlot in the fall and remain until the following calendar year. Thus, the 2018 production year included samples obtained between 1 November 2018 and 30 June 2019.

Mycoplasma bovis PG45 (*Mycoplasma bovis* ATCC® 25523) was resequenced and possessed the equivalent 23S rRNA genotype at positions 748, 2059, and 2060, as reported in the published reference genome [76, CP002188.1]. Compared to the published reference genome, no nonsynonymous mutations in L4 and L22 ribosomal proteins were observed in this resequenced isolate. The following minimum inhibitory concentration (MIC) values, defined as the lowest concentration of antimicrobial to visibly inhibit growth, were determined from AST of five PG45 replicates: GAM, 8–16; TIP, 4–8; TIL, 1; TUL, 0.25; and TYLT, 1–2 µg/mL. Due to these genotypic and phenotypic findings, *M. bovis* PG45 was considered to be a susceptible wildtype isolate.

Table 3.1 *Mycoplasma bovis* isolates ($n = 126$) by anatomical location, health status, and production year.

	Production Year								
	2006	**2007**	**2008**	**2014**	**2015**	**2016**	**2017**	**2018**	**Total**
Anatomical Location									
Joint		1	1	1	1	14	11	6	35
Lung				3	3	17	15	6	44
Nasopharynx	5	28					9	5	47
Total	5	29	1	4	4	31	35	17	126
Health Status									
Healthy	2	14					9	5	30
Diseased	3	12							15
Dead		3	1	4	4	31	26	12	81
Total	5	29	1	4	4	31	35	17	126

Table 3.2 Number of *Mycoplasma bovis* isolates ($n = 126$) with a resistant (R) or susceptible (S) phenotype by health status.

Health Status		Phenotype (% Resistant)					
		GAM	**TIL**	**TIP**	**TUL**	**TYLT**	**Total**
Healthy	R/S	19/11	30/0	30/0	11/19	22/8	30
	(%R)	(63.3)	(100)	(100)	(36.7)	(73.3)	
Diseased	R/S	9/6	15/0	15/0	9/6	11/4	15
	(%R)	(60.0)	(100)	(100)	(60.0)	(73.3)	
Dead	R/S	78/3	80/1	81/0	72/9	78/3	81
	(%R)	(96.3)	(98.8)	(100)	(88.9)	(96.3)	
Total	R/S	106/20	125/1	126/0	92/34	111/15	126
	(%R)	(84.1)	(99.2)	(100)	(73.0)	(88.1)	

GAM—gamithromycin, TIL—tilmicosin, TIP—tildipirosin, TUL—tulathromycin, and TYLT—tylosin.

3.4.2 Genome Sequencing and Assembly

Draft genomes of the 126 isolates were assembled from an average 210113 paired reads (range: 55951 to 414042); average read length of 217 bp (range: 166 to 233 bp). This produced assemblies with an average N50 of 18690 bp (range: 1780 to 34113 bp), an average coverage depth of 45.3 (range 12.2 to 89.1), and an average of 579 contigs (range: 171 to 1639).

3.4.3 23S rRNA gene

Among the 126 isolates analyzed, mutations (single nucleotide polymorphisms, SNPs) were located in hairpin 35 of domain II (nucleotide 748; E. coli numbering used throughout) and the peptidyl transferase loop of domain V (nucleotide 2059 and 2060) of the 23S rRNA (rrl) gene alleles. The 23S rRNA genotype was assigned based on alleles observed at position 748, 2059, and 2060 (Table 3.3). As there are up to two copies of the rrl gene reported for *M. bovis*, an isolate was categorized as having a change (Δ) if a mutation occurred in at least one copy of the gene. The *M. bovis* PG45 reference genome was considered as the reference (wildtype) and two isolates (1.6%) were identical to this 23S rRNA genotype. Most isolates (73.0%; 92/126) had mutations in domains II and V (Δ748Δ2059 or Δ748Δ2060); whereas, 25.4% (32/126) had a single mutation in domain II (Δ748). There were no isolates with lone mutations in domain V.

All isolates with a single mutation at Δ748 were susceptible to TUL (MIC ≤ 16 μg/mL); resistant to TIP and TIL (MIC ≥ 8 μg/mL); and either susceptible (MIC ≤ 4 μg/mL) or resistant (MIC ≥ 8 μg/mL) to GAM and TYLT (Figure 3.1a). Isolates that had accumulated mutations in both domain II and V (Δ748Δ2059 or Δ748 Δ2060) were resistant to all five macrolides (Table 3.3 and Figure 3.1b).

No dose dependent effect was apparent within a given genotype (i.e., Δ748 only) for those with a single mutant allele (i.e., G748A) or a combined mutant/wildtype allele (i.e., G748, G748A). The MIC values for isolates with Δ748 only 23S rRNA genotype, with a single mutant allele, ranged from 1 to 128 μg/mL for GAM and TYLT, and 0.25 to 8 μg/mL for TUL. Isolates with combined alleles had consistently lower MIC values of 8, 8–16, and 0.25 μg/mL for GAM, TYLT, and TUL, respectively, which were within the MIC range for isolates with a single mutant allele. For isolates with the Δ748Δ2059 23S rRNA genotype, those with combined alleles had MIC values ranging from 8 to ≥ 128 μg/mL for TYLT compared to ≥ 128 μg/mL with a single mutant allele. Regardless of allelic composition, the MIC values for isolates with Δ748Δ2060 23S rRNA genotypes were ≥ 64 μg/mL.

Table 3.3 Comparison of 23S rRNA genotypes and the number (%) of *Mycoplasma bovis* isolates ($n = 126$) resistant to the five macrolides tested.

Genotype	23S rRNA gene alleles[+]			Percent (n) of isolates	Phenotype[#] (% Resistant)				
	Domain II	Domain V			GAM	TIL	TIP	TUL	TYLT
	748	2059	2060						
Wildtype*	**G748**	**A2059**	**A2060**	**1.6 (2)**	**1 (50)**	1 (50)	2 (100)	0 (0)	0 (0)
	Total			1.6 (2)	1 (50)	1 (50)	2 (100)	0 (0)	0 (0)
Δ748 only	G748, G748A[‡]			4.0 (5)	5 (100)	5 (100)	5 (100)	0 (0)	5 (100)
	G748A			21.4 (27)	8 (29.6)	27 (100)	27 (100)	0 (0)	14 (51.9)
	Total			25.4 (32)	13 (40.6)	32 (100)	32 (100)	0 (0)	19 (59.4)
Δ748Δ2059	G748, G748A[‡]	A2059, A2059G[‡]		0.8 (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	G748A	A2059, A2059G[‡]		2.4 (3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
	G748A	A2059, A2059C[‡]		2.4 (3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
	G748A	A2059, A2059T[‡]		7.1 (9)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
	G748A	A2059G		49.2 (62)	62 (100)	62 (100)	62 (100)	62 (100)	62 (100)
	Total			61.9 (78)	78 (100)	78 (100)	78 (100)	78 (100)	78 (100)
Δ748Δ2060	G748A		A2060, A2060C[‡]	0.8 (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	G748A		A2060C	7.9 (10)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)
	G748A		A2060G	2.4 (3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
	Total			11.1 (14)	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)

* *Mycoplasma bovis* PG45 is designated as wildtype genotype. [#] GAM—gamithromycin, TIL—tilmicosin, TIP—tildipirosin, TUL—tulathromycin, and TYLT—tylosin. [‡] Representative of a combined wildtype and mutant allele.[+] Positioning of the alleles is based on *Escherichia coli* numbering.

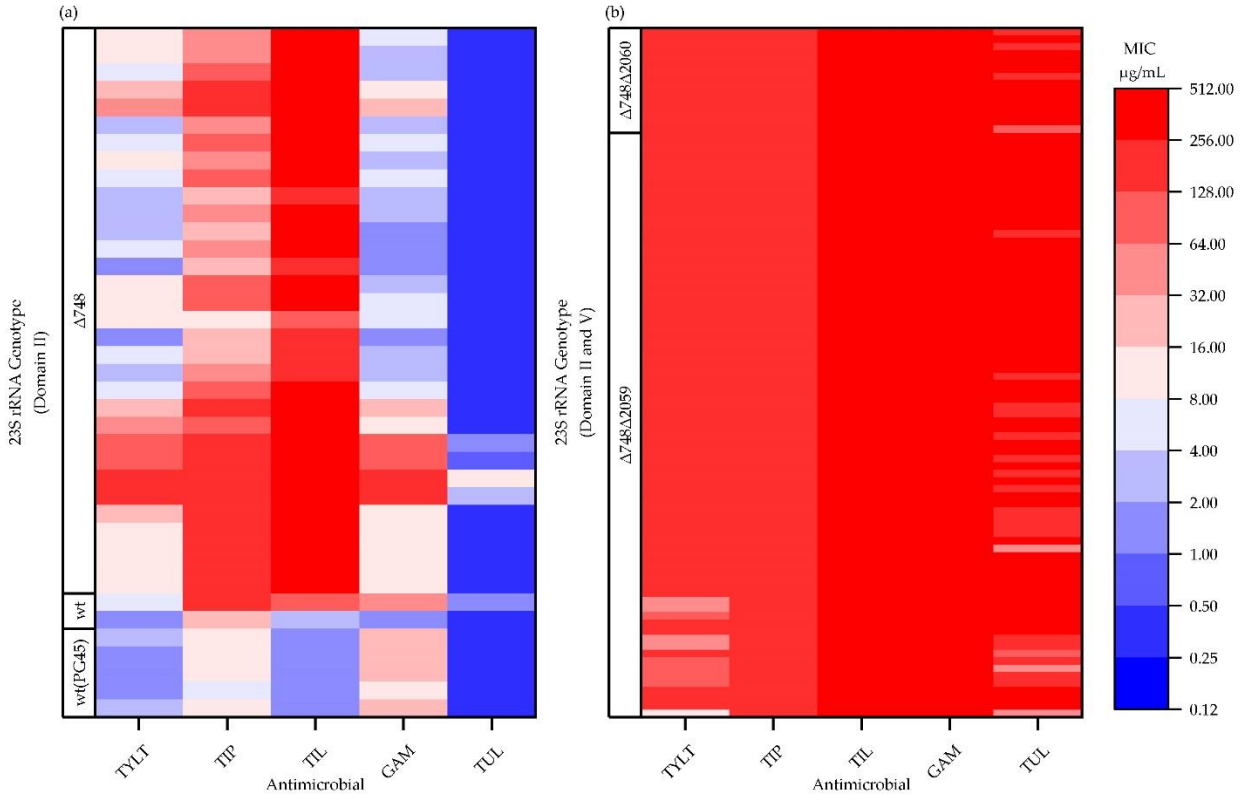

Figure 3.1 Minimum inhibitory concentrations (MIC) of *Mycoplasma bovis* isolates ($n = 126$) for tylosin (TYLT), tilmicosin (TIL), tildipirosin (TIP), gamithromycin (GAM), and tulathromycin (TUL), and the corresponding 23S rRNA genotype: a) wildtype (wt) or mutations in domain II only (Δ748); b) mutations in domain II and V (Δ748Δ2059, Δ748Δ2060). The MIC values for the five *M. bovis* PG45 replicates [wt(PG45)] are presented. TYLT, TIL, and TIP have a 16-membered core structure; whereas, GAM and TUL have a 15-membered core structure

The 23S rRNA genotypes were grouped based on the presence of mutations in domain II only versus domain II and V. The results were reported as % resistant with 95% confidence interval (95% CI) for a proportion (Table 3.4). The two isolates with wildtype 23S rRNA genotypes were susceptible to TUL and TYLT (0, 0–0.66), and 1 of 2 were resistant to GAM and TIL (0.5, 0.09–0.91). Isolates with mutations in domain II only (Δ748 only) had a similar proportion of isolates resistant to GAM (0.41, 0.26–0.58) and TYLT (0.59, 0.42–0.74) compared to TUL (0, 0–0.11). An additional mutation in domain V at positions 2059 (Δ748Δ2059) or 2060 (Δ748Δ2060) resulted in all isolates being resistant to all five macrolides. All isolates were resistant to TIP, regardless of genotype.

Table 3.4 Number and proportion of *Mycoplasma bovis* isolates ($n = 126$) resistant (R) or susceptible (S) by 23S rRNA genotype. The 95% binomial proportion confidence interval (Wilson score) is an interval estimate of the probability of the isolate being resistant if it has a particular 23S rRNA genotype.

		23S rRNA Genotype[+]			
		Wildtype	**Δ748 only**	**Δ748Δ2059**	**Δ748Δ2060**
TUL	R/S	0/2	0/32	78/0	14/0
Proportion (95% CI)		0 (0 – 0.66)	0 (0 – 0.11)	1 (0.95 – 1)	1 (0.78 – 1)
GAM	R/S	1/1	13/19	78/0	14/0
Proportion (95% CI)		0.50 (0.09 – 0.91)	0.41 (0.26 – 0.58)	1 (0.95 – 1)	1 (0.78– 1)
TYLT	R/S	0/2	19/13	78/0	14/0
Proportion (95% CI)		0 (0 – 0.66)	0.59 (0.42 – 0.74)	1 (0.95 – 1)	1 (0.78 – 1)
TIL	R/S	1/1	32/0	78/0	14/0
Proportion (95% CI)		0.50 (0.09 – 0.91)	1 (0.89 – 1)	1 (0.95 – 1)	1 (0.78 – 1)
TIP	R/S	2/0	32/0	78/0	14/0
Proportion (95% CI)		1 (0.34 – 1)	1 (0.89 – 1)	1 (0.95 – 1)	1 (0.78 – 1)
Total		2	32	78	14

GAM—gamithromycin, TIL—tilmicosin, TIP—tildipirosin, TUL—tulathromycin, and TYLT—tylosin.[+] Positioning of the alleles is based on *Escherichia coli* numbering.

The level of agreement in the classification of resistance between the presence of a mutation in domain V in the 23S rRNA genotype and phenotype (MIC values) varied by macrolide. The kappa correlation coefficient was perfect (1.000) for TUL, moderate (0.676) for GAM, weak (0.536) for TYLT, essentially nonexistent (0.042) for TIL, and could not be determined for TIP because all isolates were resistant regardless of the genotype. Despite these differences, all isolates with a mutation in domain V of the 23S rRNA genotype (Δ748Δ2059 and Δ748Δ2060) were resistant to all macrolides. However, mutations in domain V also occurred in the presence of a mutation in domain II at position 748.

3.4.4 L4 and L22 Ribosomal proteins

All isolates had a nonsynonymous mutation Gln93His (*M. bovis* PG45 number; equivalent to Gln90His using *E. coli* numbering) in the L22 ribosomal protein. There were multiple nonsynonymous L4 mutations: Ser18Thr, Thr43Ala, Ala44Thr, Glu50Thr, Ala51Thr, Ala51Ser, Ser55Ala, Thr57Ala, Val69Ala, Ala70Thr, Glu75Ala, Ala86Thr, and Ala101Thr (*M. bovis* PG45 numbering) with three different nonsynonymous mutations at two positions in

proximity to the MLSB binding pocket Gly185Arg, Gly185Ala, Thr186Pro (*M. bovis* PG45 numbering; equivalent to position 64 and 65 using *E. coli* numbering, respectively).

Twelve isolates had a nonsynonymous mutation in the L4 ribosomal protein in residues proximal to the MLSB binding pocket. Four had two nonsynonymous mutations Gly185Ala and Thr186Pro, and eight had a single nonsynonymous mutation Gly185Arg (Table 3.5). All isolates had Gln93His mutations in L22 as well. All isolates with the two nonsynonymous mutations (Gly185Ala and Thr186Pro) also had mutations in domain II of the 23S rRNA gene (Δ748). The eight isolates with a single nonsynonymous mutation (Gly185Arg) had various 23S rRNA genotypes: wildtype ($n = 1$), Δ748 ($n = 1$), and Δ748Δ2059 ($n = 6$). Overall, isolates with a Δ748 mutation in the 23S rRNA gene and mutations in L4 and L22 near the MLSB binding pocket were resistant (MICs ≥16 µg/mL) to GAM, TIL, TIP and TYLT; but susceptible (MICs ≤8 µg/mL) to TUL.

Table 3.5 Presence of ribosomal protein mutations in different 23S genotype groups and corresponding minimum inhibitory concentrations (MIC) values.

23S rRNA Genotype[+]	MIC (µg/mL)					Ribosomal Proteins[‡]	
	GAM	TIL	TIP	TUL	TYLT	L4	L22
wildtype (PG45)	8–16	1	4–8	0.25	1–2	Gly185, Thr186	Gln93
wildtype	32	64	>128	1	4	Gly185Arg	Gln93His
Δ748	128	>256	>128	2	128	Gly185Ala, Thr186Pro	Gln93His
	128	>256	>128	8	128	Gly185Ala, Thr186Pro	Gln93His
	64	>256	>128	0.5	64	Gly185Ala, Thr186Pro	Gln93His
	64	>256	>128	1	64	Gly185Ala, Thr186Pro	Gln93His
	16	256	128	0.25	32	Gly185Arg	Gln93His
Δ748Δ2059	>256	>256	>128	>256	>128	Gly185Arg	Gln93His
	>256	>256	>128	256	>128	Gly185Arg	Gln93His
	>256	>256	>128	>256	>128	Gly185Arg	Gln93His
	>256	>256	>128	128	64	Gly185Arg	Gln93His
	>256	>256	>128	128	64	Gly185Arg	Gln93His
	>256	>256	>128	32	64	Gly185Arg	Gln93His

[+] Positioning of the alleles is based on *Escherichia coli* numbering.

[‡] Positioning of amino acids is based on *Mycoplasma bovis* PG45 numbering.

3.5 Discussion and Conclusion

This study was unique in that it assessed the concordance between the genotypes and phenotypes of *M. bovis* for antimicrobial resistance (AMR) to five macrolides used to control and treat bovine respiratory disease in feedlot cattle. Of note was the inclusion of TUL, which is the most commonly used antimicrobial for BRD prophylaxis, but a pharmaceutical that has not been assessed in previous genotype–phenotype AMR studies [22,67,69,71]. This is salient because even though macrolides have a similar antibacterial mode of action, they differ in the size of the macrocyclic lactone ring and associated side-chains [59]. As a result, each macrolide has a slightly different binding affinity for domains II and V of 23S rRNA. Thus, one or more mutations within these domains may lead to very different antimicrobial susceptibility testing (AST) results. Exemplars are TUL, TIL, and TIP, where a single mutation in domain II (Δ748) conferred resistance to TIL and TIP, but not to TUL. This is consistent with the modeling of the *E. coli* ribosome, wherein TUL interacts primarily at A2058 of 23S rRNA, but is too small to

span the ribosomal tunnel and interact with G748 in domain II [59]. This finding is of interest because previous genotype studies did not include TUL.

Within the 23S rRNA gene, mutations in domain V occurred at position 2059 or 2060, but not both. In contrast, Lerner et al. [71] identified two isolates with mutations in both *rrl* alleles in domain V, but at different positions (2058 and 2059). Furthermore, others have reported mutations at position 2058 in *M. bovis* [69,71,156], an outcome that was not found in the current study. Isolates with differing alleles at a given position in domain V were resistant to all five macrolides, which is consistent with a previous study in which *Mycoplasma* spp. having a heterozygous mutation in domain V conferring resistance [138]. Additionally, mutations at position 2060 have been reported in *M. bovis* isolates that are resistant to lincomycin [67], an antimicrobial with a mechanism of action similar to macrolides [20,135]. These differences in position, albeit in close proximity to one another, could reflect differences in the selective pressure of specific antimicrobials as a result of differences in use across production systems. Despite these differences, the increased resistance of *M. bovis* to macrolides as a result of mutations in domain II and domain V is consistent with previous reports [22,67,69,71].

Overall, concordance was observed between 23S rRNA genotype and AMR phenotype, which highlights the utility of molecular targets as a viable alternative to in vitro AST. Isolates with combined mutations in domain II and V binding sites of 23S rRNA gene (Δ748Δ2059 and Δ748Δ2060) all demonstrated resistance to TUL, GAM, and TYLT. Whereas regardless of genotype, >99% of all isolates were resistant to TIP and TIL. The accumulation of SNPs in domain II and V of the 23S rRNA gene and the relationship to increasing MIC values, and therefore inferred resistance, has been reported for TYLT and TIL in *M. bovis* by Hata et al. [22]. Lui and Douthwaite [157] also demonstrated that monomethylation at positions G748 and A2058 acted synergistically to increase TYLT resistance. In both the Lerner et al. [71] study and the current study, the existence of mutations in both the II and V domains correlated with MICs for TYLT and TIL that were indicative of clinical resistance. However, it has also been reported that some *M. bovis* isolates with elevated MICs to TYLT and TIL only have a mutation in domain V, without a concurrent mutation at position 748 [69,71], while others had a change at 748 without a mutation in domain V [67].

Given that TIP and TIL are both derivatives of TYLT, the similarities in the level of resistance to these macrolides is not surprising. These three macrolides vary in the groups that decorate C5, C6, and C14 of their shared 16-membered core structure. As high levels of resistance (>99%) to both TIL and TIP was present, it was difficult to correlate phenotype and genotype. However, as per previous reports [11,13,17,18] the very high MIC values for these two antimicrobials indicate that they are unsuitable for treating mycoplasmosis in cattle.

The associations of mutations in the L4 and L22 ribosomal proteins with susceptibility phenotypes were less clear than those of domains II and V of the 23S rRNA gene. Zhao et al. [158] reported that mutations in these ribosomal proteins lead to increased macrolide resistance in *M. pneumonia*. In the current study, mutations in L4 and L22 were associated with elevated MICs for GAM, TYLT, TIP, and TIL. Given that these ribosomal proteins form the narrowest constriction of the protein exit tunnel [159], with both having loops that extend adjacent to macrolide binding sites [160], the presence of mutations is consistent with the AST phenotypes. All isolates (n = 126) also had mutations in L22 relative to the type strain, a result more prevalent than reported by Lerner et al. [71], where the nonsynonymous mutation Gln90His (*E. coli* numbering) in L22 was observed in 75% of isolates. Again, these differences across studies are likely related to increased antimicrobial selection pressure placed on the western Canadian isolates.

There was a very low prevalence of the *M. bovis* type strain PG45 genotype (1.6%) in this study compared to Hata et al. [22], who observed this genotype in 12.3% of 203 bovine isolates from Japan. Lerner et al. [71] found that this genotype in about half of the 54 isolates from cattle originating in the Middle East, Europe, and Australia. Variation in the proportion of wildtype *M. bovis* isolates circulating within cattle populations is undoubtedly related to differences in cattle production systems and antimicrobial use. In western Canada, most beef calves are weaned in the fall of the year and sold at auctions where they are commingled with cohorts from other farms. These newly weaned calves are then transported to feedlots where they are processed on-arrival. In addition to these stressors, these events occur when the weather canalso be inclement. Therefore, calves deemed to be at high-risk of developing BRD are administered macrolides, often TUL, on-arrival. Our data indicate that over time this practice has selected against wildtype genotypes and for the emergence of macrolide resistant genotypes.

Significantly, not only has macrolide resistance in western Canadian feedlot cattle been increasing over the last few decades,it is also not uncommon to recover macrolide resistant *M. bovis* isolates from the nasopharynx of healthy cattle at feedlot arrival [12]. While feedlots could rotate macrolides with tetracyclines or florfenicol, as a strategy to reduce resistance, this practice requires timely AST data or otherwise it may exacerbate antimicrobial resistance.

The study had a number of potential weaknesses. The wildtype 23S rRNA genotype essentially served as a control group; however, there were only two isolates in this group. This was unfortunate since one of two wildtype isolates were resistant to GAM and TIL, and both resistant to TIP. Additionally, control testing of healthy animals was not performed at the time of sampling diseased or dead animals. However, this study was not intended as a survey of macrolide susceptibility, but rather an investigation of the relationship between genotype and phenotype. Therefore, the healthy animals were sampled with the intent of culturing phenotypically susceptible isolates to serve as a basis of comparison to the abundance of resistant isolates derived from dead cattle. The other weaknesses were that the isolates were not uniformly spread over all 12 production years, and most isolates came from dead animals that had received extensive antimicrobial therapy prior to death. The study, however, also had some notable strengths. The relatively large number of isolates came from cattle that were sourced from a broad geographic area; samples were collected over 12 production years; from multiple anatomical locations; and from healthy, diseased and dead cattle.

Conventional antimicrobial susceptibility testing for *M. bovis* is time-consuming and technically demanding, making it unsuitable for monitoring antimicrobial resistance in real-time within a feedlot. This is an issue because prudent use guidelines for antimicrobial use are predicated on AST. This study, and others, have shown a clear linkage between genotypes and macrolide resistance, providing an avenue for developing a rapid, accurate, and cost-effective molecular based test for *M. bovis*, similar to what has been done for *Mycoplasma genitalium* [139,161,162]. This test could be used to assess *M. bovis* isolates obtained from nasopharyngeal swabs, or for conducting pen-level AST surveillance by testing isolates found in shared watering bowls.

Given that antimicrobials are the primary preventative and therapeutic tool to combat *M. bovis* infections in feedlot cattle, ongoing assessment of antimicrobial susceptibility remains

crucial to maintaining the utility of these drugs and facilitating antimicrobial stewardship practices. However, the comparatively slow growth of *M. bovis* yields longer turn-around times when exclusively using culture-based methods of assessment, which can impede timely decision making on antimicrobial use. In our study, we were able to identify mutations in domains II and V of the 23S rRNA genes that are associated with increased resistance to macrolides which show a clear linkage between genotype and phenotypic macrolide resistance (AST). These findings add strong support for utilizing rapid, accurate, and cost-effective molecular based tests for assessing the susceptibility of *M. bovis* to macrolides.

Not only can WGS be used to investigate genotypes associated with AMR, but it is amenable to various *in silico* typing methods. As the ability to categorize and differentiate isolates is the cornerstone of epidemiological surveillance [120], the study outlined in Chapter 4 utilizes four *in silico* genotyping methods (MLST, cgMLST, cgSNV, wgSNV) to assess the phylogenetic relationship of isolates and the ability to resolve phenotypic differences based on genotype.

4 APPLICATION OF FOUR GENOTYPING METHODS TO *MYCOPLASMA BOVIS* ISOLATES DERIVED FROM WESTERN CANADIAN FEEDLOT CATTLE

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Author Contributions: Conceptualization, A.K., M.W., M.D.J. ; Methodology, A.K, M.W., R.Z., K.R. M.D.J.; Visualization, A.K., M.W., T.A.M., R.Z., M.D.J. ; Validation, M.W., Formal Analysis, A.K, M.W., R.Z. ; Investigation, A.K, M.D.J. ; Resources, M.D.J., T.A.M. ; Data Curation, A.K,. M.W. ; Writing—Original Draft Preparation, A.K., M.W. ; Writing—Review and Editing, A.K., M.W., T.A.M., R.Z., K.R., M.D.J. ; Supervision, M.D.J., T.A.M. ; Investigation, T.A.M. ; Project Administration, T.A.M, M.D.J. ;Funding Acquisition, M.D.J., T.A.M.

This chapter has been accepted in the peer-reviewed *Journal of Clinical Microbiology* and published as an open-access journal article. The manuscript is included in its entirety, but has been reformatted for the purposes of this thesis.

Citation: **Kinnear, A**.; Waldner, M.; McAllister, T.A.; Zaheer, R.; Register, K.; Jelinski, M. Application of Four Genotyping Methods to *Mycoplasma bovis* Isolates Derived from Western Canadian Feedlot Cattle. *J. Clin. Microbiol.* **2021**, 59, doi:10.1128/JCM.00044-21.

4.1 Abstract

Mycoplasma bovis is a significant pathogen of feedlot cattle, responsible for chronic pneumonia and polyarthritis syndrome (CPPS). *M. bovis* isolates ($n = 129$) were used to compare four methods of phylogenetic analysis, and to determine if the isolates' genotypes were associated to phenotypes. Phenotype (metadata) included the health status of the animal from which an isolate was derived (healthy, diseased, dead); anatomical location (nasopharynx, lung, joint); feedlot; and production year (2006 to 2018). Four i*n-silico* phylogenetic typing methods were used in the analyses: multilocus sequence typing (MLST), core genome MLST (cgMLST), core genome single nucleotide variant (cgSNV) analysis, and whole genome SNV (wgSNV) analysis. Using Simpson's Diversity Index (D) as a proxy for resolution, MLST had the lowest resolution (D = 0.932), with cgSNV (D = 0.984) and cgMLST (D = 0.987) generating comparable results, while wgSNV (D = 1.000) provided the greatest resolution. Visual inspection of the minimum spanning trees found that the memberships of the clonal complexes and clades shared a similar structural appearance using cgMLST, cgSNV and wgSNV analyses. Although MLST had the lowest resolution, this methodology was intuitive, easy to apply, and the PubMLST database facilitates comparison of sequence types across studies. The cg methods had improved resolution over the MLST and the graphical interface software was user-friendly for non-bioinformaticians, but the proprietary software is relatively costly. wgSNV was the most robust for processing poor quality sequence data, while offering the highest resolution, but application of its software required specialized training. None of the four methods could associate genotypes to phenotype.

4.2 Introduction

Mycoplasma bovis is associated with a plethora of diseases in cattle, with reviews on this subject commonly emphasizing its role in chronic pneumonia, mastitis, and arthritis [4,6,45] . While the incidence of *M. bovis*-mastitis in Canada is relatively low [163,164], it is considered an emerging disease of dairy cattle [165]. The incursion of *M. bovis* into New Zealand, and the country's considerable efforts to eradicate this organism, underscores its potential to cause significant economic losses to the dairy industry. In the feedlot industry, *M. bovis* is associated with bovine respiratory disease (BRD) and chronic pneumonia and polyarthritis syndrome (CPPS) [3]. However, its role in the pathogenesis of BRD has been equivocal. In the early 1990s, it was hypothesized that *M. bovis* was unlikely to be a primary pathogen, but rather it potentiates

the pathogenesis of other bacterial BRD agents [166, 167]. It is now considered a primary pathogen of pneumonia in preweaned calves [99], and an opportunistic pathogen of BRD in feedlot cattle [3,6,45]. Cattle are frequently asymptomatic carriers, with the organism being recovered from the nasal passages of healthy cattle [99,168, 169]. It is hypothesized that stressors such as transport, commingling, and adverse climactic conditions trigger a stress response, resulting in elevated levels of glucocorticoids that may impair the immune response, allowing for increased bacterial shedding and clinical disease [170].

A number of molecular techniques have been used for typing *M. bovis* isolates. Formative techniques include pulsed field gel electrophoresis (PFGE) [2,171,172], random amplified polymorphic DNA (RAPD) [2,173], and amplified fragment length polymorphism (AFLP) [2,174]. These methods have been largely replaced by PCR-based methods such as multilocus sequence typing (MLST) and multiple loci variable number tandem repeat analysis (MLVA) (175,176,177). While MLVA has greater discriminatory power than MLST [176], the latter method isunambiguous, reproducible and scalable for characterizing isolates of bacterial species using universally acceptable schema [178; https://pubmlst.org/). These attributes make MLST well-suited for epidemiological studies that span multiple research laboratories, and for comparing isolates over time [122,123,176,179].

Core genome MLST (cgMLST) is an extension of MLST, but provides higher resolution. Whereas a typical MLST scheme uses the alleles of seven house-keeping genes, cgMLST analyzes alleles from hundreds of genes, an approach facilitated by whole genome sequencing (WGS). cgMLST has been used for typing poultry mycoplasmas [125], investigating outbreaks of *M. bovis* in dairy cattle [111,180], and examining the genetic relatedness and evolution of isolates obtained from cattle in Denmark and neighbouring countries [181]. Authors of the Denmark study noted that cgMLST and WGS-typing techniques offered greater discriminatory power than MLST, and hence may become the new standard in phylogenetic typing. However, these methods do have disadvantages, namely cost, time, and the need for technical expertise for conducting the analyses and interpreting the results.

A further progression into higher resolution strain typing involves genome-wide comparisons of single nucleotide variants (SNVs). This method can be applied to SNVs in the core (cgSNV) or whole genome (wgSNV). Using WGS, Australian researchers determined that

75 *M. bovis* isolates collected between 2006 and 2015 were of the same lineage, suggesting few, if any, incursions of new strains over the study duration. Similarly, an Israeli study used cgSNV to evaluate the genomic diversity of *M. bovis* isolates from mastitis cases between 1994 and 2017, and compared these to BRD isolates from local feedlot cattle and from calves imported from Europe and Australia [182]. There was a clear genetic distinction between the isolates from Europe and Australia, with a dominant genotype associated with mastitis. wgSNV was also used to compare the relatedness of 250 *M. bovis* isolates originating from seven countries [183]. These isolates formed six clades, with USA isolates exhibiting the greatest genetic diversity and clustering with Canadian isolates.

The objective of this study was to assess the level of concordance between four different molecular genotyping methods (*in silico* MLST, cgMLST, cgSNV, wgSNV), using a dataset of 129 *M. bovis* isolates. Secondly, to determine if one or more methods could resolve genotypic differences among isolates derived from cattle of varying health status (healthy, sick and dead cattle), from different anatomical locations (nasopharynx, lungs, and joints), different feedlots, and over a 12-year period (2006 to 2018).

4.3 Materials and Methods

4.3.1 Sample Collection

A series of cross-sectional studies, spanning the years 2006 to 2018, provided 129 *M. bovis* isolates, which were recovered from the nasopharynges, lungs, and joints of feedlot cattle, as previously described [12]. Five of these isolates were recovered from cattle imported from Idaho, USA, while the rest originated in western Canada. All deep nasopharyngeal (DNP) swabs were obtained from healthy and morbid cattle. The animal's health status was determined by the timing of the disease and presentation of clinical signs; BRD is the most common disease of feedlot cattle, with cases peaking within 21 days post-arrival to the feedlot. Thus, a putative BRD diagnosis was based upon the timing of disease, and a constellation of the clinical signs consistent with this disease (i.e. febrile, depressed, nasal discharge, dyspnea). Health status was determined by trained feedlot personnel. The DNP swabs from live cattle were obtained in accordance with Animal Use Protocols (#20070023; #20170021) approved by the University of Saskatchewan's Animal Research Ethics Board and Lethbridge Research Center's Animal Care Committee (#1641). Lung and joint samples were obtained at the time of postmortem

examination from cattle having gross pathological findings consistent with a caseonecrotic bronchopneumonia and, in some instances, concurrent septic arthritis.

4.3.2 Culture and isolation

Small changes in media and isolation methods occurred over the 12-year period, with the 2006 to 2008 isolates being cultured in Hayflick's medium (prepared in-house) [184], and all subsequent isolates cultured with pleuropneumonia-like organism (PPLO) broth and agar (BD Difco, Fisher Scientific, Waltham, MA, USA). The PPLO media were supplemented with 10 g/L yeast extract (BD Diagnostic Systems, Fisher Scientific, Waltham, MA, USA) and 20% horse serum (Invitrogen, Fisher Scientific), as previously described [12]. Supplemented media also contained 0.05% thallium (I) acetate, 500 U/mL penicillin G, and/or 0.5% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA).

DNP samples and swabs of fresh cut tissues were used to inoculate PPLO starter cultures. Cultures were serial filtered through 0.45 and 0.20 µm filters (Basix, VWR International, Radnor, PA. USA) to remove other bacteria such as co-infecting BRD pathogens (0.45 µm) and select for M*ycoplasma* spp. (0.20 µm). Filtrates were inoculated into supplemented PPLO broth, and grown in a 5% CO_2 atmosphere with 75% humidity at 37°C. Culture growth was visualized by agitating the culture tube to elicit a perceptible mass of organisms at the bottom of the tube. Cultures with visible growth were subcultured onto PPLO agar and incubated for 3-6 days. Single colonies exhibiting a "fried-egg" morphology were picked and plated on PPLO agar, and incubated for 72 h. One to three individual colonies per culture were used to inoculate separate aliquots of PPLO broth. After 48 h, each culture was stored in PPLO with 20% (v/v) glycerol at -80°C. A single culture was chosen to inoculate PPLO medium for DNA extraction.

4.3.3 DNA extraction and identification

Isolates were grown in PPLO medium for 48 h and genomic DNA extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), per manufacturer's instructions, with the exception that the final elution buffer was replaced with 10 mM Tris (pH 8.5). The extracted genomic DNA was assessed for quality using gel electrophoresis and quantified fluorometrically using a Qubit analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The isolation of high molecular weight DNA with a yield ≥1 ng/µL was sufficient to proceed. Cultures were confirmed as *M. bovis* by the presence of the *uvrC* gene via PCR [35] and sequencing of the V3-

V4 16S rRNA gene [129]. The 16S rRNA amplicon was purified using a QIAquick PCR kit (Qiagen, Nevlo, Netherlands) and submitted for Sanger sequencing (Macrogen, Seoul, South Korea). Forward and reverse sequences were assembled and edited using the Staden Package (version 1.6-r, http://staden.sourceforge.net/), and compared to the National Center for Biotechnology Information (NCBI) nonredundant nucleotide database (nr) using BLASTn run with default settings; species was assigned based on the highest match identity [185].

4.3.4 Whole genome sequencing and assembly

Sequencing libraries of genomic DNA were prepared using an Illumina Nextera XT DNA Library Preparation kit (Illumina Inc., San Diego, CA, USA), and sequenced on an Illumina MiSeq platform using the MiSeq v2 Reagent Kit to generate 250 bp paired-end reads. The raw paired-end reads are available from SRA under BioProject IDs PRJNA642970 and PRJNA708306. Genomes were assembled for the MLST, cgMLST, and cgSNV methods using Ridom SeqSphere+ in pipeline mode [186]. Raw paired-end reads were imported and preprocessed by down-sampling to 180X coverage and trimming at the 5' and 3' ends until an average quality of 30 in a window of 20 bases was achieved. Reads were assembled using SKESA [187]. Genome assembly for the wgSNV method was performed using Trimmomatic v0.39 [147] for read trimming and SPAdes v3.14.1 [188] for *de novo* assembly of the contigs. Trimmomatic was run with settings slidingwindow:5:15, leading:5, trailing:5, and minlen:25, and SPAdes was run with settings -careful and -k 127. Contigs with less than 1000 nucleotides were removed from the analysis.

4.3.5 Genotyping methods

De novo assemblies were used to assign allelic profiles and STs as per the PubMLST reference method (https://pubmlst.org/mbovis/). The *M. bovis* PG45 reference genome (NC_014760.1) was included in each genotyping method with the 129 isolates. The MLST scheme included alleles of the following genes: *dnaA*, *gltX*, *gpsA*, *gyrB*, *pta-2*, *tdk*, and *tkt* [123; version update March 15, 2021).

The same *de novo* assemblies were used to develop an *ad hoc* cgMLST scheme using Ridom Seqsphere+ (version 6.0.2) [186]. The reference strain *M. bovis* PG45 (NC_014760.1) was used as the seed genome with the following criteria: minimum length of ≥50 bases; start and end codons on each end of the gene; no multiple copies of genes with a BLAST overlap of ≥100

bp with identity ≥90%; and no overlap with genes of >4 bases. Genes identified in the seed genome were queried against the following ten penetration genomes to identify genes for inclusion in the final *ad hoc* cgMLST scheme: NC_015725.1, Hubei-1; NC_018077.1, HB0801; NZ_CP005933.1, CQ-W70; NZ_CP011348.1, NM2012; NZ_CP007589.1, HB0801-P115; NZ_CP019639.1, 08M; NZ_CP023663.1, Ningxia-1; NZ_LT578453.1, JF4278; NZ_CP038861.1, 16M; and NZ_CP045797.1, XBY01. Penetration genomes were queried using BLAST (version 2.2.12) and required to have equivalent targets that met the BLAST hit overlap = 100% with identity of ≥90% in all query genomes. The following criteria were used: word size = 11; mismatch penalty = -1; match reward = 1; gap open costs = 5; and gap extension costs = 2. Targets were also required to have a single stop codon at the end of the gene in >80% of penetration query genomes. The resulting cgMLST scheme consisted of 506 genes (loci) and covered 55.1% of the *M. bovis* PG45 genome.

The distance matrix used for cgMLST phylogenetic analysis omitted genomes missing >10% of distance columns and removed columns with missing values. This resulted in isolates being typed based on the alleles of 296 loci.

SNVs for the cgSNV method were determined from 283 gene targets (loci) of the *M. bovis* PG45 reference genome (NC_014760). Comparing all genomes to these targets yielded 6,408 SNV positions, which were filtered to 3,925 SNVs in 283 loci by only including substituted SNV positions (hide insertion/deletions), and having no neighbouring SNV positions in a window of ten bases. SNVs for the wgSNV method were identified within the 130 genomes by kSNP v3.1 [189], which yielded a SNV matrix file. The settings used for kSNP were -core and -k 31. The SNV matrix contained 14,383 SNVs across all genomes.

Simpson's diversity index [190] compared the discriminatory power of each strain typing method, based upon the clustering of isolates within the minimum spanning trees (MSTs) for the MLST, cgMLST, and cgSNV methods. The wgSNV method did not cluster any two of the isolates into a single type. Therefore, each isolate was classified as a unique type, as defined by a unique genotype for each isolate.

4.3.6 Data presentation

Neighbor joining (NJ) trees were created with iTOL [191] and the MSTs with Ridom SeqSphere+. A maximum-likelihood tree of the wgSNV matrix was generated with the Tamura-

Nei substitution model, using uniform rates and the nearest-neighbour-interchange heuristic method with MEGAX v10.1.1 [192]. The tree was visualized with iTOL and GrapeTree [193]. Isolates were grouped into clonal complexes (CCs) or clades (Cs), as appropriate. A CC was defined as a group of isolates with STs that differed by no more than two alleles from at least one other ST in the group. A singleton was a clonal group that differed from all other STs by at least three alleles. A clade was defined as a group of strains having a common biological ancestor. The CCs were assigned to MLST analyses, while clades comprised the MST of the cgMLST, cgSNV, and wgSNV analyses. These MSTs were determined by visual assignment based on the root of a tree or a ST central to the tree that served as a common ancestor.

4.4 Results

4.4.1 Isolates and assembly

M. bovis isolates ($n = 129$), spanning 12 production years (2006-2018) and 21 feedlots (A-U), were recovered from 98 individual feedlot cattle (Appendix C). All 21 feedlots were located in western Canada; 45.0% ($n = 58$) of isolates originated from two feedlots (N, Q), each having a capacity of >20,000 head. Isolates were recovered from the nasopharynges ($n = 49$), lungs ($n = 45$), and joints ($n = 35$). Most isolates were derived from dead ($n = 82$) versus healthy ($n = 32$) or diseased ($n = 15$) cattle. DNP swabs ($n = 49$) were obtained from healthy ($n = 32$), diseased ($n = 15$), and dead ($n = 2$) animals. All isolates ($n = 129$) underwent WGS and *de novo* assembly: mean N50 = 18,448 (range 997-32908), contig count = 186 (range 79-797), coverage = 84 (range 12-177), and approximate completed genome size = 90% (60-100%) relative to the PG45 reference genome.

4.4.2 MLST

A total of 126 of 130 genomes (129 isolates plus PG45 reference genome) were assigned an existing ST. Four isolates could not be typed due to missing allele(s) and are designated as ST 'Unknown' (one also had a novel allele at the *pta2* locus), a reflection of their low-quality assemblies (N50: 997-2,494; contig count: >500; coverage: 12-51, approximate genome size: ≤80%). The MLST scheme typed the 125 isolates into 24 known STs, and six newly identified STs (ST149 to ST154) (Table 4.1). The PG45 reference genome included in the analysis was assigned ST12, as expected. Simpson's diversity index was 0.932, indicating reasonably strong separation of isolates. Two previously uploaded isolates that had been assembled using SPAdes

[188] had differing MLST STs than those assigned by SKESA [187] and Ridom SeqSphere+. Two of these were within the set of four isolates that had missing alleles. These differences are likely due to low quality sequencing of these isolates and the use of alternate assembly processes.

Table 4.1 Number of MLST sequence types (STs) of *Mycoplasma bovis* isolates ($n = 125$) by production year (2006 – 2018) and health status (healthy, diseased and dead). Total represents the number of STs by production year and health status. PG45 (ST12) is excluded from this table.

Sequence Type (ST)	Production Year								Health Status			Total
	2006	2007	2008	2014	2015	2016	2017	2018	Healthy	Diseased	Dead	
2		2				1				1	2	3
14							1		1			1
21	1	4					2		2	3	2	7
24		12			1				5	6	2	13
27								3			3	3
40		5							3	2		5
42	3	2		1	1				3	2	2	7
43								1	1			1
44							9				9	9
45							3	2	4		1	5
48		1							1			1
52		1				3					4	4
60				1		13	1	8			23	23
61						1					1	1
62		1							1			1
65						6	1		1		6	7
66							2		2			2
67							12				12	12
70		1			2					1	2	3
75			1								1	1
76				2							2	2
77						2					2	2
79						1					1	1
80						3					3	3
149								1			1	1
150								1	1			1
151	1								1			1
152							1		1			1
153								2	2			2
154							2		2			2
Total	5	29	1	4	4	30	34	18	31	15	79	125

The most prevalent STs were ST60 (23/130 or 17.69%), ST24 (13/130 or 10.00%), and ST67 (12/130 or 9.23%), which is much higher than what is reported in the PubMLST database (accessed 12 March, 2021): ST60 (12/1,139 or 1.05%), ST24 (9/1,139 or 0.79%) and ST67 (9/1,139 or 0.79%). Of the ST60 isolates, 12 of 23 had been used in another unrelated study [123], and are represented in the PubMLST database, but under isolate identifiers (see Appendix C). The STs with the highest frequency in the PubMLST database were ST52 (127/1,139 or 11.15%), ST62 (71/1,139 or 6.23%) and ST21 (31/1,139 or 2.72%), all of which are frequently identified in North America, but were infrequent in the current study.

Isolates (n = 35) recovered from feedlot N, between the years 2016 to 2018, represented nine STs, with ST60 (n = 10; 28.6%) being the most prevalent in all years. The 22 isolates recovered from feedlot Q in 2007 were categorized into six STs, 12 (54.5%) being ST24. Two STs (ST2 and ST21) were of particular interest because they were separated in time and space. ST2 was isolated from feedlot Q in 2007 and from N in 2016, while ST21 was recovered from feedlot Q in 2007 and from feedlot N in 2017. These feedlots were separated by a distance of approximately 500 km. Five isolates were derived from cattle imported from the northern United States.

Isolates clustered into two clonal complexes (CC1, CC2) and as four singletons including the PG45 reference strain (ST12, ST42, ST43, ST75) (Figure 4.1). The ST52 and ST60 isolates formed the foci of the CCs in the MLST minimum spanning tree (Figure 4.2). Two isolates (MPLM0830, MPLM0608) from 2018 had allelic combinations that had not been previously described. Five STs persisted within the western Canadian cattle population for many years: ST2 (2007-2016), ST21 (2006-2017), ST24 (2007-2015), ST42 (2006-2015), and ST70 (2007-2015). Three of these STs (ST2, ST21, ST24) were grouped in CC2, whereas ST70 was grouped with CC1 and ST42 was a singleton.

The dataset included 31 pairs of lung-joint samples obtained from individual animals, with 28 lung-joint pairs having both isolates successfully typed using MLST. In 18 (64.3%) instances, the ST recovered from the lung matched the ST found in the joint of the same animal.

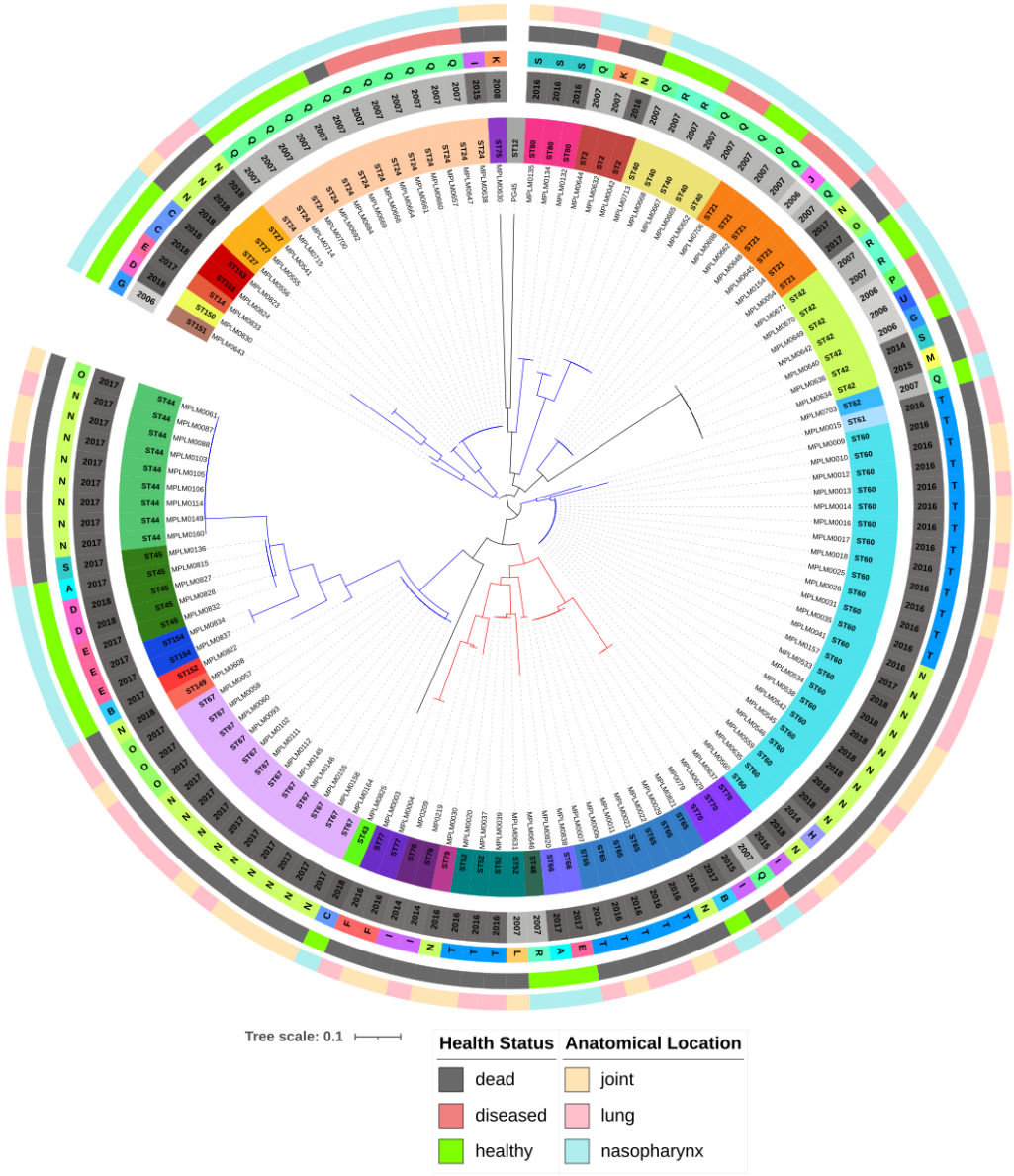

Figure 4.1 MLST neighbor joining tree of 126 *Mycoplasma bovis* isolates typed by MLST. Starting from the innermost ring, the rings contain information on isolate identifiers, MLST sequence type (ST), production year, feedlot (A-U), the anatomical sampling location, and health status at sampling. Two clonal complexes are depicted with red (CC1) and blue (CC2) branch lines. Four singletons (ST12, ST42, ST43, ST75) are depicted as black branch lines.

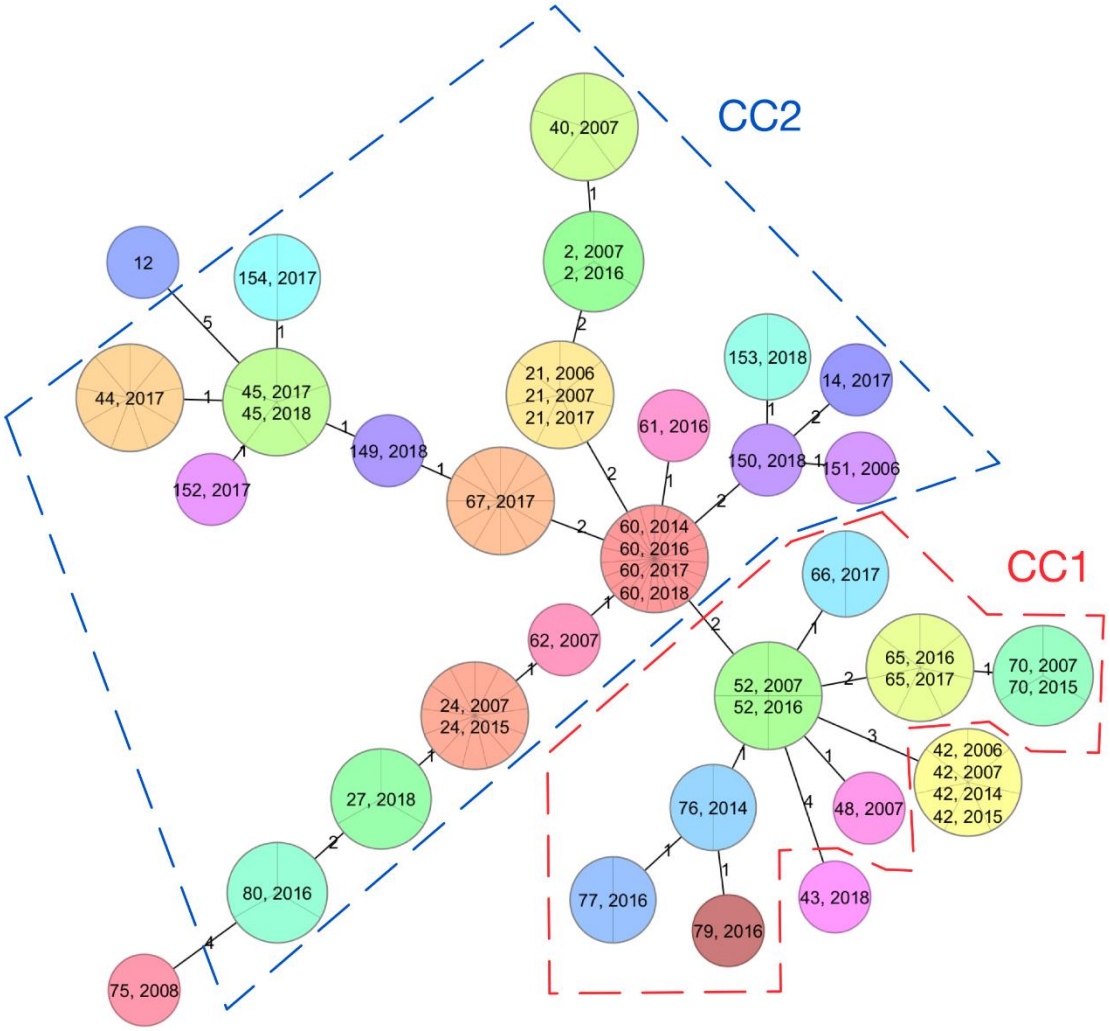

Figure 4.2 Minimum spanning tree of 126 *Mycoplasma bovis* genomes (125 field isolates plus PG45) typed by MLST. Clonal complexes 1 and 2 are surrounded by red and blue lines, respectively. Sequence types are colour coded with the size of the circle reflecting the number of isolates, with the partitioning lines within a circle delineating isolates with an identical genotype. The number of different alleles is indicated over the line connecting the sequence types. The PG45 reference genome is identified as the singleton with ST12.

4.4.3 cgMLST and cgSNV analyses

A total of 102 genomes (101 isolates and PG45) were typed by the cgMLST (Figure 4.3, Figure 4.4) and cgSNV (Figure 4.5, Figure 4.6) methods. Figures 4.4 and 4.6 provide the MSTs of the isolates, with three clades (C1, C2, C3) branching from a single focus consisting of an isolate with MLST ST62. Isolates from these clades tended to cluster together in the neighbour joining (NJ) tree, but small subclades (Figures 4.3 and 4.5) branched from the root and contained isolate specific clades with the MSTs. Both cg analyses grouped the five STs previously discussed (ST2, ST21, ST24, ST42, ST70) into two clades, with ST2, ST21, ST24, and ST42 grouped in C3, and ST70 allocated to C2 (Figures 4.4 and 4.6). Although there were minor differences observed in overall clade membership between the MLST and the two cg methods, results were fairly consistent. Simpson's diversity index was 0.987 for cgMLST and 0.984 for cgSNV, indicative of strong separation of the isolates into individual STs, with most isolates assigned a unique ST.

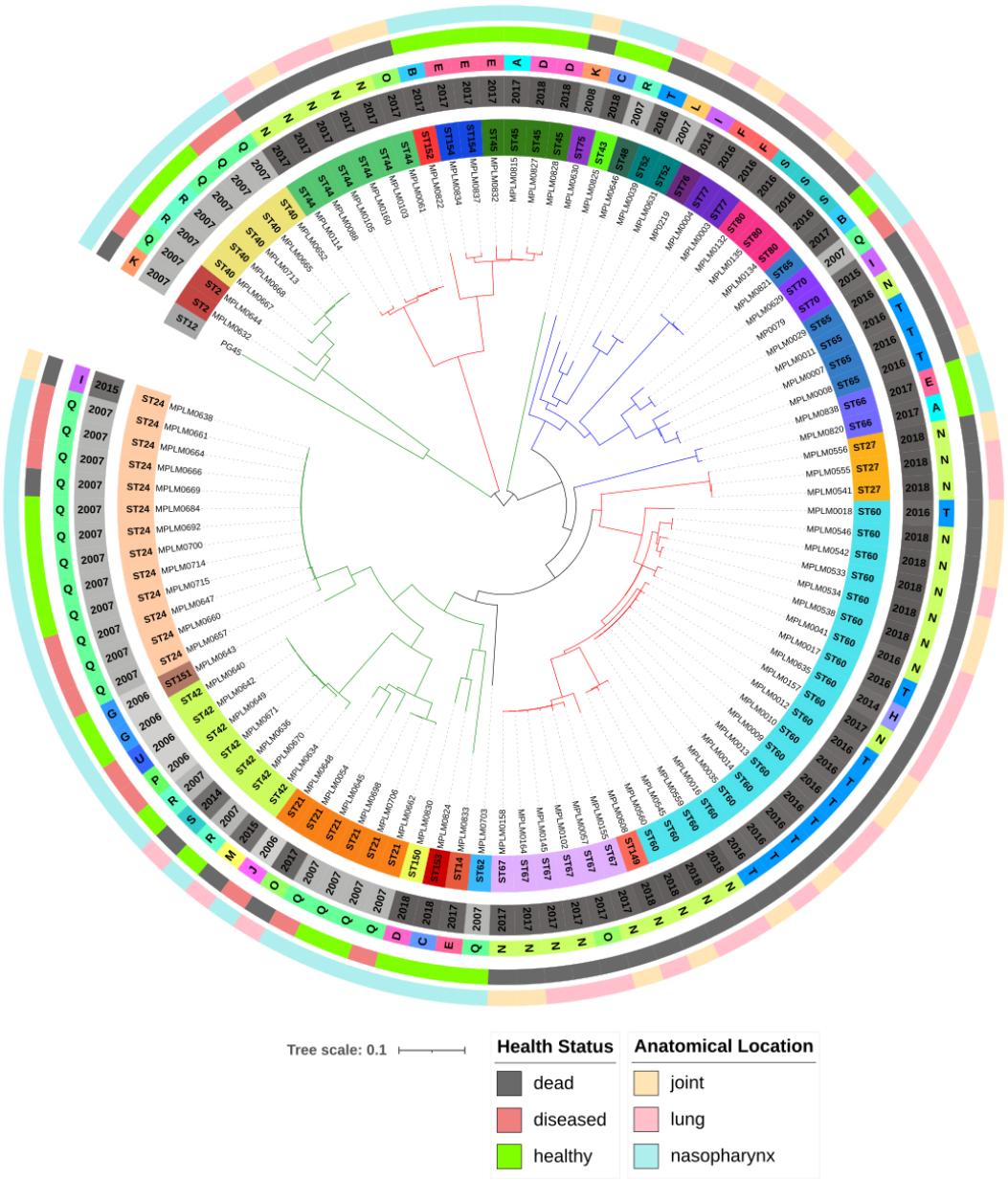

Figure 4.3 cgMLST neighbor joining tree of 102 *Mycoplasma bovis* genomes based on alleles at 296 core genome loci. Starting from the innermost ring, the rings contain information on isolate identifiers, MLST sequence type (ST), production year, feedlot (A-U), anatomical sampling location and animal health status at sampling. The branches are coloured in accordance with the three clades (C1, red; C2, blue; C3, green) identified in Figure 4.4, highlighting the differences between the tree construction methods.

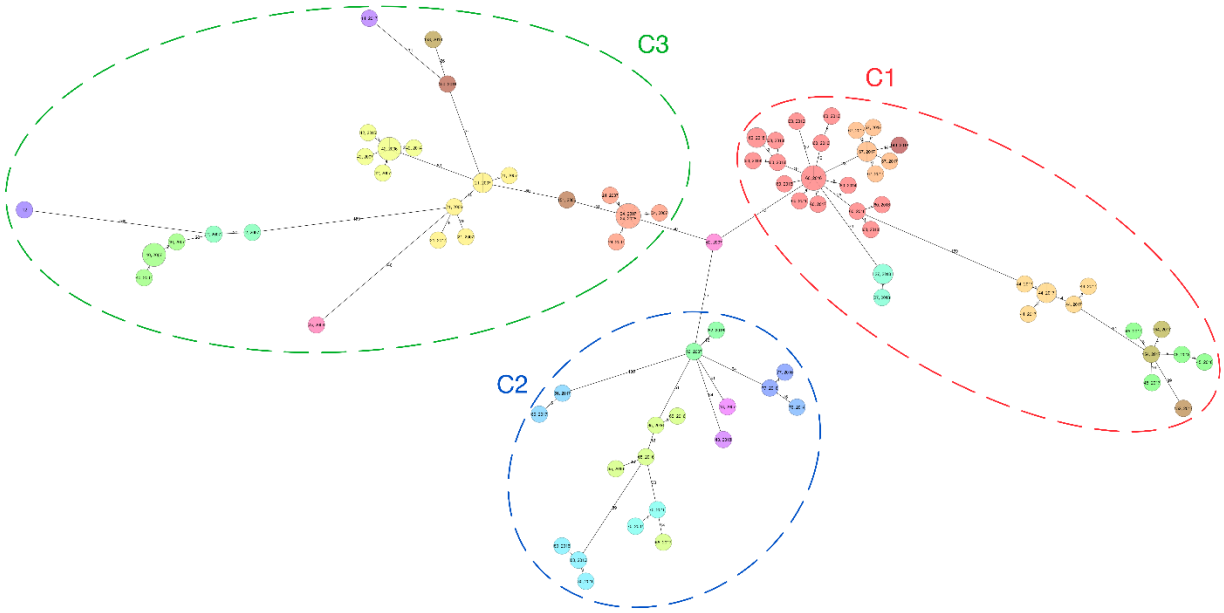

Figure 4.4 Minimum spanning tree of 102 *Mycoplasma bovis* isolates (101 field isolates plus PG45) created with cgMLST, based on alleles at 296 core genome loci. Clades 1 - 3 are denoted by a surrounding circle. The isolates are identified by MLST sequence type and production year (ST, Production Year). Sequence types are colour coded with the size of the circle reflecting the number of isolates, with the partitioning lines within a circle delineating isolates with an identical genotype. The number of different alleles is indicated over the line connecting the sequence types.

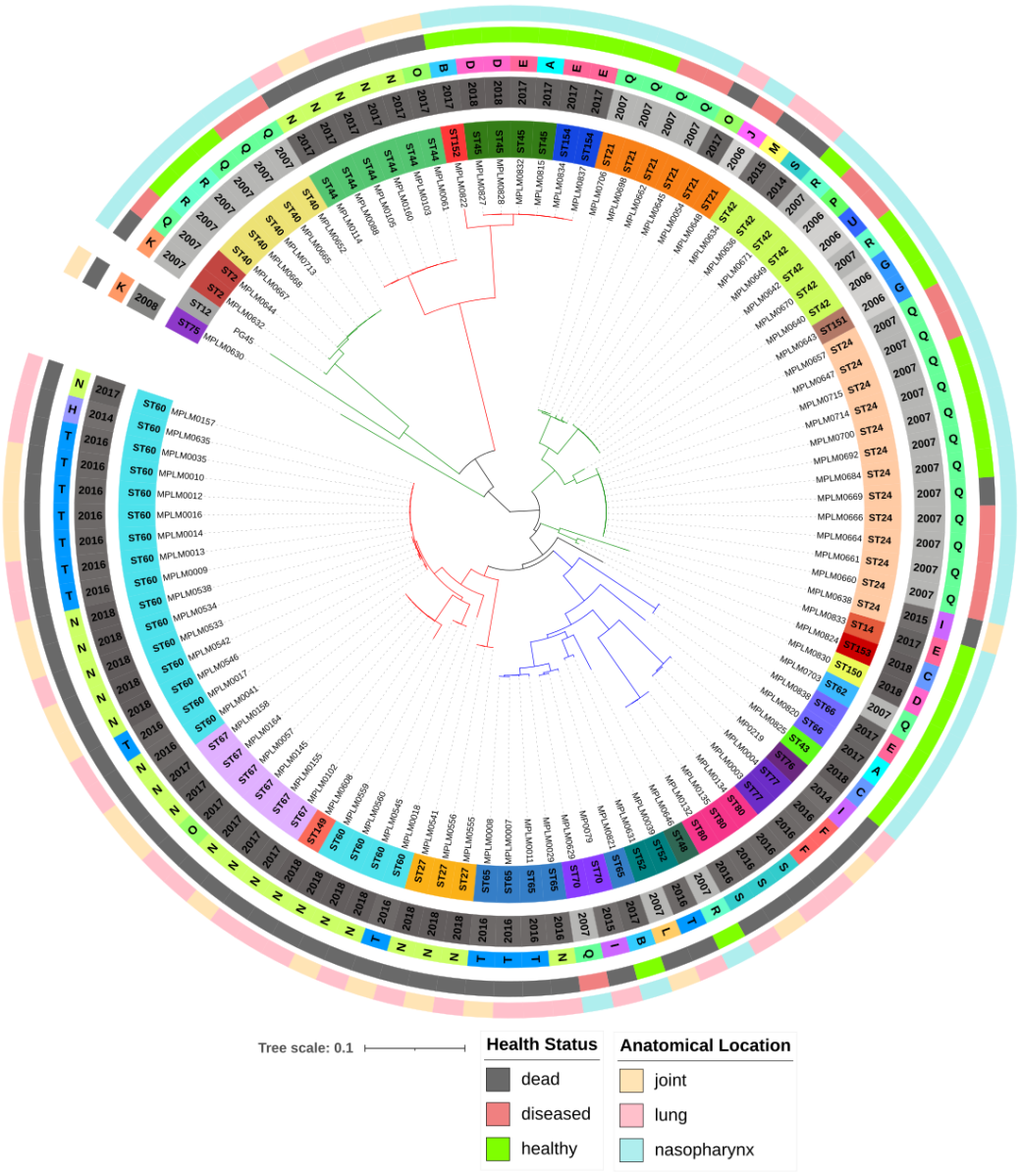

Figure 4.5 cgSNV neighbor joining tree of 102 *Mycoplasma bovis* isolates based on 283 core genome loci (3,925 SNVs). Starting from the innermost ring, the rings contain information on isolate identifiers, MLST sequence type (ST), production year, feedlot (A-U), anatomical sampling location, and health status at sampling. The branches are coloured to match the clades (C1, red; C2, blue; C3, green) identified in Figure 4.6, highlighting the differences between the tree construction methods.

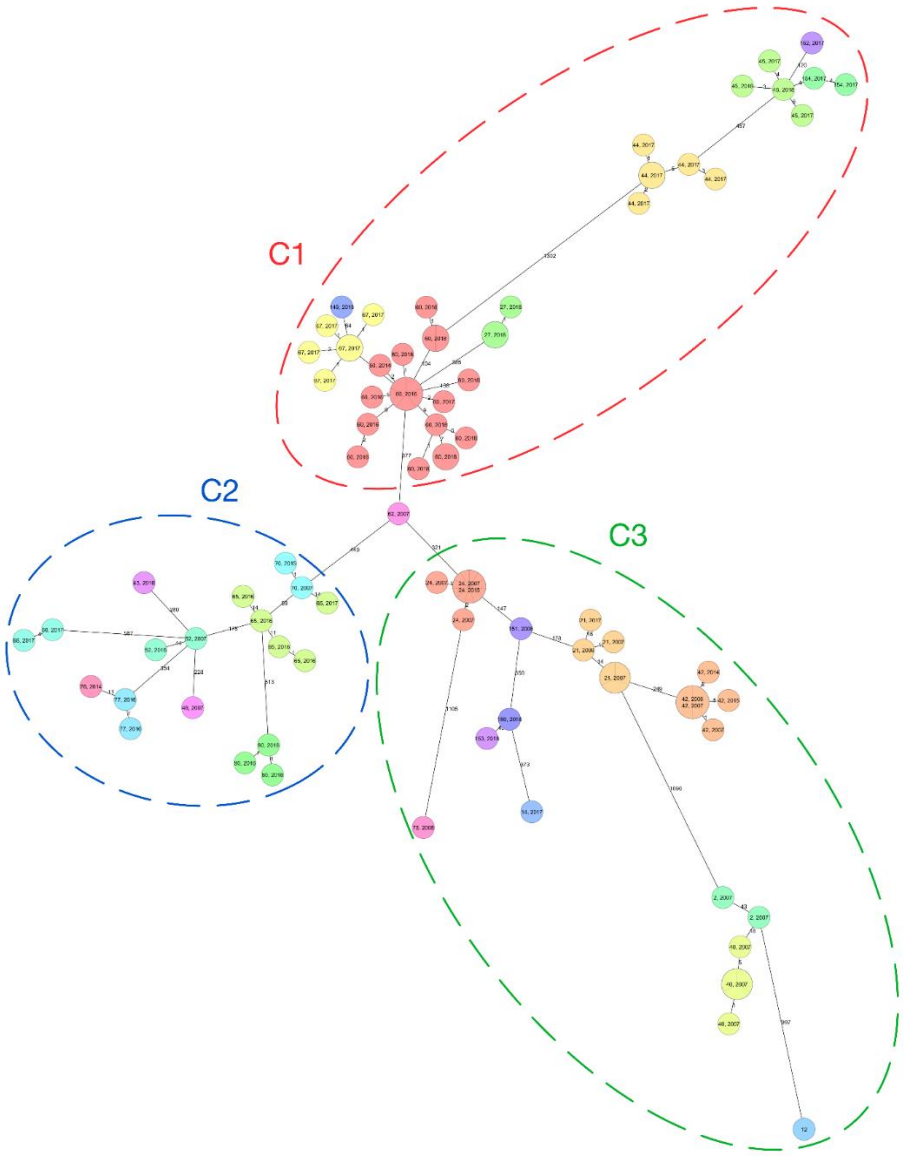

Figure 4.6 Minimum spanning tree of 102 *Mycoplasma bovis* isolates (101 field isolates plus PG45) based on 283 core genome loci, using cgSNV, based on 3,925 SNVs. The clades are indicated by an encompassing circle. The 102 *M. bovis* isolates are identified by MLST sequence type and production year (ST, Production Year). The nodes are colour coded by the MLST sequence type. The size of the circle reflects the number of isolates represented, with the partitioning lines within a circle delineating isolates with an identical genotype. The number of different alleles is indicated over the line connecting the sequence types.

4.4.4 wgSNV

All 130 genomes were typed by wgSNV and no two isolates shared an identical SNV matrix, hence each isolate was unique (Figure 4.7, Figure 4.8). As a result, Simpson's diversity index was 1.000. Two primary clades (C1, C2) branched into two sub-clades of approximately equal size. A single isolate (ST62) was positioned evenly between the two clades. wgSNV was able to resolve isolates with the same MLST ST assignment compared to the cgMLST and cgSNV methods. Similar to cgMLST and cgSNV, wgSNV grouped ST2, ST21, ST24, ST42 into C1, while the ST70 isolates grouped with a separate lineage (C2) (Figure 4.8).

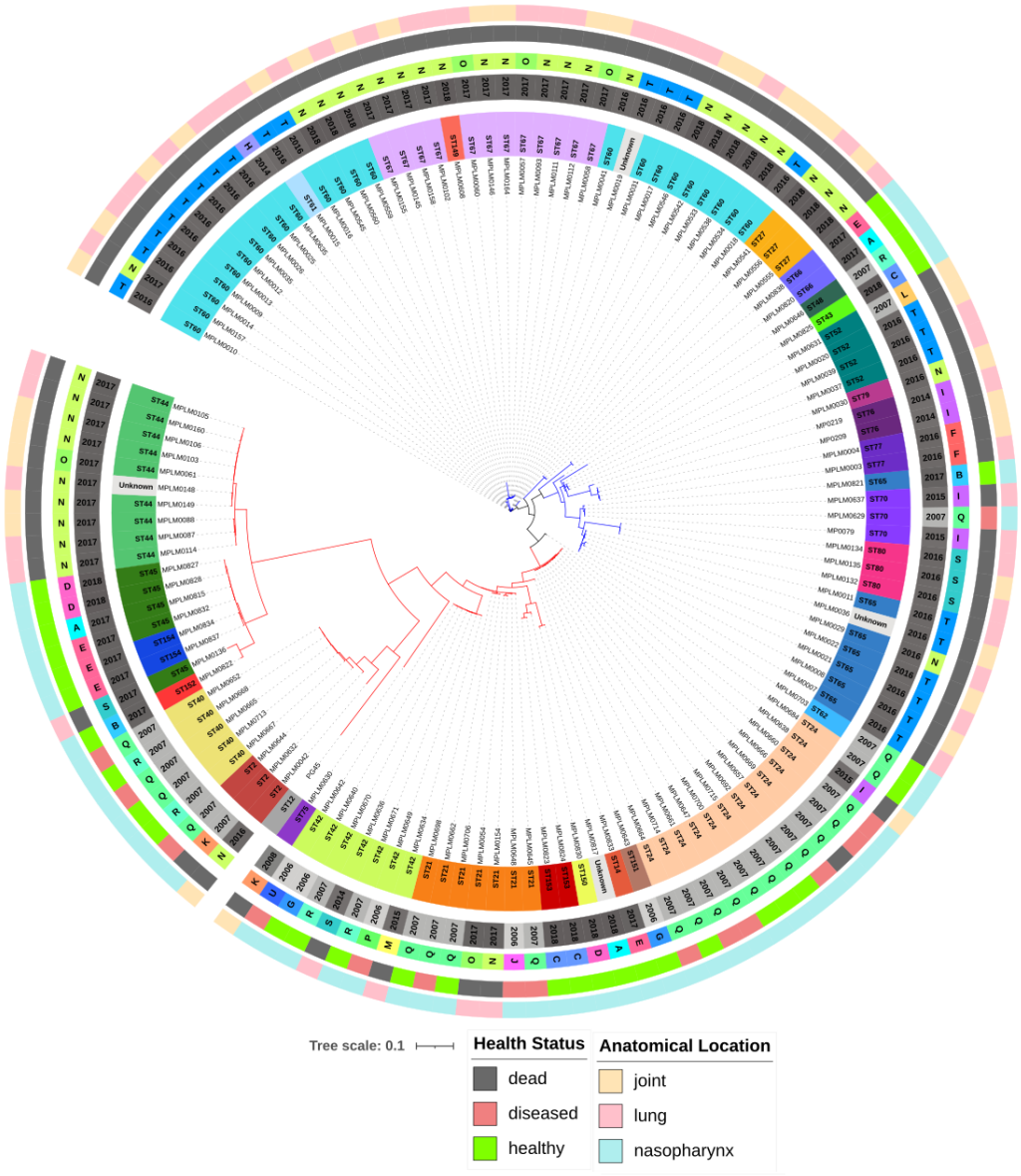

Figure 4.7 wgSNV maximum-likelihood tree of 130 *Mycoplasma bovis* genomes based on 14383 SNVs in the core and accessory genomes. Starting from the innermost ring, the rings contain information on isolate identifiers, MLST sequence type (ST), production year, feedlot (A-U), anatomical sampling location, and health status at sampling. Clades 1 and 2 are indicated by blue and red, respectively. Isolate MPLM0703 (ST62) falls evenly between both clades and as a result was not included in either. These clades match the clades identified Figure 4.8.

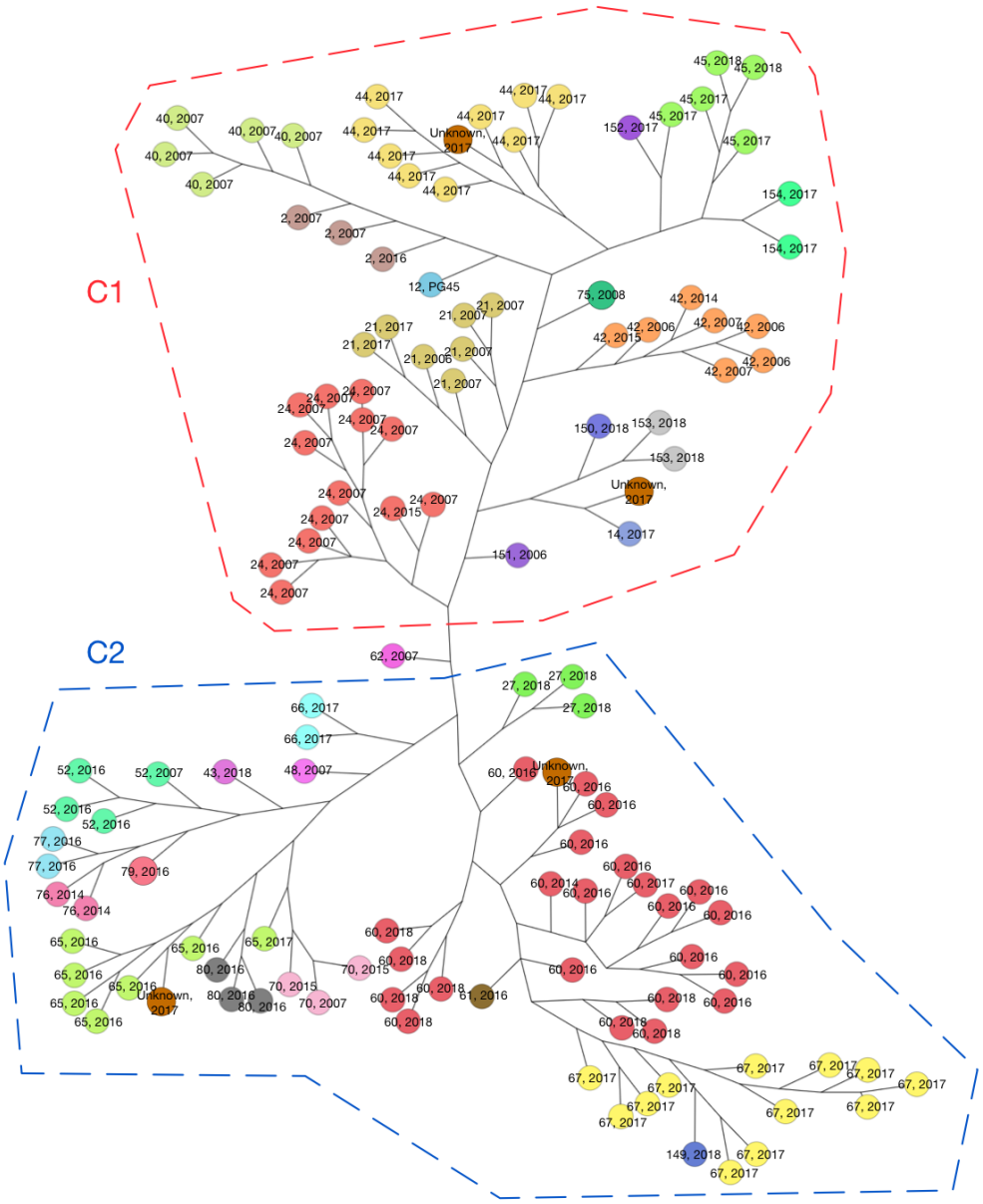

Figure 4.8 Maximum-likelihood tree of 130 *Mycoplasma bovis* isolates (129 field isolates plus PG45) based on 14383 SNVs in the core and accessory genomes typed by wgSNV. Clades 1 and 2 are indicated by a surrounding line in red and blue, respectively. The isolates are labelled with MLST sequence type and production year (ST, Production Year), and colour coded with MLST sequence type. Each individual node represents a single isolate. No two isolates had identical SNV matrices.

4.5 Discussion and Conclusion

This is the first study to compare four different phylogenetic typing methods using a relatively large and diverse set of *M. bovis* isolates derived from western Canadian feedlot cattle. Overall, a greater number of genomes were typed using the MLST scheme ($n = 126$) compared to the cgMLST or cgSNV methods ($n = 102$), which required assembly of hundreds of loci constituting a large portion of the genome. The wgSNV method successfully typed all 130 genomes, highlighting the robustness of the SPAdes and kSNP software, even with inputs of variable sequencing depth. All four methods had a high degree of discriminatory power, as judged by Simpson's diversity index. MLST had the lowest discriminatory power, while cgMLST had a modestly higher index than cgSNV. Despite some differences in the phylogenetic outputs of the four methods, the MLST method generated a pattern of clonal complexes (CC) comparable to the clades generated from the cgMLST, cgSNV and wgSNV methods. This is noteworthy since the MLST scheme relied on seven housekeeping genes, representing <1% of the *M. bovis* PG45 reference genome. In contrast, the cgMLST scheme was derived from 506 loci, covering approximately 55% of the genome. The cgSNV method analyzed 3,925 SNVs from 283 loci, while the wgSNV matrix utilized 14,383 SNVs, generating the highest genotypic resolution.

Since core genome and whole genome methods are based on a larger representation of the genome, theoretically they should have a greater potential to resolve relationships than the seven-loci MLST scheme. This makes the cgMLST, cgSNV, and wgSNV method ideally suited for epidemiological investigations where small variations between STs may be consequential. Parker et al. [103] applied wgSNV analysis to Australian *M. bovis* ($n = 75$) isolates and found a very high level of homogeneity among isolates; the maximum number of single nucleotide polymorphisms (SNPs) between any two isolates was 50 [103]. This level of resolution led the researchers to conclude that a single strain of *M. bovis* was circulating within Australia's cattle population. This is quite unique, since a number of other country-level studies have found multiple clusters of genetically distant *M. bovis* STs within the population of cattle [181,182,183,194]. Furthermore, these studies provided some insight into the movement of specific STs from country to country, and overtime. These higher resolution methods have also been applied at the farm-level, being used to examine transmission between cows and calves

within the same dairy farm [180], and the introduction of *M. bovis* into dairy herds via contaminated semen [111].

The higher concordance and resolution of the cgMLST, cgSNV, and wgSNV methods was evident when focusing on five STs (ST2, ST21, ST24, ST42, ST70), representing 33 isolates obtained between 2006 to 2017. The MLST scheme grouped three STs (ST2, ST21, ST24) to clonal complex 2 (CC2); ST70 to CC1; and ST42 as a singleton. Whereas both the cgMLST and cgSNV analyses assigned four STs (ST2, ST21, ST24, ST42) to clade 3 (C3), and ST70 to C2. The wgSNV analysis yielded results that were similar to the cgMLST and cgSNV methods. These findings underscore that all four methods had similar assignments for four of the five STs. However, because ST42 was a triple locus variant, it became an outlier or singleton. As the cgMLST, cgSNV and wgSNV methods utilize hundreds to thousands of discrete data points to compare the genetic relatedness of isolates, it grouped ST42 alongside other STs into a clade.

The MLST method identified 30 different STs, which underscores the genetic diversity of *M. bovis* in western Canada. This is most likely related to the underlying structure of the Canadian cattle industry and how feedlot cattle are procured. At the time of the last agricultural census (2016), Canada had approximately 54,000 beef cattle farms, with 38,700 (72%) located in western Canada (https://www150.statcan.gc.ca/n1/en/type/data?text=40221). Some western Canadian feedlots also import cattle from the United States. Since most feeder cattle are sold at auction, extensive commingling of cattle from multiple owners occurs during procurement. Once sold, the cattle are assembled, transported, and then processed and further commingled at the feedlot. This is also occurring in the autumn months, when inclement weather conditions arise. Conceivably, commingling, transport, and changing environmental conditions all contribute to stress, which facilitates increased shedding from carrier animals [170]. Thus, given the broad catchment from which cattle are sourced and mixed, it is understandable that feedlots had multiple STs circulating during the same time period. Interestingly, ST2 was isolated from two feedlots in 2007 (feedlots Q and K), and then not until nine years later from feedlot N. Similarly, ST21 was isolated from feedlots J in 2006, Q in 2007, and then from feedlot N and O in 2017. Not only were these isolates separated in time, but feedlots N and Q were geographically located approximately 500 km apart. This separation by time and space suggests that some STs may be more dominant and widespread than others.

While Canada's feedlot sector is concentrated in Alberta, there is bilateral trade in cattle between Canada and the United States, which is noteworthy because five isolates from the American cattle were evenly distributed amongst the Canadian isolates within the NJ trees and MSTs (identified in Appendix C). These results support the findings of a recent study wherein wgSNV analysis found a high degree of genetic diversity within the American isolates, with Canadian isolates clustering within the same clade as the American isolates [192].

There were 30 MLST STs dispersed over 12 production years, with two strains (ST21, ST52) having been reported in bovine isolates outside of North America: ST21 reported in Europe and Asia, whereas ST52 was reported in Europe, Asia, and Oceania (PubMLST isolate database accessed 12 March, 2021). ST21 is of particular interest in that it has a worldwide distribution, and a recent European study found it to be a common ancestor to isolates recovered after 2010. This worldwide distribution underscores the international trade in cattle, and the need for biosecurity measures to mitigate the transmission of *M. bovis* and other potential production-limiting pathogens. It is also noteworthy that ST21 has also been isolated from bison (PubMLST isolate database accessed 12 March, 2021).

WGS allows typing by multiple methods to be done *in silico*, with Ridom Seqsphere+ making higher resolution typing methods more accessible compared to the knowledge required to construct a customized whole genomic analysis pipeline. Established typing methods, such as MLST will invariably continue to support *in silico* efforts to conduct comparative studies using historical and contemporary data. WGS of isolates, particularly when analyzing with Ridom Seqsphere+ provides the opportunity to merge established methods (MLST) with more robust core genome approaches (i.e. cgMLST and cgSNV). Additionally, given that the cost of WGS is comparable to the sequencing of seven PCR amplicons, particularly for a small 1-Mbp genome like *M. bovis*, WGS is likely to become the standard for detailed phylogenetic studies. The ability to cost-effectively generate high quality *M. bovis* assemblies from long reads will enable greater use of cgMLST, cgSNV, and wgSNV phylogenetic typing methods [195].

A caveat for using the cg methods is the need for greater sequencing depth and fewer sequencing artifacts in order to generate more complete, higher quality assemblies. This was evident from the fact that more isolates were typed by MLST than cgMLST or cgSNV, while the wgSNV software pipeline was able to generate a phylogenetic tree for all isolates. wgSNV was

better able to process poorer quality sequencing data because it analyzes the entire genome to a greater degree than the other methods. However, it is not without its faults. It is possible that the assembly software may have erroneously assembled the small subsets of the genome resulting in false SNPs contributing to the uniqueness of the genotypes. However, misassembles occur only infrequently and in sections of an assembly with poor coverage, making this occurrence in our dataset unlikely. Care must also be taken when choosing analysis software, and associated input parameters. This is exemplified in the NJ trees MSTs, which were similar trees, but with differences in the position of ST2, ST40, ST44, ST45, and ST75 within the trees. This highlights the need for high quality sequence data as the analysis moves from MLST to more complex methods such as cgSNV or wgSNV.

The study design was appropriate for comparing the four genotyping methods. However, it was equivocal as to whether the lack of association between genotypes (STs) and phenotypes (year, health status, anatomical location) was real, or was related to the limited number of isolates collected. Obtaining a complete set of DNP swabs, lung, and joint samples from each animal would have helped in determining whether STs exhibited a tropism for specific tissues. On this point, it was salient that the dataset included paired lung-joint samples from 28 animals, 18 (64.3%) of which had the same ST in the lung and joint, suggesting the absence of a single tissue tropism. However, in ten cases, the genotype of lung and joint isolates within the same animal differed, underscoring the need for polyvalent vaccines. Others have also suggested that a polyvalent vaccine maybe required to cover the broad functional diversity found amongst isolates [192].

The lack of association between genotype and phenotype is certainly not unique, but rather is the emerging consensus. A number of studies using higher resolution typing methods have been unable to show linkages between clusters and anatomical sample locations [103,194] or to health status [103,182]. However, one of the issues is that these studies have not been specifically designed to investigate these associations. This is problematic as the lack of association may be related to not only an inadequate number of samples, but an unbalanced study design. Many of the studies have biases in sampling related to year, anatomical location, geographical location and health status. These confounding factors may result in type I and II errors. This is particularly true when investigating the association between genotype and health

status in feedlot cattle. Animals deemed healthy on-arrival and at the time of sampling, may develop BRD within days. Conversely, BRD is a polymicrobial disease, and hence clinical disease does not always equate to mycoplasmosis. This conundrum is best addressed by comparing isolates from healthy animals to those obtained at the time of postmortem examination from tissues (lungs and joints) exhibiting a pathology consistent with mycoplasmosis.

Each genotyping method has strengths and weaknesses depending on the research question. MLST is best suited as an initial screening method for detecting the presence of genetically distinct strains, and is amenable to both PCR and *in silico* methods. Furthermore, the *M. bovis* PubMLST database is curated and accessible through a publicly available website. While cgMLST, cgSNV and wgSNV analyses allow for typing of strains, they also provide a higher level of genetic resolution, which may be used to discern clinically relevant differences such as tissue tropisms, antimicrobial resistance, or virulence

The wgSNV method successfully typed all 129 field isolates, whereas the cg methods only typed 101 isolates, an outcome that may be rectified with greater sequencing depth. Overall, clade membership between the MLST and higher resolution methods was similar on visual assessment of the NJ trees and MSTs, with cgMLST and cgSNV having the highest degree of concordance. The wgSNV provided an incrementally higher level of genomic resolution and detail, which may have utility in some epidemiological investigations and for addressing research questions relating to gene function and characterization. Although the wgSNV method was very powerful and robust, it is less user-friendly, requiring specialized training in bioinformatics. Conversely, the cg analyses were performed using Ridom SeqSphere+, which is graphical interface software that non-bioinformaticians can use with moderate background knowledge. Thus, it provides greater resolution than MLST and requires less specialized training, but it is relatively expensive, particularly if only being used on a limited number of isolates. None of the methods could show a clear association between genotype and phenotype, which may reflect the limitations of these methods, or it could be related to a relatively small sample size.

5 GENERAL SUMMARY AND CONCLUSION

5.1 Discussion

The overall elevated level of antimicrobial resistance in *Mycoplasma bovis* reported in this study is consistent with trends reported in other Canadian studies [10-13] and worldwide [14- 22]. An average administration of 3 different antimicrobial classes to feedlot cattle at the time of death was also comparable to the two classes reported by another Canadian study [11]. Isolates in the current study were largely resistant to all macrolides assessed (gamithromycin, tilmicosin, tildipirosin, tylosin, and tulathromycin), which was not unexpected given their metaphylactic administration for BRD in western Canadian feedlot cattle [44]. The predominate susceptibility to florfenicol is encouraging, as it is classified as a lower category III (medium importance) antimicrobial by Health Canada [55]. However, macrolides are classified as a higher category II (high importance) antimicrobial. The elevated level of macrolide resistance underscores the global issue with antimicrobial and reinforces the importance in surveillance of antimicrobial resistance and the appropriate usage of antimicrobials to reduce the selection pressure of resistance and maintain their utility.

In the absence of a standardized AST protocol and established clinical breakpoints for *M. bovis* by Clinical and Laboratory Standard Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), interpretation of MIC values and comparison of AST results between studies can be difficult if not impossible. This, combined with the fastidious nature of culturing *M. bovis*, makes querying the genotype to determine AMR more amenable to expeditious assessment of AST than culture dependent methods, a facet of antimicrobial stewardship. When investigating the genotypic basis for macrolide resistance in chapter 3, isolates with mutations in both domain II and V of 23S rRNA gene alleles correlated with macrolide resistance in all 5 macrolides tested, however nearly all isolates were resistant to tildipirosin and tilmicosin regardless of genotype. Resistance was also observed in isolates with mutations in domain II and L4 and L22 ribosomal proteins, with the exception of tulathromycin. These findings highlight the utility of assessing antimicrobial susceptibility based on the genotypic composition of *M. bovis*. Genotypic assessment of antimicrobial susceptibility of *M. bovis* would aid in surveillance of antimicrobial susceptibility in a more cost effective and expeditious manner, allow for surveillance and evidence-based selection of antimicrobials.

Through phylogenetic analysis of *M. bovis* isolates in chapter 4, the genetic diversity of *M. bovis* isolates in western Canada was underscored by identification of 30 STs in 125 isolates. This finding was not entirely surprising given the movement and commingling of cattle during transport and at auction-marts prior to arrival at the feedlot. Interestingly, two MLST strain types (ST 2 and 21) were found separated in both time and space, suggesting dominance of certain strain types. Type STs (ST21 and ST 52), had also been reported in isolates derived from cattle outside of North America. Unfortunately, none of the different *in silico* typing methods were able to correlate genotype to phenotype, which was consistent with findings in other studies [103,182,194]. However, the phylogenetic relationship of the isolates using each of the four methods were comparable with resolution increasing with complexity of analysis.

Limitations of the studies included unequal representation of isolates over production years. Unequal representation of production years could be due in part to the expense and extensive time associated with sampling of cattle and culturing of *M. bovis*. Furthermore, *M. bovis*-associated disease is seasonal as it largely occurs weeks to months following fall entry of cattle into feedlots, limiting when sampling is likely to yield *M. bovis*. Due to this, there was a sampling bias towards dead cattle that had received antimicrobials prior to death in an effort to isolate *M. bovis*. In chapter 3, there was a low number ($n = 2$) of isolates with a wildtype 23S rRNA genotype to serves as the basis of comparison for macrolide resistance and in chapter 4 the inability to discern a clear association between genotype and phenotype could be due to method selection, small sample size, or the fact that isolates predominately came from cattle that had died from *M. bovis* related disease.

Overall, assessment of antimicrobial susceptibility via a genotypic approach is highly amenable to *M. bovis* specifically given the requirement for specialized conditions and laborious nature of culturing *M. bovis in vitro*. Furthermore, given the increased level of AMR in *M. bovis* in western Canadian feedlot cattle warranting routine surveillance, a more rapid assessment of AMR utilizing a genotypic approach could become incorporated in a feedlot production setting in the future. This would not necessarily be directly done by the producer, but rather incorporated as part of the development and review of antimicrobial use protocols put into place at feedlots in consultation with a veterinarian.

5.2 Future Research

The development of a collection of isolates with paired antimicrobial susceptibility data and WGS for this study provides an opportunity to bioinformatically interrogate the *M. bovis* genome to potentially elucidate new information regarding mechanisms of antimicrobial resistance and virulence without the requirement for additional sample collection and processing. Given the limitation of the current and previous studies [103,182,194] to definitively determine whether there was an association between genotype and tissue tropism or health status, a study could be designed to investigate those relationship specifically. This could potentially be applied to assess whether strain types (eg. MLST STs) can act as a proxies for clinically relevant parameters such and antimicrobial resistance or virulence. Furthermore, given the successful correlation between macrolide resistance and phenotype, there could be utility in developing a robust PCR based assay to assess antimicrobial resistance of *M. bovis* from a single nasal swab, despite the mixed population of microbes associated with BRD.

5.3 Conclusion

In conclusion, AMR was observed to macrolides and antimicrobials in general, with isolates remaining predominately susceptible to a single antimicrobial (florfenicol). This suggests that AMU practices should not necessarily focus on how best to apply florfenicol to current antimicrobial use strategies, but rather how current practices could be adapted to best preserve its utility otherwise its utility could be lost. Given the fastidious nature of culturing *M. bovis* and the need for expeditious antimicrobial susceptibility information, assessing antimicrobial resistance of *M. bovis* genotypically was shown to be highly comparable to phenotypic (AST) data. Given the increasing global concern of *M. bovis*, implementation of strain typing will become increasingly important in the surveillance of *M. bovis*. However, appropriate selection of the strain typing method is contingent on the end application, as pros and cons were identified for all *in silico* methods applied.

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APPENDIX A – CHAPTER 2: *MYCOPLASMA BOVIS* ISOLATES

Table A2.1. Summary of mycoplasma isolates discussed in Chapter 2. Isolates were derived from different anatomical locations (nasopharynx, lung, joint) from cattle with different health statuses (healthy, disease, dead) collected from feedlots A to Z from 2006 to 2018. The cattle identifier associated with each isolate is indicated, as more than one isolate was derived from a single animal in some cases.

Isolate Identifier	Production Year	Feedlot	Anatomical Location	Health Status	Cattle Identifier
MP0064	2014	Y	lung	dead	C073
MP0209	2014	I	joint	dead	C056
MP0219	2014	I	lung	dead	C056
MPLM0636	2014	S	lung	dead	C074
MYCO062	2014	Z	lung	dead	C065
MYCO066	2014	W	lung	dead	C067
MYCO076	2014	Z	lung	dead	C066
MYCO081	2014	X	joint	dead	C070
MYCO096	2014	W	lung	dead	C068
MP0057	2015	X	lung	dead	C069
MP0058	2015	I	lung	dead	C058
MP0063	2015	I	lung	dead	C059
MP0070	2015	Z	lung	dead	C064
MP0071	2015	I	lung	dead	C060
MP0073	2015	I	lung	dead	C061
MP0075	2015	V	lung	dead	C071
MP0077	2015	I	lung	dead	C062
MP0079	2015	I	lung	dead	C055
MP0183	2015	I	joint	dead	C063
MPLM0637	2015	I	lung	dead	C057
MPLM0638	2015	I	joint	dead	C055

MPLM0003	2016	F	lung	dead	C072
MPLM0004	2016	F	joint	dead	C072
MPLM0007	2016	T	lung	dead	C045
MPLM0008	2016	T	joint	dead	C045
MPLM0009	2016	T	lung	dead	C046
MPLM0010	2016	T	joint	dead	C046
MPLM0011	2016	T	lung	dead	C047
MPLM0012	2016	T	joint	dead	C047
MPLM0013	2016	T	lung	dead	C048
MPLM0014	2016	T	joint	dead	C048
MPLM0015	2016	T	lung	dead	C049
MPLM0016	2016	T	joint	dead	C049
MPLM0017	2016	T	lung	dead	C050
MPLM0018	2016	T	joint	dead	C050
MPLM0019	2016	T	lung	dead	C051
MPLM0020	2016	T	joint	dead	C051
MPLM0025	2016	T	lung	dead	C052
MPLM0026	2016	T	joint	dead	C052
MPLM0029	2016	N	lung	dead	C001
MPLM0030	2016	N	joint	dead	C001
MPLM0031	2016	T	lung	dead	C053
MPLM0033	2016	N	lung	dead	C022
MPLM0034	2016	N	joint	dead	C022
MPLM0035	2016	T	lung	dead	C054
MPLM0036	2016	T	joint	dead	C054
MPLM0037	2016	T	lung	dead	C043
MPLM0039	2016	T	lung	dead	C044
MPLM0040	2016	T	joint	dead	C044
MPLM0041	2016	N	lung	dead	C002
MPLM0042	2016	N	joint	dead	C002

MPLM0064	2017	N	joint	dead	C023
MPLM0066	2017	N	lung	dead	C024
MPLM0067	2017	N	joint	dead	C024
MPLM0069	2017	N	lung	dead	C025
MPLM0070	2017	N	joint	dead	C025
MPLM0073	2017	N	joint	dead	C026
MPLM0084	2017	N	lung	dead	C027
MPLM0085	2017	N	joint	dead	C027
MPLM0087	2017	N	lung	dead	C003
MPLM0088	2017	N	joint	dead	C003
MPLM0090	2017	N	lung	dead	C028
MPLM0091	2017	N	joint	dead	C028
MPLM0093	2017	N	lung	dead	C004
MPLM0094	2017	N	joint	dead	C004
MPLM0102	2017	N	lung	dead	C005
MPLM0103	2017	N	joint	dead	C005
MPLM0105	2017	N	lung	dead	C006
MPLM0106	2017	N	joint	dead	C006
MPLM0108	2017	N	lung	dead	C029
MPLM0111	2017	N	lung	dead	C007
MPLM0112	2017	N	joint	dead	C007
MPLM0114	2017	N	lung	dead	C008
MPLM0143	2017	N	joint	dead	C030
MPLM0145	2017	N	lung	dead	C009
MPLM0146	2017	N	joint	dead	C009
MPLM0148	2017	N	lung	dead	C010
MPLM0149	2017	N	joint	dead	C010
MPLM0154	2017	N	lung	dead	C011
MPLM0155	2017	N	joint	dead	C011
MPLM0157	2017	N	lung	dead	C012

MPLM0158	2017	N	joint	dead	C012
MPLM0160	2017	N	lung	dead	C013
MPLM0164	2017	N	joint	dead	C014
MPLM0533	2018	N	lung	dead	C015
MPLM0534	2018	N	joint	dead	C015
MPLM0535	2018	N	nasopharynx	dead	C015
MPLM0537	2018	N	lung	dead	C016
MPLM0538	2018	N	joint	dead	C016
MPLM0539	2018	N	nasopharynx	dead	C016
MPLM0541	2018	N	lung	dead	C017
MPLM0542	2018	N	joint	dead	C017
MPLM0543	2018	N	nasopharynx	dead	C017
MPLM0545	2018	N	lung	dead	C018
MPLM0546	2018	N	joint	dead	C018
MPLM0547	2018	N	nasopharynx	dead	C018
MPLM0549	2018	N	lung	dead	C031
MPLM0552	2018	N	lung	dead	C032
MPLM0553	2018	N	nasopharynx	dead	C032
MPLM0555	2018	N	lung	dead	C019
MPLM0556	2018	N	joint	dead	C019
MPLM0559	2018	N	lung	dead	C020
MPLM0560	2018	N	joint	dead	C020
MPLM0566	2018	N	lung	dead	C033
MPLM0567	2018	N	nasopharynx	dead	C033
MPLM0569	2018	N	lung	dead	C034
MPLM0578	2018	N	lung	dead	C035
MPLM0579	2018	N	nasopharynx	dead	C035
MPLM0582	2018	N	nasopharynx	dead	C036
MPLM0584	2018	N	lung	dead	C037
MPLM0587	2018	N	lung	dead	C038

MPLM0588	2018	N	nasopharynx	dead	C038
MPLM0593	2018	N	lung	dead	C039
MPLM0608	2018	N	lung	dead	C021
MPLM0609	2018	N	nasopharynx	dead	C021
MPLM0611	2018	N	lung	dead	C040
MPLM0622	2018	N	nasopharynx	dead	C041
MPLM0624	2018	N	lung	dead	C042
MPLM0625	2018	N	nasopharynx	dead	C042

APPENDIX B – CHAPTER 3: *MYCOPLASMA BOVIS* ISOLATES

Table A3.1. Summary of mycoplasma isolates discussed in Chapter 3. Isolates were derived from different anatomical locations (nasopharynx, lung, joint) from cattle with different health statuses (healthy, disease, dead) collected from feedlots A to Z from 2006 to 2018. The cattle identifier associated with each isolate is indicated, as more than one isolate was derived from a single animal in some cases.

Isolate Identifier	Production Year	Feedlot	Anatomical Location	Health Status	Cattle Identifier
MPLM0640	2006	G	nasopharynx	healthy	C091
MPLM0643	2006	G	nasopharynx	healthy	C092
MPLM0648	2006	J	nasopharynx	diseased	C094
MPLM0649	2006	P	nasopharynx	diseased	C102
MPLM0642	2006	U	nasopharynx	diseased	C134
MPLM0632	2007	K	nasopharynx	dead	C096
MPLM0631	2007	L	joint	dead	C097
MPLM0629	2007	Q	nasopharynx	diseased	C103
MPLM0644	2007	Q	nasopharynx	diseased	C104
MPLM0645	2007	Q	nasopharynx	diseased	C105
MPLM0647	2007	Q	nasopharynx	diseased	C106
MPLM0652	2007	Q	nasopharynx	diseased	C107
MPLM0657	2007	Q	nasopharynx	diseased	C108
MPLM0660	2007	Q	nasopharynx	diseased	C109
MPLM0661	2007	Q	nasopharynx	diseased	C110
MPLM0662	2007	Q	nasopharynx	diseased	C111
MPLM0664	2007	Q	nasopharynx	diseased	C112
MPLM0665	2007	Q	nasopharynx	diseased	C113
MPLM0666	2007	Q	nasopharynx	diseased	C114
MPLM0669	2007	Q	nasopharynx	dead	C115
MPLM0684	2007	Q	nasopharynx	healthy	C116

MPLM0692	2007	Q	nasopharynx	healthy	C117
MPLM0698	2007	Q	nasopharynx	healthy	C118
MPLM0700	2007	Q	nasopharynx	healthy	C119
MPLM0703	2007	Q	nasopharynx	healthy	C120
MPLM0706	2007	Q	nasopharynx	healthy	C121
MPLM0713	2007	Q	nasopharynx	healthy	C122
MPLM0714	2007	Q	nasopharynx	healthy	C123
MPLM0715	2007	Q	nasopharynx	healthy	C124
MPLM0646	2007	R	nasopharynx	healthy	C125
MPLM0667	2007	R	nasopharynx	healthy	C126
MPLM0668	2007	R	nasopharynx	healthy	C127
MPLM0670	2007	R	nasopharynx	healthy	C128
MPLM0671	2007	R	nasopharynx	healthy	C129
MPLM0630	2008	K	joint	dead	C095
MP0209	2014	I	joint	dead	C056
MPLM0635	2014	H	lung	dead	C093
MP0219	2014	I	lung	dead	C056
MPLM0636	2014	S	lung	dead	C074
MPLM0634	2015	M	lung	dead	C098
MP0079	2015	I	lung	dead	C055
MPLM0637	2015	I	lung	dead	C057
MPLM0638	2015	I	joint	dead	C055
MPLM0003	2016	F	lung	dead	C072
MPLM0004	2016	F	joint	dead	C072
MPLM0007	2016	T	lung	dead	C045
MPLM0008	2016	T	joint	dead	C045
MPLM0009	2016	T	lung	dead	C046
MPLM0010	2016	T	joint	dead	C046
MPLM0011	2016	T	lung	dead	C047
MPLM0012	2016	T	joint	dead	C047

MPLM0013	2016	T	lung	dead	C048
MPLM0014	2016	T	joint	dead	C048
MPLM0015	2016	T	lung	dead	C049
MPLM0016	2016	T	joint	dead	C049
MPLM0017	2016	T	lung	dead	C050
MPLM0018	2016	T	joint	dead	C050
MPLM0020	2016	T	joint	dead	C051
MPLM0025	2016	T	lung	dead	C052
MPLM0026	2016	T	joint	dead	C052
MPLM0029	2016	N	lung	dead	C001
MPLM0030	2016	N	joint	dead	C001
MPLM0031	2016	T	lung	dead	C053
MPLM0035	2016	T	lung	dead	C054
MPLM0036	2016	T	joint	dead	C054
MPLM0037	2016	T	lung	dead	C043
MPLM0039	2016	T	lung	dead	C044
MPLM0041	2016	N	lung	dead	C002
MPLM0042	2016	N	joint	dead	C002
MPLM0132	2016	S	lung	dead	C130
MPLM0134	2016	S	lung	dead	C131
MPLM0135	2016	S	joint	dead	C131
MPLM0021	2016	T	lung	dead	C133
MPLM0022	2016	T	joint	dead	C133
MPLM0815	2017	A	nasopharynx	healthy	C075
MPLM0820	2017	A	nasopharynx	healthy	C077
MPLM0821	2017	B	nasopharynx	healthy	C078
MPLM0822	2017	B	nasopharynx	healthy	C079
MPLM0832	2017	E	nasopharynx	healthy	C086
MPLM0833	2017	E	nasopharynx	healthy	C087
MPLM0834	2017	E	nasopharynx	healthy	C088

MPLM0837	2017	E	nasopharynx	healthy	C089
MPLM0838	2017	E	nasopharynx	healthy	C090
MPLM0087	2017	N	lung	dead	C003
MPLM0088	2017	N	joint	dead	C003
MPLM0093	2017	N	lung	dead	C004
MPLM0102	2017	N	lung	dead	C005
MPLM0103	2017	N	joint	dead	C005
MPLM0105	2017	N	lung	dead	C006
MPLM0106	2017	N	joint	dead	C006
MPLM0111	2017	N	lung	dead	C007
MPLM0112	2017	N	joint	dead	C007
MPLM0114	2017	N	lung	dead	C008
MPLM0145	2017	N	lung	dead	C009
MPLM0146	2017	N	joint	dead	C009
MPLM0148	2017	N	lung	dead	C010
MPLM0149	2017	N	joint	dead	C010
MPLM0154	2017	N	lung	dead	C011
MPLM0155	2017	N	joint	dead	C011
MPLM0157	2017	N	lung	dead	C012
MPLM0158	2017	N	joint	dead	C012
MPLM0160	2017	N	lung	dead	C013
MPLM0164	2017	N	joint	dead	C014
MPLM0054	2017	O	lung	dead	C099
MPLM0057	2017	O	lung	dead	C100
MPLM0058	2017	O	joint	dead	C100
MPLM0060	2017	O	lung	dead	C101
MPLM0061	2017	O	joint	dead	C101
MPLM0136	2017	S	lung	dead	C132
MPLM0824	2018	C	nasopharynx	healthy	C081
MPLM0825	2018	C	nasopharynx	healthy	C082

MPLM0827	2018	D	nasopharynx	healthy	C083
MPLM0828	2018	D	nasopharynx	healthy	C084
MPLM0830	2018	D	nasopharynx	healthy	C085
MPLM0533	2018	N	lung	dead	C015
MPLM0534	2018	N	joint	dead	C015
MPLM0538	2018	N	joint	dead	C016
MPLM0541	2018	N	lung	dead	C017
MPLM0542	2018	N	joint	dead	C017
MPLM0545	2018	N	lung	dead	C018
MPLM0546	2018	N	joint	dead	C018
MPLM0555	2018	N	lung	dead	C019
MPLM0556	2018	N	joint	dead	C019
MPLM0559	2018	N	lung	dead	C020
MPLM0560	2018	N	joint	dead	C020
MPLM0608	2018	N	lung	dead	C021

APPENDIX C – CHAPTER 4: *MYCOPLASMA BOVIS* ISOLATES

Table A4.1. Summary of mycoplasma isolates discussed in Chapter 4. Isolates were derived from different anatomical locations (nasopharynx, lung, joint) from cattle with different health statuses (healthy, disease, dead) collected from feedlots A to Z from 2006 to 2018. The cattle identifer associated with each isolate is indicated, as more than one isolate was derived from a single animal in some cases.

Isolate Identifier	MLST ST	Notes	Production Year	Feedlot	Anatomical Location	Health Status	Cattle Identifier
MPLM0632	2		2007	K	nasopharynx	dead	C096
MPLM0644	2		2007	Q	nasopharynx	diseased	C104
MPLM0042	2	MJ259	2016	N	joint	dead	C002
MPLM0833	14	USA Isolate	2017	E	nasopharynx	healthy	C087
MPLM0648	21		2006	J	nasopharynx	diseased	C094
MPLM0645	21		2007	Q	nasopharynx	diseased	C105
MPLM0662	21		2007	Q	nasopharynx	diseased	C111
MPLM0698	21		2007	Q	nasopharynx	healthy	C118
MPLM0706	21		2007	Q	nasopharynx	healthy	C121
MPLM0154	21	MJ287	2017	N	lung	dead	C011
MPLM0054	21	MJ260	2017	O	lung	dead	C099
MPLM0647	24		2007	Q	nasopharynx	diseased	C106
MPLM0657	24		2007	Q	nasopharynx	diseased	C108
MPLM0660	24		2007	Q	nasopharynx	diseased	C109
MPLM0661	24		2007	Q	nasopharynx	diseased	C110
MPLM0664	24		2007	Q	nasopharynx	diseased	C112
MPLM0666	24		2007	Q	nasopharynx	diseased	C114
MPLM0669	24		2007	Q	nasopharynx	dead	C115
MPLM0684	24		2007	Q	nasopharynx	healthy	C116
MPLM0692	24		2007	Q	nasopharynx	healthy	C117
MPLM0700	24		2007	Q	nasopharynx	healthy	C119
MPLM0714	24		2007	Q	nasopharynx	healthy	C123

MPLM0715	24		2007	Q	nasopharynx	healthy	C124
MPLM0638	24		2015	I	joint	dead	C055
MPLM0541	27		2018	N	lung	dead	C017
MPLM0555	27		2018	N	lung	dead	C019
MPLM0556	27		2018	N	joint	dead	C019
MPLM0652	40		2007	Q	nasopharynx	diseased	C107
MPLM0665	40		2007	Q	nasopharynx	diseased	C113
MPLM0713	40		2007	Q	nasopharynx	healthy	C122
MPLM0667	40		2007	R	nasopharynx	healthy	C126
MPLM0668	40		2007	R	nasopharynx	healthy	C127
MPLM0640	42		2006	G	nasopharynx	healthy	C091
MPLM0649	42		2006	P	nasopharynx	diseased	C102
MPLM0642	42		2006	U	nasopharynx	diseased	C134
MPLM0670	42		2007	R	nasopharynx	healthy	C128
MPLM0671	42		2007	R	nasopharynx	healthy	C129
MPLM0636	42	MJ292	2014	S	lung	dead	C074
MPLM0634	42		2015	M	lung	dead	C098
MPLM0825	43		2018	C	nasopharynx	healthy	C082
MPLM0087	44	MJ267	2017	N	lung	dead	C003
MPLM0088	44	MJ268	2017	N	joint	dead	C003
MPLM0103	44	MJ272	2017	N	joint	dead	C005
MPLM0105	44	MJ273	2017	N	lung	dead	C006
MPLM0106	44	MJ274	2017	N	joint	dead	C006
MPLM0114	44	MJ278	2017	N	lung	dead	C008
MPLM0149	44	MJ286	2017	N	joint	dead	C010
MPLM0160	44	MJ289	2017	N	lung	dead	C013
MPLM0061	44		2017	O	joint	dead	C101
MPLM0815	45		2017	A	nasopharynx	healthy	C075
MPLM0832	45		2017	E	nasopharynx	healthy	C086
MPLM0136	45	MJ280	2017	S	lung	dead	C132
MPLM0827	45		2018	D	nasopharynx	healthy	C083
MPLM0828	45		2018	D	nasopharynx	healthy	C084
MPLM0646	48		2007	R	nasopharynx	healthy	C125

MPLM0631	52		2007	L	joint	dead	C097
MPLM0020	52	MJ246	2016	T	joint	dead	C051
MPLM0037	52	MJ255	2016	T	lung	dead	C043
MPLM0039	52	MJ257	2016	T	lung	dead	C044
MPLM0635	60	MJ291	2014	H	lung	dead	C093
MPLM0009	60	MJ237	2016	T	lung	dead	C046
MPLM0010	60	MJ238	2016	T	joint	dead	C046
MPLM0012	60	MJ240	2016	T	joint	dead	C047
MPLM0013	60	MJ241	2016	T	lung	dead	C048
MPLM0014	60	MJ242	2016	T	joint	dead	C048
MPLM0016	60		2016	T	joint	dead	C049
MPLM0017	60	MJ244	2016	T	lung	dead	C050
MPLM0018	60	MJ245	2016	T	joint	dead	C050
MPLM0025	60	MJ249	2016	T	lung	dead	C052
MPLM0026	60	MJ250	2016	T	joint	dead	C052
MPLM0031	60	MJ253	2016	T	lung	dead	C053
MPLM0035	60		2016	T	lung	dead	C054
MPLM0041	60	MJ258	2016	N	lung	dead	C002
MPLM0157	60		2017	N	lung	dead	C012
MPLM0533	60		2018	N	lung	dead	C015
MPLM0534	60		2018	N	joint	dead	C015
MPLM0538	60		2018	N	joint	dead	C016
MPLM0542	60		2018	N	joint	dead	C017
MPLM0545	60		2018	N	lung	dead	C018
MPLM0546	60		2018	N	joint	dead	C018
MPLM0559	60		2018	N	lung	dead	C020
MPLM0560	60		2018	N	joint	dead	C020
MPLM0015	61	MJ243	2016	T	lung	dead	C049
MPLM0703	62		2007	Q	nasopharynx	healthy	C120
MPLM0007	65	MJ235	2016	T	lung	dead	C045
MPLM0008	65	MJ236	2016	T	joint	dead	C045
MPLM0011	65	MJ239	2016	T	lung	dead	C047
MPLM0029	65	MJ251	2016	N	lung	dead	C001

MPLM0021	65	MJ247	2016	T	lung	dead	C133
MPLM0022	65	MJ248	2016	T	joint	dead	C133
MPLM0821	65		2017	B	nasopharynx	healthy	C078
MPLM0820	66		2017	A	nasopharynx	healthy	C077
MPLM0838	66	USA Isolate	2017	E	nasopharynx	healthy	C090
MPLM0093	67	MJ269	2017	N	lung	dead	C004
MPLM0102	67	MJ271	2017	N	lung	dead	C005
MPLM0111	67	MJ276	2017	N	lung	dead	C007
MPLM0112	67	MJ277	2017	N	joint	dead	C007
MPLM0145	67	MJ282	2017	N	lung	dead	C009
MPLM0146	67	MJ283	2017	N	joint	dead	C009
MPLM0155	67		2017	N	joint	dead	C011
MPLM0158	67	MJ288	2017	N	joint	dead	C012
MPLM0164	67		2017	N	joint	dead	C014
MPLM0057	67	MJ261	2017	O	lung	dead	C100
MPLM0058	67	MJ262	2017	O	joint	dead	C100
MPLM0060	67	MJ263	2017	O	lung	dead	C101
MPLM0629	70	MJ290	2007	Q	nasopharynx	diseased	C103
MP0079	70		2015	I	lung	dead	C055
MPLM0637	70	MJ293	2015	I	lung	dead	C057
MPLM0630	75		2008	K	joint	dead	C095
MP0209	76		2014	I	joint	dead	C056
MP0219	76		2014	I	lung	dead	C056
MPLM0003	77	MJ234	2016	F	lung	dead	C072
MPLM0004	77		2016	F	joint	dead	C072
MPLM0030	79	MJ252	2016	N	joint	dead	C001
MPLM0132	80		2016	S	lung	dead	C130
MPLM0134	80		2016	S	lung	dead	C131
MPLM0135	80		2016	S	joint	dead	C131
MPLM0608	149		2018	N	lung	dead	C021
MPLM0830	150	USA Isolate	2018	D	nasopharynx	healthy	C085

MPLM0643	151		2006	G	nasopharynx	healthy	C092
MPLM0822	152		2017	B	nasopharynx	healthy	C079
MPLM0823	153		2018	C	nasopharynx	healthy	C080
MPLM0824	153		2018	C	nasopharynx	healthy	C081
MPLM0834	154	USA Isolate	2017	E	nasopharynx	healthy	C088
MPLM0837	154	USA Isolate	2017	E	nasopharynx	healthy	C089
MPLM0817	Unknown	Missing gpsA, Novel pta2	2018	A	nasopharynx	healthy	C076
MPLM0019	Unknown	Missing dnaA, gltX, gpsA, gyrB, tdk	2016	T	lung	dead	C051
MPLM0036	Unknown	MJ254, Missing gpsA	2016	T	joint	dead	C054
MPLM0148	Unknown	MJ285, Missing gyrB	2017	N	lung	dead	C010

APPENDIX D – ANTIMICROBIAL SUSCEPTIBILITY PLATE MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	ENRO 0.12	ENRO 0.25	ENRO 0.5	ENRO 1	ENRO 2	ENRO 4	ENRO 8	ENRO 16	ENRO 32	ENRO 64	ENRO 128	TYLT 1
B	TIP 0.12	TIP 0.25	TIP 0.5	TIP 1	TIP 2	TIP 4	TIP 8	TIP 16	TIP 32	TIP 64	TIP 128	TYLT 2
C	GAM 0.25	GAM 0.5	GAM 1	GAM 2	GAM 4	GAM 8	GAM 16	GAM 32	GAM 64	GAM 128	GAM 256	TYLT 4
D	TUL 0.25	TUL 0.5	TUL 1	TUL 2	TUL 4	TUL 8	TUL 16	TUL 32	TUL 64	TUL 128	TUL 256	TYLT 8
E	FFN 0.25	FFN 0.5	FFN 1	FFN 2	FFN 4	FFN 8	FFN 16	FFN 32	FFN 64	FFN 128	FFN 256	TYLT 16
F	OXY 0.5	OXY 1	OXY 2	OXY 4	OXY 8	OXY 16	OXY 32	OXY 64	OXY 128	OXY 256	PEN 2	TYLT 32
G	CTET 1	CTET 2	CTET 4	CTET 8	CTET 16	CTET 32	CTET 64	CTET 128	CTET 256	POS	PEN 4	TYLT 64
H	TIL 1	TIL 2	TIL 4	TIL 8	TIL 16	TIL 32	TIL 64	TIL 128	TIL 256	POS	PEN 8	TYLT 128

Figure D1. Custom Sensititre™ plate for micro-dilution. Units are in µg/mL. ENRO, Enrofloxacin; TIP, Tildipirosin; GAM, Gamithromycin; TUL, Tulathromycin; FFN, Florfenicol; OXY, Oxytetracycline; CTET, Chlortetracycline; TIL, Tilmicosin; PEN, Penicillin; TYLT, Tylosin tartrate; and POS, Positive Control.