PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF *MYCOPLASMA BOVIS* AND *PASTEURELLA MULTOCIDA* ISOLATED FROM ALBERTAN FEEDLOT CATTLE

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Prevalence and antimicrobial susceptibility of *Mycoplasma bovis* and *Pasteurella multocida* isolated from Albertan feedlot cattle

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Dedication

This work is dedicated to my son Sadiq Shahriar - thank you for your support, inspiration and smiles; to my beloved husband - thank you for your love, sacrifice, support, inspiration and understanding. Without your support, I may not be here today; my adoring mother, Shamsun Nahar -for your encouragement, prayers and support; and my caring father, Md. Eunus Ali - for your guidance, motivation and prayers. I want to thank everyone for their unwavering support, love, and encouragement that I experienced every day. Above all, I dedicate this work to the all-powerful, the most merciful Allah, who has enabled me to conduct this research work.

ABSTRACT

Bovine respiratory disease (BRD) is a significant health problem for the Canadian feedlot industry. While often polymicrobial in nature, *Mycoplasma bovis* and *Pasteurella multocida* are considered important respiratory pathogens for BRD. This study aimed to evaluate the prevalence and antimicrobial resistance of *M. bovis* and *P. multocida* isolated from Albertan feedlot cattle that were sampled 8 years apart. In the first study, nasopharyngeal swabs from cattle sampled at feedlot entry and after 60 days on feed were collected in 2008-2009 (Cohort 1). In a second study conducted in 2015-2016 (Cohort 2), nasopharyngeal swabs were collected from cattle diagnosed with BRD and matching healthy controls. Trans-tracheal samples were also collected from Cohort 2 cattle for *M. bovis* evaluation. For Cohort 1, the prevalence of *M. bovis* was lower in cattle at entry compared to when the same individuals were sampled ≥60 days later ($P<0.05$). For Cohort 2, the prevalence of *M. bovis* was greater in both nasopharyngeal and tracheal samples from cattle diagnosed with BRD, compared to controls ($P<0.05$). Similarly, *P. multocida* was more frequently isolated from the nasopharynx of BRD cases. Antimicrobial-resistant patterns changed broadly over the 8-year time period with resistance being lower ($P<0.05$) in Cohort 1 bacteria for florfenicol and tulathromycin. When evaluated for resistance genes by PCR, the majority (98%) of oxytetracycline-resistant isolates carried *tet*(H) while only 16 (15%) out of 106 tulathromycin-resistant isolates from Cohort 2 carried a known macrolide resistance gene. The genomes of nine tulathromycin-resistant isolates were sequenced, leading to the identification of a conserved gene cluster that was present in all isolates with tulathromycin-resistance but unknown macrolide resistance genes. One of the genes was a novel putative methylase, which doubled the minimum inhibitory concentration against tulathromycin when cloned into *Escherichia coli*. This study showed that macrolide resistance in *M. bovis* and *P. multocida* increased over an 8-year span, coinciding with the approval and adoption of tulathromycin to prevent BRD in Canadian cattle.

Additionally, a novel putative macrolide resistance gene was identified and shown to be widespread in *P. multocida* from the feedlots enrolled in this study. The rapid development of resistance to a newly used antimicrobial indicates the need to reserve essential antimicrobials for the treatment of cattle, to maintain their efficacy.

PREFACE

Two manuscripts are included in this thesis. Chapter 2 has been submitted to Veterinary Microbiology Journal**,** which is currently under review. Chapter 3 has been prepared to submit to the Microbiology spectrum journal.

Manuscript-1 (Chapter 2):

Razia Sultana, Roniele P. Cordeiro, Edouard Timsit, Tim A. McAllister, Trevor W. Alexander. Prevalence and antimicrobial susceptibility of *Mycoplasma bovis* from the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bovine respiratory disease.

Contribution of authors: Razia sultana was involved in establishing the SOP for *M. bovis* sensitivity testing, performed the laboratory experiments, and prepared the draft manuscripts under the guidance of Trevor W. Alexander. Roniele P. Cordeiro, Edouard Timsit and Tim A. McAllister contributed to the manuscript preparation.

Manuscript-2 (Chapter 3):

Razia Sultana, Roniele Peixoto Cordeiro, Matthew Waldner, Edouard Timsit, Tim A. McAllister, Long Jin and Trevor W. Alexander. Characterization of antimicrobial resistance in *Pasteurella multocida* isolated from Alberta feedlot cattle.

Contribution of authors: Razia sultana contributed to the laboratory work, result analysis and prepared the draft manuscripts under the supervision of Trevor W. Alexander. Matthew Waldner and Long Jin assisted with bioinformatics analysis. Roniele Peixoto Cordeiro, Edouard Timsit and Tim A. McAllister contributed to the manuscript revision.

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LIST OF ABBREVIATIONS

Symbols	Definition
AMA	Antimicrobial agents
AMR	Antimicrobial resistant
BRD	Bovine Respiratory Disease
BHV-1	Bovine herpes virus 1
BVDV	Bovine viral diarrhoea virus
BPI3V	Bovine parainfluenza 3 virus
BRSV	Bovine respiratory syncytial virus
CPPS	Chronic pneumonia and polyarthritis syndrome
CHL	Chlortetracycline
CLI	Clindamycin
DOF	Days on feed
ENR	Enrofloxacin
FLO	Florfenicol
XDR	Extensively drug-resistant
ICE	Integrative conjugative elements
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentrations
MGE	Mobile genetic elements
NP	Nasopharyngeal
OXY	Oxytetracycline
OMPs	Outer membrane proteins
PPLO	Pleuropneumonia-like organisms
PFGE	Pulsed-field gel electrophoresis
SPE	Spectinomycin
TSA	Tryptic soy agar
TTA	Trans-tracheal aspiration
TIL	Tilmicosin
TUL	Tulathromycin
TYL	Tylosin

Chapter one

Literature review

1.1 Background

Bovine respiratory disease (BRD) commonly refers to bacterial lung infection of cattle and is the most significant disease affecting the North American beef industry. Compared to other livestock, the life cycle of cattle is longer, and results in cattle being exposed to pathogens and circumstances over an extended period that can cause BRD. Cattle production typically lasts between 24 and 28 months, depending on the particular production system, comprising nine months for gestation, seven to eight months of nursing before weaning, four to six months for stocker/backgrounding, and five to eight months for fattening in feedlots (Peel, 2020).

BRD is the principal cause of morbidity and fatality in North American feedlot cattle (Holman et al., 2015). It is the single major health problem affecting the feedlot industry, leading to significant financial losses because of the expense of treatment, lower production, and death (Blakebrough-Hall et al., 2020; Klima et al., 2014). Since cattle are exposed to a variety of pathogens and stresses (e.g. weaning, transportation, commingling, and primary viral infections) at the time of their first sixty days at feedlots, BRD mainly affects cattle at this time. (Timsit et al., 2017). The disease costs the American beef sector between $800 million and $900 million yearly in economic losses (Brooks et al., 2011). According to estimates, BRD causes more than $3 billion in annual global losses (Watts and Sweeney, 2010), totalling more than the total cost of all other cattle diseases (Highlander, 2001).

1.1.1 Significance and prevalence of BRD

BRD accounts for 65-80% of morbidities and over 50% of the fatalities in North American feedlots (Edwards, 1996). In the United States BRD was detected in 16.2% of all feedlot cattle

at some time throughout production (Holman et al., 2015; USDA, 2013). Delaying BRD diagnosis and treatment raises the risk of secondary bacterial infections, serious illness, and death. BRD is thought to affect more than 22% of all pre-weaned calves in dairy operations and is responsible for approximately 20% of all animal fatalities in this population (Dubrovsky et al., 2020). An analysis of 20 years of from data of the US Meat Animal Research Center, observed 10.5% BRD diagnosed cattle (Pal et al., 2020). To control and treat BRD, significant resources have been used in the improvement of technologies and management strategies, but during the past 45 years, morbidity and fatality rates have remained mostly stable (Smith et al., 2020).

1.2 Symptoms of BRD

The clinical signs of BRD include temperature above 40° C, depression, anorexia, an elevated heart rate, rhinitis with mucosal discharge or a dry encrusted muzzle, lacrimation, and cough (Zecchinon et al., 2005). Breathing problems like dyspnea may occur in the early stages of infection (Zecchinon et al., 2005). While synonymous with "Shipping Fever" BRD may not always be associated with shipping and it can be seen after stock attendants have minimized their supervision of the newly arrived animals. The physical symptoms of BRD can vary according to a bacterial pathogen, with some, like *Mannheimia haemolytica* and *Pasteurella multocida*, resulting in more obvious symptoms. However, some pathogenic organisms, such as *Mycoplasma bovis*, do not generate the toxins like other bacteria, so animals affected with *M. bovis* may not appear depressed (Woodbury, 2015). Consequently, it is easier to ignore these animals. Persistently, when infected with *M. bovis*, cattle show less interest to move, obvious breathing difficulty, diminished or no appetite, and an inadequate or extended response to treatment.

1.3 Predisposing factors

1.3.1 General factors

BRD is a polymicrobial disease, that develops from infections from multiple bacteria and viruses (Gaudino et al., 2022). While a single risk factor may not result in BRD, a combination of risk factors may increase an animal's susceptibility to BRD. Host factors play an essential role in BRD. Age, nutritional state, immunological status, prior exposure to the infections, genetics (for example, crossbred cattle typically do better in terms of health than straightbred cattle), and others are host factors. BRD has long been connected to management factors. Close contact between cattle enhanced the possibility of BRD pathogen transmission (Loneragan et al., 2005). Transport, routine handling and sorting (for example, while processing), and intensive grazing methods may raise the possibility of spreading disease. No matter the size of the herd, outbreaks of BRD (20% of herds) were most prevalent in herds that had bought ten or more bulls, used shared pastures, purchased cows, or neglected to vaccinate recently acquired animals (Wennekamp et al., 2021). The occurrence of BRD may be affected by nutrition. According to Wilson et al, 1985, high grain levels were linked to elevated BRD. Another study found that feeding concentrates at higher levels (90%) increased morbidity (Lofgreen et al., 1975). Other dietary strategies, such as the addition of potassium, thiamine, and other B vitamins, as well as rumen bypass protein, have been suggested to reduce BRD (Brethour and Duitsman, 1972; Hutcheson et al., 1984).

1.3.2 Environmental factors

BRD affects cattle mostly during the period after feedlot entry (Larson et al., 2004). Cattle affected with BRD spread abundant pathogens into the environment which could be a significant exposure source for all other calves (Murray et al., 2018). In addition, healthy animals can also carry BRD associated bacteria, which are typically opportunistic pathogens

(Hodgins et al., 2002; Murray et al., 2018). Stress plays a significant impact in the development of BRD by suppressing immunity in calves (Ackermann et al., 2010; Dabo et al., 2007; Murray et al., 2018). Both dietary and non-nutritional variables can cause weaning distress in calves, making it a stressful period for them (Murray et al., 2018). Some other factors like disconnecting the maternal ties and changing in the social group (e.g. commingling with new pen mates in feedlots) are crucial conditions for cattle affecting host stress response and immunity (Murray et al., 2018). In addition, weather conditions, temperature difference, dust, stocking volume, moisture, airflow, and travel time are all environmental risk variables (Snowder, 2009). The transmission of BRD infections can result in exposure and, possibly, disease due to factors such as poor ventilation and transportation (Murray et al., 2018). The occurrence of BRD has also been linked to the weather. According to a study evaluating 288,388 head of cattle entered at nine U.S. commercial feedlots from September to November between 2005 and 2007, wind chill and temperature fluctuation were linked to an elevated prevalence of BRD (Cernicchiaro et al., 2012). Sub-zero temperatures to roughly 15° C are within the range of the ideal ambient temperature for beef cattle performance (Hahn, 1999). However, younger animals, aged less than a month, have a limited comfort range between 10 and 25° C (Hahn, 1999). Cattle may become stressed as the ambient temperature falls or rises to these levels, depending on additional climatic factors including relative humidity wind speed, shade, and wind protection (Cernicchiaro et al., 2012). Temperature change, wind, and precipitation have an impact on cattle (Hahn, 1999). During gradual changes, beef cattle may adapt to cooler temperatures (Cernicchiaro et al., 2012).

1.3.3 Viral agents

There are numerous viral agents linked to BRD, but the most prevalent ones are the bovine herpes virus 1 (BHV-1), bovine viral diarrhoea virus (BVDV), bovine parainfluenza 3

virus (BPI3V), and bovine respiratory syncytial virus (BRSV) (Grissett et al., 2015). BRD can manifest through primary viral infections predisposing to secondary bacterial infection of the lower respiratory tract.

1.3.3.1 Bovine herpes virus 1 (BHV-1)

BHV-1 is a double-stranded DNA virus causing infectious bovine rhinotracheitis, revealed by ulceration in the upper respiratory tract and acute inflammation, conjunctivitis, genital disorders, and immune suppression (Ellis, 2009; Jones and Chowdhury, 2007; Yates, 1982). BHV-1-associated BRD cases can promote bacterial infections leading to severe bronchopneumonia (Narita et al., 2000). A minimum of three proteins are encoded by BHV-1 that can block particular immune system components: The UL41.5 protein prevents viral peptides from reaching the cell surface, which reduces CD8+ T-cell identification of infected cells, the bICP0 protein inhibits interferon-dependent transcription and glycoprotein G which is a chemokine-binding protein that stops lymphocytes from homing to sites of infection (Jones and Chowdhury, 2007). BHV-1 can infect and cause a significant amount of CD4+ T-cell apoptosis after acute infection of calves. Consequently, BRD Complex may result from BHV-1's capacity to weaken the immune response (Jones and Chowdhury, 2007). As BHV-1 accounts for the disappearance of ciliated cells in affected airways the natural non-specific defensive mechanisms may be disrupted, thus, this pathogenesis could play an important role in secondary bacterial bronchopneumonia (Narita et al., 2000).

1.3.3.2 Bovine viral diarrhea virus (BVDV)

BVDV is a single-stranded RNA virus and is an important pathogen of the dairy and beef cattle populations, causing primary infection of the bovine lung (Fulton et al., 2002). BRD may occur in both acute and postnatal BVDV infections, mostly by immunosuppression and

the interaction of several pathogens, which speeds up secondary infection. (Ridpath, 2010). Based on antigenic and genetic variations, BVDV is categorized into two genotypes, BVDV1 and BVDV2, and then into cytopathic and non-cytopathic (ncp) biotypes depending on a strain's capacity to cause cell death in cell culture (Vilcek et al., 2005). The fetus can be infected during early gestation, as the infection can be transferred by persistently infected animals (Lanyon et al., 2014). The contribution of BVDV infections to the progression of clinical respiratory disease depends on the pathogenicity of the infecting BVDV strain, the nature of infection (acute or chronic), the period of exposure (fetal or postnatal), and the involvement of secondary pathogens (Ridpath, 2010). The respiratory tract's epithelial surface may get damaged from BVD infection, and lymphoid tissues may become depleted (Ridpath, 2010).

1.3.3.3 Bovine respiratory syncytial virus (BRSV)

BRSV is an enveloped and single-stranded RNA virus. It causes lower respiratory tract infections in calves (Hoppe et al., 2018). It can account for 70% of respiratory infections in cattle, with mortality rates between 2% and 20% (Gershwin, 2007). BRSV infection typically affects young calves between the ages of two weeks and nine months in dairy cattle (Hoppe et al., 2018). Respiratory symptoms, such as nasal and ocular discharges, are clinical signs of BRSV infection. However, in calves, acute and chronic infection can occur, if BRSV infection is not successfully protected against by maternal antibodies (Hoppe et al., 2018). Infection does not spread away from,past the respiratory epithelium (Ellis, 2009) and virus starts shedding after three days of infection. Usually, the virus is no longer detectable after 10 days (Gershwin, 2007).

1.3.3.4 Parainfluenza type 3 virus (BPI3V)

BPI3V is a single-stranded RNA virus. Infection is widespread and typically non-clinical (Ellis, 2009). BPI3V infections are usually associated with a secondary bacterial agent in BRD. The acute infection disrupts the ciliated cells but also significantly reduces the ability of infected macrophages to cause cytotoxicity (Ellis, 2010). It is difficult to detect through isolation (Henrickson, 2003) and is regarded as a BRD synergist and present in several BRD combination vaccinations (Ellis, 2010)**.**

1.3.4 Bacterial agents

1.3.4.1 *Pasteurella multocida*

Pasteurella species are opportunistic pathogens in both domestic and wild animals (Wilson and Ho, 2013). According to the World Animal Health Organization (OIE) (http://www.oie.int/), Pasteurellosis (symptomatic infection with *Pasteurella*) has a strong effect on cattle. *P. multocida* can cause both acute and chronic infections (manifested as pasteurellosis, pneumonia, atrophic rhinitis, dermonecrosis, cellulitis, abscesses, meningitis, and/or hemorrhagic septicemia), particularly in animals, and can lead to significant morbidity and mortality rates as pasteurellosis cases in the United States have risen recently (Wilson and Ho, 2013).

The gram-negative coccobacillus *Pasteurella multocida* is a member of the *Pasteurellaceae* family, and is a common inhabitant of the upper respiratory tract of both wild and domestic animal species (Aida et al., 2019). *P. multocida* can cause disease in most domestic and wild animals including poultry and wild birds, pigs, cattle, buffalo, rabbits, small ruminants, cats, dogs, and other mammals (Peng et al., 2018). *P. multocida* is the cause of several economically significant diseases in the world, including hemorrhagic septicemia, pulmonary pneumonia, and inflammation in poultry and livestock, as well as enzootic

bronchopneumonia in cattle and sheep (He et al., 2019; Tang et al., 2009a). It can also cause zoonotic disease in humans (Masafumi Seki, 2016). The pathogenesis of *P. multocida* is influenced by numerous virulence factors which assist in host colonization. The most important virulence factors are genes that affect capsule development, lipopolysaccharide, fimbriae and adhesins, toxins, iron-regulated and iron acquisition proteins, sialic acid metabolism, hyaluronidase, and outer membrane proteins (Peng et al., 2018). Several outbreaks in humans have been linked to *P. multocida*, particularly in Australia, Vietnam, Canada, and the United States (Tang et al., 2009a). Depression, inappetence, coughing, nasal discharge, fever, or a combination of these, are frequently observed as pulmonary pneumonia symptoms in cattle (S. M. Dabo, 2008) which, can lead to severe morbidity or death (Narcana et al., 2020).

1.3.4.2. *Mannheimia haemolytica*

The primary bacterial cause of BRD is thought to be the *Pasteurellaceae* family member *M. haemolytica* (Rice et al., 2007), as it is frequently isolated from newly received calves suffering from pleuropneumonia (Klima et al., 2011c). *M. haemolytica* colonization at feedlot entry has been associated with BRD (Holman et al., 2017). The virulence and pathogenesis of *M. haemolytica* are highly linked with serotypes. *M. haemolytica* has twelve serotypes, among them serotype 2 is a commensal of the normal respiratory flora and is typically thought to be non-pathogenic to cattle (Klima et al., 2011c). Serotype 1 is thought to be infectious since it is commonly identified from cattle pneumonic lesions, although serotype 6 has also been linked to respiratory illness. (Highlander, 2001; Klima et al., 2011c). Leukotoxin (Lkt) is the main virulence component of *M. haemolytica* (Dassanayake et al., 2009) and causes leukocyte cell lysis. The recent discovery of pan-resistant *M. haemolytica* in beef calves is concerning (Eidam et al., 2015; Lubbers and Turnidge, 2015), and raises questions about how to preserve the therapeutic effectiveness of antimicrobials for beef cattle.

It has been shown that after receiving metaphylactic antimicrobial therapy, the recovery of extensively drug-resistant (XDR) *M. haemolytica* isolates from groups of stocker cattle increased quickly (Snyder et al., 2017), highlighting the linkage between antimicrobial use and development of resistance in BRD-associated bacterial pathogens.

1.3.4.3: *Histophilus somni*

Histophilus somni is a member of the *Pasteurellaceae* family and can cause meningitis, ecchymotic hemorrhage on serous membranes of subcutaneous tissues and muscles, necrotic foci in the liver, bacterial thrombosis, and necrotizing vasculitis (Ward et al., 2006). *H. somni* is also a causative agent of infectious meningoencephalitis, respiratory disease, abortion, arthritis, septicemia, laminitis, mastitis, and myocarditis in feedlot cattle (Humphrey and Stephens, 1983; Ward et al., 2006). Respiratory infections typically contain many organisms, and *H. somni* is frequently discovered along with other BRD pathogens. In a study conducted in 2002–2003 (Fulton, 2003), *H. somni* was identified in 10% of the lungs of animals that died because of BRD. *H. somni* possess multiple virulence factors including lipooligosaccharide, immunoglobulin binding proteins, outer membrane, and major outer membrane proteins and exopolysaccharides (Corbeil, 2007), which contribute to pathogenesis and host defence resistance. In some feedlots, 50-60% of cattle morbidity can be linked to histophilus (J Van Donkersgoed, 2000).

1.3.4.4: *Mycoplasma bovis*

M. bovis is a member of the *Mollicutes* family and is mainly transmitted by direct contact with *M. bovis* infected animals (Razin et al., 1998). The antigenic profile of *M. bovis* is highly variable (Bürki et al., 2015) and depends on a number of significant membrane proteins that are amphiphilic and include cross-reactive epitopes that serve as important

immunogens. (Bürki et al., 2015). Due to these antigenic traits, *M. bovis* can resist the host immune system. For this reason, the host's defence against this bacterium is typically weak, and results in the persistent manifestation of diseases driven by *M. bovis*. (Buchenau et al., 2010; Bürki et al., 2015). *M. bovis* produces biofilms, which help them persist in the environment and inside the host. Furthermore, biofilms may worsen host tissue injury by protecting bacteria against host defences or antimicrobials. (Bürki et al., 2015). *M. bovis* can affect tissues and organs causing bronchopneumonia otitis, mastitis, genital disorders, arthritis, and meningitis (Bürki et al., 2015; Caswell and Archambault, 2007; Heuvelink et al., 2016). This bacterium poses a significant danger to livestock productivity as an emerging disease in industrialised nations' cattle populations (Nicholas, 2011).

M. bovis is frequently found along with other microorganisms. The most prevalent microorganisms isolated with *M. bovis* are *P. multocida, M. haemolytica, H. somni, BRSV, BHV-1, BVDV, and* BPI3V. Despite *M. bovis* being an important causative agent of BRD (Aebi et al., 2015; Caswell et al., 2010; Maunsell et al., 2011), its fastidious nature and the need for specialized medium and methods for its isolation and growth make it the least characterized BRD pathogen. According to estimates, *M. bovis* is responsible for a quarter to a third of economic losses in the cattle industry that are associated with BRD (Sulyok et al., 2014b). When cattle are mixed and placed in a commercial feedlot, the incidence of *M. bovis* in bronchoalveolar lavage fluid has been observed to increase from 1.7% at feedlot entry to 72.2% 15 days afterwards. (Castillo-Alcala et al., 2012b). Although *M. bovis* can persist in cattle without causing disease, in a recent study, 28% of cattle necropsied in three Canadian feedlots had chronic pneumonia and polyarthritis syndrome (CPPS), a disease associated largely with *M. bovis* (Maunsell et al., 2011). Thus, control of this pathogen in feedlots is important for cattle health.

1.4 Characterization of BRD bacterial pathogens

1.4.1 Pulsed-field gel electrophoresis

Before the widespread use of genome sequencing, pulsed-field gel electrophoresis (PFGE) was considered the "gold-standard" for phylogenetic analysis. Due to its benefits for identifying bacterial subgroups, bacterial DNA fingerprinting is currently the primary PFGE application. PFGE can be used to determine the clonal source of isolates in an infectious outbreak or to monitor spread within a population (Goering and Evolution, 2010; Lopez-Canovas et al., 2019). By employing PFGE, a restriction enzyme detects certain DNA sequences in the genome and cleaves bacterial genomic DNA into a number of smaller fragments. More than 90% of the bacterial genome may be resolved by PFGE in band patterns using macro-restriction DNA fragments in a single experiment, providing the DNA fingerprint of each isolate tested. This allows for each bacterial isolate to be given a specific subtype based on the PFGE study of its genome. The results of PFGE show excellent agreement between isolates epidemiological relationships (Popovic et al., 2001). Typically, identical pulsotypes can be considered as clones and pulsotypes >90% similar describe groups that are highly related genetically.

1.4.2 Comparative genomics

1.4.2.1 Pan and core genome

Comparative genomics is used to look at various characteristics, such as overall sequence similarity, gene organization, and gene transfer (Wei et al., 2002). The term "pan-genome" refers to the total number of genes in a species that code for the entire range of proteins that a species can produce (Tettelin et al., 2008). The core genome is the collection of genes that are present in all strains, can reveal valuable data on the evolution and lifecycle of a bacterial species and is likely to contain functions linked to basic biology and phenotypic

(Donati and Rappuoli, 2013). Recently, the genomes of three avian *P. multocida* strains (Pm70, x73, and 1059) were compared to each other and revealed 61 genes that were lacking from one non-pathogenic strain (Pm70) but shared by two pathogenic strains (x73 and 1059), showing the utility of genome sequencing in comparing *Pasteurella* bacteria (Hurtado et al., 2018). Genotyping analysis can therefore be employed to identify virulence genes. It has also been used in characterizing antimicrobial resistance (AMR) genes in BRD bacterial pathogens (Cameron et al., 2018). However, the identification of resistance genes by genome analysis is limited to those in databases being used for cross reference. As such, novel resistance genes may be missed in initial genome screening, and additional characterization based on phenotype may be required to identify genes conferring resistance.

1.4.2.2 Mobile genetic elements

In bacterial genomes, horizontal gene transfer of mobile genetic elements (MGE) is responsible for acquiring about three-quarters of all genes (Juhas, 2015). Plasmids, phage, genomic islands, and genomic modules are some examples of these that spread horizontally via conjugation, transduction, or transformation (Wozniak and Waldor, 2010). In order to recognize the mechanisms of virulence and to determine the genes responsible for resistance, it is important to identify mobile genetic components inside genomes, such as bacteriophage and integrative conjugative elements (ICE). In a previous study, *P. multocida* had acquired resistance to 11 different antimicrobial drug classes, and the genes conferring resistance were associated with MGE (Smith et al., 2021).

1.4.2.3 Integrative conjugative elements

The process of conjugation is important for the dissemination of AMR genes in microbial communities (Wilcks and Jacobsen, 2010). Conjugative plasmids and ICE, such as conjugative

transposons, are the components that cause conjugation. Integrative conjugative elements are one of the most significant groups of mobile genomic components in bacteria. These elements are special because they can transmit themselves and contain all the mechanics needed for integration, excision, and transfer (Wozniak and Waldor, 2010). ICEs enable bacteria to quickly adapt to shifting environmental conditions and colonize new habitats (Burrus and Waldor, 2004), by facilitating the transfer of accessory genes that affect phenotypic, such as virulence and antimicrobial resistance, (Wozniak and Waldor, 2010). Many ICEs can be transferred to a variety of species and are not host-specific (Garriss et al., 2009), however the exact parameters influencing host range have not been completely investigated (Wozniak and Waldor, 2010). In the chromosomes of *Pasteurellaceae*, resistance genes frequently congregate within ICEs (Beker et al., 2018). Transposons are repeated DNA sequences that are able to move (transpose) from one area of the genome to another. Transposon migration can affect gene expression and cause mutations (Gao et al., 2015). Analysis of ICE*Pmu1* from *P. multocida* showed that it was comprised of 12 AMR genes, highlighting the importance of ICE in conferring resistance in BRD pathogens (Michael et al., 2012).

1.5 Management of BRD

1.5.1 Preconditioning

Pre-conditioning is an idea created to adopt weaning management measures that minimize stress and enhance the animal's nutritional and immune systems (Lalman and Ward, 2005). Vaccinations, antihelminthic therapy, castration, dehorning, and adaptation to feed bunks and water troughs are typically included in pre-conditioning programs, however, they can vary (Taylor et al., 2010a). It is recommended to perform stressful treatments including weaning, castration, and dehorning at least one month prior to entering a feedlot (Hay et al., 2016), and if possible to spread the stressors our across time. Feeding can be increased and the

requirement for BRD treatments can be minimized by minimal stress weaning techniques (such as two-stage, fence-line weaning) (BCRC, 2022). When calves are transported to a feedlot that mostly buys preconditioned calves, these preconditioning programs have significantly reduced morbidity and mortality (Cravey, 1996; Taylor et al., 2010a).

1.5.2 Vaccines

The performance of cow herds in terms of reproduction is improved by administering vaccines against BRD pathogens through maternal antibodies and reduced incidence of disease, and it also helps keep pre-weaned calves healthy (Stokka, 2010). Canadian herd health programs heavily rely on vaccinations, but they rarely offer perfect protection. Inadequate nourishment, poor biosecurity, and poor environmental management can all reduce their effectiveness. There are commercially available inactivated (killed) and active (modified-live) forms of multivalent viral vaccinations against BHV-1, BVDV, BRSV, and PI-3V (Richeson and Falkner, 2020). Additionally, the formulations of bacterin and/or leukotoxoid against *P. multocida*, *H. somni*, and *M. haemolytica* are available. However, despite using vaccines, the prevalence of BRD in feedlot cattle has remained steady or increased (Hilton, 2014; Zhang et al., 2019). This may be due to the limited efficacy of current vaccines against *M. haemolytica*, *P. multocida,* and *H. somni* and the lack of an effective vaccine against *M. bovis* (Larson and Step, 2012, Richeson and Falkner, 2020).

1.5.3 Antimicrobials

The main focus of BRD prevention and control in big commercial feedlots in North America is bacterial pathogens, using antimicrobial agents (AMA) and vaccination programs. From birth until weaning, respiratory illnesses account for most antimicrobial treatments in calves. In 77% of cow-calf operations in Western Canada, at least one calf received treatment

for respiratory disease (Waldner et al., 2019). Upon arrival at the feedlot, cattle thought to be at a high risk of developing BRD are frequently administered metaphylactic AMA (Ives and Richeson, 2015a). Metaphylaxis is the treatment of a cohort of cattle considered to be at high-risk (e.g., low weight, sourced from an auction market, and recently weaned) of developing BRD. Approximately 39.2% of high-risk cattle in the United States received an injectable antimicrobial agent following feedlot placement (USDA, 2013). In Canada, 20% to 50% of cattle have been reported to be injected with a metaphylactic AMA at feedlot entry, to control BRD (Checkley et al., 2010a).

Metaphylactic treatment of cattle has previously been shown to lower the incidence of BRD in cattle and thus enhance performance compared to cattle that are not treated (Ives and Richeson, 2015a). However, the efficacy of AMA can be negatively impacted by resistant bacteria, which may account for rates of BRD remaining constant in feedlots. The use of AMA may result in the emergence of bacterial resistance and there is evidence of increased resistance rates in respiratory bacteria from cattle over the past 10 years (Cameron and McAllister, 2016; Portis et al., 2012a).

1.6 Antimicrobial resistance:

1.6.1 Antimicrobial used in livestock and development of resistance

A major concern to human and animal health is antimicrobial resistance in pathogenic bacteria from food-producing animals and environmental sources (Bronzwaer et al., 2002; Tang et al., 2009b). Multiple national surveillance systems have been established to identify and track alterations in the antimicrobial susceptibility profiles of organisms significant to both the food business and human health. To maintain efficient antimicrobial selection for BRD management and therapy, accurate and current knowledge of pathogen susceptibility patterns

and resistance mechanisms is required (Lubbers and Turnidge, 2015). Most of the important classes (i.e., relevant human health) of antimicrobials used in the beef industry are for treating and preventing BRD. For example, medications used to manage BRD-related bacterial infections in feedlots include florfenicol (classified as phenicols), ceftiofur (classified as cephalosporin), oxytetracycline (classified as tetracycline), and tulathromycin (classified as macrolide) (Portis et al., 2012a). Recently, it was reported that isolates of *M. haemolytica* and *P. multocida* had high levels of resistance (>70%) to tulathromycin and oxytetracycline, and isolates of *H. somni* from morbid cattle in Alberta had high levels of resistance to oxytetracycline (>67%) and penicillin (52%) (Timsit et al., 2017). Increasingly there is evidence linking agricultural antimicrobial usage to the development and spread of antimicrobial-resistant microorganisms in humans (Fey et al., 2000, Manyi-Loh et al., 2018). Additionally, epidemiological proof demonstrates that feedlot management techniques have facilitated the development of veterinary pathogen resistance (Portis et al., 2012a). Bacteria may be naturally resistant to antimicrobial agents or can develop resistance to these agents by *de novo* mutations in their genomes or via horizontal gene transfer via conjugation, transformation, or transduction. Several MGE including plasmids and ICE have been connected to resistance spreading (Wozniak and Waldor, 2010). These elements frequently contain collections of accessory genes, such as recombinant multidrug resistance cassettes. Once they have been created, multidrug-resistant elements can be established and survive in bacterial populations by using a single antimicrobial that co-selects for the whole element. The resistance gene portions (including *tet*(R)-*tet*(H), *erm*(42), and *msr*(E)-*mph*(E)) of the integrative and conjugative element ICEPmu1, may cross genus boundaries and activate its resistance genes in a variety of hosts, such as *P. multocida* and *M. haemolytica* (Klima et al., 2014b; Michael et al., 2012). Because AMA is often selected as a first line of defense for both

animal and human bacterial diseases, the development of resistance to these AMA has considerable relevance to both cattle and human health.

1.6.2 Antimicrobial resistance in *M. bovis*:

M. bovis cells do not possess a cell wall or periplasmic space and are intrinsically resistant to AMA targeting the peptidoglycan cell wall, such as the beta-lactam ceftiofur (Heuvelink et al., 2016; Sulyok et al., 2014b). Additionally, as *mycoplasmas* do not produce folic acid, they are naturally resistant to sulfonamides. *Mycoplasmas* are generally susceptible to AMA that interferes with protein (aminoglycosides, lincosamides, macrolides, tetracyclines, and florfenicol) or DNA (fluoroquinolones group) synthesis (Heuvelink et al., 2016; Sulyok et al., 2014b). Resistance against these antimicrobials has been reported (Jelinski et al., 2020b). *M. bovis* can lead to persistent BRD (Booker et al., 2008). The effect of persistent *Mycoplasmosis* is significant because the cattle receive repeated antimicrobial treatment to control chronic *Mycoplasmosis* (Booker et al., 2008). *M. bovis* develops resistance through DNA mutations, instead of developing resistance via acquired genes (Sulyok et al., 2017).

1.6.3 Antimicrobial resistance in *P. multocida*

Antimicrobials are still regarded as the primary method of treatment for *P. multocida* infections (Amaral et al., 2019). *P. multocida* isolates from BRD-affected and unaffected feedlot calves collected in 2015 exhibited high rates of tetracycline and macrolide resistance (Timsit et al., 2017). Over the period 2012-2017, resistance patterns of *P. multocida* isolated from cattle showed an increase in tetracycline ($p > 0.001$), tilmicosin ($p > 0.001$), flumequine ($p = 0.003$), and enrofloxacin ($p = 0.008$) (Bourély et al., 2019). The prevalence of extensively drug-resistant (XDR) *P. multocida* strains in cattle has increased recently (Snyder and Credille, 2020a). Resistance in *P. multocida* typically occurs through the acquisition of resistance genes.

Tetracycline resistance can occur by various mechanisms such as resistance via active efflux pumps [e.g., *tet*(H)] the synthesis of ribosomal protection proteins, enzymatic degradation, reduced drug permeability, or mutation in target gene (Thaker et al., 2010). Macrolide resistance occurs typically due to the acquisition of genes coding for rRNA methylases, [e.g., *erm* (42)] or macrolide efflux proteins (Kadlec et al., 2011; Zhong and Shortridge, 2000). A study identified an ICE in the chromosomal DNA of *P. multocida* that contained 11 genes conferring resistance to streptomycin and spectinomycin (*aadA*25), streptomycin (*str*A and *str*B), gentamicin (*aad*B), kanamycin/neomycin (*aph*A1), tetracycline [*tet*R-*tet*(H)], chloramphenicol/florfenicol (*flo*R), sulphonamides (s*ul*2), tilmicosin/clindamycin [*erm*(42)] or tilmicosin/tulathromycin [*msr*(E)-*mph*(E)](Michael et al., 2012).

1.7 Conclusion

The prevention and treatment of BRD depend on the use of antimicrobials (Amaral et al., 2019). In western Canada, injectable metaphylactic antimicrobials are administered to 20% to 50% of calves arriving at feedlots to prevent BRD (Checkley et al., 2010a). Antimicrobial resistance in pathogenic bacteria from environmental sources and animals used for food production is one of the biggest risks to human and animal health (Tang et al., 2009a). For successful antimicrobial selection for BRD control and therapy, accurate and current knowledge of pathogen susceptibility patterns is required (Lubbers and Turnidge, 2015). Due to fastidious nature, *M. bovis* is less investigated than other BRD bacterial pathogens. Although *P. multocida* is the most prevalent pathogen causing BRD, it has also been underexplored. This is why these two pathogens have been selected for investigation in this thesis.

1.8 Hypothesis

It is hypothesized that:

1. The prevalence of *M. bovis* and *P. multocida* will be greater in cattle diagnosed with BRD compared to healthy cattle.
2. Antimicrobial resistance will increase in *M. bovis* and *P. multocida* isolated from cattle in 2015-2016, compared to those from cattle sampled in 2008-2009.
3. Antimicrobial use will select for genetically related *P. multocida* that encode resistance genes.

1.9 Objectives

1. To evaluate differences in prevalence of *M. bovis* and *P. multocida* throughout feedlot placement and between healthy and sick cattle.
2. To compare antimicrobial susceptibility patterns in *M. bovis* and *P. multocida* isolated 8 years apart.
3. To characterize genetic relatedness of *P. multocida* and identify genes that confer antimicrobial resistance.

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Chapter two

Chapter two has been submitted to the Veterinary Microbiology journal.

Prevalence and antimicrobial susceptibility of *Mycoplasma bovis* from the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bovine respiratory disease

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2.1. Introduction

Bovine Respiratory Disease (BRD) is the leading cause of death and illness in newly received feeder calves, resulting in significant economic losses to the beef industry due to treatment costs, reduced performance, and mortality (Larson, 2005; Snowder et al., 2007). The cause of BRD is multifactorial with several prominent viral agents (bovine viral diarrhea virus, bovine respiratory syncytial virus, bovine herpesvirus 1, parainfluenza 3 virus) and opportunistic bacterial pathogens (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*) being implicated (Klima et al., 2014b).

The efficacy of vaccines against BRD bacterial pathogens is variable (Larson and Step, 2012; Perez-Casal et al., 2017; Sulyok et al., 2014a). Consequently, antimicrobials are often the primary management practice to control BRD-associated pathogens in large feedlots. Between 20% and 50% of calves arriving at feedlots receive injectable metaphylactic antimicrobials to prevent BRD in western Canada (Checkley et al., 2010b). Because antimicrobial use selects for resistance in bacteria, it is important to have accurate information on susceptibility patterns of pathogens to ensure that antimicrobials remain effective for BRD control and treatment (Lubbers and Turnidge, 2015). In 2015, we showed that resistance to macrolides and tetracyclines was high in *M. haemolytica* and *P. multocida* isolated from BRD-afflicted and healthy feedlot calves (Timsit et al, 2017). For *M. haemolytica*, rates of resistance to these classes of antimicrobials increased as compared to earlier studies (Klima et al., 2011b). Similarly, over ten years, the minimum inhibitory concentrations of macrolides were shown to increase for *M. haemolytica*, *P. multocida*, and *H. somni* isolated from feedlot cattle (Portis et al., 2012b), highlighting the role that surveillance studies can play in gaging the efficacy of antimicrobials.

Of the BRD bacterial pathogens, *M. bovis* is often less studied due to its fastidious nature. However, economically, *M. bovis* is an important etiological agent of BRD. Losses to the US beef industry resulting from reduced weight gain and carcass value because of *M. bovis* have been estimated at $32 million per year, and in the United Kingdom, *M. bovis* accounts for a quarter of economic losses due to BRD (Maunsell and Donovan, 2009). In addition to BRD, *M. bovis* is also associated with other clinical diseases in cattle such as otitis media, mastitis, and arthritis, leading to chronic conditions that respond poorly to antimicrobial therapy (Sulyok et al., 2014a). No effective vaccine is commercially available for *M. bovis* thus antimicrobials are critical for its control.

The lack of a cell wall in *M. bovis* limits the use of antimicrobials to those that do not target cell wall synthesis (Perez-Casal et al., 2017). While a recent review highlighted that resistance may be increasing in *M. bovis*, variation in resistance can arise due to differences in geography, livestock species, and animal health status (Lysnyansky and Ayling, 2016). In the present study, we evaluated *M. bovis* in respiratory samples collected from two Cohorts of feedlot cattle in Alberta, Canada. The first study was conducted in 2008-2009, with nasopharyngeal swabs (NP) collected from cattle at feedlot entry and after more than 60 days on feed (Cohort 1). In a second 2015-2016 study, nasopharyngeal and trans-tracheal samples were collected from cattle diagnosed with BRD and matching healthy controls (Cohort 2). The objectives were to evaluate differences in prevalence and resistance i) throughout feedlot placement, ii) between healthy and sick cattle, and iii) between the upper and lower respiratory tract. In addition, antimicrobial susceptibility patterns were compared between isolates from Cohort 1 and Cohort 2 to detect changes in resistance that may have occurred over an 8 year period.

2.2. Material and methods

2.2.1 Animals and study design

Cohort 1: as part of a previous surveillance study to evaluate the anti-microbial susceptibility of *M. haemolytica*, NP swabs were collected from cattle upon entry to four feedlots and again from the same animals at ≥60 days on feed (DOF) as previously described (Klima et al., 2011b). Swabs were stored in a cryopreservative (brain heart infusion:glycerol, 0.8:0.2 mixture) at −80°C. In total, approximately 3,000 swabs from 1,500 calves were stored from September 2008 to February 2009. For the present study, only cattle that were not treated for clinical disease during placement were enrolled for selection. Of these, NP swabs were

randomly selected from cattle that were administered metaphylaxis tulathromycin (high risk for developing BRD; 2.5 mg/kg body weight; N = 120 cattle) or oxytetracycline (medium risk for developing BRD; 30 mg/kg body weight; N = 77 cattle) at feedlot entry.

Cohort 2: in a case-controlled study, NP and trans-tracheal aspiration (TTA) samples were collected from cattle across four feedlots between November 2015 and January 2016 (Timsit et al, 2017). All cattle from this study were ranked as high-risk for developing BRD and were administered tulathromycin at feedlot entry. Approximately one healthy pen-mate (i.e., control) was pulled for every two BRD cases and clinically examined by a licensed veterinarian prior to study enrollment. In total, 104 healthy and 215 BRD cases were sampled. These samples were cultured for *M. bovis* within 24 h after being collected. The diet of Cohort 2 cattle contained 25 ppm of monensin and 35 ppm of chlortetracycline. In addition, all cattle received two pulses of chlortetracycline within the first 21 days on feed to prevent histophilosis (Timsit et al, 2017).

2.2.2 Bacteria isolation

The NP and TTA samples were processed for *M. bovis* culturing as previously described (Anholt et al., 2017b) with slight modifications with pleuropneumonia-like organisms (PPLO) agar used instead of heart infusion agar. For Cohort 1, the banked swab/cryopreservative mixture was vortexed and 150 µl of the suspension was added to 1.5 ml of PPLO broth amended with 500 µg/mL ampicillin (AMP). The swabs from Cohort 2 cattle were processed within 24 h of collection and were vortexed in 1.2 ml of brain heart infusion: glycerol (0.8:0.2) and 150 µl of the suspension was added to 1.5 ml PPLO+AMP broth. For the TTA samples from Cohort 2, 150 µl of aspiration (physiological saline) was directly added to 1.5 ml PPLO+AMP broth. An aliquot of the filtrate was plated onto PPLO+AMP agar and

plates were incubated for 5 days at 37° C. Isolates displaying morphology (fried egg shape) typical of *M. bovis* were confirmed by PCR as described below.

2.2.3 DNA extraction and real-time PCR

The isolates that showed typical *M. bovis* morphology were selected for PCR for confirmation. Bacterial colonies were selected from plates, mixed with Tris-EDTA buffer (TE) buffer and lysed at 98 °C for 3 min and centrifuged at 5200 x g for 2 min. PCR was performed in a 25 µL reaction volume containing SSO Advanced probes suspension (BIO-RAD), 0.25µM of each primer (MbovF2024: TCTAATTTTTTCATCATCGCTAATGC, MbovR2135: TCAGGCCTTTGCTACAATGA
AC), 0.15 µM probe (Mbov uvrC: FAM-AACTGCATCATATCACATACT), and 2.5 µL of DNA template (Clothier et al., 2010). Reactions were performed in a real-time PCR cycler (C1000 Touch Thermal cycler, BIO-RAD). The PCR reaction was carried out using the following cycling parameters: polymerase-activation for 3 min at 95° C followed by 40 cycles of denaturing at 95° C for 15 seconds and annealing and amplification at 60° C for 45 seconds. Isolates were considered *M. bovis* if the cycle threshold value was less than 35.

2.2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by microdilution using a customized commercial plate (Oxoid, Nepean, ON, Canada) on a subset of PCR-confirmed *M. bovis* isolated from Cohort 1 (n=68; Oxy = 33, Tul = 35) and Cohort 2 (n=131; BRD NP =41, BRD TTA=62, Control NP=14, Control TTA=14). Cohort 1 isolates were all from the second sampling of cattle (≥ 60 days on feed). Cultures were suspended in PPLO to a final concentration of 10^3-10^5 CFU/ml. This final suspension was used to inoculate the antimicrobial plates of the following antimicrobial agents in round-bottom 96-well plates at concentrations

of 1 µg/ml – 256 µg/ml for chlortetracycline, clindamycin, oxytetracycline, tilmicosin, tulathromycin, and tylosin and 0.12 µg/ml – 256 µg/ml for enrofloxacin, florfenicol, and spectinomycin. The plates were sealed using perforated film and incubated with 5% CO_2 at 37°C for 48-96 h. The minimum inhibitory concentrations (MIC) were assigned by visual assessment as outlined in the Clinical and Laboratory Standards Institute (Clinical Laboratory Standards, 2015). The lowest concentration of antimicrobial with no growth was recorded as the MIC. At the time of the experiment, CLSI resistance breakpoints for all antimicrobials tested were not available for *M. bovis*. Therefore, MIC values established as breakpoints for bovine respiratory pathogens within *Pasteurellaceae* (Clinical Laboratory Standards, 2015; CLSI, 2020a) were used to define resistant *M. bovis* isolates. This strategy has previously been implemented for *M. bovis* (Anholt et al., 2017b; Jelinski et al., 2020a). The breakpoints for tulathromycin (≥64 µg/ml), enrofloxacin (≥2 µg/ml), florfenicol (≥8 µg/ml), oxytetracycline (≥8 µg/ml), chlortetracycline (≥8 µg/ml), tilmicosin (≥32 µg/ml), spectinomycin (≥ 128µg/ml) (CLSI, 2020a), tylosin (≥16µg/ml) and clindamycin (≥4 µg/ml) (Clinical Laboratory Standards, 2015) were used. The custom plates had wells for positive controls. Quality control required that the positive controls showed the growth of *M. bovis*. Reference strains *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 also served as quality controls.

2.2.5 Statistical analyses

The statistical analyses were carried out using R (R core team 2022). The prevalence of *M. bovis* was calculated and described for Cohort 1 and 2 using Microsoft Excel. Generalized linear modeling was done using SAS PROC GLIMMIX (SAS version 9.4, SAS Institute Inc., Cary, NC) to test the null hypothesis that the proportions of *M. bovis* isolate that were persistent in the experimental groups (effects of feedlot placement, i.e., entry and exit and antimicrobial

treatment i.e., oxytetracycline and tulathromycin of Cohort 1, effect of health status i.e., healthy and BRD and days on feed for Cohort 2) were the same. The prop. test function in R was used to test the null hypothesis that the proportions of *M. bovis* MIC from Cohort 1 and Cohort 2 were the same over time; and the null hypothesis that the proportions of *M. bovis* resistant isolates were the same in the metaphylactic treatment groups for Cohort 1, the health status groups for Cohort 2, and for the two Cohorts. Significance was declared at an P value of 0.05.

2.3. Results

For Cohort 1, the prevalence of *M. bovis* in NP swabs increased during the feeding period, regardless of antimicrobial treatment (Table 2.1). These differences in prevalence at entry and exit indicated that cattle sampled at ≥ 60 days on feed were 66.67 times more likely to be positive for *M. bovis* (OR = 66.67; 95% CI: 20.62-215.58; P<0.05; Table 2.2) compared to cattle at arrival. The prevalence of *M. bovis* was not different between the antimicrobial treatments (P=0.596; Table 2.2). However, compared to oxytetracycline-treated cattle (OR=32.93: 7.54-143.76), tulathromycin-treated cattle had an increased chance of being colonized by *M. bovis* after ≥ 60 days on feed (OR=136: 18.4-1005.44) (Table 2.2).

For Cohort 2, *M. bovis* was more frequently recovered from cattle with BRD than healthy cattle, regardless of the sample source (Table 2.3). The likelihood of *M. bovis* recovery in NP samples was 2.12-foldgreater in cattle with BRD, as compared to healthy animals (OR=2.12; 95% CI: 1.15-3.9; P < 0.05; Table 2.4). Similarly, the likelihood of recovery in TTA samples from BRD cases was 3.22-foldmore than that of healthy animals (OR=3.22; 95% CI: 1.79-5.79; P < 0.05; Table 2.4). Days on feed had a limited effect on *M. bovis* prevalence for both NP and TTA samples (Table 2.4).

The antimicrobial MIC frequencies for all *M. bovis* isolates are shown in Figure 2.1. Except for tilmicosin, there was a trend of increased MIC of antimicrobials in isolates collected from Cohort 2 (2015-2016) compared to Cohort 1 (2008-2009). When evaluated at resistance breakpoints, resistance to all antimicrobials was detected in isolates from Cohort 1 cattle, except for enrofloxacin and spectinomycin (Figure 2.2). More than 85% of *M. bovis* from both cattle administered either oxytetracycline or tulathromycin at feedlot entry were resistant to chlortetracycline, oxytetracycline, and tilmicosin at ≥ 60 DOF. For all antimicrobials evaluated, there were no differences in resistance when comparing oxytetracycline- versus tulathromycin-treated cattle.

Regardless of animal health status, the majority of *M. bovis* isolated from Cohort 2 cattle were resistant to tilmicosin, tulathromycin, and tylosin (Figure 2.3, >92%). For chlortetracycline, florfenicol, and oxytetracycline, resistance was greater for *M. bovis* from BRD cases, compared to healthy cattle. However, none of the isolates were resistant to spectinomycin. Unlike Cohort 1 isolates, in Cohort 2 *M. bovis*, resistance to enrofloxacin was detected (11% and 6% from control or BRD cattle, respectively). When compared to Cohort 1, *M. bovis* from Cohort 2 animals had a greater prevalence of isolates resistant to clindamycin (18% vs 86%), enrofloxacin (0% vs 6.8%), florfenicol (18% vs 37%), tulathromycin (19% vs 99%), and tylosin (75 % vs 98%) ($P<0.05$; Figure 2.4). There were no differences in the frequency of *M. bovis* resistance between Cohorts for chlortetracycline and tilmicosin, of which resistance was high for both groups (>85%), or spectinomycin, of which no resistance was detected (Figure 2.4).

2.4 Discussion

M. bovis has emerged as an important feedlot pathogen and is associated with both acute and chronic BRD, yet there is still a lack of information detailing how this pathogen impacts feedlot cattle and how resistance is related to antimicrobial use (Caswell et al., 2010; Hendrick et al., 2013). A strength of our study was the *M. bovis* isolates originated from moderate-high risk feedlot cattle in Alberta, with a known history of antimicrobial use. The study was limited however by the cattle in Cohorts 1 and 2 having different in-feed antimicrobial use, with Cohort 2 receiving pulses of chlortetracycline followed by subtherapeutic administration. Thus, caution is needed in comparing tetracycline-based resistance between the two Cohorts. Additionally, the history of feedlot antimicrobial use in the time between the collection of Cohort 1 and 2 isolates was unknown. However, all cattle did receive metaphylaxis treatment as the only injectable antimicrobial administered before sampling, and only *M. bovis* from the ≥60 daytime point for Cohort 1 were evaluated for resistance, to similarly reflect isolates collected after metaphylaxis treatment for both Cohorts.

Typically, incoming cattle are classified according to the risk of developing BRD (low, medium, high, ultra-high), based on criteria established by feedlot managers, such as body weight, vaccination status, transportation distance, and source (Avra et al., 2017a). The selection of an antimicrobial for metaphylaxis will vary according to risk, with oxytetracycline typically used for low to medium- and tulathromycin for high-risk calves (Brault et al., 2019a). Interestingly, the prevalence of *M. bovis* was low in both oxytetracycline- and tulathromycin-treated cattle upon feedlot entry of Cohort 1, suggesting that BRD risk identified by the feedlots was not related to *M. bovis* colonization on arrival. *M. bovis* colonization then increased substantially in both antimicrobial treatments for Cohort 1, after ≥ 60 days on feed. This is supported by previous studies that have shown *M. bovis* prevalence to increase from less than

2% in cattle before or upon arrival to feedlots, to 9% (Nobrega et al., 2021a) or 85.5% (Castillo-Alcala et al., 2012a) after feedlot placement. Both newly-arrived and previously-placed cattle can be sources of *M. bovis* transmission in feedlots (Timsit et al., 2012a). We observed that cattle administered tulathromycin had a greater odds ratio of *M. bovis* colonization after ≥60 days of placement, compared to oxytetracycline-treated cattle. While difficult to explain, metaphylaxis treatment has been shown previously to enrich *Mycoplasma* in the nasopharynx of cattle, though it varied for different antimicrobials and the authors attributed it to potential alterations in the microbiota (Holman et al., 2019a). Regardless, it is clear that the feedlot environment increases the chance of colonization by *M. bovis*, which may occur through the animal-to-animal transmission of clonal strains (Timsit et al., 2012).

Among Cohort 2 cattle, the agreement between nasal and tracheal *M. bovis* colonization was low, with <50% of the cattle positive for tracheal colonization and also being positive for nasopharyngeal colonization. Despite this, similar colonization trends were observed for both sample types, with increased prevalence in BRD cases and after animals sampled ≥40 days on feed. The effect of time on *M. bovis* colonization was less evident in Cohort 2 cattle, compared to Cohort 1. Only cattle that had been placed for ≥40 DOF had an increased chance of colonization when sampled from the NP or trachea, but the effect was limited. This was likely a result of cattle from Cohort 2 already being placed in feedlots before sampling, and no sampling is done at feedlot entry for comparison. In support of this, a previous study showed that the prevalence of *M. bovis* increased from 1.7% at arrival to 72.2% after ≤15 days on feed (Castillo-Alcala et al., 2012a).

Cohort 2 cattle diagnosed with BRD had a greater chance of being positive for *M. bovis* than healthy animals, for both NP and TTA samples. The direct role *M. bovis* has in the onset

of acute BRD is still difficult to ascertain. While cattle positive for *M. bovis* in nasal swabs have been shown to be more likely to have fever compared to calves not shedding *M. bovis* (Wiggins et al., 2007), the concentration of *M. bovis* in bronchoalveolar lavage samples has been reported to not correlate with disease status (Castillo-Alcala et al., 2012a). In a study by Castillo-Alcala and colleagues (Castillo-Alcala et al., 2012a), the authors suggested host factors and coinfection with other BRD pathogens have a role in the degree that *M. bovis* contributes to BRD. Indeed, the same cattle in Cohort 2 of the current study were also analyzed for *P. multocida*, *M. haemolytica*, and *H. somni* in TTA samples (Timsit et al, 2017). In cattle with BRD, *P. multocida* was the most frequent bacterium isolated (54.8%), followed by *M. haemolytica* (30.5%) and *H. somni* (22.9%) (Timsit et al, 2017). Thus, when including *M. bovis* as part of the evaluation, it was the second most isolated pathogen from TTA collected from cattle with BRD (39%).

Although CLSI guidelines for resistance breakpoints do not exist for *M. bovis*, and care should be taken in interpreting data, resistance breakpoints have been suggested previously (Anholt et al., 2017b; Jelinski et al., 2020a) and applied in studies (Andrés-Lasheras et al., 2021a; Nobrega et al., 2021a). The bacteria evaluated for antimicrobial susceptibility from Cohort 1 were all isolated from the second sampling time point, ≥60 DOF. This was done to have isolates from cattle after metaphylaxis, for comparison to the Cohort 2 isolates, which were also collected after metaphylaxis. Despite not measuring resistance in isolates collected at entry, there was no apparent short-term effect of metaphylaxis on resistance after ≥60 DOF in Cohort 1 *M. bovis*, as both oxytetracycline- and tulathromycin-treated cattle were colonized with *M. bovis* displaying high levels of resistance to chlortetracycline, oxytetracycline, tilmicosin, and tylosin. A recent study in western Canadian feedlots from 2008 to 2012 (Brault et al., 2019a) found that tetracyclines were the most widely used antimicrobials for both

injectable (oxytetracycline) and in-feed (chlortetracycline) forms. *M. bovis* acquires resistance through DNA mutations, rather than resistance genes. While it was not known if resistance to chlortetracycline and oxytetracycline were due to similar or unique mutations, it was apparent that widespread repeated use of these antimicrobials likely resulted in the high prevalence of tetracycline resistance in *M. bovis*. However, reports of tetracycline resistance in *M. bovis* from Canadian feedlots have varied and ranged from levels similar to ours (69.5-80.1%; (Anholt et al., 2017b) to levels far lower (<21.4%; (Nobrega et al., 2021a). This may reflect differences in both the extent and long-term use of antimicrobials by individual feedlots.

The prevalence of tulathromycin-resistant *M. bovis* did not differ between the metaphylaxis groups in Cohort 1. This suggests that tulathromycin-resistant strains may have already been in the feedlots and colonized cattle from both treatments, rather than resistance developing through selective pressure from tulathromycin that was administered during the short study period. Compared to other macrolides tested, tulathromycin resistance was substantially lower in cattle from Cohort 1. Tulathromycin was approved for use in Canada to treat and prevent BRD in cattle in 2006 and came to market in 2007 (Schunicht et al., 2007). Thus, the feedlots in Cohort 1 could only have been utilizing tulathromycin for metaphylaxis for 1-2 years prior to when these cattle were sampled. In contrast, tilmicosin was registered for use in North America in 1992 and was commonly used for BRD until the introduction of tulathromycin (Andrés-Lasheras et al., 2022). This is likely why tilmicosin resistance was greater than tulathromycin in isolates from Cohort 1. Similarly, in a study evaluating *M. bovis* isolated from cattle in western Canada and sampled in 2007 and 2008, the MIC_{50} for tilmicosin was 128 µg/ml, whereas it was 2 µg/ml for tulathromycin, supporting the linkage between the use of these antimicrobials and resistance in *M. bovis*. It is interesting to note that tulathromycin-resistant *M. haemolytica* isolated from the same cattle in Cohort 1 were reported

to account for less than 0.3% of the population (Alexander et al., 2013b), indicating that resistance may develop at different rates in BRD pathogens.

In Cohort 2, resistance to chlortetracycline, florfenicol, and oxytetracycline was greater in isolates from cattle diagnosed with BRD than in healthy controls. While mycoplasmosis can be a chronic disease requiring several courses of antimicrobial therapy, potentially selecting for resistance in bacteria (Jelinski et al., 2020a), each of the cattle in Cohort 2 was sampled at the first pull for acute BRD and sampled prior to treatment with antimicrobials. Thus, at least while in the feedlot, antimicrobial use was similar for both BRD cases and healthy controls at the time of sampling. While it is difficult to explain what might have promoted increased resistance to antimicrobials in isolates from BRD cases, others have reported similar high levels of resistance to macrolides and tetracyclines in *M. bovis* from healthy cattle (Kinnear et al., 2020a). It is possible that inter-animal transfer of resistant strains contributed to the detection of resistant *M. bovis* in untreated healthy cattle (Timsit et al., 2012). Data on the genetic relatedness of resistant strains in feedlots, and potential linkage to virulence, would help identify how resistance is selected in future studies.

The most notable change over the 8 years was a large increase in resistance to tulathromycin. Macrolides were the second most commonly used class of antimicrobials for individual (tulathromycin) and in-feed (tylosin) administration from 2008 to 2012 in Western Canada feedlots. Tulathromycin accounted for 88% of individual macrolide use (Brault et al., 2019a). Assuming a similar trend from 2013 onwards, it is evident that tulathromycin use from 2009 to 2016 resulted in an increase in resistance, with almost all *M. bovis* displaying resistance in Cohort 2 cattle. Although tylosin resistance was already relatively high in Cohort 1 isolates, resistance to tylosin was greater in Cohort 2. We also observed that tilmicosin resistance

remained high in Cohort 2 cattle, showing that resistance to this antimicrobial may be endemic in *M. bovis* from certain feedlots in Alberta and that selective pressure from tilmicosin use is not needed to retain resistance. While macrolide resistance in *M. bovis* results from point mutations in the 50S rRNA subunit (Andersen et al., 2012b; Poehlsgaard et al., 2012), differences in structure alter how these antimicrobials bind, thus cross-resistance does not necessarily occur. Similar to our study, high resistances to tylosin (85-97%), tulathromycin (61-92%), and tilmicosin (98-100%) have been reported recently in Albertan feedlot calves (Anholt et al., 2017b; Nobrega et al., 2021a).

It is interesting to note that clindamycin resistance had a similar trend to tulathromycin resistance. Although clindamycin is not used in feedlots, it was added to the antimicrobial panel because of the potential for cross-resistance between lincosamides and macrolides (Leclercq, 2002). Our data show that resistance to tulathromycin in *M. bovis* might confer resistance to clindamycin as well. In contrast, spectinomycin which was also not used in any of the feedlots in our study, was the only antimicrobial for which resistance was not detected in any of the isolates. Spectinomycin is an aminocyclitol antimicrobial, and though approved for BRD in North America in 1996, it is not widely used in Canadian feedlots (Andrés-Lasheras et al., 2022). Thus *M. bovis* susceptibility to spectinomycin represents an example of a BRD-approved antimicrobial that has limited use in feedlots. Enrofloxacin and florfenicol also have less widespread use in feedlots, mainly being reserved for BRD treatment, rather than metaphylaxis (Andrés-Lasheras et al., 2022). Enrofloxacin was the only other antimicrobial where no isolates from Cohort 1 showed resistance, though resistance was observed in *M. bovis* isolated from Cohort 2. There was also an increase in resistance to florfenicol when comparing Cohort 1 *M. bovis* to those of Cohort 2. From 2008 to 2012, florfenicol use doubled in Western Canadian feedlots (Brault et al., 2019a), which if sustained, conceivably led to an increase in

florfenicol resistance in Cohort 2 *M. bovis*. Indeed, the feedlots in our study did use florfenicol for BRD treatment, but none of the enrolled animals were administered this antimicrobial. While one study showed florfenicol resistance to be present in 67.9% of *M. bovis* (Nobrega et al., 2021a), another reported lower rates of <11.6% (Jelinski et al., 2020a). Thus, it may be important to limit florfenicol use so that resistance in *M. bovis* does not continue to increase similar to that of macrolides.

In summary, we showed that *M. bovis* colonization increased after feedlot placement of cattle and that BRD cases had a greater colonization rate, though there was low agreement between prevalence in the upper and lower respiratory tracts. Resistance to tulathromycin was observed in most *M. bovis* after only 9 years of being available for use by feedlots. There were also increases in resistance against florfenicol and enrofloxacin, when Cohort 2 was compared to Cohort 1, with enrofloxacin resistance not being detected in Cohort 1 isolates. Overall, in *M. bovis* isolated from cattle in Cohort 2, there were high rates of resistance against macrolides and tetracyclines. The effectiveness of these antimicrobials in treating mycoplasmosis may therefore be limited in some feedlots. While the majority of isolates were susceptible to florfenicol and enrofloxacin, the increasing trends in resistance against these antimicrobials emphasize that they should be retained for therapeutic use, until efficacious alternatives to antimicrobials are developed.

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Table 2.1 Prevalence of *M. bovis* in samples collected from the nasopharynx of feedlot cattle administered metaphylactic antimicrobials between 2008-2009[a].

Antimicrobial treatment	**Entry**	**≥ 60 DOF**
Oxytetracycline (Oxy; N=77)	2 (2.5%)	36 (47%)
Tulathromycin (Tul; (N=120)	1 (0.8%)	64 (53%)
Oxy + Tul (N=197)	3 (1.5%)	100 (51%)

[a]Individual animals were sampled at entry into a feedlot and then ≥ 60 days on feed (DOF).

Table 2.2 Effects of time in the feedlot and antimicrobial treatment on nasopharyngeal colonization with *M. bovis* in cattle sampled between 2008-2009.

Independent variable and level[a]	Odds ratio	95% Confidence interval	*P-value*
Time			
Entry	Ref.		
≥60 Days	66.67	20.62-215.58	<0.001
Antimicrobial treatment			
Oxytetracycline	Ref.		
Tulathromycin	1.13	0.77-1.80	0.596
Oxytetracycline			
Entry	Ref.		
≥60 Days	32.93	7.54-143.76	<0.001
Tulathromycin			
Entry	Ref.		
≥60 Days	136	18.4-1005.44	<0.001

[a]Individual animals were sampled at entry into a feedlot and then ≥ 60 days on feed (DOF). Cattle were administered oxytetracycline or tulathromycin at feedlot entry. Generalized linear models fit by maximum likelihood are presented.

Table 2.3 Prevalence of *M. bovis* from nasal swab and trans-tracheal aspiration samples collected from healthy and BRD-diagnosed cattle between 2015-2016.

Health status	**Nasal**	**Tracheal**
Healthy (N=104)	16 (15%)	17 (16%)
Sick (BRD confirmed; N=215))	59 (27%)	83 (39%)
Healthy and sick (N=319)	75 (23%)	100 (31%)

Table 2.4 Effects of health status and days on feed on the prevalence of *M. bovis* from feedlot cattle nasal and tracheal samples between 2015-2016.

Sample source	Independent variable and level[a]	Odds ratio	95% Confidence interval	*P* value
Nasopharynx	Health status			
	Healthy	Ref.		
	BRD	2.12	1.15-3.9	0.016
	Days on Feed			
	< 20	Ref.		
	≥20 < 40	0.58	0.33-1.01	0.054
	≥40	0.12	0.04-0.36	<0.001
Trachea	Health status			
	Healthy	Ref.		
	BRD	3.22	1.79-5.79	<0.001
	Days on Feed			
	< 20	Ref.		
	≥20 < 40	0.70	0.42-1.19	0.189
	≥40	0.38	0.19-0.79	0.009

[a]Generalized linear models fit by maximum likelihood. BRD, bovine respiratory disease.

Figures:

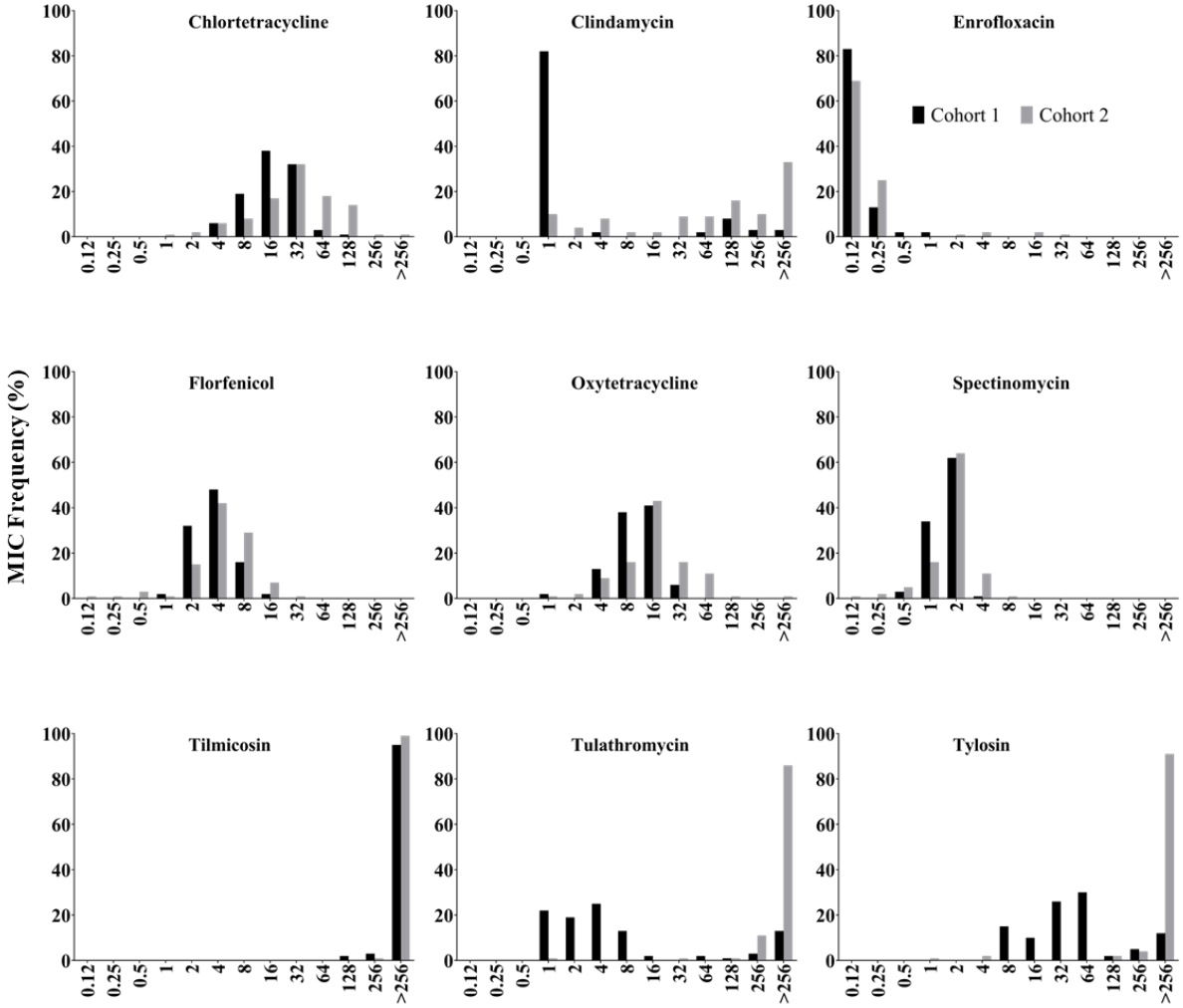

Figure 2.1 The MIC of various antimicrobials for *M. bovis* isolates from Cohort 1 (2008-2009) and Cohort 2 (2015-2016). Antimicrobial agents included (1 µg/ml – 256 µg/ml) for chlortetracycline, clindamycin (CLI), oxytetracycline (OXY), tilmicosin (TIL), tulathromycin (TUL), and tylosin (TYL), and (0.12 µg/ml – 256 µg/ml) for enrofloxacin (Enro), florfenicol (FLO) and spectinomycin (SPE).

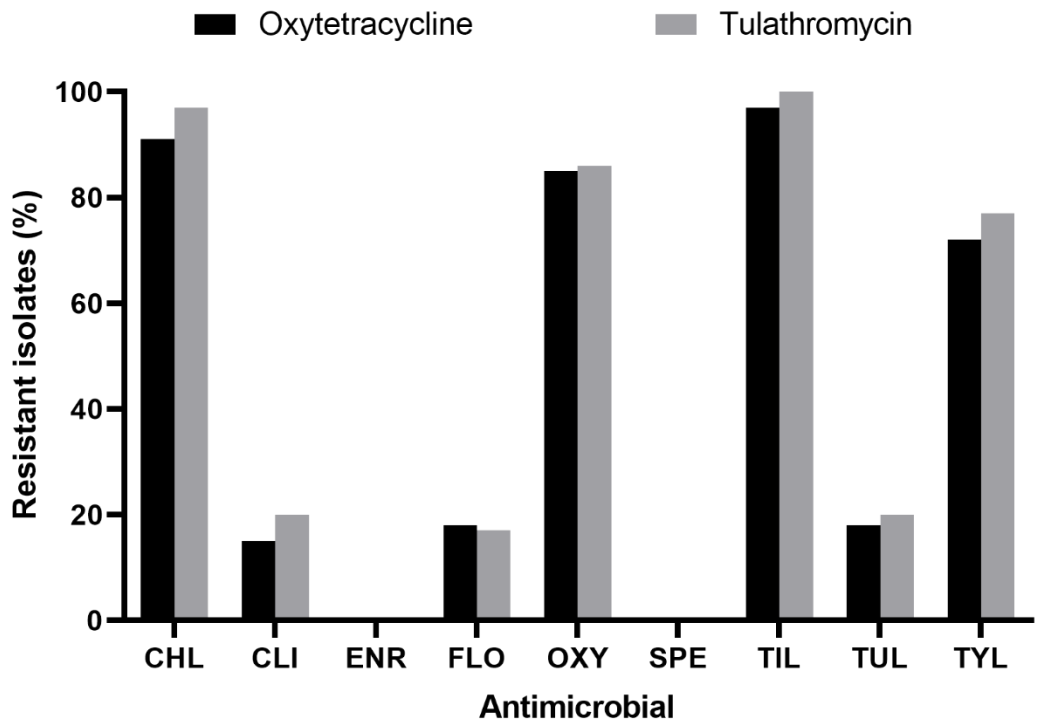

Figure 2.2 Frequency of antimicrobial-resistant *M. bovis* isolated from cattle administered either oxytetracycline (n=33) or tulathromycin (n=35) after ≥ 60 days on feed. Antimicrobial agents tested were chlortetracycline (CHL), clindamycin (CLI), enrofloxacin (ENR), florfenicol (FLO), oxytetracycline (OXY), spectinomycin (SPE), tilmicosin (TIL), tulathromycin (TUL), tylosin (TYL). Resistance breakpoints were 2 µg/ml (ENR), 4 µg/ml (CLI), 8 µg/ml (CHL, OXY, FLO), 32 µg/ml (TYL, TIL), 64 µg/ml (TUL), and 128 µg/ml (SPE).

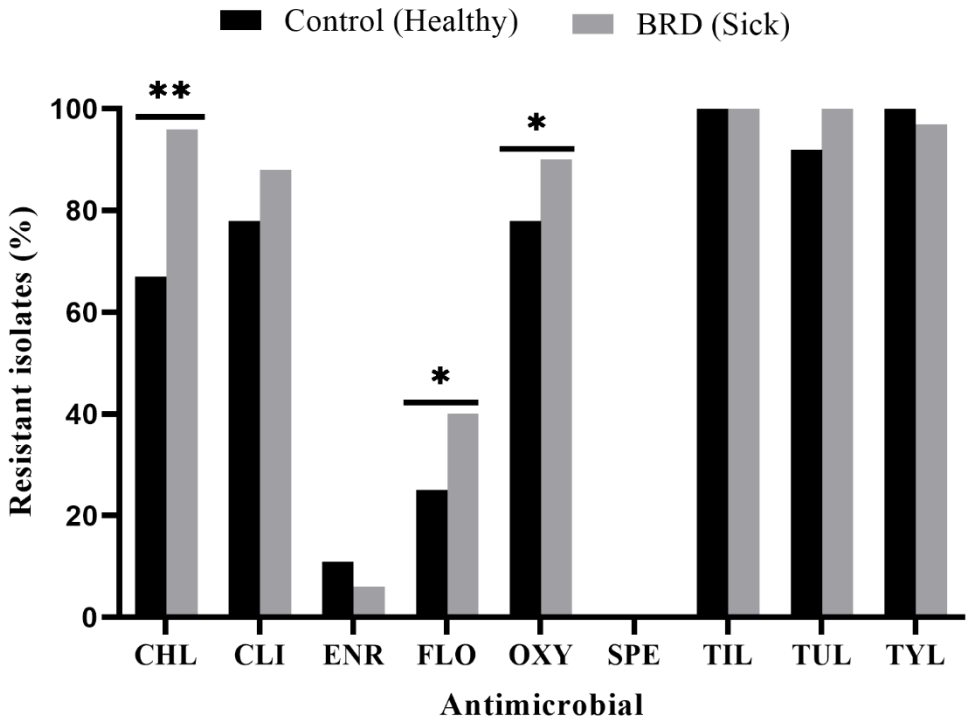

Figure 2.3 Frequency of antimicrobial-resistant *M. bovis* isolated from healthy (n=28) and BRD-affected (n=103) cattle in Cohort 2. Antimicrobial agents tested were chlortetracycline (CHL), clindamycin (CLI), enrofloxacin (ENR), florfenicol (FLO), oxytetracycline (OXY), spectinomycin (SPE), tilmicosin (TIL), tulathromycin (TUL), tylosin (TYL). * indicates $P < 0.05$ and ** indicates $P<0.005$. Resistance breakpoints were: 2 µg/ml (ENR), 4 µg/ml (CLI), 8 µg/ml (CHL, OXY, FLO), 32 µg/ml (TYL, TIL), 64 µg/ml (TUL), and 128 µg/ml (SPE).

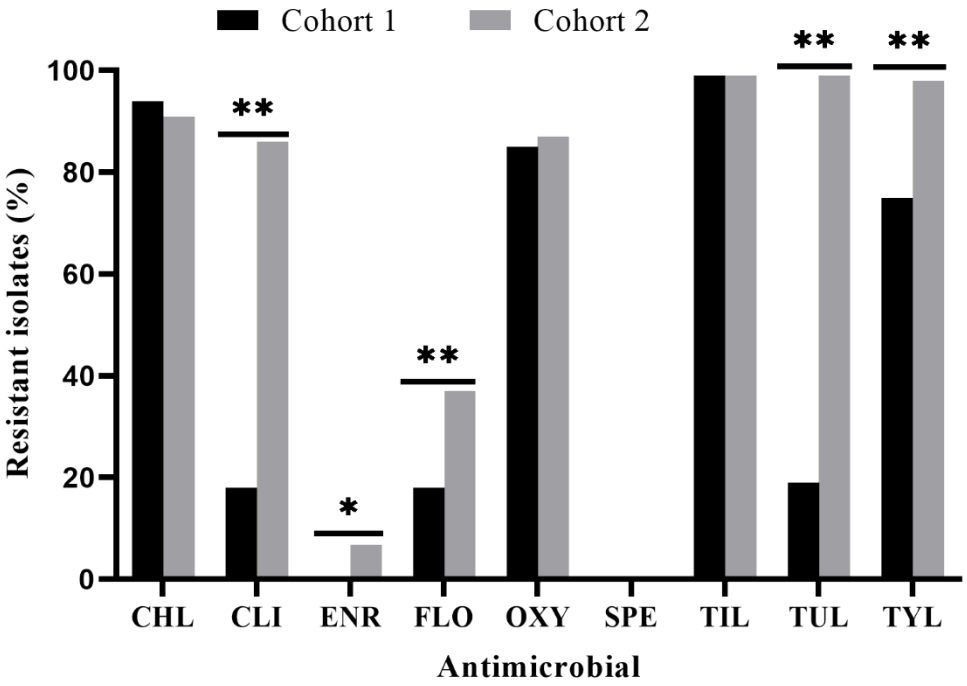

Figure 2.4 Frequency of antimicrobial-resistant *M. bovis* isolated from Cohort 1 (n=68; years 2008-2009) and 2 (n=131; years 2015-2016) cattle. Antimicrobial agents tested were chlortetracycline (CHL), clindamycin (CLI), enrofloxacin (ENR), florfenicol (FLO), oxytetracycline (OXY), spectinomycin (SPE), tilmicosin (TIL), tulathromycin (TUL), tylosin (TYL). * Indicates $P < 0.05$ and* *indicates $P<0.005$. Resistance breakpoints were: 2 µg/ml (ENR), 4 µg/ml (CLI), 8 µg/ml (CHL, OXY, FLO), 32 µg/ml (TYL, TIL), 64 µg/ml (TUL), and 128 µg/ml (SPE).

Chapter Three

This chapter has been formatted for submission to the Microbiology spectrum journal.

Characterization of antimicrobial resistance in *Pasteurella multocida* isolated from Albertan feedlot cattle

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3.1 Introduction

Bovine respiratory disease (BRD) is an important health problem in North American beef cattle production (Klima et al., 2019). BRD is multifactorial and results from infection by viral agents and bacterial pathogens, along with various stressors (e.g., weaning, transportation, commingling) that suppress immunity. Cattle are primarily affected during their first sixty days at the feedlot (Griffin et al., 2010), coinciding with exposure to bacterial pathogens including *Mannheimia haemolytica*, *Histophilus somni*, *Mycoplasma bovis*, and *Pasteurella multocida* (Klima et al., 2014b). While immunization against BRD-associated bacterial pathogens has shown variable efficacy (Ives and Richeson, 2015a; Taylor et al., 2010b), the use of antimicrobial metaphylaxis in cattle at arrival to feedlots reduces morbidity and mortality (Ives and Richeson, 2015b; Lofgreen, 1983; Step et al., 2007; Wellman et al., 2007).

P. multocida, a gram-negative coccobacilli is a member of the *Pasteurellaceae* family, and has been implicated in numerous economically important diseases in livestock (Tang et al., 2009b) and zoonotic diseases in humans (Seki et al., 2016). Among the BRD bacterial pathogens, *P. multocida* has been consistently detected in the general cattle population (Guo et al., 2020), In sick (Timsit et al., 2017) or dead (Welsh et al., 2004) cattle, prevalence of *P. multocida* has been shown to be increasing. Modifications in virulence, the potency of antimicrobial agents, or differences in the way sick cattle are managed are potential factors for this shift (Welsh et al., 2004).

Antimicrobials play a crucial role in both preventing and treating BRD infections, including those caused by *P. multocida* (Amaral A.F., 2019; Amaral et al., 2019). Approximately 20-50% of calves arriving at feedlots receive injectable metaphylactic antimicrobials to prevent BRD in western Canada (Checkley et al., 2010a). However, antimicrobial use can lead to resistance in bacteria associated with livestock, and can adversely impact human and animal health (Bronzwaer et al., 2002; Tang et al., 2009b). Previously, *P. multocida* isolated from trans-tracheal washes of BRD-afflicted and healthy feedlot calves sampled in 2015 showed high resistance to macrolides and tetracyclines (Timsit et al.,2017). More recently, multidrug-resistant *P. multocida* strains have been detected in feedlot cattle (Snyder and Credille, 2020b). It is therefore important to evaluate resistance in *P. multocida*, to manage the use of antimicrobials in a manner that ensures their efficacy against these important BRD pathogens (Lubbers and Turnidge, 2015).

Currently, there is a lack of data showing how the prevalence and mechanism of resistance of *P. multocida* in feedlot cattle have evolved over time. This study aimed to evaluate antimicrobial resistance and genetic diversity of *P. multocida* isolated from the upper

respiratory tract of Alberta feedlot cattle, that were sampled 8 years apart. For this, two Cohorts of cattle were evaluated. First, in a study conducted in 2007-2008, nasopharyngeal (NP) swabs from cattle sampled at feedlot entry and ≥ 60 days on feed were collected (Cohort-1) (Klima et al., 2011c). Second, in a study conducted in 2015-2016, NP samples were collected from cattle diagnosed with BRD and healthy controls (Cohort-2) (Timsit et al., 2017). The *P. multocida* in each cohort were assessed for resistance and subjected to comparative genomics.

3.2 Materials and methods

3.2.1 Animals and study design

Cohort 1: As part of a previous surveillance study for genetic characterization and evaluation of antimicrobial susceptibility in *M. haemolytica*, nasopharyngeal (NP) swab samples were taken when cattle entered into four feedlots and again when the same animals had been on feed for 60 days or more (Klima et al., 2011a). The swabs were cryopreserved (brain heart infusion: glycerol, 0.8:0.2 mixture) at −80° C. A total of 3,000 swabs from 1,500 calves were stored from September 2008 to February 2009. From these, 311 pairs (entry and ≥60 days swabs from individual cattle) were randomly included in the present study. The selected cattle were either administered tulathromycin (2.5 mg/kg body weight; n = 162 cattle) or oxytetracycline (30 mg/kg body weight; n = 149 cattle) at feedlot entry.

Cohort 2: In a case-controlled study, tracheal samples were collected from cattle across four feedlots between November 2015 and January 2016 (Timsit et al., 2017). From these same cattle, NP swabs were also collected and cryopreserved (brain heart infusion: glycerol, 0.8:0.2 mixture) at −80° C. A total of 208 and 104 NP swabs from BRD cases, or healthy controls respectively, were stored and used in the present study. The Cohort 2 cattle were administered tulathromycin (2.5 mg/kg body weight) at feedlot arrival. Additionally, 25 ppm

of monensin and 35 ppm of chlortetracycline were included in the diet, and throughout the first 21 days of feeding, two pulses of chlortetracycline were given to every animal. (Timsit et al., 2017).

3.2.2 Bacterial isolation, susceptibility testing, and pulsed-field gel electrophoresis.

Bacteria were isolated using a 100 mL aliquot of the nasal swab suspension. The sample was cultured on tryptic soy agar (TSA) culture plates with 5% sheep blood and 15 mg/mL of bacitracin (Dalynn Biologicals, Inc., Calgary, AB, Canada), and plates were kept on incubator overnight at 37° C. Presumptive colonies of *P. multocida* were selected based on colony morphology, including translucent, grayish in colour, and mucoid consistency. The colonies were subcultured and confirmed by PCR using primers and annealing conditions as described (Supplementary table 1), and HotStarTaq Plus master mix (Qiagen Canada, Inc., Toronto, ON) following the guidelines provided by the manufacturer. DNA template was prepared by lysing *P. multocida* in TE (tris1X-EDTA) at 98° for 5 min and adding 2 µL of this lysate to the PCR.

As previously mentioned, microdilution process was used to conduct antimicrobial susceptibility testing (AST) in a commercial sensitire plate (BOPO6F custom bovine plates, TREK diagnostic systems, Cleveland, OH, USA) (Timsit et al., 2017). Resistance breakpoints were based on CLSI (2020) guidelines (CLSI, 2020b) for *P. multocida* at the following concentrations: Ampicillin (0.25 µg/ml), Enrofloxacin (2 µg/ml), Oxytetracycline (8 µg/ml), Penicillin (1 µg/ml), Tulathromycin (64 µg/ml). For neomycin, resistance was determined at 32 µg/ml, according to Klima et al., (Klima et al., 2014a). In total, 133 and 132 isolates were evaluated for susceptibility testing from Cohorts 1 and 2, respectively.

Isolates displaying phenotypic resistance were evaluated for resistance genes against tulathromycin (*erm*(42), *msr*(E), *mph*(E)), neomycin (*aphA1*), oxytetracycline (*tet*(H)), and florfenicol (*floR*) using primers and annealing temperatures as outlined in supplementary table 1, and PCR conditions as described above. In addition, although a resistance breakpoint for sulphonamides was unavailable, the presence of *sul2* in all isolates showing phenotypic resistance to other antimicrobials was assessed, as *sul2* is often co-selected in antimicrobial resistant *Pasteurellaceae* (Kehrenberg et al., 2003).

Each of the bacterial isolates evaluated for susceptibility was also characterized for genetic relatedness by pulsed-field gel electrophoresis (PFGE), according to the method described previously (CDC). For this, ApaI was used as a restriction enzyme, and *Salmonella* serotype Braenderup was used as a control to standardize gels. Fragment analysis was carried out utilizing the software BioNumerics version 7.1 (Applied Maths Inc., Austin, Texas). The PFGE restriction patterns for individual isolates were classified as pulsotype. When pulsotypes for bacteria were completely identical, isolates were regarded as clones. When pulsotypes were ≥90% similar, bacteria were classified as belonging to the same pulsogroup.

3.2.3 Genome sequencing and analysis

The majority of isolates showing resistance to macrolides did not carry known macrolide resistance genes (*erm*(42), *msr*(E), *mph*(E)). Therefore, 10 isolates from Cohort 2 (strains 228T, 22T, 22N, 35N, 37T, 37N, 124T, 76T, 150T, 150N) were subjected to whole genome sequencing in an attempt to identify resistance genes conferring macrolide resistance. Isolates were selected based on resistant phenotype and the presence or absence of resistance genes by PCR. The isolates were tulathromycin-resistant with unknown macrolide resistance genes (strains 228T, 22T, 22N, 35N, 37T, 37N), tulathromycin-resistant with known macrolide

resistance gene (PCR positive for *erm*(42), *msr*(E), *mph*(E); strains 124T, 76T, 150T), and tulathromycin susceptible isolates (strain 150N). DNA extraction was performed by a phenol-chloroform method described previously (Klima et al., 2016b). The NEB Next Ultra II DNA library prep kit was used to create libraries (New England Biolabs, Whitby, ON, Canada) and sequenced using an Illumina Hi-seq PE150 at the University of Calgary.

As a quality control measure, the read sets were trimmed to remove low-quality regions with Trimmomatic v0.39 (Bolger et al., 2014b) using settings sliding window:5:15, leading:5, trailing:5, and minlen:75. FastQC v0.11.9 [https://github.com/s-andrews/FastQC] was used to check out the quality of the trimmed reads to ensure all read sets had acceptable quality scores, deemed as a minimum average Phred quality score of 25 for each read set. The trimmed read sets were *de novo* assembled using SPAdes v3.13.1 (Bankevich et al., 2012). Replicate read sets were concatenated into single forward and reverse FASTQ files as a form of increasing read coverage during subsequent assembly. The coding sequences of the 10 genomes were annotated with Prokka 1.14.6 (Seemann, 2014b).

The *P. multocida* 22N isolate was chosen for long-read sequencing for additional sequence verification and comparison. Cells were collected during the log phase and DNA extraction was done by following QIAGEN Genomic-tip 100/G protocol, according to the manufacturer's instructions. Library was prepared using the Ligation kit (SQK-LSK110, Oxford Nanopore Technologies, UK) protocol with the long fragment buffer option. The library was deposited into an individual MinION flowcell FLO-MIN106D and an ONT MinION Mk1B sequencer with MinKNOW version 21.05.8 (ONT) was used to sequence the data. The raw FAST5 files were processed for base-calling using Guppy (6.1.1). The output of the .FASTQ files were trimmed and assembled using porechop 0.2.4 (Kehrenberg et al., 2003) and flye 2.9 (Kolmogorov et al., 2019) respectively, to produce a completed genome. Prokka

1.14.6 was used for genome annotation (Seemann, 2014a). The raw reads for these genomes are available at PRJNA914751.

3.2.3.1 Phylogenetic analysis

A phylogenetic tree of the ten *P. multocida* isolates was created using a reference-genome-based methodology. Each concatenated read set was mapped against the *P. multocida* reference genome (CP028926.1) with BWA mem v0.7.17-r1188 (Li, 2013), and converted to a .FASTA consensus sequence with samtools v1.10 and bcftools v1.10.2 (Li et al., 2009b). A multiple alignment file was produced through the alignment of the consensus sequences with MAFFT v7.471 (Katoh et al., 2013). The MEGA11 analysis software (Tamura et al., 2021) created a maximum likelihood tree from the multiple alignment file using the Tamura-Nei model and a Nearest-Neighbour-Interchange heuristic method. iTOL was used to visualise the tree (Letunic and Bork, 2019).

3.2.3.2 Pangenome analysis and antimicrobial resistance gene identification

Pangenome analysis was performed on the 10 *de novo* assembled *P. multocida* genomes to identify novel genes associated with antimicrobial resistance. The pangenome was calculated with Roary 3.13.0 (Page et al., 2015), and a core gene phylogeny was constructed with FastTree 2.1.11 (Price et al., 2010). Scoary v1.6.16 (Brynildsrud et al., 2016) with default settings was used to associate genes within the accessory genome of the 10 isolates to tulathromycin resistance levels as determined using AST. Additionally, the 10 *de novo* assembled *P. multocida* genomes were screened for antimicrobial resistance genes (ARGs) with abricate 1.01 using the ResFinder, Card, and ARG-ANNOT databases [https://github.com/tseemann/abricate] (Gupta et al., 2014a; Jia et al., 2016; Zankari et al.,

2012). Manual screening of the pangenome analysis and ARGs included BLASTN (Camacho et al., 2009) searches of potential ARGs against the de novo assembled genomes.

3.2.4 Fosmid library preparation and analysis

Genome analysis did not identify any candidate macrolide resistance genes. Therefore, a fosmid library was constructed using strain 61T (tulathromycin-resistant isolate with unknown macrolide resistance genes). DNA was prepared using phenol-chloroform extraction (Klima et al., 2016a) and a CopyControl fosmid library manufacturing kit (Epicenter) was used to create a genomic library for *P. multocida* 61Twith modifications described previously (Donahue and Ebling, 2007). Briefly, genomic DNA was ligated into the pCC1FOs vector. The ligation mixture was packaged into phage particles and used to infect four replicates of EPI 300 T1 *Escherichia coli*. Transformants were plated onto Luria-Bertani (LB) agar amended with chloramphenicol (12.5 µg/ml), and harvested colonies were pooled.

Macrolide resistance screening was performed on transformed *E. coli* by plating on LB agar containing erythromycin (128 µg/ml). Four isolates that grew on erythromycin-amended agar were selected for fosmid sequencing. Fosmid DNA extraction was done as described (Cameron et al., 2018) and sequenced at MR DNA (Texas, USA) using an Illumina MiSeq platform. Trimmomatic v0.39 was used to trim the reads (Bolger et al., 2014a) and mapped to the *E. coli* K-12 substrain MG1655 reference genome. Non-mapping reads were extracted and assembled with Shovill 1.1.0 [https://github.com/tseemann/shovill]. Prokka 1.12 was used for annotated the assembled sequences (Seemann, 2014a). Screening of the sequences for antimicrobial resistance genes was done with abricate 0.8 [https://github.com/tseemann/abricate] using the ResFinder (Gupta et al., 2014b) database.

3.2.5 Fosmid visualization within the completed *P. multocida* genome

As an additional means to identify the genomic regions with divergent or non-shared sequences between the isolates, the trimmed reads of each of the 10 concatenated short read sets were mapped against the 22N completed genome using BWA mem v0.7.17-r1188 (Li, 2013), post-processed with samtools v1.10 (Li et al., 2009a), and annotated with Prokka 1.14.6 (Seemann, 2014a). The alignment map files were visualized against the 22N completed genome in BRIG 0.95 (Alikhan et al., 2011). The presence and location of the fosmid sequence within the 22N completed genome were confirmed by using a BLASTN (Camacho et al., 2009) comparison between the two. An 80,000 bp long sub-sequence containing the fosmid sequence, ICE, and AMR genes were visualized with the gggenomes package v0.9.5.9 in R v4.1.0. Coding sequences within the 80,000 bp annotated by Prokka as producing "hypothetical proteins" were searched against the nr database with BLASTP (Camacho et al., 2009) to further identify the protein product. The closest protein match for each hypothetical protein was substituted as the annotation.

3.2.6 Evaluation of fosmid-related genes to confirm macrolide resistance:

After analysis of the fosmids and genomes, 5 genes were identified in the genomes of tulathromycin-resistant isolates, without an identified resistance gene (228T, 22T, 22N, 35N, 37T, 37N) yet were absent in isolates with known tulathromycin resistance genes (124T, 76T, 150T, 150N). These genes were identified as a hypothetical protein, methylase, aadA31 streptomycin 3"-adenylyltransferase, hypothetical protein, helix-turn-helix domain-containing protein, hypothetical protein, permease, and ISL3 family transposase. The protein sequence of the fosmid methylase and 9 other highly-similar methylases and methyltransferases were pairwise compared using Clustal Omega (Sievers et al., 2011). This produced a distance matrix of identity values that were plotted in R v4.1.0. Additionally, a selection of highly-similar

proteins were compared against the 22N completed genome with TBLASTN (Camacho et al., 2009) to determine the relationship of the fosmid methylase to similar proteins. The methylase and permease were screened using PCR in 134 *P. multocida* isolates from Cohort 2, using primers and annealing conditions in supplementary table 1. In addition, the methylase and permease were cloned into a pUC57-Kan vector (BioBasic Inc., Markham, Canada) and transformed into *E. coli* BL21. Positive transformants, and untransformed BL21 parental strain, were tested for MIC against macrolide using a commercial susceptibility plate (BOPO6F custom bovine plates, TREK diagnostic systems, Cleveland, OH, USA), as described above. The *E. coli* isolates were tested in triplicate at three separate time points, for MIC evaluation.

3.2.7 Statistical analysis

R (Team, 2020) and Microsoft Excel were used for statistical analyses. The prevalence of *P. multocida* was calculated and described for Cohort 1 and 2 using Microsoft Excel. The prop.test function in R (Team, 2020) was used to test the null hypothesis that the proportions of *P. multocida* isolates that were persistent in the experimental groups (effects of feedlot placement, i.e., entry and ≥60 days of Cohort 1, health status groups for Cohort 2) were the same and the null hypothesis that the proportions of *P. multocida* resistant isolates were the same in the two Cohorts.

3.3 Results

3.3.1 *P. multocida* prevalence

For Cohort 1, 16% of entry and 18% of ≥ 60 day samples from oxytetracycline-treated cattle were positive for *P. multocida* (Figure 3.1), with no difference in prevalence between the two time points (P= 0.7619, OR 1.15, 95% CI -0.1136-0.7337). In contrast, the prevalence of *P. multocida* in tulathromycin-treated cattle was greater at entry (32%) than at ≥60 days (17%)

(OR=0.435; 95% CI: 0.0553-0.2532, P < 0.005; Figure 3.1). For Cohort 2 cattle, *P. multocida* was 2.45-fold more likely to be isolated from sick as compared to healthy cattle (OR=2.45; 95% CI: 6.90-35.099; P < 0.005; Figure 3.1).

3.3.2 Antimicrobial sensitivity

Antimicrobial-resistant patterns changed broadly over the 8 years with the incidence of breakpoint resistance being lower (P < 0.05) in Cohort 1 isolates, compared to those from Cohort 2, for four out of 8 antimicrobials tested (Figure 3.2). Resistance of *P. multocida* to florfenicol (1% vs 10%), oxytetracycline (3% vs 80%), neomycin (4% vs 63%), and tulathromycin (4% vs 78%) was lower in Cohort 1. The rates of resistance for ampicillin, penicillin, and enrofloxacin did not differ in isolates between cohorts, with all isolates being sensitive to enrofloxacin. The isolation time point did not affect antimicrobial resistance in Cohort 1 isolates.

3.3.3 PCR resistance gene screening and PFGE analysis

For Cohort 1, all isolates resistant to oxytetracycline or neomycin, carried the *tet*(H) and *aphA-1* gene, respectively. Macrolide resistance genes were not detected in tulathromycin-resistant isolates (data not shown). When resistant isolates from Cohort 2 were evaluated by PCR, corresponding resistance genes were detected in the majority of oxytetracycline (*tet*(H), 98%)-, neomycin (*aphA-1*, 92%)-, and florfenicol (*floR*, 92%)-resistant isolates (Table 3.1). Although phenotypic resistance to sulphonamides was not tested, the *sul2* gene was detected in 69% of the isolates that were resistant to the tested antimicrobials. Only 16 (15%) out of the 106 tulathromycin-resistant isolates from Cohort 2 carried a known macrolide resistance gene (*erm*(42), *msr* (E), *mph* (E)).

Analysis of the PFGE restriction patterns from Cohort 1 *P. multocida* showed 16 pulsogroups (>90% similarity) and 32 unique (singlets) patterns (Table 3.2). There were two dominant pulsogroups (group 6, N=16; and group 8, N=34) that accounted for the majority of isolate restriction patterns. In contrast, only 9 pulsogroups were observed for Cohort 2 *P. multocida*, and 5 singlet patterns (Table 3.3). One pulsogroup accounted for the majority of isolate restriction patterns (group 6, N=79). This pulsogroup was detected across four feedlots, with the majority of isolates being multidrug-resistant to tulathromycin, oxytetracycline, and neomycin.

3.3.4 Genome sequence analysis and antimicrobial resistance gene detection

Because only 16 of 130 tulathromycin-resistant isolates from Cohort 2 cattle were PCR-positive for known macrolide resistance genes (*erm*(42), *msr* (E), *mph* (E)), genome sequencing was performed to evaluate potential unknown genes conferring macrolide resistance. The genomes of nine tulathromycin-resistant isolates and one susceptible isolate were sequenced to analyze their phylogenetic relationships and determine the presence of antimicrobial genes. The average coverage depth of the 10 isolates was 119x, with coverages ranging from 30 (22T) to 189 (35N). The average NGA50 was 141,655 bp, ranging from 40,390 (22T) to 186,851 (35N) and the average read length was 149 bp after trimming and quality control measures. Phylogenetic analysis of the 10 genomes revealed 2 primary clades with Clade 1 consisting of four isolates (76T, 124T, 150N, 150T) and Clade 2 of five isolates (35N, 22N, 37N, 37T, 228T) (Figure 3.3). The remaining isolate, 22T, was an outlier in the tree, which may have resulted from the lower sequencing quality of that isolate. ARGs encoding resistance to aminoglycosides (*aph*(3')-Ia, *aph*(6)-Id), *aadA31*), macrolides (*erm*42, *msr*(E), *mph*(E)), phenicols (*flor*), tetracyclines *tet*(H), sulphonamides (*sul2*), and streptomycin (*strA*) were detected among the ten sequenced isolates after analyzing the genomes using resistance gene

databases (Figure 3.4). Distribution of macrolide resistance genes grouped the ten isolates into two groups, with the first group (76T, 124T, 150N, 150T four samples) encoding macrolide resistance genes *msr*(E), *mph*(E)and *erm*(42), and the second group (22N, 22T, 35N, 37N, 37T, 228T six samples) not possessing any known macrolide resistance genes (Figure 3.4). Isolate 150N was phenotypically sensitive to macrolides however, sequencing results showed that it carried known macrolide resistance genes *msr*(E), *mph*(E), and *erm*(42). PCR screening of resistance genes for this isolate confirmed that it carried *msr*(E), *mph*(E), and *erm*(42). One aminoglycoside resistance gene, *aadA31*, was present only in the tulathromycin-resistant isolates with unknown macrolide resistance genes. Notably, genome sequencing did not detect any additional macrolide resistance genes in the databases used for evaluation.

3.3.5 Pangenome and fosmid analysis

When the 10 short-read sequenced datasets were mapped against the completed 22N genome (Figure 3.5), it showed two gaps in tulathromycin-resistant isolates with known macrolide resistance genes, that were present in tulathromycin-resistant isolates with unknown macrolide resistance elements. One gap represented bacteriophage proteins (intA, helix turn helix domain-containing protein, Rha family transcriptional regulator, host cell division icd like protein) induced by viruses, and the other gap included several known AMR genes, an unknown methylase, a permease, and ISL3 family transposase (Figure 3.5). These genes and surrounding sequences matched with the fosmid sequence and are further characterized in Figure 3.6. Of the genes identified in the fosmid, 5 were only present in the genomes of isolates that were tulathromycin-resistant without known macrolide resistance genes (isolates 22N, 22T, 35N, 37N, 37T, 228T). The five genes are further annotated in Figure 3.7. The methylase and permease were screened using PCR in *P. multocida* from Cohort 2, and the majority of tulathromycin-resistant isolates with unknown macrolide resistance genes carried both genes

(81%), while isolates that were tulathromycin-resistant and encoded known macrolide resistance genes (*emr42, msr(E), mph(E*)) or those that were tulathromycin-sensitive did not (Table 3.1).

3.3.6 Analysis of the methylase and permease genes

We speculated that the methylase identified in tulathromycin-resistant isolates but deficient in the isolates that encoded *emr42, msr(E), and mph(E*) may have conferred resistance to tulathromycin. Therefore, *E. coli* were transformed with this gene and the newly identified permease. When methylase was transformed into *E. coli* BL21, the MIC against tulathromycin doubled, while MIC was unchanged for the macrolide tilmicosin and tylosin (Table 3.4). No changes in MIC were observed when *E. coli* were transformed with a plasmid carrying the permease gene (data not shown). The sequence of the fosmid methylase shared a high percentage identity with hypothetical proteins, N-6 DNA methylases, and SAM-dependent DNA methyltransferases; and existed as a 71 amino acid (aa-s) truncated sequence of those found in these similar proteins (Supplementary figure 3.1 Panel A, Supplementary figure 3.2). The fosmid methylase has been previously annotated in the NCBI nr database as a hypothetical protein (Accession: WP_021265564.1) but has not been studied or described in-depth. Comparison of several proteins sharing high identity with the fosmid methylase to the 22N completed genome with TBLASTN revealed length and aa differences caused by mutations within the fosmid sequence (Supplementary figure 3.1 Panel B, Supplementary figure 3.3). The fosmid methylase was determined to be a subsequence of other proteins with methylase activity (e.g., NAG37415.1, WP_236043638.1, VEC36965.1). These proteins start 72 aa-s before the fosmid methylase subsequence and continue for tens to hundreds of aa-s. The later start and earlier end of the fosmid methylase relative to other proteins were due to the mutation of stop codons within the greater sequence shared by other methylating proteins; notably, a mutation

of a Glutamine (Q) in a stop codon (*) 26 aa into the shared sequence and a (Q) to (*) mutation introduced at the144th aa. These mutations resulted in a 25 aa long pseudo-protein sequence that was not able to be annotated and caused the fosmid methylase to start at Methionine (M) 73 aa into the sequence and end at 144th aa. Substitution mutations are also present within the fosmid methylase, relative to the other sequences.

3.4 Discussion

P. multocida is a significant feedlot pathogen and is linked with acute BRD (Griffin et al., 2010). Commercial vaccines against *P. multocida* are limited, and as a result, antimicrobial metaphylaxis is frequently used to target this bacterium and mitigate BRD. The type of antimicrobial used for metaphylaxis varies with selection, and is often based on the disease risk classification of incoming cattle (low, medium, high, ultra-high) according to criteria that include vaccination status, body weight, and transportation distance (Avra et al., 2017b). Typically, oxytetracycline and tulathromycin have been used for medium- and high-risk cattle, respectively (Brault et al., 2019b). Interestingly, for Cohort 1 oxytetracycline-treated cattle, *P. multocida* prevalence at entry and ≥60 days on feed did not differ, whereas tulathromycin-treated cattle had higher prevalence at feedlot entry, followed by a reduction after ≥60 days on feed. While the reasons for the feedlot classification of the tulathromycin-treated cattle are unknown, it appeared those cattle had an increased chance of colonization at feedlot entry. A previous study showed that transportation stress increased the prevalence of the BRD pathogen *M. haemolytica,* indicating that certain respiratory pathogens respond to those stress factors that are used to identify BRD risk (Frank and Smith, 1983), a response that may also be relevant to *P. multocida*. The reduction in the prevalence of *P. multocida* in tulathromycin-treated cattle may have been due to metaphylaxis. Tulathromycin has been shown to reduce the relative abundance of *Pasteurella* for up to 12 days after injection (Holman et al., 2019b). Similarly, *P.*

multocida numerically decreased from 40 to 31% in cattle sampled at weaning or feedlot placement, as compared to those sampled at reprocessing (Nobrega et al., 2021b). However, others have shown an increase in *P. multocida* prevalence after feedlot placement, potentially as a result of the selection of resistant strains after antimicrobial use (Guo et al., 2020). Thus, the prevalence of *P. multocida* can be variable and affected by different management strategies and bacterial responses to selective pressures.

In Cohort 2 cattle, *P. multocida* was more frequently recovered from nasopharyngeal samples from cattle with BRD as compared to healthy controls. Few studies have compared BRD pathogen carriage in healthy and morbid cattle within the same feedlot pen. In the study from which our swabs were collected, analysis of transtracheal washes of the cattle previously indicated that lung prevalence of *P. multocida* was greater in BRD cases (55%) than in healthy cattle (25%) (Timsit et al., 2017). Thus, both upper and lower respiratory tract samples have increased rates of *P. multocida* in BRD-afflicted cattle, supporting that disease progression results from bacterial translocation to the lungs from the upper respiratory tract (Timsit et al., 2017). In a multi-feedlot study within Alberta, recovery of *P. multocida* was reported to be 17.9% from mortality lung and 21.1% from morbidity deep nasal swabs (Anholt et al., 2017a). While previously considered to be more commonly implicated in enzootic calf pneumonia in dairy calves (Angen et al., 2009), *P. multocida* is clearly a significant pathogen of feedlot cattle in North America. The fact that both healthy and BRD-inflicted cattle can be colonized by *P. multocida*, has been reported previously (Allen et al., 1991), highlighting the multifactorial nature of BRD and the limitation of reporting only prevalence data. Primary viral infection, host immunity, and abundance of *P. multocida* are factors that may affect BRD outcomes due to *P. multocida* colonization (Allen et al., 1991).

While a strength of our study was the use of moderate-risk feedlot cattle originating from Alberta with a known antimicrobial use history, some caution is warranted in comparing resistance between the two Cohorts. In-feed antimicrobial use differed, with Cohort 2 cattle receiving pulses of chlortetracycline followed by subtherapeutic administration. In addition, antimicrobial use by the feedlots in the period between sampling the Cohorts was unknown, and *P. multocida* isolates from Cohort 1 were collected before and after metaphylaxis, while those in Cohort 2 were only isolated after metaphylaxis. However, sampling time did not affect antimicrobial resistance in Cohort 1 isolates, and resistance patterns in isolates from varying cattle sources with unknown history have been used previously to provide historical context with regard to antimicrobial susceptibility (Kinnear et al., 2020b; Portis et al., 2012c). The most notable changes in antimicrobial susceptibility between isolates from the two Cohorts were increases in multidrug resistance (7.5% to 83%), particularly to oxytetracycline and tulathromycin.

A recent analysis of antimicrobial use data in Canadian feedlots between 2008 to 2012 showed that tetracyclines were the most widely used injectable (oxytetracycline) and in-feed (chlortetracycline) antimicrobials in feedlots during that period (Brault et al., 2019b). Additionally, macrolides were the second most common class of antimicrobial, with tulathromycin accounting for 88% of injectable macrolide use (Brault et al., 2019b). In Canada, tulathromycin was first marketed for BRD treatment and prevention in 2007 (Schunicht et al., 2007). Assuming similar antimicrobial use trends from 2012 onward, our study suggests that tulathromycin use led to *P. multocida* resistance substantially increasing within 8 years of its availability. When BRD morbidities and mortalities were sampled from a network of feedlots in Alberta from 2014-2015, *P. multocida* resistance to oxytetracycline and tulathromycin was 55.6% and 29.9%, respectively (Anholt et al., 2017a). Rates were lower in a study that sampled

cattle at feedlot reprocessing in 2017 (tetracycline, 15.8%; tulathromycin, 5.3%), though fewer *P. multocida* were evaluated (n=38) (Nobrega et al., 2021b). Similar to our study, no *P. multocida* were found to be resistant to enrofloxacin in those studies, and resistance to florfenicol was low (0-1.7%) (Anholt et al., 2017a; Nobrega et al., 2021b). This is likely due to enrofloxacin and florfenicol mainly being used for BRD treatment as opposed to prevention in feedlots. It is noteworthy that florfenicol use doubled in Western Canadian feedlots between 2008 and 2012 (Brault et al., 2019b), which may account for increased resistance to this antimicrobial in *P. multocida* from Cohorts 2 vs 1. Thus, it is important that the use of fluoroquinolones and phenicols for BRD treatment be limited, so as to not accelerate the development of resistance to these antimicrobials. Although neomycin is not used in Canadian feedlots, we observed increased resistance to this antimicrobial, and others have reported high resistance rates in *P. multocida* as well (65.8%, (Anholt et al., 2017a)). This is likely due to the linkage of aminoglycoside resistance genes to macrolide and tetracycline resistance genes through mobile genetic elements, resulting in co-selection for neomycin resistance (Guo et al., 2020; Klima et al., 2014a). It is noteworthy that *M. haemolytica* was evaluated in cattle from which the samples in our study originated, and similar to *P. multocida*, its resistance to oxytetracycline (3.8%) and tulathromycin (0 - 0.4%) prior to 2011 was low (Alexander et al., 2013a; Klima et al., 2011c) but increased in 2015-2016 (oxytetracycline, 83%; tulathromycin, 79.3%) (Timsit et al., 2017). Thus, increased resistance to oxytetracycline and tulathromycin over time occurred in both of these prominent respiratory pathogens.

The reduced genetic diversity observed in Cohort 2 *P. multocida* was an important finding. Although Cohort 2 isolates were collected at single time points, the identification of highly related strains being concomitant with multidrug resistance would suggest that antimicrobial use was selected for these bacteria due to their enhanced fitness as a result of

encoded resistance genes. This resulted in fewer strains dominating cattle colonization in Cohort 2, compared to Cohort 1 which had a greater heterogeneity of *P. multocida* and resistant isolates being sporadic within pulsogroups or not belonging to a pulsogroup. In a repeated sampling study, (Guo et al., 2020) observed that several multi-drug resistant *P. multocida* strains dominated the respiratory tract microbiome after feedlot placement and antimicrobial administration. Increased homogeneity and strain dominance of multi-drug resistant *M. bovis* have also been reported in France (Becker et al., 2015) and Nordic countries (Tardy et al., 2020), with selection thought to have resulted from antimicrobial use. In our study, clonal strains identified by PFGE were found in both BRD cases and healthy cattle in the same feedlot. Large feedlots in North America have consistent inputs of cattle and typically clean pen floors and water bowls once to twice per year. It has been reported that *M. haemolytica* persists in animal housing (Burriel, 1997; Neupane et al., 2019), and we have recently isolated multi-drug resistant *P. multocida* from feedlot water bowls (unpublished). Thus, the feedlot environment may be conducive to spreading antimicrobial-resistant *P. multocida* among cattle. In several instances, clonal PFGE types were observed in different feedlots. It is more difficult to explain how transmission occurred between feedlots, but cattle are often sourced from auction markets, and perhaps spread between cattle destined for different feedlots occurred at the market. Clonal expansion of fluoroquinolone-resistant *M. haemolytica* in Japan (Katsuda et al., 2009) and multi-drug resistant *M. bovis* in Nordic European countries (Tardy et al., 2020) support the potential for widespread transmission of BRD pathogens.

The *tet*(H) gene has consistently been detected in *Pasteurellaceae* (Guo et al., 2020), and was detected in oxytetracycline-resistant *P. multocida* from Cohort 1. None of the tulathromycin-resistant isolates from Cohort 1 carried *erm*(42), *msr*(E), or *mph*(E), which were first reported in tulathromycin-resistant *P. multocida* in 2011 (Desmolaize et al., 2011; Kadlec

et al., 2011). Similarly, tulathromycin-resistant *M. haemolytica* isolated before 2011 did not carry these genes (Alexander et al., 2013a), suggesting that other mechanisms such as mutations in the 23S rRNA gene (Andrés-Lasheras et al., 2021b) were responsible for early emergence of resistance to tulathromycin. It was surprising that the majority of tulathromycin-resistant *P. multocida* from Cohort 2 did not carry *erm*(42), *msr*(E), or *mph*(E) genes. Given the widespread occurrence of tulathromycin-resistant isolates among different pulsogroup lineages, we hypothesized that resistance was due to the carriage of genes on a mobile element that could be transferred between bacteria. Initial genome analysis did not identify a macrolide resistance gene in isolates 22N, 22T, 35N, 37N, 37T, or 228T. However, genome analysis did confirm the presence of *erm*(42), *msr*(E), and *mph*(E) in those isolates that were positive for these genes as measured by PCR (124T, 76T, 150N, and 150T). Interestingly, genome sequencing showed that the tulathromycin-susceptible isolate we included for analysis (150N) carried *erm*(42), *msr*(E), and *mph*(E). Thus, isolate 150N had the genetic potential for resistance but the phenotypic expression was limited. Similar findings have been reported previously for *H. somni* (Owen et al., 2017b) and *Mycobacterium tuberculosis* (Ahmad et al., 2016).

Notably, two gene regions were observed in the genomes of tulathromycin-resistant isolates without known macrolide resistance genes. The first was a set of genes of prophage origin. While intact prophages can account for up to 11% of *Pasteurellaceae* genomes (Klima et al., 2016a), the genes we identified were limited and did not belong to an intact prophage as indicated by the absence of proximate additional phage genes, including those that encoded for tail or head proteins. While it is common to find phages within bacterial genomes that are not intact and lack function (Delavat et al., 2017), it was interesting that these phage genes were common to all the sequenced *P. multocida* isolates with unknown macrolide resistance. The

second set of genes common to these isolates was located in close proximity to an integrative and conjugative element (ICE), as indicated by adjacent genes coding for a type IV secretion system, integrase, and relaxase (Klima et al., 2016a). However, initial referencing to antimicrobial resistance gene databases did not link any of these genes to tulathromycin resistance.

Lower concordance between metagenomic (Klima et al., 2016a) or genomic (Owen et al., 2017b) identification of resistance genes and antimicrobial phenotype of BRD isolates has highlighted the importance of having complementary isolate phenotypes for evaluation. As we were unable to identify macrolide resistance genes by PCR or genome sequencing, a fosmid library was prepared. The inserted fosmid sequence shared >98% pairwise identity to a previously identified integrative and conjugative element that was a variant of ICE*Mh1*, and found in *P. multocida* and *H. somni* (Cameron et al., 2018). The fosmid insert contained the second set of 5 genes identified as common to only tulathromycin-resistant isolates, without known resistance genes. The PCR identification of two of these genes in almost all tulathromycin-resistant isolates with unknown resistance genes, and their absence in isolates with *erm*(42), *msr*(E), and *mph*(E) implied that they may have had a role in tulathromycin resistance. Therefore, a BLASTP search of the protein sequences of these 5 genes against the NCBI nr database was performed and revealed a fosmid methylase that was 100% identical to hypothetical proteins in *P. multocida* (WP_199764665.1) and *H. somni* (Cameron et al., 2018), and 86-92% identical to N-6 methylases from *Klebsiella pneumoniae*, *E. coli,* and *Aeromonas salmonicida*. The *erm* family of genes encodes for methyltransferases that target the *E. coli* N-6 position of nucleotide A2058 within 23S rRNA and are implicated in macrolide resistance in numerous bacteria (Desmolaize et al., 2011). Thus, the methylase was considered a candidate tulathromycin resistance gene. Additionally, we also considered the permease as a potential

tulathromycin resistance gene, as permeases are membrane transport proteins that have a broad range of efflux and uptake roles in bacteria (Tseng et al., 1999). Initially, the methylase and permease were cloned for transforming *P. multocida*, however, transformation attempts were not successful (data not shown), and *E. coli* was selected for expression. Despite intrinsic macrolide resistance in this species, *E. coli* has been used previously to characterize tulathromycin resistance genes from *P. multocida* (Kadlec et al., 2011). The permease did not alter MIC, but the methylase did double the MIC of tulathromycin for *E. coli*, indicating that it has the potential to confer tulathromycin resistance.

The size of the methylase identified indicated that it unlikely acts alone as an enzyme. The smallest known enzyme is 62 aa-s in length (Chen et al., 1992), while the smallest known methylase is 152 aa-s (Strassler et al., 2022). The methylase in *P. multocida* was a partial sequence of the HsdM protein in *E. coli*, an N-6 methylase, and subunit of a type I restriction enzyme. Type I restriction endonucleases have three subunits, including M (modification), S (specificity), and R (restriction) units that form enzymes with restriction, methylase, and ATPase multifunctional activity (Loenen et al., 1987; Meselson and Yuan, 1968). HsdM has been shown to induce drug resistance in *Mycobacterium tuberculosis* through multiple methylating actions (Chu et al., 2021). Therefore, we hypothesize that the *P. multocida* methylase may act as a subunit of a greater protein that affects pathways similar to HsdM in *M. tuberculosis*, though this needs further evaluation.

With the methylase being observed in *P. multocida* from multiple feedlots in our study and others (Cameron et al., 2018), it appears that this gene is widespread. Additionally, its presence in *H. somni* (Cameron et al., 2018) highlights the potential for inter-species spread. Bacterial transfer could potentially be propagated by the adjacent transposase which was only

identified in *P. multocida* with the methylase. It is more difficult to hypothesize the roles of the permease and helix-turn-helix genes, but both have been implicated in antimicrobial resistance previously by impacting cell influx/efflux (Davin-Regli et al., 2008) and transcription (Alekshun et al., 2001; Chu et al., 2022), respectively. Whether there is a cumulative effect of these genes on tulathromycin resistance, above and beyond that for the methylase, would be interesting to evaluate.

3.5 Conclusion

Multiple factors affect disposition towards BRD, including management strategies and primary viral infections that impact host immunity. However, *P. multocida* appears to be increasingly important in BRD pathogenesis. We observed that changes in *P. multocida* occurred as a result of metaphylaxis, including the development of resistance against tulathromycin, which reduced the diversity of the *P. multocida* population. These effects may vary by management production practices and result in variation in *P. multocida* prevalence and resistance across feedlots. Consistent findings of low resistance to enrofloxacin highlight the importance of maintaining this antimicrobial for the treatment of BRD only. The identification of a novel candidate methylase also highlighted that sequence-based surveillance of resistance is limited by comparisons being made to only genes with known functions in databases. The methylase was hypothesized to act as a subunit to a larger enzyme, given its small size, but this needs to be verified. Regardless, conservation of the methylase in almost all tulathromycin-resistant *P. multocida* lacking previously identified macrolide resistance genes, revealed its potential role in conferring an antimicrobial phenotype.

3.6 Reference

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Table 3.1 PCR analysis of resistance genes in Cohort 2 isolates (n=130)

Phenotyping result[a]	Macrolides				Permease	Tetracycline	Aminoglycoside	Sulfonamide	Phenicol
	erm(42)	*mph*(E)	*msr*(E)	methylase[b]	permease[c]	*tet*(H)	*aphA*-1	*sul2*	*floR*
Sensitive (n=13)	0	na	na	0	0	na	na	na	na
Neo, Oxy, Tul (n=67)	2	7	8	64	64	67	63	66	na
Ffe, Neo, Oxy (n=4)	0	1	1	0	0	4	1	4	4
Ffe, Neo (n=2)	0	na	na	0	0	2	2	2	2
Amp, Pen, Neo, Oxy, Tul (n=1)	0	0	0	1	1	1	1	1	na
Ffe, Neo, Oxy (n=1)	0	na	na	0	0	1	1	1	1
Ffe, Neo, Oxy, Tul (n=3)	3	1	1	0	0	3	3	3	na
Ffe, Neo, Oxy (n=2)	2	na	na	0	0	2	2	2	1
Oxy, Tul (n=24)	0	1	2	21	21	23	1	0	Na
Tul (n=11)	0	0	0	2	2	na	3	0	Na
Neo, Oxy (n=2)	0	na	na	0	0	1	2	2	1
Total (n=130)	7	10	12	88	88	104	79	81	9

[a]Sensitive, isolates were sensitive to all antimicrobials; Neo, neomycin; Oxy, oxytetracycline; Tul, tulathromycin; Ffe, florfenicol; Amp, Ampicillin; Pen, Penicillin; na, Not evaluated. The number of resistant isolates is indicated in brackets. Methylase, permease *erm(42)*, *mph*(E), *msr*(E), *tet*(H), *sul2*, *aphA-1*, *floR* were analyzed by PCR. The corresponding phenotypes for the resistance gene are listed in Supplementary Table 1.[b] The methylase was a putative novel gene identified in this study potentially conferring tulathromycin resistance. [c]This gene is a permease, not categorized as a resistance element. Permease was included in PCR screening because it was in close proximity to the methylase gene.

Table 3.2 Genetic relatedness and antimicrobial resistance of *P. multocida* from Cohort 1 (n=129) analyzed using pulsed-field gel electrophoresis.

Pulsogroup[a]	TUL[b]	OXY[c]	Entry[d]	≥60[e]	Total no. of feedlots[f]	Phenotypic resistance (no. of isolates)[g]
1 (1,1)	2	0	1	1	1	
2 (1,1)	2	0	1	1	1	
3 (2,1)	2	1	0	3	3	
4 (3,3,1)	4	3	4	3	4	Neo-Oxy(1)
5 (2,1)	2	1	2	1	2	
6 (14,1,1)	10	6	6	10	4	
7 (2,1)	2	1	2	1	3	
8 (13,2,1,1,2,1,7,7)	23	11	17	17	4	Neo(3),Oxy(1)
9 (1,1)	0	2	1	1	1	
10 (2)	0	2	0	2	1	Tul(1)
11 (1,1)	0	2	2	0	2	Tul(1),
12 (2)	2	0	2	0	1	
13 (2,1)	3	0	3	0	1	Oxy-Tul(1)
14 (5,1)	6	0	3	3	1	
15 (1,1)	0	2	0	2	1	
16 (2)	1	1	1	1	2	
Singlets (32)	21	11	24	8	4	Oxy(1),Tul(1), Ffe(1)

[a]Pulsogroups were defined as restriction patterns having ≥ 90% similarity. Sub-groups of isolates that had identical pulsotypes (100% similarity, considered clones) are indicated in brackets; the numbers indicate how many isolates were clonal within a subgroup, Singlets: Unique pulsotype refers to singlets, [b]Isolates derived from cattle that were administered tulathromycin. [c]Isolates derived from cattle that were administered oxytetracycline, [d]Isolates derived from cattle that were collected at feedlot entry. [e] Isolates derived from samples that were collected at ≥60 days in a feedlot, [f]Total no. of feedlots, the total number of feedlots where pulsogroup identified, [g]Phenotyping resistance: Neo, neomycin; Oxy, oxytetracycline; Tul, tulathromycin; Ffe, Florfenicol. The number of resistant isolates is indicated in brackets.

Table 3.3 Genetic relatedness and antimicrobial resistance of *P. multocida* from Cohort 2 (n=118) analyzed using pulsed-field gel electrophoresis.

Pulsogroup[a]	Healthy[b]	BRD[c]	No. of feedlots[d]	Phenotypic resistance (no. of isolates)[e]
1(5)	3	2	2	Ffe(4), Neo(5), Oxy(5),Tul(1)
2(5)	0	5	2	Tul(5)
3(1,1)	1	1	1	Tul(2)
4(2,2,8)	2	10	3	Chltet(1),Ffe(3), Neo(5), Oxy(8)
5(4,1)	2	3	2	Chtet(3), Ffe(3), Neo(5),Oxy(5),Tul(2)
6(70, 5,2,1,1)	14	65	4	Amp(1),Chltet(3),Ffe(3),Neo(56),Oxy(75),Tul(61)
7(2)	0	2	1	Neo(2),Oxy(2),Tul(2)
8(2)	1	1	1	Neo(2),Oxy(2),Tul(2)
9(1)	0	1	1	
Singlets (5)	4	1	3	Neo(4),Oxy(4),Tul(2)

[a]Pulsogroups were defined as having ≥ 90% similarity. Sub-groups of isolates that had identical pulsotypes (100% similarity, considered clones) are indicated in brackets; the numbers indicate how many isolates were clonal within a subgroup. Singlets, a unique pulsotype refer to singlets. [b]Isolates derived from healthy cattle. [c]Isolates were derived from cattle that were diagnosed with bovine respiratory disease.[d]No of feedlots where pulsogroup were identified, [e]Phenotypic resistance: Neo, neomycin; Oxy, oxytetracycline; Tul, tulathromycin; Ffe, florfenicol. The number of resistant isolates is indicated in brackets.

Table 3.4 Minimum inhibitory concentration (MIC) of *E. coli* BL21 when transformed with the putative methylase[a].

Antimicrobial agent	MIC (µg/ml) in the *E. coli*		
	***E. coli* BL21**	***E. coli* BL21+ pUC57**	***E. coli* BL21+ pUC57 + methylase**
Tulathromycin	4	4	8
Clindamycin	>16	>16	>16
Tilmicosin	64	64	64
Tylosin tatrate	>32	>32	>32

[a]The methylase gene was synthesized and cloned into the pUC57-Kan vector and the resultant construct was transformed into *E. coli* BL21. The pUC57-Kan vector without methylase was also transformed into *E. coli* BL21 to evaluate any potential plasmid effect.

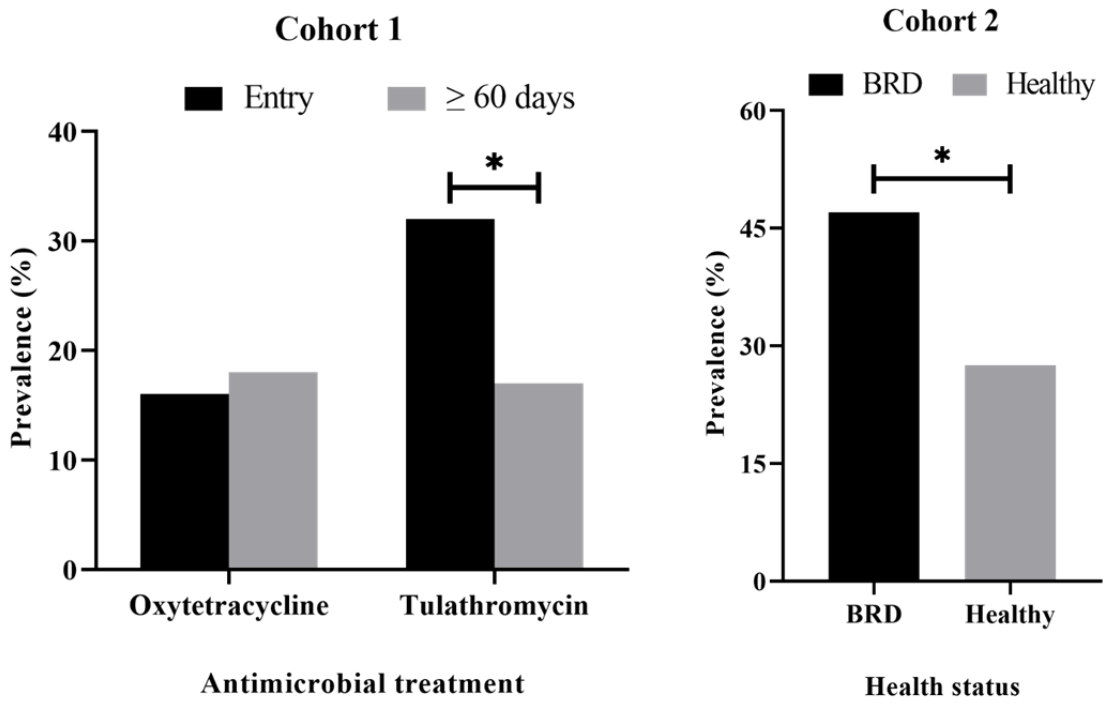


Figure 3.1 Prevalence of *P. multocida* from Cohorts 1 and 2. For Cohort 1 (2008-2009), *P. multocida* was isolated from nasopharyngeal swabs collected from cattle treated with oxytetracycline (n=149) or tulathromycin (n=162) at feedlot entry and then ≥60 days on feed. For Cohort 2 (2015-2016), nasopharyngeal swabs were collected from cattle diagnosed with bovine respiratory disease (BRD; n=208), before treatment, and matching healthy controls (n=104).

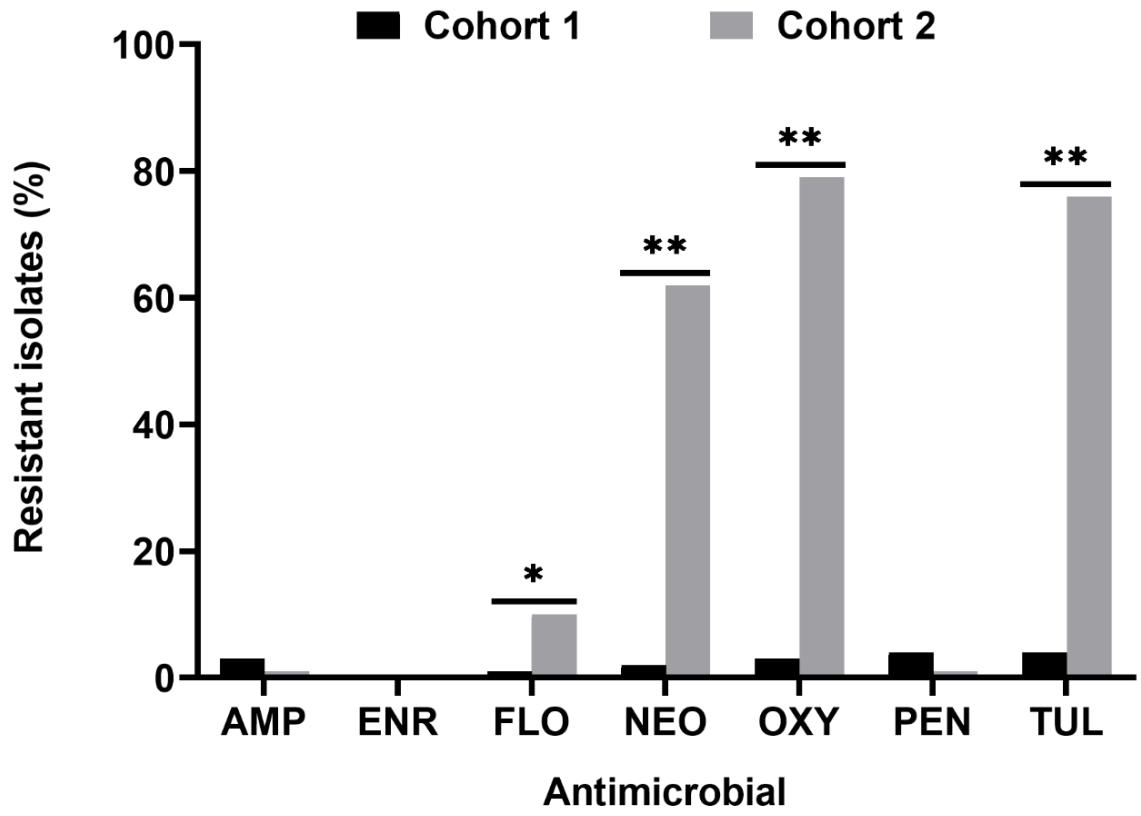


Figure 3.2 Antimicrobial-resistant *P. multocida* isolated from Cohort 1 (n=133; years 2008-2009) and Cohort 2 (n=132; years 2015-2016) cattle. Antimicrobials tested were ampicillin (AMP), enrofloxacin (ENRO), florfenicol (FLO), penicillin (PEN), neomycin (NEO), oxytetracycline (OXY) and tulathromycin (TUL)). Resistance breakpoints were based on CLSI (2020) guidelines, described in the methods. * indicates $P < 0.05$ and ** indicates $P<0.005$.

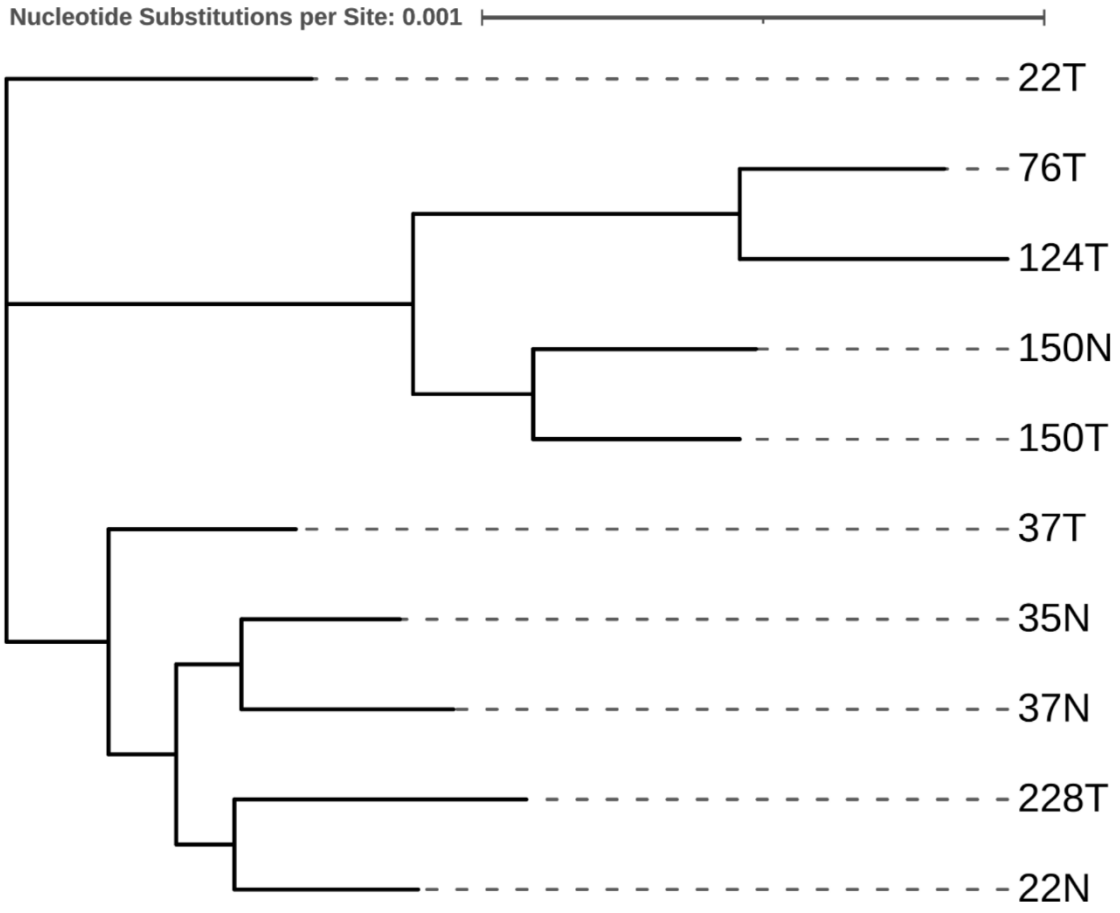


Figure 3.3 Phylogenetic tree of genome-sequenced *P. multocida*. Isolates were tulathromycin-resistant with unknown macrolide resistance genes (strains 22T,37T,35N, 37N,228T,22N), tulathromycin-resistant with known macrolide resistance gene (PCR positive for (*erm*(42), *msr*(E), *mph*(E)); 76T, 124T, and 150T strains), and macrolide-susceptible (strain 150N).

Isolate	Phenotyping resistant	Macrolide			Tetracycline	Aminoglycoside			Sulfonamide	Phenicol
		erm(42)	*mph*(E)	*msr*(E)	*tet*(H)	*strA*	*aph(3)-Ia*	*aph(6)-Id*	*aadA31*	*sul2*	*flor*
228T	Neo,oxy,Tul										
22T	Neo,oxy,Tul										
22N	Neo,oxy,Tul										
35N	Neo,oxy,Tul										
37T	Neo,Oxy,Tul										
37N	Neo,oxy,Tul										
124T	Flor,Neo,Oxy,Tul										
76T	Flor,Neo,Oxy,Tul										
150T	Flor,Neo,Oxy,Tul										
150N	Flor,Neo,Oxy										

Figure 3.4 Resistant genes identified in *P. multocida* from genome sequencing analysis. Isolates were resistant to neomycin (Neo), oxytetracycline (Oxy), florfenicol (Flor), or tulathromycin (Tul).Light orange indicates the presence of the gene and Light blue color indicates the absence of the gene. NB, despite being susceptible to tulathromycin, resistance genes *erm*(42), *msr*(E), and *mph*(E)were identified in isolate 150N.

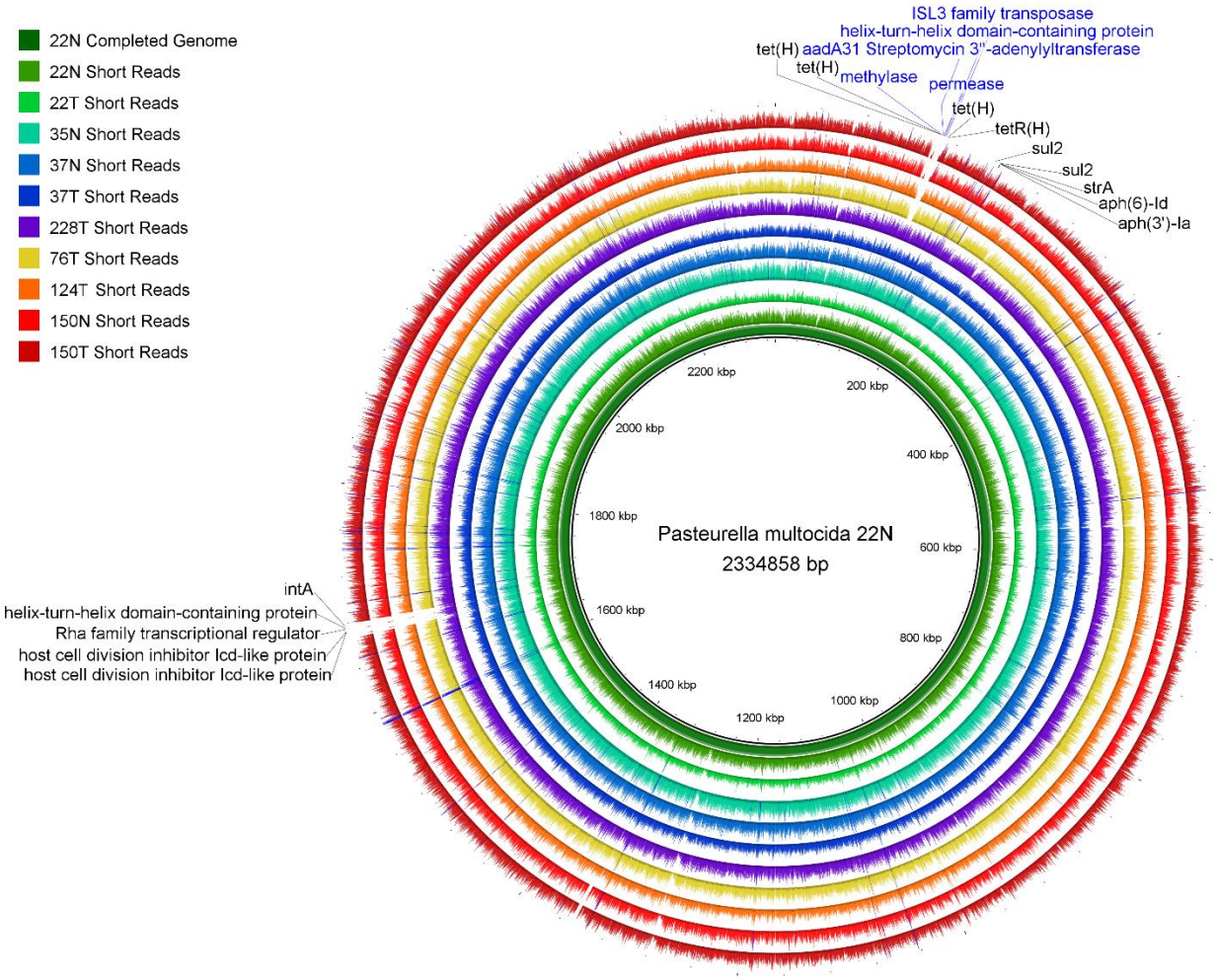


Figure 3.5 Pangenome analysis of *P. multocida* from Cohort 2 cattle. The 10 short-read sequenced datasets were mapped against the completed 22N genome. Each genome is represented by a colored ring, with the innermost ring representing the 22N genome. Relative coverage of reads mapping to the 22N genomes is represented by the peaks within each ring. The blue lines that occur within the rings represent points of exceptionally high coverage. Two notable clusters of genes were present only in isolates that had tulathromycin-resistant phenotype but unknown resistance gene (strains 22N, 22T, 35N, 37N, 37T, 228T) but not present in isolates that were tulathromycin-resistant with known macrolide resistance gene (PCR positive for (*erm*(42), *msr*(E), *mph*(E)), strains 124T,76T,150T), and macrolide susceptible isolate (strain 150N).

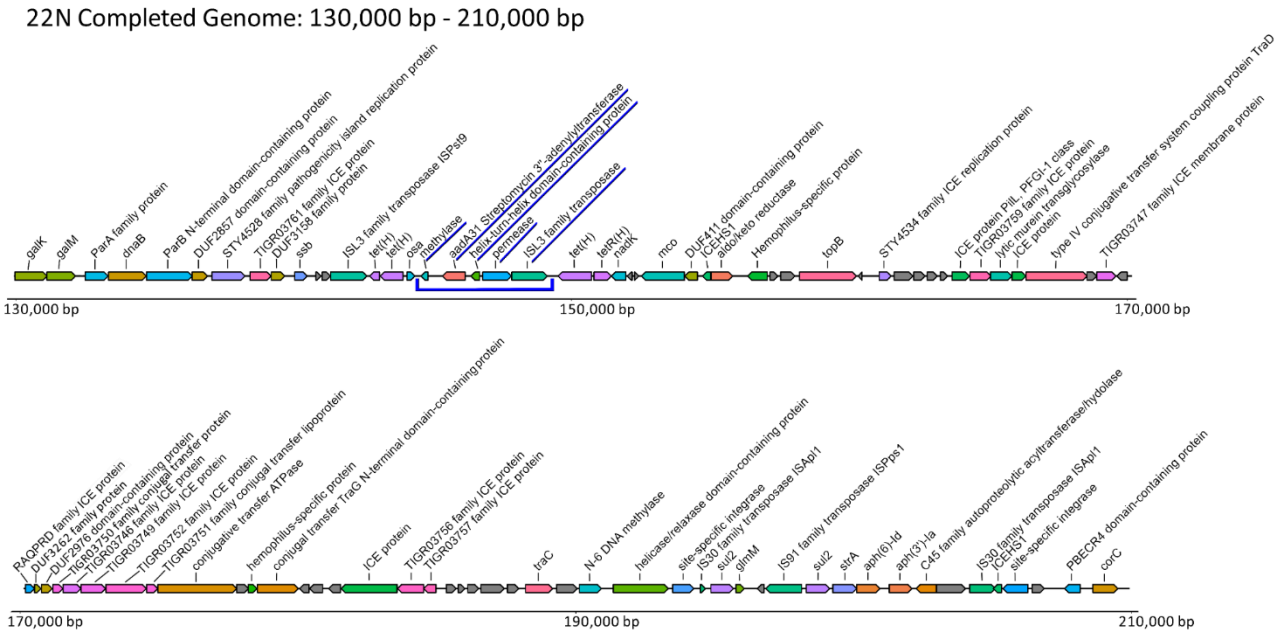


Figure 3.6 Gene annotation of the fosmid sequence aligned against the completed genome of isolate 22N. The fosmid sequence is underlined in blue. The five double-underlined genes were only present in isolates with tulathromycin-resistant phenotype and unknown tulathromycin resistance genes (22N, 22T, 35N, 37N, 37T, 228T).

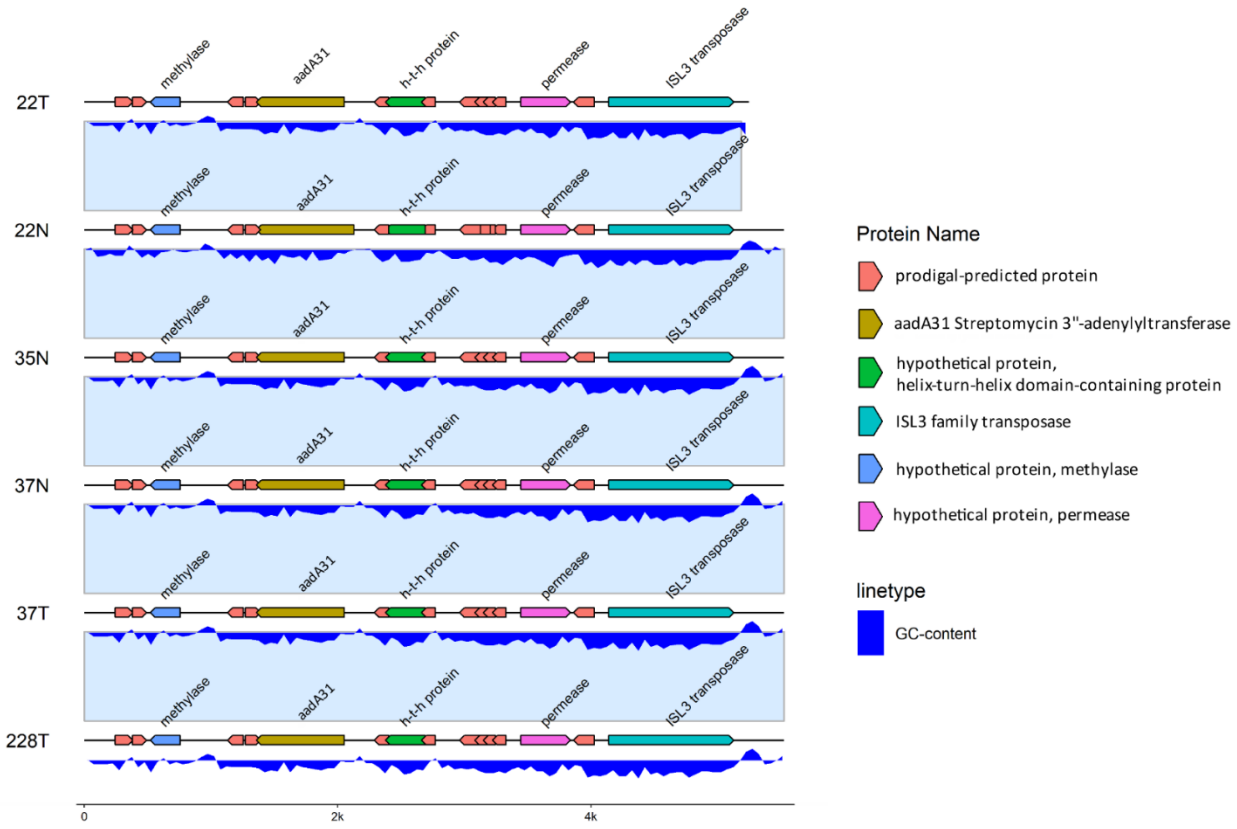
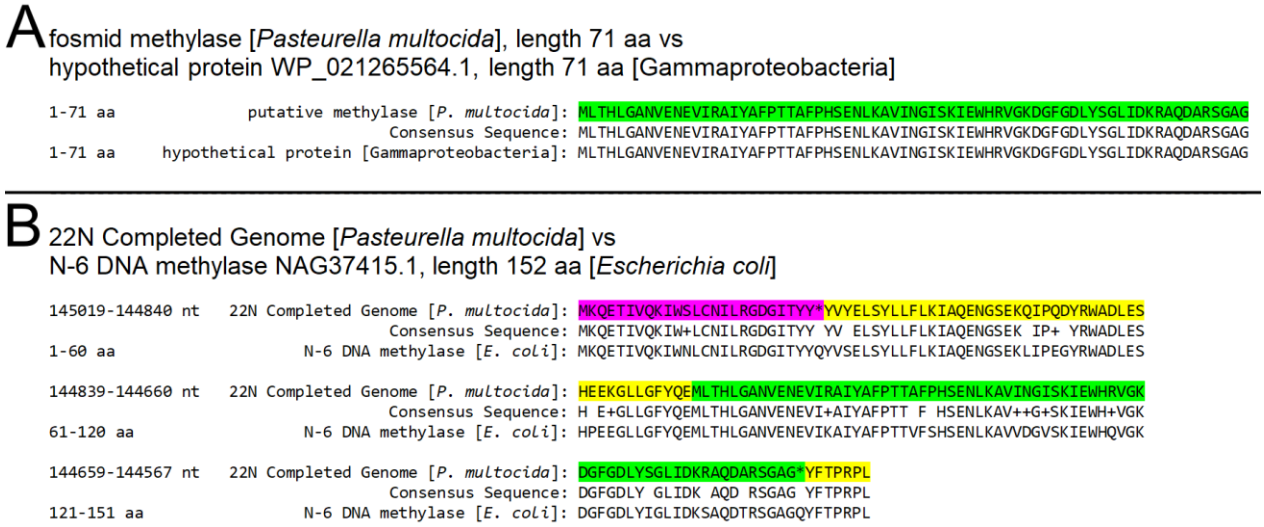


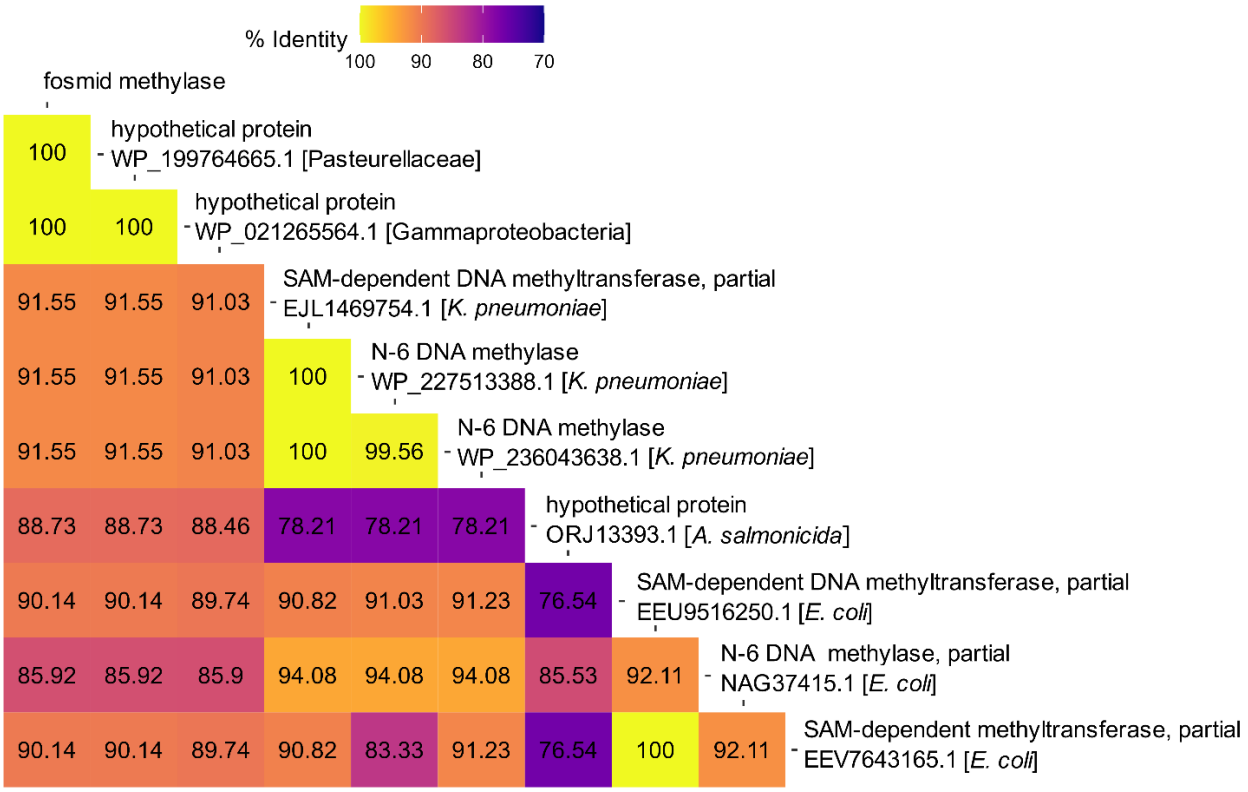
Figure 3.7 Five common genes were found in tulathromycin-resistant isolates with unknown macrolide resistance genes (22N, 22T, 35N, 37N, 37T, 228T) from sequencing results. The pairwise alignments of each contig are represented by the light blue rectangles between each sequence. These alignments are represented as rectangles because the contigs are nearly identical. The relative GC content is presented as a blue ribbon. A positive ribbon (above the baseline) represents a higher GC content level than AT, and a negative ribbon represents the opposite. The nucleotide scale is represented at the bottom of the figure.

Supplementary table 3.1 Primers used for PCR analysis of *P. multocida*.

PCR target/Phenotype	Target gene	PCR primer sequence Forward/Reverse (5'-3')	Amplicon size	Annealing temperature (° C)	Primer reference
Pasteurella multocida	23SrRNA	GGCTGGGAAGCCAAATCAAAG/CGAGGGAC TACAATTACTGTAA	1432	52	(Miflin and Blackall, 2001)
	erm(42)	GGGTGAAAAGGGCGTTTATT/ACGTTGCACT TGGTTTGACA	1254	60	(Rose et al., 2012)
Macrolide	*msr(E)*	ACCAGCCACCTTGATCTCAATG/GTTCCATT CGATCCAGTTATAGCG	620	60	(Kadlec et al., 2011)
	mph(E)	TCTGTAGCGGGTTTCCAATTGC/AATGGTTG CTGCGTATTCCTCG	401	60	(Kadlec et al., 2011)
	methylase	CCGCTATAGAGGTCACCAAACC/TACGCATT TCCTACAACGGC	119	52	This study
Aminoglycoside	*aphA1*	TTATGCCTCTTCCGACCATC/GAGAAAACTC ACCGAGGCAG	489	54	(Poppe et al., 2002)
Tetracycline	*tet(H)*	ATACTGCTGATCACCGT /TCCCAATAAGCGACGCT	1076	60	(Klima et al., 2011c)
Sulfonamide	*Sul2*	CCAATACCGCCAGCCCGTCG/TGCCTTGTCG CGTGGTGTGG	489	64	(Klima et al., 2014b)
Phenicol	*floR*	GACGGTTCGCGACGTTTATG/GAAGACGAA GAAGGTGCCCA	320	58	(Klima et al., 2014b)
Permease	-	TTGCCTTACCTGCTCATCGG/CAGCGCGAAT ATACAACGGC	148	55	This study



Supplementary figure 3.2 Panel A: An amino acid sequence alignment of the fosmid methylase to the hypothetical protein WP_021265564.1 using BLASTP. The sequence of the fosmid methylase is highlighted in green and is identical to that of the hypothetical protein WP_021265564.1. **Panel B: An alignment of the N-6 DNA methylase NAG37415.1 to the 22N completed genome.** The sequence of the fosmid methylase is highlighted in green. Non-coding regions surrounding the fosmid methylase are highlighted in yellow. The 25 amino acid pseudo-protein that is truncated by the introduction of the stop codon (*) is highlighted in pink. Position information is given in nucleotides (nt) for the 22N completed genome and amino acids (aa) for NAG37415.1. NAG37415.1 has an additional Valine (V) at the end of the sequence not shown by the alignment.



Supplementary Figure 3.2 Heatmap of the Pairwise identities of the fosmid methylase compared among highly similar proteins. Pairwise identities are represented by a triangle heatmap with labels indicating both the row and columns of each sequence. Each protein apart from the fosmid methylase is represented by a protein name, accession identifier, and the name of the species of origin.

A 22N Completed Genome [*Pasteurella multocida*] vs
N-6 DNA methylase WP_236043638.1, length 228 aa [*Klebsiella pneumoniae*]

Result 1, 22N Completed Genome Frame -3:

```
145019-144840 nt   22N Completed Genome [P. multocida]: MKQETIVQKIWSLCNILRGDGITYY*YVYELSYLLFLKIAQENGSEKQIPQDYRWADLES
                                 Consensus Sequence: MKQETI+QKIWSLCNILRGDGITYY YV ELSYLLFLKIAQENGSEK IP+ YRWADLES
1-60 aa              N-6 DNA methylase [K. pneumoniae]: MKQETIIQKIWSLCNILRGDGITYYQYVSELSYLLFLKIAQENGSEKLIPKGYRWADLES

144839-144660 nt   22N Completed Genome [P. multocida]: HEEKGLLGFYQEMLTHLGANVENEVIRAIYAFPTTAFPHSENLKAVINGISKIEWHRVGK
                                 Consensus Sequence: HEE+GLLGFYQEMLTHLGANVENEVIRAIYAFPTT F HSENLKAVI+GISKIEWH+VGK
61-120 aa            N-6 DNA methylase [K. pneumoniae]: HEEEGLLGFYQEMLTHLGANVENEVIRAIYAFPTTVFSHSENLKAVIDGISKIEWHQVGK

144659-144567 nt   22N Completed Genome [P. multocida]: DGFGDLYSGLIDKRAQDARSGAG*YFTPRPL
                                 Consensus Sequence: DGFGDLYSGLIDK AQD RSGAG YFTPR L
121-151 aa           N-6 DNA methylase [K. pneumoniae]: DGFGDLYSGLIDKSAQDTRSGAGQYFTPRSL
```

Result 2, 22N Completed Genome Frame -2:

```
144565-144383 nt   22N Completed Genome [P. multocida]: VNTIVRLIQPSLGELIQDPATGSGGFLVSADEYIRKNNSREKYKINPPQYQGVEIEKNTRK
                                 Consensus Sequence: VNTIVRLIQPSLGELIQDPA GSGGFLVSAD +IR   S ++Y+ NPP+YQGVEIEKNTR+
152-212 aa           N-6 DNA methylase [K. pneumoniae]: VNTIVRLIQPSLGELIQDPAIGSGGFLVSADSFIRNKYSHKEYQTNPPKYQGVEIEKNTRR
```

B 22N Completed Genome [*Pasteurella multocida*] vs
type I restriction enzyme StySJI M protein (hsdM_1 gene) VEC36965.1, length 511 aa [*Escherichia coli*]

Result 1, 22N Completed Genome Frame -3:

```
145019-144840 nt   22N Completed Genome [P. multocida]: MKQETIVQKIWSLCNILRGDGITYY*YVYELSYLLFLKIAQENGSEKQIPQDYRWADLES
                                 Consensus Sequence: MKQ+T++QKIWSLCNILRGDGITYY YV ELSYLLFLKIAQENGSEK IP+ YRWADLES
1-60 aa                    StySJI M protein [E. coli]: MKQDTVIQKIWSLCNILRGDGITYYQYVSELSYLLFLKIAQENGSEKLIPKGYRWADLES

144839-144660 nt   22N Completed Genome [P. multocida]: HEEKGLLGFYQEMLTHLGANVENEVIRAIYAFPTTAFPHSENLKAVINGISKIEWHRVGK
                                 Consensus Sequence: HEE+GLLGFYQEMLTHLGANVENEVIRAIYAFPTT F HSENLKAVI+GISKIEWH+VGK
61-120 aa        StySJI M protein [E. coli]: HEEEGLLGFYQEMLTHLGANVENEVIRAIYAFPTTVFSHSENLKAVIDGISKIEWHQVGK

144659-144567 nt   22N Completed Genome [P. multocida]: DGFGDLYSGLIDKRAQDARSGAG*YFTPRPL
                                 Consensus Sequence: DGFGDLYSGLIDK AQD RSGAG YFTPR L
121-151 aa                 StySJI M protein [E. coli]: DGFGDLYSGLIDKSAQDTRSGAGQYFTPRSL
```

Result 2, 22N Completed Genome Frame -2:

```
144565-144383 nt   22N Completed Genome [P. multocida]: VNTIVRLIQPSLGELIQDPATGSGGFLVSADEYIRKNNSREKYKINPPQYQGVEIEKNTRK
                                 Consensus Sequence: VNTIVRL QP+LGELIQDPA GSGGFLVSAD YIR   +REKYK NPP+YQGVEIEKNTR+
152-212 aa                 StySJI M protein [E. coli]: VNTIVRLTQPNLGELIQDPAAGSGGFLVSADSYIRSKYTREKYKTNPPKYQGVEIEKNTRR
```

Supplementary figure 3.3 Panel A: An alignment of the N-6 DNA methylase WP_236043638.1 [*Klebsiella pneumoniae*] to the 22N completed genome. Panel B: An alignment of the type I restriction enzyme StySJI M protein VEC36965.1 [*Escherichia coli*] to the 22N completed genome. The gene coding for VEC36965.1 is annotated as hdsM_1. Both panels have highlighted sections that show the sequence of the fosmid methylase in green, non-coding regions surrounding the fosmid methylase in yellow, and the 25 amino acid pseudo-protein that is truncated by the introduction of a stop codon (*) in pink. 'Result 1' in each panel refers to the alignment of the first 151 amino acids of each protein to nucleotides 145019-144567 in reading frame -3. An insertion nucleotide mutation is present at nucleotide 144566 in the 22N completed genome causing a frameshift mutation relative to the two protein sequences. Therefore, 'Result 2' in each panel is the continued alignment of each protein sequence's amino acids 152-212 with the 22N completed genome nucleotides 144565-144383, but with the nucleotides in reading frame -2 instead of frame -3.

Chapter four

General Discussion

4.1 Summary of Thesis:

In this study, the prevalence and antimicrobial susceptibility of *M. bovis* and *P. multocida* were evaluated from two cohorts of feedlot cattle, spanning an 8-year period. For both bacteria, the most significant difference in antimicrobial susceptibility between isolates from the two cohorts was increased resistance to tulathromycin, which is used as a metaphylactic treatment to control BRD. When *P. multocida* was evaluated by PCR, only 16 out of the 106 tulathromycin-resistant isolates from Cohort-2 carried a known tulathromycin resistance gene. Genome sequencing of a subset of *P. multocida*, in combination with the preparation of a fosmid library, led to the identification of a conserved set of genes in tulathromycin-resistance isolates, including a novel putative methylase. It was shown that the methylase conferred resistance against tulathromycin and was widespread in the population of tulathromycin-resistant *P. multocida*. Overall, significant increases in resistance against tulathromycin in both *P. multocida* and *M. bovis* occurred after only 8 years of its commercialization to mitigate BRD in incoming feedlot cattle. Low resistance to antimicrobials mainly used for BRD treatment, such as enrofloxacin and florfenicol, highlighted the importance of reserving those antimicrobials for treatment, to prevent the development of resistance against them.

4.2 Strengths and Limitations:

The research presented in this thesis has several strengths. First, both *M. bovis* and *P. multocida* were isolated from moderate-to-high-risk feedlot cattle in Alberta, with a known history of antimicrobial use. Thus, the animal population evaluated was representative of commercial

feedlots employing typical health management protocols for incoming cattle, including vaccination and antimicrobial metaphylaxis. Second, for Cohort 2 cattle, before enrolment and sampling, two skilled veterinarians performed clinical examinations on each animal chosen by pen checkers to verify its health status. This confirmation improved the validity of the BRD diagnosis in comparison to pen-checking alone (Timsit et al., 2016), which is a persistent challenge in bovine respiratory studies because there is no definitive method for BRD diagnosis (Buczinski and Pardon, 2020). Third, broth microdilution, the standard procedure for assessing the activity of an antimicrobial against a bacterial strain, was used to examine the antimicrobial susceptibility (Lubbers and Turnidge, 2015). The antimicrobial resistance of *M. bovis* was determined by the microdilution method using a customized commercial plate. This method allowed for the evaluation of multiple antimicrobial compounds concurrently with an increased range of antimicrobial concentrations. The Sensititre plate BOPO6F, which represents a wide range of antimicrobials frequently used in beef cattle production, was used for *P. multocida* sensitivity testing. Lastly, the resistance phenotype in *P. multocida* was confirmed through the identification of resistance genes, and in the case of tulathromycin resistance, a novel methyltransferase was repeatedly shown to increase the MIC against tulathromycin in *E. coli*. The main limitation of this study was that feedlot antimicrobial use throughout the period spanning Cohorts 1 and 2 was unknown. Thus, assumptions about antimicrobial use were used to define increases in the resistances of bacteria in Cohort 1 compared to Cohort 2. Additionally, antimicrobial use differed for the cattle studied, with Cohort 2 cattle being administered in-feed chlortetracycline. Finally, no clinical breakpoints for resistance in *M. bovis* exist, which required the assumption of resistance breakpoints defined by previous publications (Anholt et al., 2017a; Jelinski et al., 2020b), and

genotyping was not used to confirm resistance in *M. bovis*, which acquire resistance through mutations (Sulyok et al., 2017).

4.3 Implications:

Both *M. bovis* and *P. multocida* were evaluated from the same study animals. When analyzed in Cohort 1 cattle, *M. bovis* increased significantly in the upper respiratory tract of cattle after ≥60 days on feed, whereas *P. multocida* decreased or remained similar across time. This highlights the highly transmissible nature of *M. bovis* through animal-to-animal or environment-to-animal transmission in feedlots (Timsit et al., 2012b). It is noteworthy, however that when comparing BRD cases in Cohort 2, *P. multocida* was more frequently detected than *M. bovis*, indicating the importance of *P. multocida* as a BRD pathogen in acute cases. Although strain transfer of *M. bovis* was not evaluated, it has been shown before that feedlot clonal transmission occurs (Timsit et al., 2012b). For *P. multocida*, previous studies have shown carriage in feedlot cattle to either increase or decrease from entry to reprocessing (Nobrega et al., 2021b) and *Pasteurellaceae* can persist in farm or feedlot environments and spread among cattle (Burriel, 1997; Katsuda et al., 2009; Neupane et al., 2019). While *P. multocida* spread appeared limited in Cohort 1, there was strong evidence of expanded clonal transmission between cattle in Cohort 2. This was likely related to antimicrobial-resistant *P. multocida* outcompeting susceptible strains after antimicrobial administration, as a far greater number of *P. multocida* were multidrug-resistant in Cohort 2 animals. In support of this, the genetic diversity of *P. multocida* in Cohort 2 cattle was greatly reduced compared to those from Cohort 1 cattle. Similarly, horizontal transmission has previously been shown to be related to animal antimicrobial use and bacterial resistance in *P. multocida* (Guo et al., 2020). It was interesting that although the prevalence of *M. bovis* and *P.*

multocida was greater in BRD cases compared to healthy cattle in Cohort 2, resistance patterns were largely similar in isolates from healthy or sick cattle. Thus, the spread of resistant BRD pathogens in feedlots occurs regardless of animal health status, and this was confirmed by observing *P. multocida* clones in both healthy and sick cattle. Similar findings have been reported previously (Allen et al., 1991), emphasizing the complexity of BRD and the notion that additional factors such as host immunity affect susceptibility to infection. Overall, this thesis showed that prevalence among the general and BRD-afflicted population of feedlot cattle varies for different BRD pathogens and can be affected by bacteria conferring resistance to metaphylactic antimicrobials.

Compared to Cohort 1 isolates, multidrug resistance increased in bacteria from Cohort 2, with most Cohort 2 *M. bovis* being resistant to tulathromycin, tylosin tartrate, clindamycin, and *P. multocida* being resistant to oxytetracycline and tulathromycin. Of particular interest was the increase in resistance to tulathromycin, which became available for veterinary feedlot use in 2006 in Canada. On average, bacterial resistance to antimicrobials occurs approximately 8 years after the initial use of a respective antimicrobial (Schmieder and Edwards, 2012). In bacteria from humans, resistance to the macrolide erythromycin was observed three years after its introduction (Schmieder and Edwards, 2012). Similarly, resistance to tulathromycin was identified in several *M. bovis* and *P. multocida* from Cohort 1, which corresponded to approximately three years after tulathromycin availability. After 10 years of tulathromycin use, resistance occured in the majority of *M. bovis* (99%) and *P. multocida* (76%) evaluated. While a direct correlation between tulathromycin use and resistance was not possible to evaluate, resistant bacteria coinciding with increased tulathromycin use by Albertan feedlots (Holman et al., 2019b) suggest a causal

relationship. This was also supported by lower resistance being observed for antimicrobials that are primarily used for treatment, specifically, enrofloxacin and florfenicol. It was noteworthy that increases in antimicrobial resistance were similar for *M. bovis* and *P. multocida*, studied in this thesis, and *M. haemolytica*, reported previously (Klima et al., 2011c; Timsit et al., 2017). Thus, antimicrobial selective pressures are shared across major BRD pathogens that colonize the bovine respiratory tract. This may also be related to the interspecies transfer of mobile genetic elements carrying resistance genes, as has been shown to occur for *M. haemolytica* and *P. multocida* (Klima et al., 2014b). However, variation in rates of resistant BRD pathogens can vary greatly by feedlot (Jelinski et al., 2020b; Klima et al., 2019; Timsit et al., 2017) highlighting that animal management practices have an impact on the development of resistance.

Genes conferring tulathromycin resistance (*erm* (42), *msr(E), mph(E)*) in *P. multocida* were first identified in isolates from BRD mortalities in the United States in 2011 (Desmolaize et al., 2011; Kadlec et al., 2011). PCR analysis revealed that only a small percentage of tulathromycin-resistant isolates evaluated in Chapter 3 carried these genes. Therefore, the genomes of several *P. multocida* strains were sequenced and compared against resistance gene databases. However, analysis of the genomes did not identify candidate tulathromycin resistance genes. Similarly, metagenomic and genomic approaches to characterizing BRD pathogen resistance have shown low concordance to phenotype (Freeman et al., 2022; Owen et al., 2017a) highlighting the limitation of sequencing-based methods to identify resistance elements in bacteria. A fosmid library was therefore prepared using macrolide selection, to provide sequencing information in addition to bacterial genomes. This led to the identification of an inserted cluster of 5 genes in tulathromycin-resistant *P. multocida,* with one of the genes being candidate methylase. The

methylase showed homology to an N-6 methylase from *K. pneumonia* but was unique in that the sequence only encoded a 71 AA protein. Despite this, transformation of *E. coli* with the methylase repeatedly doubled the MIC against tulathromycin. Thus, it was proposed that the methylase may act as a subunit part of a functional unit, rather than an independent enzyme, that alters tulathromycin resistance in bacteria. The 5-gene cluster in tulathromycin-resistant isolates also included a transposon and was within an ICE segment, indicating the potential for the spread of these genes between bacteria. ICE have been shown to be important mode of resistance gene transfer within *Pasteurellaceae*, with some cassettes encoding resistance to all available antimicrobials to treat BRD (Klima et al., 2014b). The methylase was present in almost all tulathromycin-resistant *P. multocida* that did not encode known macrolide resistance genes, emphasizing its role as a novel tulathromycin resistance gene that was widespread among the feedlot bacteria evaluated.

4.4 Future directions:

Future work should examine the mechanism of the identified methylase in conferring tulathromycin resistance. Biochemical analysis of methylase can be used to confirm the methylation activity. Additionally analysis of the methylase structure using software prediction programs (IntFOLD, Phyre) and crystallization may aid in predicting how the protein acts to reduce susceptibility to tulathromycin. Protein structure evaluation has helped define the role of other macrolide resistance proteins (Andersen et al., 2012a). BRD remains an important limitation of cattle production and is complicated by multiple management strategies and pathogens being implicated in predisposition. Although *M. bovis* and *P. multocida* were identified in healthy and sick animals, it is noteworthy that strain pathogenicity and its impact on BRD have not been

studied. It appears that *P. multocida* is becoming increasingly important as a BRD pathogen, and the reasons for this, including virulence and antimicrobial resistance, should be explored. Additionally, alternatives to antimicrobials are greatly needed to reduce resistance and spare the efficacy of currently available antimicrobials. Thus, research into novel management strategies including bacterial therapeutics targeting the respiratory microbiota (Amat et al., 2019) or vaccines against major BRD pathogens (Larson and Step, 2012) is needed. Coinciding with this would be an economic evaluation of those strategies to reduce antimicrobial use and also the cost for the beef industry when antimicrobials lose efficacy. Lastly, an ongoing effort to describe antimicrobial resistance in feedlots is necessary, particularly given that this thesis showed resistance increasing shortly after the commercialization of an antimicrobial indicated for metaphylaxis to manage BRD. This will provide producers and veterinarians with updated information on antimicrobial efficacy and aid in the judicial use of antimicrobials in the future.

This study highlighted the ongoing requirement for AMR surveillance of respiratory pathogens in feedlot cattle as antimicrobial treatment is critical for the prevention, control, and treatment of BRD in cattle. This will help veterinarians make treatment protocol decisions that encourage effective drug use. In addition, a novel putative macrolide resistance gene was discovered and found to be common in the feedlots included in this investigation.

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