CHARACTERIZATION OF THE GENETIC DIVERSITY AND ANTIMICROBIAL RESISTANCE IN *MANNHEIMIA HAEMOLYTICA* FROM FEEDLOT CATTLE

CASSIDY L. KLIMA
Bachelor of Science, University of Lethbridge, 2007

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

MASTER OF SCIENCE
(Molecular Biology/ Microbiology)

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

DEDICATION

This work is in dedication to all of the friends and family that have been an amazing support over the past years. I am sure I will look back at this experience in all fondness and they will look back and say “Thank goodness that it is over with!”

I love you all!

ABSTRACT:

Characterization of the genetic diversity and antimicrobial resistance in *Mannheimia haemolytica* from feedlot cattle

Mannheimia haemolytica is an opportunistic pathogen in cattle and the main bacterial agent in bovine respiratory disease. Despite its economic importance, few studies have characterized the genetic diversity of *M. haemolytica*, particularly from feedlots. Three genotyping techniques (BOX-PCR, $(GTG)_5$-PCR and PFGE) were compared to discriminate *M. haemolytica* and strains from the family *Pasteurellaceae*. PFGE was the most discriminating and repeatable, although BOX-PCR was most accurate in clustering isolates together according to species.

Mannheimia haemolytica was isolated from nasal swab samples collected from cattle upon entry and exit from two feedlots in southern Alberta. These were characterized by PFGE and antimicrobial susceptibility using a disk-diffusion assay. Select gene determinants were screened for using PCR. PFGE analysis revealed the isolates to be highly diverse. Ten percent of the isolates exhibited resistance. At present, the development and spread of antimicrobial resistance in *M. haemolytica* observed within the feedlots examined appears to be low.

ACKNOWLEDGMENTS

To Dr. Tim McAllister, heartfelt thanks for both the lesson that a person can not but succeed with three things: the desire to do so, the opportunity to do so, and the support to do so; and for his provision of the latter two. To Dr. Brent Selinger whom was gracious and committed enough to still offer a hand up when I was stubborn and wanted to fall all on my own. To Dr. Trevor Alexander who dealt with it *all*. And to Shaun Cook who shared the greatest gifts, both his time and knowledge.

Thank you also to all of the staff at Feedlot Health Services who collected, recorded and shipped samples on a weekly basis, and in particular to the ladies in charge of data management, Maria Fuchs and Chelsea Flaig. Yours is the kind of thankless task that is essential to the work here and I am grateful for the time and effort you put in.

And a last, and not in anyway the least, emphatic thank you to Shaun Cook, Sherry Hunt and Lorna Selinger who spear headed the technical support needed on this project. From isolate collection, identification and storage, to all of the associated data management in between. The following document is sourced in their very hard work. Thanks guys!

Financial support for this study was provided from the Advancing Canadian Agriculture and Agri-Food (ACAAF) Program, the Beef Cattle Research Council, the Alberta Beef Producers and the Agriculture and Agri-Food Canada Matching Investment Initiative. Their support is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 3

Chapter 4

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

AIP	acute interstitial pneumonia
AMCL	amoxicillin/clavulanic acid
AMPI	ampicillin
AMR	antimicrobial resistance
AMU	antimicrobial use
ATCC	American type culture collection
BHV-1	bovine herpesvirus
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BVDV	bovine viral diarrhea virus
CCUG	University of Göteborg culture collection
CTIO	ceftiofur
dhfr	dihydrofolate reductase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ENRO	enrofloxacin
FLOR	florfenicol
GENT	gentamicin
IBRV	infectious bovine rhinotracheitisvirus
Lkt	leukotoxin
LPS	lipopolysaccharide
MANOVA	multivariate analysis of variance
OXYT	oxytetracycline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PI-3V	parainfluenza-3 virus
RNA	ribonucleic acid
TBE	tris-borate-EDTA
TILM	tilmicosin
TMSZ	sulfamethoxazole/trimethoprim
TSA	tryptic soy agar
TULA	tulathromycin
UBRD	undifferentiated bovine respiratory disease
UF	undifferentiated fever
UGCC	University of Guelph culture collection

CHAPTER ONE

Introduction

Bovine respiratory disease (BRD) is currently one of the most economically important health problems for cattle producers. Financial losses due to the condition exceeded the combined costs of all other diseases of cattle (Highlander, 2001) and are incurred through treatment cost, production loss and death. Control strategies for this disease are limited to feed management and treatment with vaccines and antimicrobials. The latter has lead to concerns regarding the development and spread of antimicrobial resistance. Many viral and bacterial agents contribute to the BRD complex, but *Mannheimia haemolytica* is identified as the primary bacterial pathogen. Development of antimicrobial resistance in this pathogen holds consequence for maintaining efficacy of antimicrobial therapies and management of the disease.

1.1 Bovine respiratory disease

1.1.1 Economics and prevalence

In terms of economics, BRD is the most important disease affecting feedlot cattle throughout North America (Gardner et al., 1999; Griffin, 1997). It is the primary cause for loss associated with sickness and death (Gagea et al., 2006; Larson, 2005) and is estimated to be responsible for up to 75% of morbidity and up to 70% of mortality in received cattle (Edwards, 1996; Encinias et al., 2006). Mean monthly mortality rates have been shown to range from 0.128% (Vogel and Parrott, 1994) to 0.71% of occupancy (Edwards, 1996), however, prevalence is reported to be variable based upon both

geography (Gagea et al., 2006) and season (Vogel and Parrott, 1994). In the USA, death losses due to BRD were observed to have increased between 1994 and 1999 with an average incidence rate of 1.42% documented from the twelve states surveyed in 1999 (Dedonder, 2008; Snowder et al., 2006). Further extension of this study revealed that this rate reached 1.75% in 2003 (Thomson and White, 2006).

An accurate evaluation of the exact economic losses accountable to BRD is difficult to find. Estimates within the USA place the value between 640 million and 1 billion US dollars annually (Babcock et al., 2008; Bowland and Shewen, 2000; Snowder et al., 2007) although factoring in treatment costs, which vary from $13.90 to $37.90 per head (Sanderson et al., 2008; Smith, 1998; Snowder et al., 2006), can inflate that value to 3 billion US dollars annually (Snowder et al., 2007). Despite the enormity of these numbers they are in all likelihood an underestimation of the true cost of BRD to the industry. These values are primarily based upon treatment costs and death losses which are routinely monitored. However, there are additional losses associated with BRD that are not as easily determined including those attributed to increased labor expenditures, performance losses and reduction in carcass value (Bateman et al., 1990; Encinias et al., 2006; Gagea et al., 2006; Larson, 2005)

1.1.2 History and definition

Documenting the history of BRD is a difficult task. This is mostly due to the state of progression of both the knowledge base and nomenclature surrounding the condition. Yates (1982) has argued that the lack of a clear, workable definition for the condition has allowed etiological theories and vague generalizations to affect the nomenclature describing it, ultimately, hindering the chronological advancement of knowledge. The

result of this has been an amassing of data and various terminologies that have focused primarily on the etiology of the condition resulting in a description rather than diagnosis of the condition. This is still a large concern as a variety of terms and definitions are present in the literature. In order to proceed in good standing the following section has been created to present a clear working definition of BRD for use within the context of this document, to provide a brief account of the history behind the condition and to clarify the various terminologies that may be encountered in current literature.

1.1.3 Bovine respiratory disease complex vs. bovine respiratory disease

The term bovine respiratory disease (BRD) complex was originally applied as an expansive term to describe three disease conditions that are responsible for pneumonia in cattle; enzootic pneumonia of calves, shipping fever complex and atypical interstitial pneumonia (Lillie, 1974). Although this definition is still used on occasion (Bowland and Shewen, 2000), it is becoming more common to see the term BRD complex used exclusively with the term shipping fever (Booker et al., 2008; Gardner et al., 1999; Schunicht et al., 2007; Wildman et al., 2008). This situation has in all likelihood been induced and perpetuated by the similarity of the term BRD complex to the term BRD, which is a name that is conventionally used as a synonym for shipping fever (Apley, 2006; Larson, 2005). As it is a potential point of confusion, any reference to the term BRD within this document is meant to be in association with the condition and ailments of shipping fever and should not be confused with the term BRD complex of which it should be considered a component.

It has recently been argued that acute interstitial pneumonia (AIP) is a syndrome that is not linked to the characteristic respiratory disease pathogens and as such should be

considered a separate disease entity from the BRD complex (Woolums et al., 2005). Consequently, it may be more accurate to define BRD complex as a term encompassing the entities of enzootic pneumonia in calves and shipping fever alone. However, for the sake of simplicity, the working definition for BRD complex applied in the present paper will be held in accord with that previously outlined by Lillie (1974).

1.1.4 History of shipping fever and current terminology

The term 'shipping fever' has been in use for over six hundred years, and was initially used to describe a variety of sicknesses in horses ranging from influenza to pink eye to pneumonia (Rehmtulla and Thomson, 1981). With the turn of the 19th century, shipping fever became recognized as a complex of three different conditions; strangles, influenza and contagious pneumonia (Rehmtulla and Thomson, 1981), but it wasn't until the 1950s that the term was used to describe fever and pneumonia in cattle following transport to feedlots (Adams et al., 1959; Graham, 1953; Ryff and Glenn, 1957). Association of this term with cattle generated confusion, causing both the vernacular and description of symptoms surrounding the disease to be varied (Rehmtulla and Thomson, 1981). Some of the various terms documented include transit fever, stockyard's pneumonia, exposure disease, transport fever, hemorrhagic septicemia (now reserved for systemic infections), fibrinous pneumonia, and exposure disease (Rehmtulla and Thomson, 1981; Yates, 1982).

In the 1970's it became clear that *Pasteurella* spp. were a key component of the condition. This led to a general acceptance of the term bovine pneumonic pasteurellosis (Yates, 1982) which has since been modified to bovine pneumonic mannheimiosis to reflect the taxonomic amendments made to the *Pasteurella* genus in 1999 (Angen et al.,

1999). Although recognized, this term is employed less commonly in publications than the terms shipping fever or BRD. This is in all probability due to the complex etiology of the condition (discussed below) which has made it difficult for many to justify naming the condition after one causative agent (Yates, 1982).

As a result of the discord over nomenclature, there is presently an array of terms used to discuss shipping fever/BRD. Included are; bovine respiratory disease (BRD) (Apley, 2006; Larson, 2005), undifferentiated bovine respiratory disease (UBRD) (Bateman et al., 1990), undifferentiated fever (UF) and/or bovine respiratory disease (BRD) complex (Booker et al., 2008; Schunicht et al., 2007; Wildman et al., 2008), shipping fever and bovine pneumonic mannheimiosis. For the purposes of this document, the terms BRD, shipping fever and bovine pneumonic mannheimiosis will be used synonymously.

1.1.5 Symptoms and etiology

The etiology of BRD is complex. When described in general terms, BRD is created by a suite of environmental stressors, typically associated with transport and marketing, that combined are sufficient to compromise the host immune system and allow for pulmonary invasion by viral and/or bacterial pathogens that are ubiquitous within the feedlot environment (Cusack et al., 2003). In most cases the disease is characterized by a primary viral infection, followed by a secondary bacterial infection, the agent of which ultimately colonizes the lower respiratory tract of the animal and causes severe fibrinonecrotic pneumonia (Kehrenberg et al., 2001b; Whiteley et al., 1992). Consequently, transient illness, progression to chronic pneumonia or death of the animal ensues (Welsh et al., 2004).

Calves are most often affected by pneumonia shortly after arrival in feedlots (Gagea et al., 2006; Rice et al., 2007). Acute outbreaks resulting in death last approximately 2 to 3 days although those that survive this period can become chronically ill (Mohamed and Abdelsalam, 2008). Clinical symptoms can include fever, nasal or ocular discharge, cough and respiratory distress, depression, lethargy, along with loss of both weight and appetite (Duff and Galyean, 2007; Friend et al., 1977). The main cause of death is acute fibrinous pleuropneumonia (Confer et al., 1988; Rice et al., 2007) also termed multifactorial fibrinonecrotizing pneumonia (Highlander et al., 2000; Jeyaseelan et al., 2002; Whiteley et al., 1992) or bovine pneumonic mannheimiosis when associated with *M. haemolytica*. The duration, distribution and severity of infection is unequal between populations (Bateman et al., 1990) and potential risk factors for the condition can include age, body weight, procurement method, amount of commingling before and after arrival, and previous vaccinations (Wildman et al., 2008). Typically, the condition is centered within pens and does not sweep through the entire feedlot (Rice et al., 2007).

Despite advancements in our understanding of BRD, the intricacies of the disease are still disputed and the precise contributions of each agent involved are unclear. There are multiple environmental stressors that contribute to the condition and an array of infectious organisms, both viral and bacterial, that have been recovered from pneumonic animals. The following section provides a brief description of some of the key factors that are associated with or contribute to the condition of BRD. However, the focus of this review is of the role of one of the primary bacterial pathogens associated with the condition, *M. haemolytica*, and the remainder of the document will focus its involvement with BRD.

1.1.6 Environmental/ host factors

Environmental stressors play a key role in predisposing cattle to BRD. It is commonly accepted that stress negatively impacts the immune system (Duff and Galyean, 2007) which can lead to a propensity for viral/bacterial infection. Cattle experience various stressors as they are transitioned into the feed yard. Typically these are linked to transport and marketing, however, feedlot management practices may also play a role. Stressors can be either psychological or physical in nature. Psychological stressors are largely a result of handling technique but may also be linked to the effects of commingling, movement through marketing channels, weaning and novelty (Bowland and Shewen, 2000; Thomson and White, 2006). Physical stressors are more numerous and may include hunger, thirst, fatigue, injury, thermal extremes, crowding, poor ventilation, castration, vaccinations, and horn tipping (Bowland and Shewen, 2000; Duff and Galyean, 2007; Thomson and White, 2006). Stress provides the intiation of BRD by generating animals with compromised immune function while they are situated in an environment where the chance of exposure to infectious agents is high (Jericho, 1979).

1.1.7 Viral factors

Viral agents are potentiators for BRD infection. They are immunosuppressive agents that act synergistically with other respiratory pathogens to permit bacterial colonization of the lower respiratory tract (Loneragan et al., 2005). Multiple viral agents have been associated with BRD ((Bowland and Shewen, 2000; Duff and Galyean, 2007); Table 1.1) but BHV-1, BRSV, BVDV and PI-3V are the key viral pathogens identified in the literature (Apley, 2006). Complete details of the mechanics of viral infection are not within the scope of this paper, although generally speaking, these agents are linked with

diminished neutrophil and lymphocyte function (Brown et al., 1991; Brown and Ananaba, 1988; Woldehiwet and Sharma, 1992), an increase in the abundance of bacteria in the nasal pharynx, a decrease in ciliary activity of epithelial cells in the trachea (Rossi and Kiesel, 1977) and a reduction in clearance from the lung (Filion et al., 1984; Lopez et al., 1976). All of these factors contribute to impairing host defences and allow the opportunity for bacteria to gain access to the lower respiratory tract possibly leading to bacterial pneumonia.

Table 1.1
Viral and bacterial agents linked to occurrence of bovine respiratory disease

Viral	Bacterial
infectious bovine rhinotracheitisvirus (IBRV)	*Mannheimia haemolytica*
bovine herpesvirus (BHV-1)/(BHV-4)	*Pasteurella multocida*
bovine viral diarrhea virus (BVDV)	*Histophilus somnus*
bovine respiratory syncytial virus (BRSV)	*Actinomyces pyogenes*
parainfluenza-3 virus (PI-3V)	*Mycoplasma bovis*
malignant catarrhal fever virus	*Mycoplasma dispar*
bovine adenovirus	*Mycoplasma hyorhinis*
bovine rhinovirus	*Chlamydia spp*
bovine reovirus	*Ureaplasma diversum*
bovine calicivirus	*Streptococcus pneumoniae*
bovine enteric coronavirus	*Stapylococcus aureus*
bovine parvovirus	
bovine enterovirus	

1.1.8 Bacterial factors

As is the case with the viral agents, there are multiple bacterial pathogens associated with BRD (Table 1.1), *M. haemolytica* (formerly *Pasteurella haemolytica*), *Pasteurella multocida*, *Haemophilus somnus* and *Mycoplasma bovis* the most common bacterium isolated from morbid or deceased cattle (Apley, 2006; Thomson and White, 2006). There is still discussion concerning the significance of each of these pathogens in

the condition. Perpetuating the debate is the consideration that all of these bacteria are ubiquitous commensals in cattle (Cusack et al., 2003) and although *M. haemolytica* is the most frequently recovered bacterial pathogen from cattle with BRD, many other species can perpetuate the condition (Cusack et al., 2003). The fastidious nature of many of these organisms plays a large role in their detection ultimately making any argument linking prevalence of a pathogen to disease a difficult one (Dabo et al., 2008).

In Europe, *M. bovis* is considered to be responsible for at least 25 to 33% of all pneumonia cases in calves presenting BRD (Gevaert, 2006). Others feel the role of this organism in BRD is still unclear and argue that there is still challenge in the interpretation of its isolation from chronic cases (Apley, 2006). *H. somnus* is also recovered less frequently than other organisms from post-mortem lung tissue but this finding is also argued to be a reflection of its fastidiousness nature (Cusack et al., 2003). Pneumonia caused by *H. somnus* is generally more subacute or chronic than that caused by *M. haemolytica* or *P. multocida* (Hodgins et al., 2002). *P. multocida* and *M. haemolytica* are the most commonly recovered bacteria from cattle exhibiting BRD with the latter considered to be found most frequently (Cusack et al., 2003). *P. multocida* is usually associated with subacute to chronic bronchopneumonia, rather than acute to subacute fibrinous pleuropneumonia (Welsh et al., 2004) and its role in the disease is more evident in dairy calves than feedlot cattle (Dabo et al., 2008). It is considered less virulent than *M. haemolytica* as more cells are required to induce primary pneumonia in experimental challenge studies (Hodgins et al., 2002).

1.2 *Mannheimia haemolytica*

1.2.1 *M. haemolytica* the organism

Presently, *M. haemolytica* is considered to be the primary bacterial pathogen associated with shipping fever (Gioia et al., 2006; Katsuda et al., 2007; Rice et al., 2007; Welsh et al., 2004; Whiteley et al., 1992; Zecchinon et al., 2005). This is because it is the bacterium most frequently recovered from cattle exhibiting BRD, can be isolated in pure culture from pneumonic lungs and can reproduce the pneumonic lesions characteristic of the BRD through endobronchial, intratracheal or intrapulmonary inoculation with serotype A1 of the bacterium (Jeyaseelan et al., 2002). Although *M. haemolytica* is considered to be a primary pathogen in BRD it is important to reaffirm that the condition is a complex of factors and as this document switches its focuses to *M. haemolytica* it is important to be reminded that it is only one of the many factors involved.

M. haemolytica is classified as a Gram-negative, non-motile, non-spore forming, fermentative, oxidase-positive, facultative anaerobic coccobacillus (Rice et al., 2007). Its complete taxonomy is: domain Bacteria; phylum Proteobacteria; class Gammaproteobacteria; order Pasteurellales; family Pasteurellaceae; genus *Mannheimia* (Zecchinon et al., 2005). It is typically found in association with ruminants (Angen et al., 1999) and is often a commensal organism in the upper respiratory tract of healthy cattle (Catry et al., 2005; Lillie, 1974). However, when an animal's immune system becomes compromised the organism has shown the ability to shift to a pathogenic state, infect the lower respiratory tract and induce acute fibrinonecrotizing pleuropneumonia (Gioia et al., 2006; Highlander, 2001). This shift in pathogenicity appears to have a link with capsular serotype. Serotypes A1 and A2 are the ones most commonly recovered in cattle, though

it is serotype A1 that is frequently found in association with pneumonic lesions (Dassanayake et al., 2007; Gioia et al., 2006; Katsuda et al., 2007; Lo, 2001). Serotype A2 is generally not considered to be pathogenic to cattle (McVey et al., 1990), and more regularly causes pneumonia in sheep (Highlander, 2001; McVey et al., 1990; Rice et al., 2007). Recently, increasing amounts of serotype A6 have been recovered from cases of bovine pneumonic mannheimiosis in Germany (Ewers et al., 2004), France, the United Kingdom (Kehrenberg et al., 2001b) and North America (Al-Ghamdi et al., 2000; Purdy et al., 1997). Despite these trends, serotypes A2, A5, A6 and A9 have been shown to induce lesions experimentally and can be recovered from pneumonic tissue (Al-Ghamdi et al., 2000). *Mannheimia* generated pneumonias have only been found in association with cattle, sheep and goats (Zecchinon et al., 2005).

1.2.2 Reclassification

The taxonomy of *M. haemolytica* has been elaborate and continues to evolve. Previous to the appreciation of BRD as a multifactoral condition, focus was directed towards the identification of a single causative agent for the disease. The first noted was *Bacterium bipolare multocidum* by Theodore Kitt (1885). It was renamed *Bacillus bovisepticus* by Flugge in 1896 (Highlander, 2001) and was subdivided into three groups based upon immunological, fermentation and virulence tests by Jones(1921). Of these groups, two were associated with hemorrhagic septicemia (now recognized as *P. multocida*) and the other with bovine pneumonia. Jones (1921) recommended the latter be classified as *Pasteurella bovisepticas*.

Further examination of *P. bovisepticus* by Newsom and Cross (1932), lead to its reclassification as *P. haemolytica* and division into two biotypes, designated A and T,

based upon a strain's ability to ferment L-arabinose or trehalose, respectively. Inconsistencies were found with regard to L-arabinose fermentation from multiple strains of biotype A (Bisgaard, 1984; Mraz, 1969). Consequently, it has become convention to describe isolates as either trehalose-negative or trehalose-positive rather than as biotype A or biotype T.

Although the trehalose-positive and trehalose-negative classification scheme has been adopted in the literature, other biovariates have been described. A total of 12 biogroups have been characterized based upon various morphological and fermentative characteristics (Tefera, 2001). However, biotyping has not been commonly employed in laboratories. This is no doubt due to inconsistencies regarding both delineation of the existent biotypes and the lack of consistency obtained from phenotypic analysis. Thus, little has been documented about the scope and prevalence of the described biogroups or their significance in veterinary medicine (Angen et al., 1999).

In the 1960's, Biberstein et al. (1960) developed an indirect haemagglutination test (IHA) that revealed a consistent relationship between capsular serotype and biotype. Seventeen serotypes within the *P. haemolytica* complex were subsequently identified, a total of thirteen within the trehalose-negative biotype (serotypes A1, A2, A5-9, A11-14, A16 and A17) and four within the trehalose-positive biotype (serotypes 3, 4, 10 and 15) (Angen et al., 2002; Highlander, 2001; Younan and Fodar, 1995). Despite the successes of the IHA method, approximately 10% of the isolates retrieved from ruminants are considered untypeable (Adamu, 2007; Jaworski et al., 1998). Multiple untypeable isolates have been characterized through alternate methods including counter-current

immunoelectrophoresis and rapid plate agglutination tests (Donachie et al., 1984; Frank, 1980).

It was with the development of genomic analysis that further division in the *P. haemolytica* complex was observed. In 1986, Mutters et al. (1986) excluded P. *haemolytica* from the genus *Pasteurella* sensu stricto based upon DNA-DNA hybridization. This was supported by additional molecular analysis (De Ley et al., 1990; Dewhirst et al., 1992, 1993), but neither a new genus nor new genera was assigned at that time. Multiple works also indicated that the two biotypes were incorrectly grouped into one species (Biberstein and Francis, 1968; Mannheim, 1983; Sneath and Stevens, 1985). It was not until 1990 that the members of the trehalose-positive group were reclassified as *Pasteurella trehalosi* (Bingham et al., 1990; Sneath and Stevens, 1990). *P. trehalosi* has since been assigned to a new genus, *Bibersteina trehalsoi* comb. nov., based upon phylogenetic analysis conducted by Blackall et al. (2007).

Angen et al. (1999) developed the current taxonomic scheme applied to organisms formerly recognised as the trehalose-negative biotype of the *P. haemolytica* complex. Based upon extensive evaluation of both phenotypic and genomic characteristics, the new genus *Mannheimia* was formed, under which five species currently reside (*M. haemolytica, Mannheimia glucosida, Mannheimia granulomatis, Mannheimia ruminalis* and *Mannheimia varigena*) as well as currently un-named taxa such as Bisgaard Taxon 39 (Blackalu et al., 2001). Of the 17 serovars initially described within the *P. haemolytica* complex, 3, 4, 10 and 15 are associated with *B. trehalosi*, A11 with *M. glucosida* and the remaining 12 with *M. haemolytica* (Adamu, 2007).

It is important to note that within the current classification system, molecular typing techniques still show significant levels of heterogeneity within strains of the same serovar (Davies et al., 1997a, b; Kodjo et al., 1999). Also, it is clear that limitations are present with the utilization of either serotyping or phenotypic characterisation alone for identification of both *Pasteurella* and *Mannheimia* species. However, recent development of both a multiplex PCR assay (Alexander et al., 2008b) and a real-time PCR assay (Guenther et al., 2008) have provided molecular means to aid in the accurate identification of the currently recognized species within the *Mannheimia* genus.

Due to the link with pathogenicity there has been considerable effort devoted to characterising the differences and similarities between serotypes. Multilocus enzyme electrophoresis has revealed *M. haemolytica* isolates to be clonal and has shown they can be placed into three distinct lineages that are associated with electrophoretic type, host species, capsule, lipopolysaccharide and outer membrane protein types (Davies et al., 1997b). Ribosomal RNA sequencing has revealed all serotypes to have identical rRNA sequences with the exception of serotype A2 and A7 for which the former differs at two positions and the latter data is not yet available (Davies et al., 1996). Three structural capsule groups have been characterised, one that is serotype A1-like, one that is A2-like and a final that is A7-like (Highlander, 2001) and nuclear magnetic resonance spectroscopy has shown the O-chain polysaccharide of serotypes A1, A6 and A9 to be identical (Lacroix et al., 1993). Curiously, despite these groupings, typing sera is generally found not to be cross-reactive showing a large degree of specificity for each serotype (Frank and Wessman, 1978).

1.2.3 Pathogenicity

M. haemolytica possesses multiple virulence factors which promote its adhesion, colonization and proliferation within the respiratory tract (Mohamed and Abdelsalam, 2008). These factors also aid it in circumventing host defences and clearance (Zecchinon et al., 2005). There are many reviews that describe in great detail what is known about the virulence factors of *M. haemolytica* (Highlander, 2001; Mohamed and Abdelsalam, 2008; Thumbikat et al., 2005; Zecchinon et al., 2005). A few of the key points are summarized below but a detailed account of each factor is not within the scope of this review.

1.2.4 Virulence Factors

M. haemolytica is thought to have several factors that aid in its attachment and colonization within the respiratory tract of calves. These include adhesion proteins, capsular polysaccharide, fimbriae, sialoglycoprotease and neuraminidase (Rice et al., 2007).

1.2.4.1 Adhesions

Adhesions are thought to play a significant role in colonization (Zecchinon et al., 2005); however, little is known about the specific adhesions characteristic of *M. haemolytica*. Although a locus encoding for adhesion proteins in *M. haemolytica* is yet to be documented, a putative adhesion-like sequence has been identified within the draft sequence of *M. haemolytica* (Highlander, 2001).

1.2.4.2 Fimbriae

Fimbriae are small appendages on the surface of some Gram-negative bacteria that either permit or enhance adherence and colonization. Two types of fimbriae, described as a large 12-nm rigid structure and a 5-nm flexible structure have been identified in *M. haemolytica* (Morck et al., 1989; Morck et al., 1987). Both are capable of enhancing mucosal attachment of *M. haemolytica* to the epithelium of the lower respiratory tract (Mohamed and Abdelsalam, 2008).

1.2.4.3 Capsule

The capsule plays a significant role in defence and is integral in both the resistance of complement-mediated lysis (Chae et al., 1990; Lo, 2001) and the prevention of phagocytosis by macrophages and polymorphonuclear leukocytes (Chae et al., 1990; Lo, 2001; Mohamed and Abdelsalam, 2008). The polysaccharide capsule has been shown to interact with pulmonary surfactants that regulate the surface tension of the mucosal layer lining the alveoli and thus is also implicated with facilitating adhesion of the bacteria to the respiratory tract epithelium (Brogden et al., 1989; Whiteley et al., 1990).

1.2.4.4 Neuraminidase

Production of neuraminidase by *M. haemolytica* facilitates colonization through reducing the viscosity of respiratory mucus, allowing for closer bacterial apposition to the cell surface (Zecchinon et al., 2005). Although the exact mechanisms are not understood, the enzyme is thought to remove terminal sialic residues from mucin, a respiratory mucus and thereby modifying host immunity (Mohamed and Abdelsalam, 2008).

1.2.4.5 Outer membrane proteins

Outer membrane proteins and lipoproteins are believed to be important protective antigens of *M. haemolytica* (Highlander, 2001). Many are of interest as potential vaccine candidates as they have been implicated in protecting against phagocytosis and complement-mediated killing (Pandher et al., 1998). They are also thought to be involved in remodelling of the host cytoskeleton (Highlander, 2001).

1.2.4.6 Proteases

All serotypes of *M. haemolytica* produce a zinc metalloglycoprotease which can cleave cell-surface glycoproteins from macrophages or other leukocytes (Abdullah et al., 1991; Sutherland et al., 1992). This enzyme has the ability to act at the host-cell surface and enhance adhesion (Highlander, 2001). Its activity has been shown to be enhanced by incubation with leukotoxin (Nyarko et al., 1998).

M. haemolytica serotype A1 also produces an IgG1-specific protease (Lee and Shewen, 1996). As IgG is the primary secretory antibody in the lower respiratory tract of cattle (Highlander, 2001), the IgG1-specific protease is thought to reduce the effectiveness of antibodies and thus contribute significantly to the pathogenesis of bovine pneumonic mannheimiosis (Lee and Shewen, 1996).

1.2.4.7 Lipopolysaccharide (LPS) and leukotoxin (Lkt)

The two most important virulence factors possessed by *M. haemolytica* are its endotoxin (Lipopolysaccharide or LPS) and exotoxin (leukotoxin or Lkt) (Dassanayake et al., 2007). Both have been well studied, particularly in serotype A1.

1.2.4.7.1 Lipopolysaccharide endotoxin

LPS is one of the main virulence factors involved with bovine pneumonic mannheimiosis. It represents 10-25% of the dry matter of *M. haemolytica* (Keiss et al., 1964) and eight different variants have been described for serotype A1 alone (Davies and Donachie, 1996). Both a rough and smooth form of LPS exist and serotypes A2 and A8 have been shown to possess the rough variety while the remaining serotypes possess the smooth form (Lacroix et al., 1993). LPS is directly toxic to epithelial cells and can alter leukocyte function and cause lysis of blood platelets (Mohamed and Abdelsalam, 2008). It is an inducer of inflammation and initiates both coagulation (clotting) and complement (immune system) cascades (Cusack et al., 2003). More specifically, LPS induces macrophage production of proinflammatory cytokines, which can induce localized haemorrhage, edema and thrombosis (Breider et al., 1990). It can also increase vascular permeability which can lead to an accumulation of inflammatory cells, particularly neutrophils, and both intravascular and extravascular fibrin deposition in the lung (Corbeil et al., 1985). In addition to this, LPS can form complexes with leukotoxin which can enhance both Lkt stability and cytotoxicity (Li and Clinkenbeard, 1999).

1.2.4.7.2 Leukotoxin

The *M. haemolytica* leukotoxin (Lkt) is a pore forming, calcium-dependant cytotoxin of the RTX family of toxins (Highlander et al., 2000; Jeyaseelan et al., 2002; Li and Clinkenbeard, 1999). It has specificity for ruminant leukocytes and platelets (Clinkenbeard and Upton, 1991; Shewen and Wilkie, 1982) and is considered the primary virulence factor produced by all serotypes (Burrows et al., 1993; Saadati et al., 1997). There are eleven distinct forms described (Davies and Baillie, 2003) and although the

function of *M. haemolytica* Lkt is highly conserved, considerable heterogeneity in its production rate, molecular weight and biological activity has been described even within strains of the same serotype (Saadati et al., 1997). *M. haemolytica* Lkt is typically secreted in abundance during the logarithmic phase of growth (Zecchinon et al., 2005).

The effects of Lkt range from disruption of cell function to lysis of ruminant leukocytes (Rice et al., 2007). At low concentrations, Lkt activates alveolar macrophages and neutrophils (Jeyaseelan et al., 2002), inducing the release of various inflammatory mediators, including toxic oxygen radicals, proteases (Maheswaran et al., 1992; Rice et al., 2007), eicosanoids, proinflammatory cytokines (Yoo et al., 1995), lipid mediators (Clinkenbeard et al., 1994; Henricks et al., 1992; Wang et al., 1998) and nitric oxide (Yoo et al., 1996). At higher concentrations, Lkt is cytocidal to bovine alveolar macrophages and neutrophils (Berggren et al., 1981; Maheswaran et al., 1980). Cytolysis of these leukocytes results in the release of proteolytic enzymes and proinflammatory substances that cause damage to the alveolar lining (Jeyaseelan et al., 2002). These substances are also chemotactic for various types of inflammatory cells and amplify damage to the lung tissue by increasing cell recruitment to the area (Zecchinon et al., 2005).

1.2.4.8 Other virulence factors

In addition to the above factors other intrinsic components that may contribute as virulence factors include iron-regulated outer membrane proteins, toxic outer membrane proteins, iron acquisition proteins, transferrin-binding proteins and extracelluar enzymes (Lo, 2001; Mohamed and Abdelsalam, 2008; Rice et al., 2007). Also, a quorum sensing

model was recently described for *M. haemolytica* that may play a significant role in coordinating the virulence factors just described (Malott and Lo, 2002).

1.3 Mechanics of bovine pneumonic mannheimiosis

Bovine pneumonic mannheimiosis is recognized as a condition of acute febrile respiratory disease with fulminating fibrinous lobar pneumonia or fibrinopurulent bronchopneumonia and fibrinous pleurisy (Mohamed and Abdelsalam, 2008). It is a pneumonic state characterised by edema and fibrin deposition within the lungs and pleura, typified more specifically, by the presence of degraded neutrophils and macrophages along with fibrin, blood and seroproteinaceous fluid within the alveoli, bronchi and bronchioles (Highlander, 2001). Onset occurs within hours of infection and is associated with extensive parenchymal necrosis caused by *M. haemolytica* leukotoxin, lipopolysaccharide and inflammatory factors released by neutrophils as well as other inflammatory response cells (Ackermann and Brogden, 2000; Rice et al., 2007). Thrombosis and distension are observed in the pulmonary interlobular septae due to infusion with gelatinous material containing edema, fibrin, leukocytes, and distended lymphatics (Rice et al., 2007; Zecchinon et al., 2005). Necrosis is often extended across the interlobar septae into adjacent lobules (Ackermann and Brogden, 2000). Inflammation of airways initiates at the terminal bronchioles and although bronchi maintain normal walls, necrosis and desquamation of epithelial cells may occur (Zecchinon et al., 2005).

The disease is initiated by viral and bacterial agents that break down the antimicrobial barrier of β-defensins, anionic peptides, serous and mucous secretions of

the respiratory tract, allowing *M. haemolytica* to transform from a commensal to a pathogenic state (Brogden et al., 1998). *M. haemolytica* subsequently migrates through gravitational drainage along the tracheal floor into the lower respiratory tract (Mohamed and Abdelsalam, 2008). At this point it is believed to attach itself to the alveoli through reactions between the alveolus surfactant layer and its capsular polysaccharide (Ackermann and Brogden, 2000). This binding may be further enhanced by the activity of neuraminidase whose production is accompanied by increased rates of cell replication (Frank et al., 1996; Straus et al., 1998). As a point of interest, studies suggest that *M. haemolytica* has the ability to alter its glycoconjugate expression, enhancing both its invasiveness and colonization (Frank et al., 1996; Straus et al., 1998).

Once *M. haemolytica* is resident within the lung, an inflammation response is initiated due to the release of LPS, polysaccharide and Lkt into the intra-alveolar exudates, as well as the uptake of LPS by neutrophils and the localization of polysaccharide in the alveolus and alveolar macrophages (Ackermann and Brogden, 2000). Inflammation is mediated by multiple cell types, including endothelial cells, macrophages, neutrophils, mast cells, nerve fibres and epithelial cells (Ackermann and Brogden, 2000). A series of inflammatory mediators are released during acute *M. haemolytica* pneumonia including IL-1, TNF-α and IL-8, leukotriene B4, histamine, prostaglandin E2 and platelet-activating factor (Ackermann and Brogden, 2000).

The major determinant of *M. haemolytica* pathogenesis is the host-pathogen interaction between Lkt, leukocytes and the neutrophil-mediated inflammatory response (Zecchinon et al., 2005). Lkt has no direct effect on vascular endothelial cells (Sharma et al., 1992); rather it acts as a catalyst for damage by stimulating both the activity and

cytolysis of neutrophils and macrophages (Highlander, 2001). Cytolysis of neutrophils releases enzymes such as elastase and acid hydrolases along with oxidative radicals and cytokines that cause membrane damage, vascular leakage of protein and the formation of conjugated dienes (Ackermann and Brogden, 2000). These damage the lung mucosa and expose the lung tissue to inflammatory mediators and bacteria present within the exudates (Ackermann and Brogden, 2000). Endotoxins (LPS) cause thrombosis of pulmonary veins, capillaries and lymphatics eventually leading to focal ischaemic necrosis of the pulmonary parenchyma and a severe inflammatory reaction (Mohamed and Abdelsalam, 2008).

The severity of lesions produced in bovine pneumonic mannheimiosis is dependent upon the infecting strain's virulence. Virulence determines the degree of bacterial colonization, the amount of endotoxin released and the degree to which the defences of the host are impaired (Mohamed and Abdelsalam, 2008). Once infected, the anatomic features of the lung make it difficult to alleviate the factors that lead to pneumonia. The lungs of cattle have few pores of Kohn between alveolar units and extensive interlobular septa that result in reduced collateral ventilation and alveolar expansion ultimately limiting the capacity for expulsion of alveolar exudates (Ackermann and Brogden, 2000).

1.4 Management strategies

The complex nature of BRD provides challenges for both prevention and treatment. Because there is more than one causative agent for the condition, control of just one factor does not necessarily alleviate the problem. Many feel that a complete

management program is necessary in order to manage the disease (Rice et al., 2007; Thomson and White, 2006). Presently, BRD management strategies include preconditioning programs, vaccinations and antimicrobial therapy.

1.4.1 Preconditioning/pre-weaning programs

It has been argued that the best way to manage BRD is to reduce or eliminate the stressors that predispose animals to infection in the first place (Cusack et al., 2003). Pre-conditioning is a concept designed to implement management practices around weaning that will reduce stress and optimize both the animal's immune system and nutritional status (Lalman and Smith, 2002). Pre-conditioning programs vary, but generally involve a weaning period, vaccinations, treatment with antihelmenthics, castration, dehorning and acclimatization to feed bunks and water troughs (Thomson and White, 2006). This kind of management requires commitment on the part of producers and the economic gains associated are still debated (Pritchard and Mendez, 1990; Waggoner et al., 2005). Generally, adoption of pre-conditioning programs has not been successful although pre-weaning programs have generated some interest (Bowland and Shewen, 2000). These are essentially the same as pre-conditioning but exclude any acclimatization of animals to feed or water.

1.4.2 Vaccines

The use of vaccines is common in feedlots; however there are few challenge studies that evaluate the efficacy of such programs (Bowland and Shewen, 2000). Regardless, a significant amount of the BRD literature promotes both the development and use of vaccines over the use of antimicrobials. The main priorities of vaccination

programs are to stimulate an immune response, prevent shedding of the antigens and improve performance through decreasing subclinical disease (Thomson and White, 2006). Many feedlots will utilize viral antigens for calves upon entry and to a lesser degree will use bacterial vaccines (Thomson and White, 2006). Commercial vaccines currently available for use in Canada protect against IBRV, BVDV, BRSV, PI-3V, *P.haemolytica*, and *H. somnus* (Bowland and Shewen, 2000). Vaccines against *M. haemolytica* are problematic as they require serological information of the infecting strain to be effective (Kehrenberg et al., 2001b). Because vaccination against *M. haemolytica* is often ineffective (Kehrenberg et al., 2001b), antimicrobial therapy is the most common therapy (Catry et al., 2005).

1.4.3 Antimicrobials

Antimicrobial use is considered the primary and most effective method for prevention and treatment of BRD (Watts et al., 1994). Although immunization programs are also frequently employed, in North America it is considered standard procedure to administer metaphylactic treatment to a population of calves either upon arrival into the feedlot or at some point shortly thereafter (Rice et al., 2007). Products that sustain blood levels of antibiotics for 48-72 hours or more are preferred as a means of decreasing labour costs and treatment stresses through reduced handling (Rice et al., 2007). This type of treatment is typically administered to entry populations as the early stages of BRD are too difficult to diagnose during the period of delay between the time of vaccination and the generation of a immune response (Thomson and White, 2006). Metaphylactic use of antimicrobials has been alleged to reduce morbidity by 50% (Thomson and White, 2006). It has been estimated that 80% of the antimicrobials

licensed for use in cattle are designed for treatment against agents of BRD (Bowland and Shewen, 2000). A list of some common antimicrobials administered to cattle is presented in Table 2 (Carson et al., 2008; Thomson and White, 2006) .

Table 1.2
Antimicrobials commonly administered to feedlot cattle

Antimicrobial	Trade Name	Family	Administration Method
ceftiofur	Naxcel	cephalosporin	injectable
florfenicol	Nuflor	Phenicol	injectable
tilmicosin	Micotil	Macrolide	injectable
tylosin	Tylan	Macrolide	feed
tulathromycin	Draxxin	Macrolide	injectable
oxytetracycline	Terramycin	Tetracycline	injectable, feed, water
chlortetracycline	Aureomycin	Tetracycline	feed, water
spectinomycin	Trobicin	aminocyclitol	injectable
lincomycin-spectinomycin	SpecLinx-50	lincosamide/ aminocyclitol	water
monensin	Rumensin	polyether ionophore	feed
enrofloxacin	Baytril	fluoroquinolone	injectable

1.5 Antimicrobial resistance

1.5.1 Development of antimicrobial resistance

Concerns over increasing antimicrobial resistance have led to the formation of multiple national surveillance programs designed to detect and monitor changes in antimicrobial susceptibility patterns of organisms relevant to both the food industry and human health. Programs have been established in Canada (Canadian Integrated Program for Antimicrobial Resistance Surveillance -CIPARS) and the USA (US National Antimicrobial Resistance Monitoring System - NARMS), as well as various European countries (Hendriksen et al., 2008). As a result, information regarding the antimicrobial

resistance of both *Pasteurella* and *Mannheimia* spp. are available from many countries, including France, Germany, the United Kingdom, the Netherlands, and Portugal (Kehrenberg et al., 2001b), Belgium (Catry et al., 2005), and North America (Post et al., 1991; Watts et al., 1994).

1.5.2 Antimicrobial resistance in *M. haemolytica*

Providing a synopsis of antimicrobial resistance in *M. haemolytica* is complicated. This is due to two factors. One is the tendency towards conjunctive studies that focus on both *M. haemolytica* and *P. multocida* within the same document and the second is the ambiguity generated from the taxonomic amendments made within the group over the past twenty years. The outcome of the former is the frequent culmination of resistance data into a '*Pasteurella* group' profile. Although both *M. haemolytica* and *P. multocida* are closely related, each is a unique organism and their antimicrobial resistance profiles vary substantially. The inclination to document resistance on the group level can give a misleading impression about the diversity between the two. The outcome of the latter is varying levels of uncertainty regarding the identities of the organisms examined. Studies that have supplied serological data allow for differentiation between those within the *P. haemolytica* group that are now *M. haemolytica*, however, those that don't, require the data to be interpreted at the group rather than the species level. It is important to keep in mind that those studies produced prior to 1999 can only be reported as *P. haemolytica* rather than *M. haemolytica* so both terms are encountered throughout this document.

Antimicrobial resistance in *M. haemolytica* is already well documented and the basic trend is towards increasing amounts of resistance to a large number of antimicrobials (Watts et al., 1994). The most common types of resistance reported are to

beta-lactams, tetracycline, streptomycin, chloramphenicol, sulfonamides, trimithoprim, macrolides and sulfamethazines (Catry et al., 2005; Highlander, 2001; Kehrenberg and Schwarz, 2001; Watts et al., 1994). A few studies have been conducted that survey the trends in susceptibilities of *M. haemolytica* isolates and they are discussed further. However, it is important to note that not reflected in this summary is the high degree of variability often encountered over sampling periods.

In 1989, an extensive study reviewed the antimicrobial susceptibilities of close to five hundred *P. haemolytica* isolates collected from diseased cattle in USA (Post et al., 1991). They found high levels of susceptibility to ceftiofur, gentamicin, and sulfa-chlorpyridazine, moderate susceptibility to erythromycin, and resistance to ampicillin, penicillin, sulfadimethoxine, tetracycline, and tylosin. A four-year study conducted by Watts from 1988 to 1992 (1994) revealed frequent resistance of *P. haemolytica* isolates (n= 461) to ampicillin, tetracycline, erythromycin and sulfamethazine. All isolates screened were susceptibile to ceftiofur and an increase in the resistance to timlicosin was observed, a result that was thought to be linked to cross-resistance to erythromycin (Watts et al., 1994). In a review of antimicrobial resistance in *Pasteurella* and *Mannheimia* species, Kehrenberg (2001b) summarized data collected from *P. haemolytica* isolates from both France and Germany. The data from France is strictly from 1997 ($n \leq 1015$), however the information available from Germany spans 1990-1996 (n=3524), and 1997 ($n \leq 469$) (Kehrenberg et al., 2001b). The overall susceptibility of *P. haemolytica* isolates from France tended to be lower than those from Germany and there was a clear decline over time in the susceptibility of the German isolates, particularly with regard to tetracycline, chloramphenicol, gentamicin and penicillin

(Kehrenberg et al., 2001b). Although data was not provided, the review also reported continued susceptibility to ceftiofur, cefquinome and florfenicol and in the majority of cases, susceptibility to enrofloxacin, erythromycin, spiramycin, tylosin, and tilmicosin (Kehrenberg et al., 2001b).

Isolates collected in the USA from 1994 through 2002 revealed susceptibilities for ceftiofur, cephalothin, enrofloxacin, sulfachloropyridizine and trimethoprim/sulfamethoxazole to be consistently high (85% and above), whereas susceptibility to erythromycin, florfenicol, spectinomycin, and tilmicosin (n= 390) tended to decline over the collection period (Welsh et al., 2004). Work conducted by Catry et al. (2005) between 2002 and 2003 reported the overall prevalence rate of resistant isolates from dairy, beef and veal calves to be 21.9%, with resistance to ampicillin, oxytetracycline, potentiated sulfonamides, gentamicin, tilmicosin, and enrofloxacin frequently encountered and resistance to ceftiofur and florfenicol not detected (n= 15). Most recently, the results of a three-year survey documented high levels of resistance to ampicillin, tetracycline and trimethoprim/sulphonamide in France, the Netherlands and Portugal ($n \leq 529$) (Hendriksen et al., 2008).

The monitoring of antimicrobial resistance is critical to aid practitioners in the prudent use of antimicrobials. Veterinarians typically rely upon the information available in textbooks or from reported surveillance data to guide their choices for drug therapy (Caprioli et al., 2000). It is important that information is up to date and relevant to equip them with the knowledge they need to make effective decisions. The imprudent use of antimicrobials leads to an increased risk for the selection of resistant bacteria and the

promotion of any resistance already present. This ultimately results in reduced efficacy of the agents presently available leading to potential treatment failure.

1.5.3 Genetic determinants

Most of the resistance genes identified in *M. haemolytica* are associated with mobile genetic elements such as small plasmids or transposons (Kehrenberg and Schwarz, 2001; Zecchinon et al., 2005). Of the resistance determinants characterised, few are considered indigenous to *Pasteurella* or *Mannheimia* suggesting that they have been acquired from other bacteria through horizontal gene transfer (Kehrenberg et al., 2001b). Recent data highlights the capacity of *M. haemolytica* to be naturally competent (Gioia et al., 2006) and several studies have documented its suspected propensity for horizontal gene transfer (Davies et al., 2002; Kelley et al., 2007; Wood et al., 1995). Consequently, *M. haemolytica* should be recognized as a potential candidate for the transfer and spread of antimicrobial resistance.

1.5.3.1 Resistance to β-lactams

Bla$_{\text{ROB-1}}$ is the only of three β-lactamase genes (*bla*$_{\text{ROB-1}}$, *bla*$_{\text{TEM-1}}$, and *bla*$_{\text{PSE-1}}$), to be detected in *M. haemolytica*. It is a member of the Ambler Class A (Bush class 2b) of β-lactamases and can hydrolyse penicillins and narrow-spectrum cephalosporins (Kehrenberg et al., 2006). It does not display extended spectrum β-lactamase activity or mediate resistance to the newer generations of cephalosporins such as ceftiofur or cefquinome and can be inactivated by clavulanic acid (Kehrenberg et al., 2006). The gene is encoded on small plasmids and is widely distributed among the *Pasteurelleacea* (Highlander, 2001). Not surprisingly, a strong genetic homology has been observed

between bla_{ROB-1} genes collected from *P. multocida, P. haemolytica, H, influenzae* and *A. pleuropneumoniae* (Kehrenberg et al., 2006).

1.5.3.2 Tetracyclines

Tetracycline resistance is found frequently in members of the family Enterobacteriaceae but rarely in the *Pasteurella* species (Hansen et al., 1996). This is thought to be due to the ineffectiveness of *Pasteurella* species at transcribing *Escherichia coli* genes (Hansen et al., 1996). However, some cases of tetracycline resistance have been encountered in *M. haemolytica* and are summarized below.

The tetracycline resistance observed within the *Pasteurella, Mannheimia, Actinobacillus*, and *Haemophilus* groups is diverse. There have been seven different genes identified that represent two different mechanisms, including the active efflux genes *tet*H, *tet*B, *tet*G, *tet*K, *tet*L and the ribosomal protective proteins *tet*M and *tet*O (Kehrenberg et al., 2006). Both the prevalence and occurrence of each gene varies within the *Pasteurellaceae* family with the overall frequency of tetracycline-resistance being higher in *M. haemolytica* than in *P. multocida* (Catry et al., 2003).

The *tet*H allele is most commonly observed in *M. haemolytica* (Highlander, 2001). It was first recognized in *P. multocida* in 1993 (Hansen et al., 1993) and was subsequently discovered in isolates of *P. haemolytica* recovered from cattle in North America (Hansen et al., 1996). It is assumed to be associated with a transposable element as it has been detected on plasmids and integrated into the chromosome (Hansen et al., 1996). *Tet*G was recently detected on the chromosomes of multiple related isolates of *M. haemolytica* (Kehrenberg et al., 2001a) and t*e*tL has been recovered from both plasmid and chromosomal DNA of *M. haemolytica* (Kehrenberg et al., 2005b). The *tet*B gene is

the most widely spread tetracycline resistance gene among the Enterobacteriaceae (Chopra and Roberts, 2001), however, it has only been documented once in an isolate of *P. haemolytica* collected from a bovine sample in France (Chaslus-Dancla et al., 1995).

1.5.3.4 Aminoglycosides and aminocyclitols

The *str*A gene is the most common streptomycin resistance determinant found in *Mannheimia* and *Pasteurella* species (Kehrenberg et al., 2006). It mediates resistance through production of an aminoglycoside-3'-phosphotransferase which enzymatically inactivates the drug (Kehrenberg and Schwarz, 2001). It is sometimes found in conjunction with a whole or truncated form of *str*B which codes for an aminoglycoside-6'-phosphotransferase (Kehrenberg et al., 2006). This *str*A-*str*B combination shows high resistance to streptomycin and is present on the plasmids or integrated into the chromosome of many pathogenic and commensal bacteria (Kehrenberg et al., 2006).

Spectinomycin is an aminocyclitol also used for treatment in cattle. There have been cases of high-level resistance to the drug documented in *M. haemolytica* (Kehrenberg and Schwarz, 2001) however, recent work undertaken to describe spectinomycin resistance was unsuccessful (Schwarz et al., 2004). Although the first *add*A14 gene was recently detected on a plasmid from a bovine *P. multocida* isolate, it was concluded that the genes responsible for spectinomycin and spectinomycin/streptomycin resistance in *Pasteurella* and *Mannheimia* organisms are yet to be described (Kehrenberg et al., 2005a).

1.5.3.5 Sulfonamides

Sulfonamide resistance is one of the most frequently detected resistances in *M. haemolytica* and is typically mediated by the *Sul*II gene which codes for a type II dihydropteroate synthase (Kehrenberg and Schwarz, 2001). This gene has been found on plasmids of varying size and sequence analysis shows it to display a significant degree of heterogeneity (Kehrenberg et al., 2006). It has also often been found linked to *str*A-*str*B genes (Kehrenberg et al., 2006) and has been associated with kanamycin and/or tetracycline resistance (Kehrenberg and Schwarz, 2001).

Trimethoprim resistance is usually mediated through dihydrofolate reductases which have been found located on plasmids, transposons or gene cassettes (Kehrenberg et al., 2006). Studies on trimethoprim resistance in *M. haemolytica* have exposed the lack of its association with plasmids or its ability to hybridize with gene probes specific to *dhfr*I to *dhfr*V (Escande et al., 1991). A novel trimethoprim resistance gene, *dfr*A20, has recently been described from a plasmid present in a bovine *P. multocida* isolate (Kehrenberg and Schwarz, 2005a).

1.5.3.6 Chloramphenicol and florfenicol

Chloramphenicol resistance is typically mediated by either chloramphenicol acetyltransferases or chloramphenicol exporters; the genes for which are located on plasmids, transposons or gene cassettes (Kehrenberg et al., 2006). Three types of inactivating enzymes have been described, *catA1* to *catA3*, based upon biochemical and immunological properties as well as their vulnerability to inhibition by 5,5'-dithiobis (2-nitrobenzoic acid) (Vassort-Bruneau et al., 1996). Chloramphenicol resistance has been documented in *M. haemolytica* and is attributed to the genes *catA1* and *catA3*. The *catA2*

gene has been detected in *Haemophilus* spp., but has yet to be observed in *Mannheimia* (Kehrenberg et al., 2006).

So far, the fluorinated chloramphenicol derivative florfenicol is still effective against *M. haemolytica*. However, a *pp-flo* gene encoding for the export of chloramphenicol/florfenicol has been identified in the fish pathogen *Pasteurella piscicida* (Kim and Aoki, 1996) and a similar gene, *flo*R has recently been detected in a bovine *P. multocida* isolate collected in the United Kingdom (Kehrenberg and Schwarz, 2005b).

1.6 *M. haemolytica* and the feedlot industry in Canada

Little has been published about the economics and epidemiology of BRD within Canadian feedlots and of the information available, much is out-dated. Estimates generated in 1978 place the cost of respiratory disease in Alberta feedlots to be $9.6 million annually (Church and Radostits, 1981). A more recent study reported that 29%-45% of the cases of respiratory disease related death in Ontario feedlots and 10%-57% of the cases of respiratory disease related death in western Canada were due to shipping fever pneumonia or pneumonic mannheimiosis (Gagea et al., 2006), but these values reference back to the late 70's and 80's. Indeed, the only information uncovered by the author relating to the occurrence and economics of BRD in Canada date back prior to 1999 (Booker et al., 1999; Church and Radostits, 1981; Harland et al., 1991; Martin et al., 1981; Martin et al., 1980; Ribble et al., 1995a; Ribble et al., 1995b). In addition to a general lack of information, much of what is reported is measured as total morbidity or total mortality values. Total morbidity values may be misleading as a there has been a clear lack of association described between the clinical symptoms of BRD and the lung

lesions characteristic of the condition (Wittum et al., 1996). Furthermore, the definition of each morbidity and mortality due to BRD can vary between studies making total morbidity and total mortality crude dependant variables for epidemiological studies (Ribble et al., 1995b).

Few studies in Canada have focused on *M. haemolytica* as a primary agent of BRD. Work conducted in Ontario in 1978-79 documented a 25% isolation rate of *P. haemolytica* from deceased cattle. However, the majority of recent publications are directed towards describing the efficacy of various antimicrobial treatments against this pathogen (Booker et al., 1997; Hoar et al., 1998; Jim et al., 1999; Schunicht et al., 2002). This highlights both the reliance of the Canadian feedlot industry on antimicrobial usage for the treatment of BRD, as well as the lack of enthusiasm surrounding epidemiological studies of BRD within our country. It has been proposed that part of the difficulty in amassing data pertaining to BRD in Canada is due to the confidentiality clauses put in place by feedlot managers (Church and Radostits, 1981). Given this circumstance it should not be unexpected that little progress has been made towards reducing the impact of BRD in Canada.

Understanding the ecology of *M. haemolytica* within the feedlot setting is crucial to developing a means to manage it. To our knowledge no work has been conducted examining the diversity of *M. haemolytica* populations upon arrival and exit from Canadian feed yards neither in relation to genetic profiling, nor antimicrobial susceptibility profiles. The former is of considerable concern as recent taxonomic amendments have emphasized the necessity for genetic characterization in the proper identification of *Mannheimia* species and there has been an obvious link established

between the diversity observed within the species and its potential for pathogenicity. In regards to antimicrobial susceptibility profiling, defining the relationship between antimicrobial use (AMU) and antimicrobial resistance (AMR) in the feedlot sector is necessary to identify the risks associated with treatment.

In most cases, the study of antimicrobial use in the livestock industry leads many to adopt the ostrich attitude of 'it is better not to know' with the main concern being, that documentation of antimicrobial resistance will lead to the restricted use of antimicrobials. The reality is that despite its detection, the development of antimicrobial resistance is inevitable. However, responsible use of antimicrobials within the feedlot environment can greatly reduce the speed and severity with which AMR occurs. Obtaining a clearer understanding of the interaction between AMU and AMR in cattle production will ultimately aid producers by giving them the information needed for the prudent use of antimicrobials for prevention and treatment of BRD.

A key objective of this work is to fill in the gaps in knowledge about the ecology of *M. haemolytica* within the Canadian cattle industry. The aim of the study is to characterize the diversity of *M. haemolytica* isolates collected from cattle upon arrival and exit from two feedlots in Southern Alberta, focusing on their genetic profiles through molecular analysis using pulsed-field gel electrophoresis and antimicrobial resistance profiles using a disk diffusion assay.

CHAPTER TWO

A method to improve pulsed-field gel electrophoresis analysis of *Mannheimia haemolytica*

2.1 Introduction

Mannheimia haemolytica is the primary bacterial pathogen implicated in Bovine Respiratory Disease complex (BRD), one of the most important health concerns in cattle production. Recent reclassification of the *Pasteurella haemolytica*-complex, forming the genus *Mannheimia* (Angen et al., 1999), has revealed the importance of *M. haemolytica* in the development of BRD and has also highlighted gaps in the epidemiology of this pathogen. Studies have investigated the prevalence of serotypes isolated from cattle (Angen et al., 2002; Katsuda et al., 2007), but there are few reports on the genetic relatedness of *M. haemolytica* isolates. Such information is important in order to gain a better understanding of the epidemiology of this pathogen.

Pulsed field gel electrophoresis (PFGE) is commonly used for molecular typing bacteria and several studies have already applied this technique to *M. haemolytica*. PFGE is considered both highly discriminatory and reproducible (Foxman et al., 2005; Tenover et al., 1997); however, both of these factors are dependant on the quality of the restriction profiles created. The resolution of the bands produced and the quality of the reference or size standard are critical to genotyping analyses. Size standards are of particular significance because they are an essential factor for the normalization of gels, construction of databases and comparisons of genetic profiles between laboratories.

The size standards most commonly reported in PFGE analysis of *M. haemolytica* are pre-manufactured Pulse Markers comprised of lambda DNA concatemers (Katsuda et

al., 2003; Villard et al., 2006). In our preliminary trials we found the band compression and resolution of these standards to be poor. In addition, PFGE analysis of *M. haemolytica* digested with *Sal*I restriction endonuclease (Kodjo et al., 1999; Villard et al., 2006) requires the use of both high range (50-1000kb) and low range (0.1-200kb) Pulse Markers to encompass the range of DNA fragments produced. The method described here provides an alternative to the reference standards currently used for PFGE analysis of *M. haemolytica* and also provides tactics to improve band resolution by adjusting the PFGE conditions from those currently reported in the literature. The results presented compare the use of a lambda based Pulse Marker size standard with a *Salmonella* based standard and show the effect of reduced running temperature and the addition of thiourea to running buffer on the resolution of restriction profiles.

2.2 Materials and Methods

Plugs and gels were prepared according to PulseNet USA protocols (CDC, 1996). *M. haemolytica* isolates were cultured overnight at 37 ºC on 5% sheep blood agar containing 15 µg/ml of bacitracin according to Catry et al (2007). For each isolate, colonies were suspended in 2 ml of Cell Suspension Buffer (0.1M Tris; 0.1M EDTA, pH 8.0) to the equivalent of a 5 McFarland suspension (absorbance reading between 1.25 and 1.35 at 610 nm). *Salmonella* serotype Braenderup (H9812) used for the size standard was cultured in the same conditions on Luria-Bertani agar. Plugs of both species were prepared by combining 100µL each of the culture suspension and 1% SeaKem Gold low-melting temperature agarose (Lonza Canada, Inc., Shawinigan, QC) containing 10%

sodium dodecyl sulphate (SDS) with 10µL of 20 ng/ml Proteinase K (Roche Diagnostics Canada, Ltd., Mississauga, ON).

Plugs were incubated in a water bath at 54 ºC with constant agitation at 100 rpm for 3 h in 50-ml plastic tubes containing 5 ml cell lysis buffer (0.05 M Tris pH 8.0, 0.05 M EDTA pH 8.0, 1% Sarcosyl) and 25 µl of 20 ng/ml Proteinase K. Plugs were washed twice with 10-15 ml of sterile ultra pure water (Fischer Scientific Co., Ottawa, ON) and four times with 10-15 ml sterile 1xTE (10 mM Tris, 1mM EDTA, pH 8.0) for periods of 15 min at 54 ºC and 100 rpm. The washed plugs were stored in sterile 1xTE buffer and refrigerated at 4 °C until use.

M. haemolytica plugs were digested overnight at 37 ºC with 10 units of *Sal*I (Invitrogen Canada, Inc., Burlington, ON) and 180 µl of appropriate buffer provided by the manufacturer. *Salmonella* serotype Braenderup standard plugs were digested overnight for the same duration and temperature with 10 units of *Xba*I (Invitrogen Canada, Inc., Burlington, ON) and 180 µl of buffer provided by the manufacturer. Two sizes of pre-manufactured lambda Pulse Markers were used; a low range 0.1-200 kb (Sigma-Aldrich Canada, Ltd., Oakville, ON) and a high range 50-1000 kb (Sigma-Aldrich Canada, Ltd.). Slices of Pulse Marker were cut with a blade from the end of the syringe according to manufactures specifications (approximately 0.5 µg in size) and imbedded along with digested *Salmonella* and *M. haemolytica* plugs in 1% SeaKem Gold low-melting temperature agarose (Lonza Canada, Inc.) that was dissolved in 0.5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Prior to incorporation into the gel, digested plugs were incubated with 200 uL of 0.5 x TBE at room temperature for 20 min.

The digested DNA was separated by PFGE using a CHEF DRII device (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON) according to the program outlined by Hunter et al. (2005) for *Listeria monocytogenes* with exception of an extension of the running time to 22 h to aid with separation of the top two bands of the *Salmonella* standard. Gels were run at either 14 °C or 12 °C; voltage was maintained at 6 V/cm with switch times of 4-40 s. The gels were run in 0.5 X TBE buffer with or without the addition of thiourea (0.5 mM). Gels were stained with 1 µg/ml of ethidium bromide in distilled water for 20 min followed by three washes in distilled water for the same duration. Gels were photographed with the AlphaImager gel documentation system (Alpha Innotech Corp., St Leandro, CA) with constant settings for all images. Fragment analysis was performed with BioNumerics V5.1 (Applied Maths, Inc., Austin, TX).

2.3 Results and Discussion

In all three cases (Fig. 2.1, Fig. 2.2 and Fig. 2.3), the limitations of the lambda Pulse Markers were evident. Although distinct separation was achieved with the lambda Pulse Markers, in multiple cases the spread of the bands exceeded 30 kb. In these instances we found position assignment difficult, leading to poor normalization of gels within the BioNumerics analysis program. In contrast, a *Salmonella* size standard described previously (Hunter et al. 2005) provided well-defined bands that were consistent in breadth to the *M. haemolytica* samples analyzed. We found these lanes more reliable for use in analysis, such as library creation and genotype profiling and comparisons.

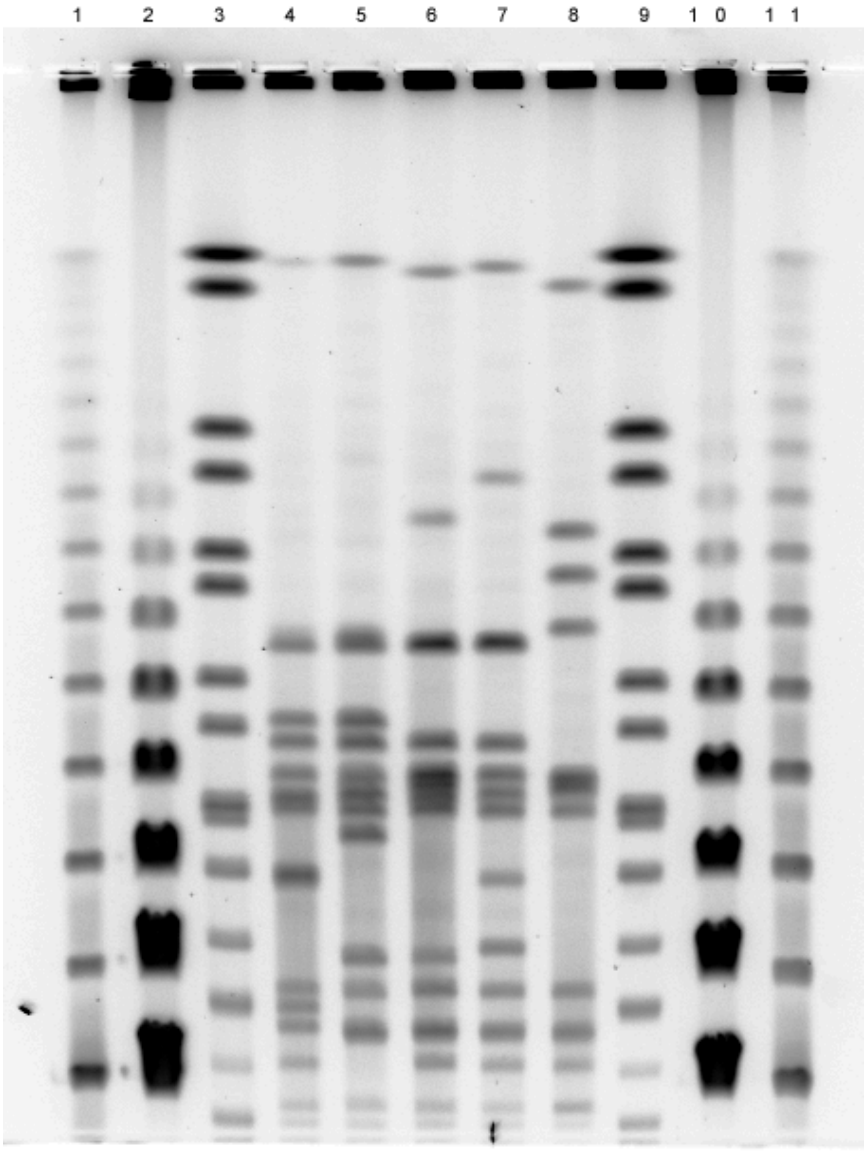


Fig. 2.1. PFGE of *M. haemolytica* isolates digested with *Sal*I endonuclease, *Salmonella* serotype Braenderup digested with *Xba*I and a high and a low Lambda Pulse Marker. Program consisted of electrophoresis for 22 h at 14°C with switch times from 4-40 seconds; no thiourea was added to the running buffer. Lanes 1 and 11: high range Pulse Marker 50-1000kb; lanes 2 and 10: low range Pulse Marker 0.1-200kb; lanes 3 and 9: *Salmonella* serotype Braenderup; lanes 4 to 8: *M. haemolytica isolates.*

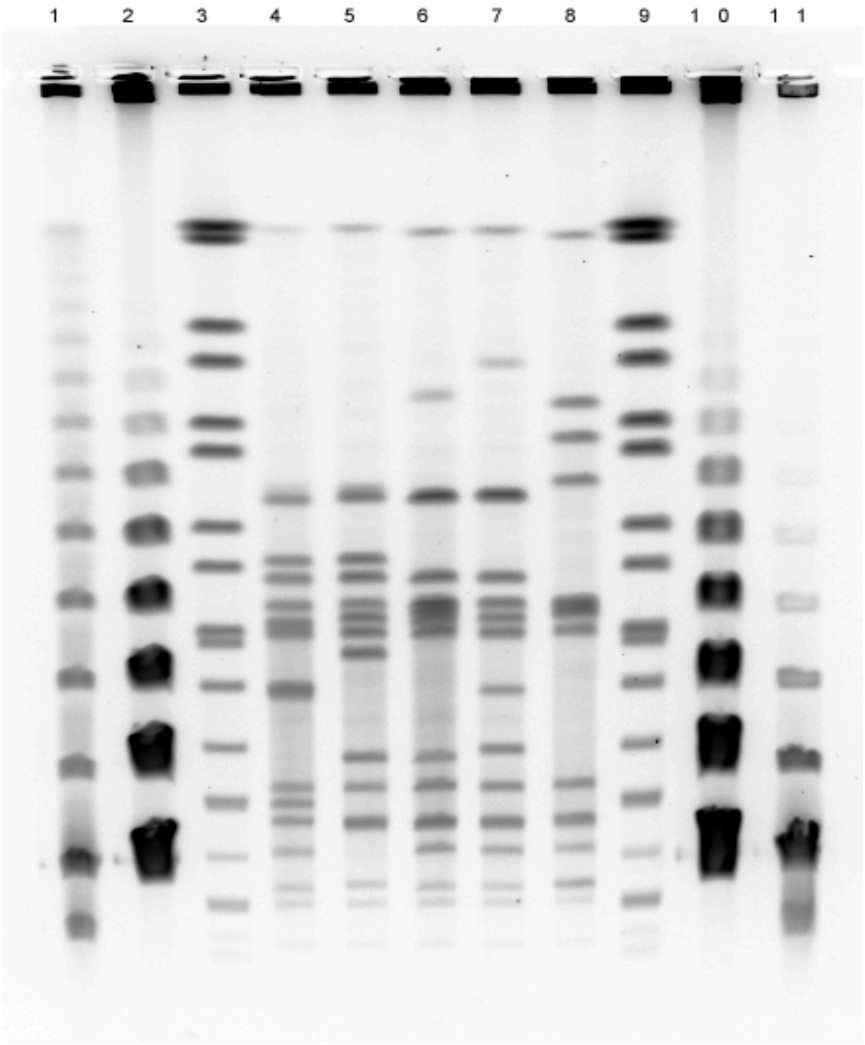


Fig. 2.2. PFGE of *M. haemolytica* isolates digested with *Sal*I endonuclease, *Salmonella* serotype Braenderup digested with *Xba*I and a high and a low Lambda Pulse Marker. Program consisted of electrophoresis for 22 h at 12°C with switch times from 4-40 seconds; no thiourea was added to the running buffer. Lanes 1 and 11: high range Pulse Marker 50-1000kb; lanes 2 and 10: low range Pulse Marker 0.1-200kb; lanes 3 and 9: *Salmonella* serotype Braenderup; lanes 4 to 8: *M. haemolytica* isolates.

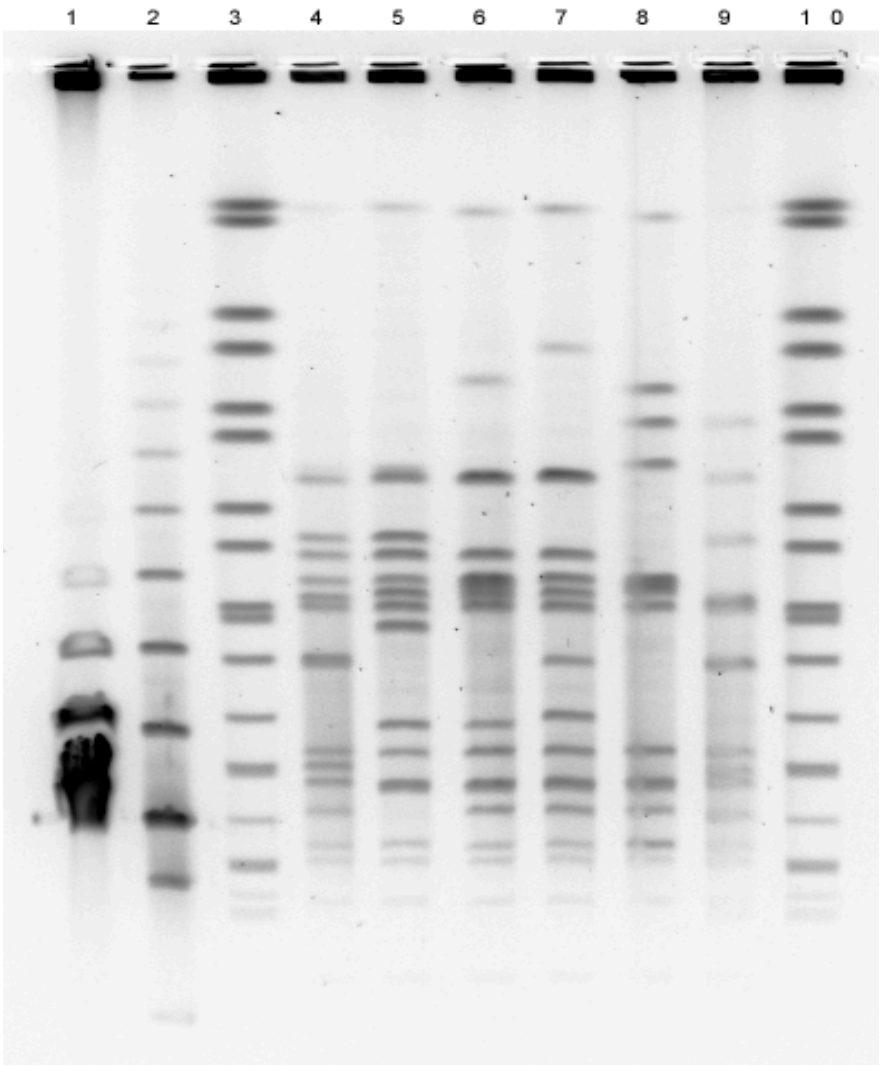


Fig. 2.3. PFGE of *M. haemolytica* isolates digested with *Sal*I endonuclease, *Salmonella* serotype Braenderup digested with *Xba*I and a high and a low Lambda Pulse Marker. Program consisted of electrophoresis for 22 h at 12°C with switch times from 4-40 seconds; thiourea was added to the running buffer. Lane 1: low range Pulse Marker 0.1-200kb; lane 2: high range Pulse Marker 50-1000kb; lanes 3 and 10: *Salmonella* serotype Braenderup; lanes 4 to 9: *M. haemolytica* isolates.

Fig. 2.1 and Fig. 2.2 contrast the effects of the reduction in running temperature from 14 °C to 12 °C, respectively. Reducing the temperature by 2 °C resulted in improved band compression and clarity. Fig. 2.3 shows the results from the addition to

thiourea to the running buffer. A slight improvement was observed in the separation of bands arising from the *Salmonella* standard, compared to when no thiourea was added (viz. bands one and ten). Although thiourea has been shown to improve the quality of PFGE profiling in other organisms (Corkill et al., 2000; Silbert et al., 2003; Zhang et al., 2004) its use here did not appear to significantly affect the resolution of *M. haemolytica* bands. However, improving the separation of the bands of the *Salmonella* standard resulted in a significant improvement in the analysis of PFGE gels. Therefore, the addition of thiourea to the electrophoresis buffer was adopted when *Salmonella* standards were used for PFGE of DNA from *M. haemolytica*.

To conclude, we found that utilization of the *Salmonella* size standard in conjunction with both a reduction in running temperature and the addition of thiourea to the running buffer to be advantageous in the PFGE analysis of DNA from *M. haemolytica.* Both resolution and clarity of restricted DNA fragments were improved by these procedures, allowing for better normalization of gels and comparison of *M. haemolytica* isolates by PFGE. The utilization of *Salmonella* as a reference standard increased the consistency of gel interpretation leading to an improvement in PFGE analysis.

CHAPTER THREE

Comparison of repetitive PCR and pulsed-field gel electrophoresis for analyses of *Mannheimia haemolytica* genetic diversity and identification

3.1 Introduction

Mannheimia haemolytica is the principal bacterial pathogen associated with bovine respiratory disease (BRD) in cattle (Rice et al. 2007). Also referred to as shipping fever, undifferentiated fever (UF) and/or bovine respiratory disease complex (Booker et al., 2008; Schunicht et al., 2007; Wildman et al., 2008), this condition is one of the most significant health problem facing the North American cattle industry (Snowder et al., 2006). The economic losses it generates surpass those incurred by all other diseases of cattle combined (Highlander, 2001) and are due to production losses, treatment costs and mortalities (Bateman et al., 1990; Gagea et al., 2006; Larson and Tyler, 2005).

Although numerous studies have focused on the role of *M. haemolytica* in the pathology of BRD (Ackermann and Brogden, 2000; Highlander, 2001; Mohamed and Abdelsalam, 2008; Zecchinon et al., 2005), little is known about the role that genetic variability plays in the biology of this pathogen. Information on environmental persistence, transmission and virulence may all be augmented if a suitable technique for characterizing the genetic diversity of *M. haemolytica* can be identified. Defining the role that these factors have in the etiology of BRD may provide insight into effectual management strategies for *M. haemolytica*-associated BRD within the feedlot sector.

Current molecular genotyping methods provide multiple means by which to identify and monitor genetic variants within populations. Typically utilized for species identification (De Vuyst et al., 2008; Masco et al., 2003), source tracking (Currie et al.,

2007) and strain discrimination (Gunawardana et al., 2000), genotyping methods have become essential tools for epidemiological investigations. Pulsed-field gel electrophoresis (PFGE) has been described as one of the most powerful microbial genotyping methods owing to its high discriminatory power (Tenover et al., 1997). However, PFGE has some drawbacks as it is a time consuming process and requires expensive, specialized equipment. Alternative methods such as repetitive PCR have been presented as an alternate approach for subspecies classification and strain delineation (Healy et al., 2005). These provide a more cost-effective and efficient typing method compared to PFGE, however they may lack discriminatory power (Foxman et al., 2005).

Understanding the strengths and weaknesses of a chosen bacterial typing technique is needed in order to accurately interpret study results (Foxman et al., 2005). The objective of this work was to compare the capabilities of PFGE and two repetitive PCR techniques in the genotypic analysis of *M. haemolytica.* Additional species within *Pasteurellaceae* were also analyzed to determine the potential for each genotyping method to identify *Mannheimia* spp.

3.2 Materials and Methods

Isolates

A total of 40 *M. haemolytica* field isolates were typed with PFGE and with the rep-PCR methods BOX-PCR (primer BOXA1R) and $(GTG)_5$-PCR (primer $(GTG)_5$). The isolates originated from bovine nasal swabs collected from feedlot cattle in the southern region of Alberta, Canada. Prior to use all isolates were positively identified as *M. haemolytica* through biochemical testing and multiplex PCR (Alexander et al., 2008b)

and stored at -80 °C. Twenty four reference strains including *Mannheimia glucosida* (strains CCUG 28375, CCUG 28376, CCUG 38454, CCUG 38456, CCUG 38467, CCUG 38459, CCUG 38460), *Mannheimia granulomatis* (strain CCUG 45422), *Mannheimia haemolytica* (strains CCUG 18142, CCUG 24141, CCUG 29694, CCUG 43453, CCUG 38454#, ATCC BAA-410, ATCC 43270, ATCC 33396, ATCC 29694, UGCC G2, UGCC G2, UGCC G5, UGCC G12), *Mannheimia ruminalis* (strains CCUG 38470, CCUG 38466#) and *Pasteurella multocida* subsp. *multocida* (strains CCUG 17976B) were also analyzed using the aforementioned genotyping methods. Reference strains used were obtained from either the American Type Culture Collection (ATCC, Manassas, VA, USA), University of Göteborg culture collection (CCUG, University of Göteborg, Sweden) or through the University of Guelph culture collection (UGCC, Guelph, ON, Canada). *Salmonella* serotype Braenderup (H9812) was used as a reference standard in PFGE. For PFGE and PCR-based analyses, *Mannheimia* spp. and *P. multocida* were cultured overnight at 37 ºC on TSA agar plates containing 5% sheep blood agar plates. *Salmonella* serotype Braenderup (H9812) was cultured under the same conditions with the exception of plating on Luria-Bertani agar.

PFGE

Plug and gel preparation were conducted according to PulseNet USA protocols (CDC, 1996). For each isolate, colonies were suspended in 2 ml of cell suspension buffer (0.1M Tris; 0.1M EDTA, pH 8.0) to the equivalent of a 5 McFarland turbidity standard (absorbance reading between 1.25 and 1.35 at a wavelength of 610 nm). Agar plugs of each species were prepared from suspensions containing 10 µl of Proteinase K (20 ng/ml in sdH_2O) (Roche Diagnostics Canada, Ltd., Mississauga, ON) combined with 100 µl of

a culture suspension and 100 µl 1% SeaKem Gold agarose (in sterile TE, pH 8) (Lonza Canada, Inc., Shawinigan, QC) containing 10% sodium dodecyl sulphate (in sterile distilled H_2O).

Plugs were incubated in 50 ml plastic tubes containing 5 ml cell lysis buffer (0.05 M Tris pH 8.0, 0.05 M EDTA pH 8.0, 1 % Sarcosyl in sdH_2O) and 25 µl of Proteinase K (20 ng/ml in sdH_2O) in a shaker water bath at 54 ºC with constant agitation at 100 rpm for 3 h. Plugs were washed twice with 10-15 ml of sterile ultra pure water and subsequently four times with 10-15 ml sterile 1 × TE (10 mM Tris, 1mM EDTA, pH 8.0) for periods of 15 min at 54 ºC and 100 rpm. Once washed the plugs were stored in sterile 1 × TE buffer and refrigerated at 4 °C until used.

Plugs of *Mannheimia* and *Pasteurella* sp. were digested overnight at 37 ºC with 10 units of *Sal*I and 180 µl of appropriate buffer provided by the manufacturer (Invitrogen Canada, Inc.). *Salmonella* serotype Braenderup standard-plugs were digested similarly with the exception of using 10 units of restriction enzyme *Xba*I (Invitrogen Canada, Inc., Burlington, ON) and 180 µl of buffer. The digested plugs were imbedded in 1% SeaKem Gold low-melting temperature agarose (Lonza Canada, Inc., Shawinigan, QC) that was dissolved in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Prior to incorporation into the gel, digested plugs were incubated with 200 µl of 0.5 × TBE at room temperature for 20 min.

The digested DNA were separated by PFGE using a CHEF DRII device (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON), according to the program outlined by Hunter *et al*, (2005) for *Listeria monocytogenes* with exception to an extension in running time to 22 h to aid with separation of the top two bands of the *Salmonella* standard. Gels

were run at 12 °C; voltage was maintained at 6 V/cm with switch times of 4-40 s. The gels were run in 2,200 ml of 0.5 × TBE buffer with the addition of 1 ml of 1 M thiourea. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) in distilled water for 20 min followed by three washes in equal volumes of distilled water for the same duration. Gels were photographed with an AlphaImager gel documentation system (Alpha Innotech Corp., St Leandro, CA, USA); the settings were maintained constant for all images obtained. Fragment analysis was performed with BioNumerics V5.1 software (Applied Maths Inc., Austin, TX, USA).

Rep PCR

Isolates were grown overnight on TSA agar plates containing 5% sheep blood (37 °C). Bacteria from approximately one half of each plate were removed using an inoculating loop. Genomic DNA was extracted and purified using the DNeasy Tissue Kit (Qiagen Canada, Inc., Mississauga, ON) according to the manufacturer's instructions with only slight modifications. Lysis was extended overnight at 4 °C and the final product was eluted with 180 µl of elution solution. DNA was quantified fluorometrically using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen Canada, Inc.,) with a VersaFluor fluorometer (Bio-Rad Laboratories Canada, Ltd.,), diluted to 5 ng/µl with sterile TE (pH 7.4), and stored at -20 °C until used.

Repetitive sequence PCR was conducted using the BOX-PCR analogous primer pair set (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and the $(GTG)_5$-PCR single oligonucleotide primer (5'-GTG GTG GTG GTG GTG-3') (Mohapatra and Mazumder, 2008). Each PCR mixture (20 µl) contained (final concentrations) 1 × HotStarTaq Master Mix (Qiagen Canada, Inc.), 1 × CoralLoad Concentrate Mix (Qiagen Canada,

Inc.), 2.0 µM primer, 0.28% dimethyl sulfoxide, and 0.1 µg/µl bovine serum albumin. DNA (10 ng) was added as template to each PCR. All PCR were performed with a Mastercycler thermal cycler (Eppendorff Canada, Ltd., Mississauga, ON) using the following conditions: 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 40 °C $(GTG)_5$-PCR or 50 °C BOX-PCR annealing temperatures for 1 min, and 72 °C for 1 min; and 72 °C for 8 min.

Following PCR, 20 µl of product was analyzed by agarose gel electrophoresis [(1.2% (w/v) SeaKem LE agarose (Lonza Canada, Inc., Shawinigan, QC)] with a setting of 40 V for a period of 12 h at 8 °C. Gels were stained, washed and visualized as described above for PFGE. One lane containing seven microlitres of Gene Rule DNA Ladder Mix (Fermentas Canada, Inc., Burlington, ON) was included with every six to seven samples to serve as a molecular size standard. Fragment analysis was performed with the BioNumerics V5.1 software (Applied Maths, Inc., Austin, TX, USA).

Method repeatability

A repeatability experiment was conducted using five randomly selected field isolates grown under identical conditions. For each isolate examined, five colonies were selected from a single plate and subjected to the procedures described above for PFGE, BOX-PCR and $(GTG)_5$-PCR.

Statistical analysis

All statistical analyses were performed using BioNumerics V5.1 software (Applied Maths, Inc.,). Dendrograms were created using UPGMA clustering of Dice coefficient values with 1.00% optimization and 1.5% position tolerance settings. Congruence values were calculated using Kendall's tau coefficient with a degree of one.

Discrimination indices were derived from the Simpson's Diversity Index script downloadable from the Applied Maths web link tool (http://www.applied-maths.com/bn/scripts/comparisons/bncomscripts.htm). Repeatability was reported as the proportion of isolates that had >95% similarity based on UPGMA cluster analysis of Dice coefficients within the BioNumerics V5.1 program (Applied Maths, Inc., Austin, TX, USA).

3.3 Results

DNA band analysis

Representative profiles from each genotyping assays are depicted in Fig. 1.3. PFGE analysis of *M. haemolytica* isolates revealed bands that ranged in size from approximately 25 to 1000 kb. PFGE band profiles consisted of 12.7 bands and when examined with Dice correlation coefficient with 1.0% optimization and 1.5% position tolerance settings, a total of 37 unique profiles were identified from the 40 field isolates.

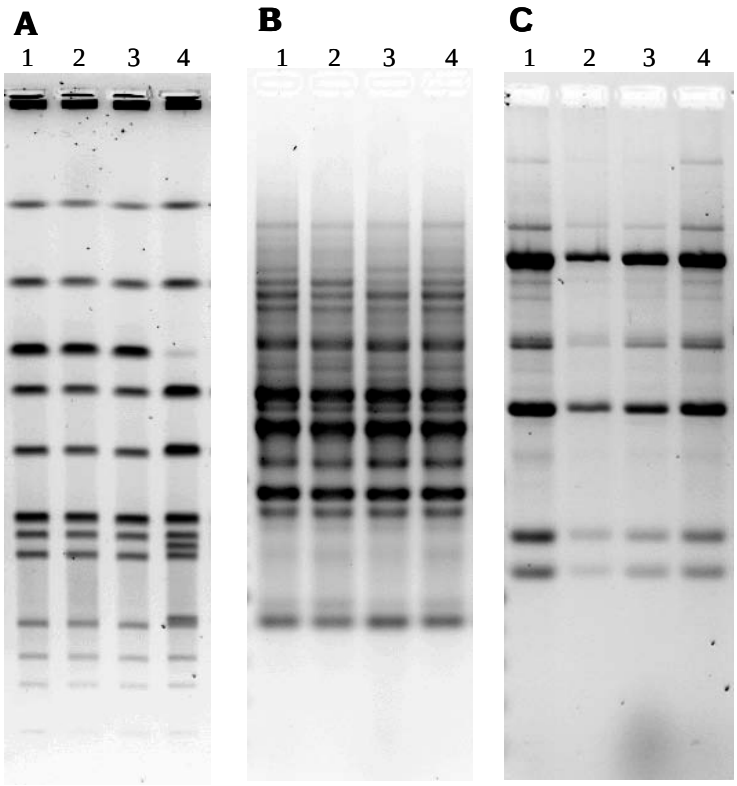


Fig. 3.1. Representative PFGE (A), $(GTG)_5$-PCR(B) and BOX-PCR (C) profiles. Lanes 1 through 4 represent replicate isolates

BOX-PCR analysis of the *M. haemolytica* isolates revealed a band range of approximately 900 to 6,000 bp while the $(GTG)_5$-PCR analysis ranged from 500 to 6,000 bp. BOX-PCR and $(GTG)_5$-PCR band profiles were 10.8 and 17.8 respectively. When examined with Dice correlation coefficient with 1.0% optimization and 1.5% position tolerance settings the 40 isolates produced 25 unique profiles with BOX-PCR and 20 unique profiles with $(GTG)_5$-PCR.

Discriminatory Indices

Although a discriminatory index >0.90 was achieved with both PFGE and BOX-PCR, only PFGE was capable of obtaining effective discriminatory powers of 0.98 and 0.92 (Hunter and Gaston, 1988) when relaxing the similarity cutoff below the 95% value

to 90% and 85% respectively (Table 3.1). $(GTG)_5$-PCR alone did not produce a discriminatory index above 0.72, however when used in combination with BOX-PCR a value >0.90 was achieved. A composite fingerprint was produced with weightings of 1:1, 3:1 and 6:1 of BOX-PCR to $(GTG)_5$-PCR, respectively, using BioNumerics software. The combined profiles increased the overall discriminatory value from that observed with $(GTG)_5$-PCR alone, but none of the combinations produced discrimination greater than what was achieved by BOX-PCR alone.

Table 3.1
Discriminatory power of PFGE, $(GTG)_5$-PCR and BOX-PCR in genotype analysis of *M. haemolytica* field isolates (n=40)

Method	Similarity cut-off	Cluster Sizes	Discriminatory Index
PFGE	95	1, 1, 2, 2, 2, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 3, 1, 1, 1, 2, 3, 1, 1, 1, 1, 1, 1, 1, 1, 1	>0.98*
	90	1, 1, 2, 2, 2, 1, 1, 2, 1, 1, 1, 1, 1, 1, 1, 1, 4, 2, 2, 3, 1, 1, 1, 1, 1, 1, 1, 1, 1	0.98
	85	2, 2, 2, 2, 1, 1, 2, 1, 1, 3, 1, 1, 1, 11, 1, 1, 1, 1, 1, 1, 2, 1	0.92
	80	2, 2, 2, 2, 1, 1, 3, 1, 3, 1, 13, 2, 1, 1, 1, 1, 2, 1	0.88
$(GTG)_5$-PCR	95	5, 2, 1, 1, 20, 5, 4, 1, 1	0.72
	90	7, 1, 1, 30, 1	0.42
	85	8, 1, 31	0.37
	80	9, 31	0.36
BOX-PCR	95	4, 2, 1, 2, 2, 1, 10, 2, 2, 1, 1, 3, 1, 1, 1, 1, 1, 2, 2	0.92
	90	4, 2, 1, 2, 2, 1, 14, 2, 4, 1, 2, 1, 2, 2	0.86
	85	7, 2, 3, 16, 5, 2, 1, 2, 2	0.80
	80	9, 3, 21, 3, 2, 2	0.67
BOX-PCR: $(GTG)_5$-PCR (1:1)	95	2, 2, 4, 3, 4, 1, 1, 2, 2, 9, 1, 3, 1, 1, 1, 1, 2	0.92
	90	4, 7, 5, 1, 8, 2, 1, 2	0.75
	85	4, 7, 24, 3, 2	0.61
	80	35, 5	0.22
BOX-PCR: $(GTG)_5$-PCR (3:1)	95	4, 3, 2, 2, 1, 3, 1, 1, 1, 2, 2, 10, 1, 2, 1, 2, 2	0.92
	90	7, 2, 2, 1, 5, 1, 15, 3, 2, 2	0.82
	85	9, 3, 6, 18, 2, 2	0.73
	80	12, 24, 4	0.55
BOX-PCR: $(GTG)_5$-PCR (6:1)	95	2, 1, 2, 4, 3, 2, 3, 1, 1, 1, 2, 1, 2, 2, 10, 1, 2	0.92
	90	2, 1, 2, 7, 2, 4, 1, 1, 3, 15, 2	0.82
	85	3, 2, 9, 6, 18, 2	0.73
	80	5, 9, 24, 2	0.59

*Clusters too numerous for calculation to be completed within the BioNumerics program. Similarity cut-off is the percent relatedness value used to define clusters. Cluster size is the number of isolates comprising each cluster defined (differentiated by comma). Discriminatory index calculated using Simpson's Diversity index

Repeatability

Fig. 3.1 shows replicate images of isolates analyzed by PFGE, $(GTG)_5$-PCR and BOX-PCR genotyping methods. While no method was found to be 100% repeatable, both PFGE and $(GTG)_5$-PCR had reproducibility values ≥0.8 (Table 3.2). When the BioNumerics software was utilized for band identification and classification, repeatability of BOX-PCR was found to be low with a value of 0.4. However, repeatability values for the same analysis increased to a mean value of 0.7 when band classification was manually modified based on visual assessment (data not shown). In one instance, an isolate analyzed by PFGE appeared to exhibit an additional restriction site (Fig. 3.1A, lane 4). Apart from this, all PFGE were reproducible.

Table 3.2
Repeatability of PFGE, $(GTG)_5$-PCR and BOX-PCR in genotyping of *M. haemolytica*

Method	Isolate #	Repeatability
PFGE	235	1.0
	553	1.0
	616	0.8
	1335	1.0
	1358	1.0
$(GTG)_5$-PCR	235	0.8
	553	0.8
	616	0.8
	1335	0.8
	1358	0.8
BOX-PCR	235	0.4
	553	0.4
	616	0.8
	1335	0.2
	1358	0.2

Repeatability calculated as the proportion of isolates >95% similar based on UPGMA cluster analysis of Dice coefficients, n=5

Congruence and agreement between methods

The congruence and regression (r) values derived from the Kendall's tau congruence analysis between PFGE, BOX-PCR and $(GTG)_5$-PCR are presented in Table 3.3. The highest congruence was seen between PFGE and $(GTG)_5$-PCR while the lowest was observed between the two rep-PCR methods. All three congruence values scored a significance value >99.99 and standard deviation of ± 2.39.

Table 3.3
Congruence analysis between PFGE, $(GTG)_5$-PCR and BOX-PCR genotypic analysis of *M. haemolytica*

	Congruence value			Regression plot r value		
Genotyping method	PFGE	$(GTG)_5$-PCR	BOX-PCR	PFGE	$(GTG)_5$-PCR	BOX-PCR
PFGE			29.8			0.61
$(GTG)_5$-PCR	39.0			0.65		
BOX-PCR		25.0			0.55	

All congruence values scored a significance >99.99 and had a standard deviation of ± 2.39

Isolates that clustered together (at a similarity value of >90%) across genotyping methods are indicated in Table 3.4. In total there were three groups of isolates that clustered together in all three typing methods examined, including the three weighted variants of the combined matrix. PFGE and $(GTG)_5$-PCR analyses exhibited the most similar cluster arrangement, an observation which is supported by the congruence analysis.

Table 3.4

Cluster agreement between PFGE, GTG_5 and BOX-PCR methods when compared against PFGE type

Isolate	GTG-5 type	PFGE type	BOX type	BOX:GTG5 type (1:1)	BOX:GTG5 type (3:1)	BOX:GTG5 type (6:1)
964A	3	1	19	15	16	3
596C	5	1	10	12	14	11
553A	1	2	18	17	17	17
1432A	1	2	19	14	16	3
1359A	2	3	5	2	4	1
1358A	2	3	5	2	4	1
235A	1	4	4	1	3	6
243A	1	4	4	1	3	6
932A	4	5	6	16	5	2
482A	1	6	18	17	17	17
1348A	8	7	8	8	10	13
1346A	7	7	14	7	9	10
1340A	5	8	8	8	10	13
916A	5	9	15	5	7	8
369A	6	10	1	3	1	4
163A	6	10	2	4	2	5
1332A	5	10	7	10	12	15
863A	5	11	17	9	11	14
1339A	5	12	7	10	12	15
164A	6	13	2	4	2	5
867A	5	14	13	9	11	14
616A	5	14	11	13	15	12
596A	5	14	7	10	12	15
394A	5	14	7	10	12	15
134A	5	14	7	10	12	15
154A	5	14	7	10	12	15
382A	5	14	9	12	12	16
254A	5	14	9	12	14	11
346A	5	14	1	3	1	4
251B	5	14	1	3	1	4
153A	6	14	3	4	2	5
1064A	5	15	12	5	6	7
1347A	9	16	7	11	13	15
596B	5	17	7	10	12	15
1333A	7	18	7	10	12	15
936A	7	19	16	6	8	9
1335A	5	20	7	10	12	15
1046A	5	21	12	5	6	7
1043A	7	21	12	5	6	7
364A	6	22	1	3	1	4

Similarity cut-off values were chosen based upon the lowest values that corresponded with a discriminatory value >90% in Table 1. Blocks highlight isolates that show consistent clustering for multiple methods of analysis. Numbers represent the cluster each isolate was grouped with within each method.

Species Identification

Dendrograms produced from cluster analysis of the reference strains by BOX-PCR, PFGE and $(GTG)_5$-PCR are presented in Fig. 2, Fig. 3, and Fig. 4, respectively. Banding patterns generated from the BOX-PCR contained between 7 and 25 bands ranging from 400 to 10,000 bp. Banding patterns generated from $(GTG)_5$-PCR contained between 15 and 23 bands ranging from 100 to 6,000 bp and PFGE produced between 8 and 17 bands that ranged from 20.5 to1,135 kb. BOX-PCR clustered all *M. haemolytica* strains together at a similarity value of 50% while PFGE did so at 36.6%. Both BOX-PCR and PFGE analyses clustered all *M. haemolytica* into a single group. With the exception of one isolate, $(GTG)_5$-PCR clustered *M. haemolytica* into one group with a relatedness of 79%. BOX-PCR and $(GTG)_5$-PCR also clustered *M. glucosida* and *M. ruminalis* isolates together.

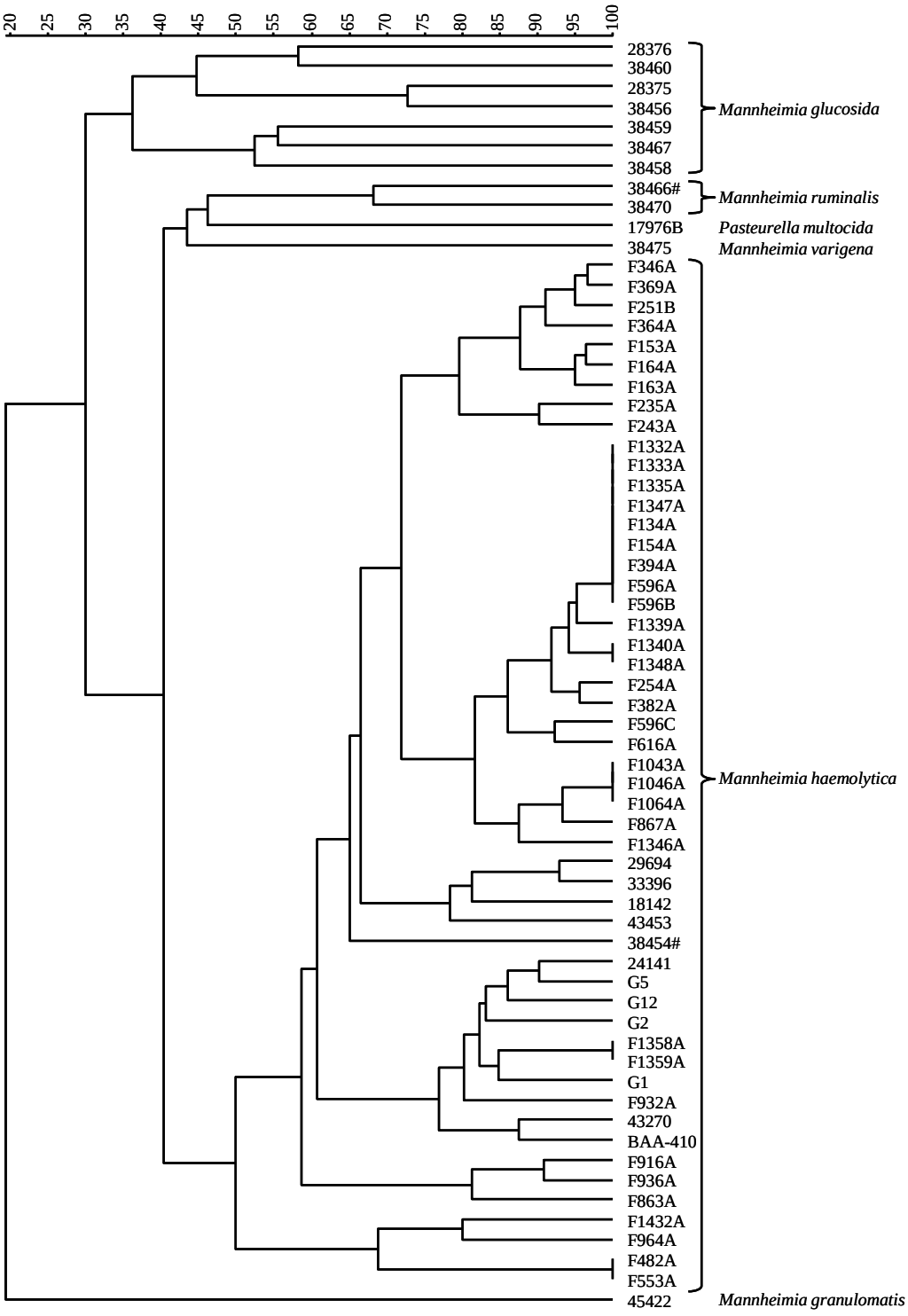


Fig. 3.2. Dendrogram of BOX-PCR fingerprints created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and position tolerance settings of 1.00% and 1.5% respectively. Sample assignments preceded by an F indicate *M. haemolytica* field isolates.

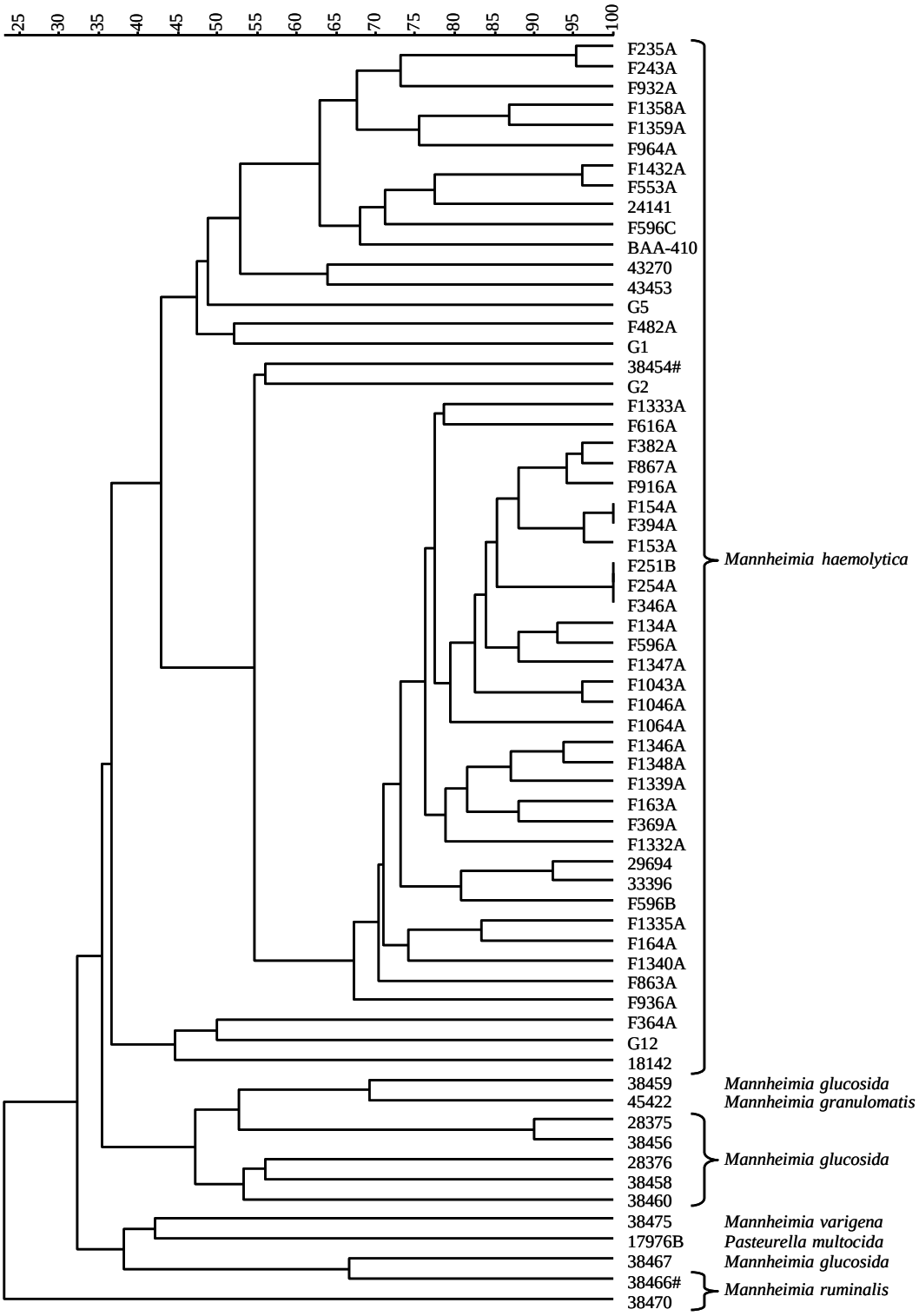


Fig. 3.3. Dendrogram of PFGE fingerprints created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and position tolerance settings of 1.00% and 1.5% respectively. Sample assignments preceded by an F indicate *M. haemolytica* field isolates.

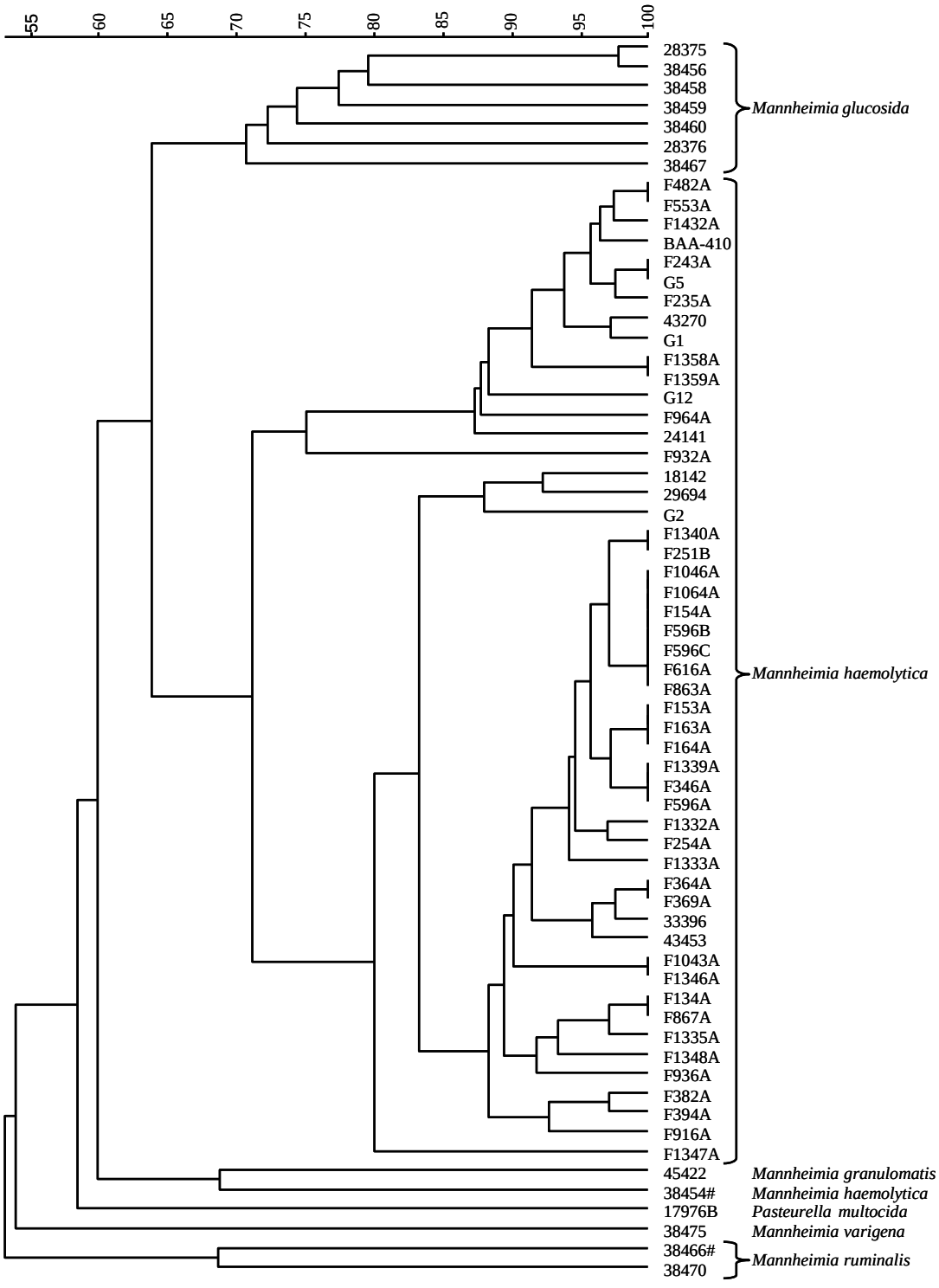


Fig. 3.4. Dendrogram of $(GTG)_5$-PCR fingerprints created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and position tolerance settings of 1.00% and 1.5% respectively. Sample assignments preceded by an F indicate *M. haemolytica* field isolates.

3.4 Discussion

Discriminatory power and repeatability

The primary aim of this study was to compare the use of two rep-PCR techniques and PFGE for the molecular typing and speciation of *M. haemolytica*. PFGE analysis is widely accepted as the gold standard for molecular typing and has previously been utilized with *Pasteurellaceae* species, including *M. haemolytica* (Gunawardana et al., 2000; Katsuda et al., 2003; Kodjo et al., 1999; Villard et al., 2006). Consequently, it was expected that PFGE analysis would perform well in comparison with the rep-PCR methods examined here. Both $(GTG)_5$-PCR and BOX-PCR have been applied to an array of species including acetic acid bacteria (De Vuyst et al., 2008), *Bifidobacterium* species (Masco et al., 2003), and *Escherichia coli* (Mohapatra et al., 2008), each claiming varying degrees of success. However, these methods have not previously been applied to the study of *M. haemolytica* making the rep-PCR performance here more difficult to predict.

Of the methods employed, $(GTG)_5$-PCR displayed the lowest discriminatory power whereas PFGE exhibited the highest. To be considered reliable, a typing method should exhibit a discriminatory index greater than 0.9 (Hunter and Gaston, 1988). Alone, $(GTG)_5$-PCR analysis was less than this value; however, a composite fingerprint that combined both BOX-PCR and $(GTG)_5$-PCR profiles was shown to increase the discriminatory index above this confidence threshold. Previous studies examining both *Listeria monocytogenes* (Chou and Wang, 2006) and *E.coli* (Johnson and O'Bryan, 2000) have shown a similar increase in discriminatory indices when producing a combined analysis indicating the potential for improved discriminatory performance with the

amalgamation of multiple typing methods. However, our study showed that BOX-PCR analysis alone provides better discrimination than $(GTG)_5$-PCR or a combination of $(GTG)_5$-PCR and BOX-PCR.

Although discriminatory power is often considered at the forefront when evaluating typing methods, repeatability of the procedure is equally important in determining its overall value. Generally, PFGE is considered a procedure with medium to high repeatability while rep-PCR is considered to have medium to low repeatability (Foxman et al., 2005). Our results are consistent with this pattern, as PFGE was highly repeatable while $(GTG)_5$-PCR and BOX-PCR exhibited low repeatability when used to genotype *M. haemolytica*.

For BOX-PCR and $(GTG)_5$-PCR, the majority of the error related to repeatability was caused by misidentification of bands by the BioNumerics software. Reproducibility of rep-PCR techniques have previously been reported to be problematic (Johnson and O'Bryan, 2000) with qualitative difference in banding patterns highlighted as a specific problem (Gevers et al., 2001). The latter issue was of a particular concern here with respect to the BOX-PCR results and to a lesser extent with the $(GTG)_5$-PCR. Although the rep-PCR banding patterns between replicate profiles were often visually identifiable as the same, variance in the intensity of the bands resulted in misidentifications when bands were identified using image analysis. Visual identification of bands improved repeatability, in particular with BOX-PCR. However, manual identification of bands negates time savings associated with these more rapid procedures and introduces the possibility of subjective bias.

In contrast to rep-PCR, individual PFGE profiles showed consistency in intensity. In the case of PFGE, the error in repeatability was due to a shifting in the band profile of one replicate isolate. This resulted in the loss of one larger fragment with the subsequent appearance of two smaller fragments, indicating that the cause was most likely the result of a point mutation that created a new restriction site (Tenover et al., 1995). Although plasticity in the genome of *M. haemolytica* has been reported (Villard et al., 2008), variation was not observed until after 15 or more repetitive subcultures. In spite of this single incident, PFGE was still found to be a very repeatable procedure and even with the shifting in a single profile, an average repeatability value >95% was maintained.

Congruence and agreement between methods

A low level of congruence was observed between the three techniques examined. Lack of agreement between typing methods has previously been documented (Weigel et al., 2004; Yokoyama et al., 2007) and should be expected as separate typing methods use different sources of genetic variation to produce genetic profiles (CLSI, 2007). Low congruence between genotyping methods may allow for increasing discriminative capability by combining the methods (Yokoyama et al., 2007). This mainly would be applicable to the rep-PCR methods, as they required less processing time, and both had lower discriminatory power compared to PFGE. However, as mentioned above, there was no benefit to combining $(GTG)_5$-PCR and BOX-PCR as the resulting discrimination did not increase to a value greater than that achieved by BOX-PCR alone.

Species Identification

Since the reclassification of the *Mannheimia* genus (Angen et al., 1999), few attempts have been made to develop molecular based methods to speciate this respiratory

pathogen. Of the PCR –based identification assays available (Alexander et al., 2008a, b; Catry et al., 2004; Guenther et al., 2008) none are able to fully speciate *M. haemolytica.* This is mainly because little information is known about the genomes of other *Mannheimia* species. In our study, both the PFGE and rep-PCR methods exhibited some capacity to separate isolates at the species level but only BOX-PCR was capable of clustering all species correctly. Other studies have presented rep-PCR as a promising tool for identification at the species level (Gevers et al., 2001; Masco et al., 2003) and the results here support BOX-PCR for future use as a means of speciation of members of the *Mannheimia* genus.

In conclusion, based on discriminatory power and repeatability, PFGE was more effective than either BOX-PCR or $(GTG)_5$-PCR at genotyping *M. haemolytica.* However, BOX-PCR was shown to provide important information in terms of discrimination and may hold potential for use in epidemiological study of *M. haemolytica* in feedlot cattle. BOX-PCR was also shown to be the most effective means of speciation for members of the *Mannheimia* genus examined. This study supports that rep-PCR holds potential as an alternative to PFGE, which was shown to be not only the most discriminating of the methods examined but also the most time consuming and costly.

CHAPTER FOUR

Genetic characterization and antimicrobial susceptibility of *Mannheimia haemolytica* isolated from the nasopharynx of feedlot cattle in western Canada

4.1 Introduction

Bovine respiratory disease (BRD) is one of the most significant health problems facing the North American cattle industry. Financial losses due to this condition exceeds the combined cost of all other diseases in cattle (Highlander, 2001) as a result of treatment costs, production losses and mortalities (Gagea et al., 2006; Larson, 2005; Snowder et al., 2006). Estimates within the USA place losses between 640 million USD (Babcock et al., 2008; Bowland and Shewen, 2000; Snowder et al., 2007) and 3 billion USD annually (Snowder et al., 2007) but these values are in all likelihood underestimated. Additional costs associated with increased labor, performance losses and reduced carcass value (Bateman et al., 1990; Encinias et al., 2006; Gagea et al., 2006; Larson, 2005) also add to economic losses associated with BRD. The etiology of BRD is complex with its onset linked to a variety of environmental stressors associated mostly with the marketing and transport of cattle. These include practices such as, weaning, castration, shipping, and co-mingling (Gagea et al., 2006) all of which are often executed just prior, or at the time that cattle first enter the feedlot. Combined, these factors may compromise the immune system enabling viral and/or bacterial pathogens to become established in the respiratory tract and contribute to BRD (Cusack et al., 2003). In most cases, illness is thought to be initiated by a viral agent, followed by a secondary bacterial infection that induces severe fibrinonecrotic pneumonia (Kehrenberg et al., 2001b; Whiteley et al., 1992).

Presently, *Mannheimia haemolytica* is considered the principal bacterial pathogen associated with BRD (Gioia et al., 2006; Katsuda et al., 2007; Rice et al., 2007; Welsh et al., 2004; Whiteley et al., 1992; Zecchinon et al., 2005). Although significant information is available that addresses the pathogenic nature of *M. haemolytica* (Ackermann and Brogden, 2000; Highlander, 2001; Mohamed and Abdelsalam, 2008; Zecchinon et al., 2005) little is understood about the epidemiology of this pathogen within the feedlot setting. Recent taxonomic amendments to the *Mannheimia* genera (Angen et al., 1999) have made works published previous to this time ambiguous about the taxa examined. As a result, both epidemiological and long-term surveillance data exclusive to *M. haemolytica* is largely unavailable.

Obtaining a better understanding of the ecology of *M. haemolytica* within the feedlot setting is necessary towards the development of an efficacious means of management. To our knowledge no work has been conducted examining the genetic diversity or nature of antimicrobial resistance in *M. haemolytica* from cattle upon arrival and exit from feed yards. The former is of considerable concern as a clear link has been established between strain type, particularly with regards to serotype A1, and its potential for pathogenicity (Highlander, 2001). The latter is of equal significance as the development of antimicrobial resistance has implications for the efficacy of antimicrobials targeted at controlling BRD. It is the objective of this work to provide epidemiological data that will aid in our understanding of the ecology of *M. haemolytica* within the feedlot sector.

4.2 Materials and Methods

Isolation and identification

Nasal samples were collected from a subset of cattle (approximately 10% from 10% of the pens) housed within two feedlots in the southern region of Alberta, Canada. Samples were taken from corresponding animals at arrival and within the same animals 30 days prior to exit over a period spanning September 2008 to February 2009. Samples were collected with commercially available deep-guarded culture swabs with a Cary-Blair agar reservoir for transport (BD Canada, Inc., Mississauga, ON). All samples were transported on ice to a central shipping location and stored at 4 °C for a period no longer than ten days prior to shipment on ice to a microbiology laboratory for analysis. Upon receipt the samples were suspended in 0.7 ml of brain heart infusion (BHI) broth. A 100 µl aliquot of the suspension was cultured overnight at 37 °C on TSA plates containing 5% sheep blood and 15 µg/ml of bacitracin (Dalynn Biologicals, Inc., Calgary, AB, Canada). A maximum of three *Mannheimia*-like colonies (i.e. white-grey, round, medium sized, non mucoid, exhibiting haemolysis) were subcultured (conditions as above) from each plate and further tested for catalase and oxidase production. Oxidase testing was performed using commercially obtained oxidase testing strips (Oxoid Canada, Inc., Nepean, ON) and catalase testing was conducted by combining a loop full of culture and approximately 10 µl of 3% hydrogen peroxide on a sterile glass slide. A positive response for catalase testing was identified by the production of gas. Those isolates that had typical *M. haemolytica* colony morphology and proved both catalase and oxidase positive were subsequently confirmed as *M. haemolytica* using a multiplex PCR assay

(Alexander et al., 2008b). *M. haemolytica* isolates were stored at -80 °C in BHI broth containing 20% glycerol until further characterization was undertaken.

Preliminary pulsed-field gel electrophoresis (PFGE) analysis was conducted on three isolates (maximum) obtained from each of a dozen randomly selected nasal samples, encompassing both arrival and exit samples. The results revealed that colonies of *M. haemolytica* collected from the same plate were largely clonal (data not shown) or strongly related (>90% similar). As a result, a single isolate from each nasal sample was used for further analysis within this study.

Pulsed-field gel electrophoresis (PFGE)

Preparation of DNA samples and pulsed-field gels adhered to the PulseNet USA protocols employed by the Centers for Disease Control and Prevention, Atlanta, USA (CDC, 1996). Briefly, overnight blood agar cultures (16-18 h at 37 °C) of *M. haemolytica* were suspended in 2 ml of cell suspension buffer (0.1 M Tris; 0.1 M EDTA, pH 8.0) to an absorbance reading between 1.25 and 1.35 at a wavelength of 610 nm. *Salmonella* serotype Braenderup (H9812) was cultured overnight on Luria-Bertani agar then diluted to be within the same OD at 610 nm as for *M. haemolytica* and was used as a reference standard. Agar plugs of each species were prepared from suspensions containing 10 µl of Proteinase K (20 ng/ml of sterile distilled H_2O) combined with 100 µl of a culture suspension and 100 µl 1% SeaKem Gold agarose (in sterile TE, pH 8) (Lonza Canada, Inc., Shawinigan, QC) containing 10% sodium dodecyl sulphate (in sterile distilled H_2O). These were lysed in 5 ml of cell lysis buffer (0.05 M Tris pH 8.0, 0.05 M EDTA pH 8.0, 1% Sarcosyl) containing 25 µl of Proteinase K (20 ng/ml of sdH_2O) at 54 ^oC and 100 rpm for 3 h. The plugs were then washed twice with 10 ml of sterile ultra

pure water and four times with 10 ml of sterile 1xTE (10 mM Tris, 1mM EDTA, pH 8.0) for periods of 15 min at 54 ºC and 100 rpm. *M. haemolytica* DNA was digested overnight at 37 º C with 10 U of *Sal*I endonuclease and 180 µl of associated buffer that was supplied by the manufacturer (Invitrogen Canada, Inc., Burlington, ON). The *Salmonella* serotype Braenderup size standards were digested overnight in a similar fashion with 10 U of *Xba*I endonuclease (Invitrogen Canada, Inc.).

Fragments of DNA were separated by PFGE using the CHEF DRII apparatus (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON) run at 6 V/cm at 12 °C with switch times of 4-40 s for 22 h. Samples were loaded into 1% SeaKem Gold agarose (Lonza Canada, Inc., Shawinigan, QC) that had been dissolved in 0.5X TBE buffer and were run in the same manner with the addition of 0.5 mM thiourea to improve band compression. The gels were stained with 1 µg/ml of ethidium bromide for 30 min followed by three washes in distilled water for the same duration and photographed on a UV transilluminator. Fragment analysis was performed with BioNumerics V5.1 (Applied Maths, Inc., Austin, TX, USA). *M. haemolytica* type strain 33396 (ATCC) was run with each group of isolates to serve as a reproducible standard for comparisons in BioNumerics.

Antibiograms

Disk susceptibility tests were conducted in accordance with the Clinical and Laboratory Standards Institute document M31-A (CLSI, 2007). Reference strains *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619 and *M. haemolytica* ATCC 33396 were used as quality controls. Briefly, Muller-Hinton blood agar cultures (16-18 h at 37 °C) were suspended

into Muller-Hinton broth to an absorbance reading between 0.130 and 0.145 at 625 nm. The prepared inocula were swabbed onto Muller-Hinton blood agar plates using sterile swabs and antimicrobial charged disks were dispensed onto the plate surface. The plates were incubated at 37 °C in ambient air, with the exception of *S. pneumoniae* ATCC 49619 which required cultivation in a 5% CO_2 atmosphere for 24 h. The resulting zones of inhibition were read using the BioMic V3 imaging system (Giles Scientific, Inc., Santa Barbara, CA, USA). The antimicrobials examined and their suppliers as well as the media and resistance breakpoints applied are listed in Table 4.1.

Table 4.1
Antimicrobial agents, suppliers, disk contents and interpretative criteria used for disk susceptibility testing

			Disk Content	Zone diameter breakpoints		
Antimicrobial	Code	Supplier	(ug)	S	I	R
Amoxicillin/Clavulanic Acid[d]	AMCL	BD Biosciences	30	≥27	n/a	≤26
Sulfamethoxazole/trimethoprim[b]	TMSZ	Benex	23.75/1.25	≥24	n/a	n/a
Ampicillin[a]	AMPI	BD Biosciences	10	≥27	n/a	n/a
Ceftiofur[a]	CTIO	BD Biosciences	30	≥21	18-20	≤17
Enrofloxacin[a]	ENRO	BD Biosciences	5	≥21	17-20	≤16
Florfenicol[a]	FLOR	Mast Diagnostics	30	≥19	15-18	≤14
Tulathromycin[a]	TULA	Pfizer/ BD Biosciences	30	≥18	15-17	≤14
Tilmicosin[a]	TILM	BD Biosciences	15	≥14	11-14	≤10
Gentamicin[c]	GENT	BD Biosciences	10	≥15	14-13	≤12
Oxytetracycline[b]	OXYT	Benex	30	≥23	n/a	n/a

[a]M31-A2: Performance Standard for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard- Second Edition

[b]M45-A: Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline

[c]BioMic Vet General

[d]BioMic Pasteurella

PCR

PCR analysis of isolates exhibiting resistance was performed to determine the presence or absence of the determinants listed in Table 4.2. Isolates exhibiting resistance to oxytetracycline were screened for six *tet* resistant genes whereas those exhibiting resistance to ampicillin and/or amoxicillin/clavulanic acid were screened for $bla_{\text{ROB-1}}$, bla_{OXA1}, $bla_{\text{TEM-1}}$ and $bla_{\text{PSE-1.}}$ Isolates that exhibited macrolide resistance (tilmicosin and/or tulathromycin resistance) were screened for the presence of six *erm* genes.

Table 4.2

Primer sequence, amplicon size, annealing temperature and gene accession numbers for resistance genes screen for by PCR

Resistance gene	PCR primer sequence 5'-3'	Amplicon size	Genbank accession No.[a]	Annealing temp (°C)
*tet*B	TTGGTTAGGGGCAAGTTTTG	659	J01830	55
	GTAATGGGCCAATAACACCG			
*tet*G	GCTCGGTGGTATCTCTGCTG	468	S52437	55
	AGCAACAGAATCGGGAACAC			
*tet*H	ATACTGCTGATCACCGT	1076	Y16103	60
	TCCCAATAAGCGACGCT			
*tet*K	TCGATAGGAACAGCAGTA	169	S67449	55
	CAGCAGATCCTACTCCTT			
*tet*L	TCGTTAGCGTGCTGTCATTC	267	U17153	55
	GTATCCCACCAATGTAGCCG			
*tet*M	GTGGACAAAGGTACAACGAG	406	X90939	55
	CGGTAAAGTTCGTCACACAC			
$bla_{\text{ROB-1}}$	AATAACCCTTGCCCCAATTC	685	X52872	60
	TCGCTTATCAGGTGTGCTTG			
$bla_{\text{OXA-1}}$	TCAACTTTCAAGATCGCA	591	N/a	56
	GTGTGTTTAGAATGGTGA			
$bla_{\text{TEM-1}}$	GTGCGGTATTATCCCGTGTT	416	N/a	58
	AACTTTATCCGCCTCCATCC			
$bla_{\text{PSE-1}}$	CGCTTCCCGTTAACAAGTAC	419	M69058	60
	CTGGTTCATTTCAGATAGCG			
*erm*A	GAAATYGGRTCAGGAAAAGG	332	X03216	55
	AAYAGYAAACCYAAAGCTC			
*erm*B	GATACCGTTTACGAAATTGG	364	X52632	58
	GAATCGAGACTTGAGTGTGC			
*erm*C	TCAAAACATAATATAGATAAA	642	M19652	50
	GCTAATATTGTTTAAATCGTCAAT			
*erm*F	CGACACAGCTTTGGTTGAAC	309	M14730	56
	GGACCTACCTCATAGACAAG			
*erm*T	CATATAAATGAAATTTTGAG	369	AF310974	51
	ACGATTTGTATTTAGCAACC			
*erm*X	GAGATCGGRCCAGGAAGC	488	X51472	58
	GTGTGCACCATCGCCTGA			

[a]n/a, not available

Isolates used for DNA extraction were cultured overnight at 37 °C on blood agar plates. Genomic DNA was purified using the DNeasy Tissue Kit (Qiagen Canada, Inc., Mississauga, ON) according to the manufacturer's instructions with slight modification to the procedure. Lysis was extended overnight at 4 °C and the final elution steps were

reduced to 180 µl to concentrate the product. Genomic DNA was diluted to 5 ng/µl with sterile TE (pH 7.4) and stored at -20 °C until use.

All PCR reaction mixtures (20 µl) contained (final concentrations): 1X HotStarTaq *Plus* Master Mix (Qiagen Canada, Inc.)**,** 2.0 µM primer and 1X CoralLoad Concentrate (Qiagen Canada, Inc.). Two microlitres of purified 5 ng/µl DNA was used as template and all PCR amplifications were performed using an Eppendorf Mastercycler (Eppendorff Canada, Ltd., Mississauga, ON) with the following conditions: 95 °C for 5 min; 30 cycles of 94 °C for 30 s, variable annealing temp (refer to Table 4.2) for 1 min and 72 °C for 1 min; and 72 °C for 8 min.

A modification of the above program was used for *erm* genes where a touchdown assay was applied as described by Chen *et al*. (2007). The program consisted of an initial activation step of 95 °C for 5 min, followed by five cycles of touchdown PCR involving: 94 °C for 30 s, 30 s of annealing temp beginning at 5 °C above the final annealing temperature (Table 4.2) with a 1 °C decrement for each cycle, and extension at 72 °C for 1 min. The five touchdown cycles were followed by regular PCR: 35 cycles of 94 °C for 30 s, respective annealing temperatures (Table 4.2) for 30 s and 72 °C for 1 min; and 72 °C for 8 min.

Statistical analysis

All PFGE related statistical analyses were performed using BioNumerics V5.1 software (Applied Maths, Inc., Austin, TX, USA). Dendrogram analysis used UPGMA clustering of Dice coefficient values with 1.0 % optimization and 1.5 % position tolerance settings. MANOVA analysis was calculated from a binary band-matching character table constructed from the 44 band classes originally identified from the

individual isolate banding profiles. The four categories used for analysis corresponded to the four sampling categories defined (feedlot A and feedlot B, arrival and exit samples) and accounting for covariance was selected within the BioNumerics software.

The Fisher's exact test was used to determine the significance of disparity found in the prevalence of *M. haemolytica* between arrival and exit samples from feedlots A and B.

4.3 Results

Prevalence data

A total of 2487 bovine nasal swabs were processed during the study period (Table 4.3). Recovery of *M. haemolytica* from nasal swabs was similar between the two feedlots, with just over 16 % of the samples being positive. In feedlot B, the prevalence of *M. haemolytica* was higher (P <0.005) in exit samples (20%) than arrival (13.16%) samples. The highest recovery rate of *M. haemolytica* was 20% (feedlot B exit) and the lowest was 13.6% in arrival samples from feedlot B.

Table 4.3
Prevalence of *M. haemolytica*

	No. nasal samples processed			No. nasal samples positive for *M. haemolytica* (%)			Fisher's Exact Test 2-Tail
Feedlot	arrival	exit	total	arrival	exit	total	(p-value)
A	605	595	1200	105 (17.36)	98 (16.48)	203 (16.92)	0.762
B	669	618	1287	88 (13.16)	125 (20.23)	213 (16.56)	0.005
Total	1274	1213	2487	193 (15.15)	223 (18.39)	416 (16.73)	0.072

PFGE Total Data Set

PFGE typing and cluster analysis revealed considerable diversity within the *M. haemolytica* isolates obtained. Of the 414 isolates genotyped, a total of 313 unique pulsotypes were detected (Table 4.4). When the similarity cut-off was set at 85%, analysis revealed 148 clusters of related isolates within the total data set. A high level of diversity was observed from all four classifications (feedlot A and B, arrival and exit) and the average cluster size was low (1.91-2.8).

Table 4.4
Results of *M. haemolytica* PFGE analysis

			similarity cut-off 85%		
	No. isolates	No. unique pulsotypes	No. Clusters	Ave. No. of isolates per cluster (±SD)	Range of isolates per cluster
Feedlot A & B	414	335	148	2.80 (3.72)	1-27
Feedlot A	203	171	96	2.14 (2.88)	1-26
arrivals	106	95	49	2.15 (2.86)	1-20
exits	97	83	48	1.95 (2.65)	1-16
Feedlot B	211	187	95	2.22 (2.26)	1-12
arrivals	82	72	43	1.91 (2.09)	1-10
exits	129	119	71	1.82 (1.65)	1-9

Dendrogram analysis of all isolate profiles identified three main clusters (A, 97 isolates; B, 288 isolates; C, 24 isolates) with divergence at 46.4 %, 48.3% and 61.7%, respectively (Fig. 4.1). A total of five isolates were observed as outliers from the three main groups. Nodes were strategically collapsed with the intention of maintaining the general structure of the dendrogram while at the same time condensing the figure into a manageable size. Visual comparison revealed no clear trend in cluster composition based upon sampling classification. Of the six largest clusters identified, five contained samples from each of the four sample classifications while the remaining cluster was comprised mostly of *M. haemolytica* from arrival cattle sampled at feedlot A.

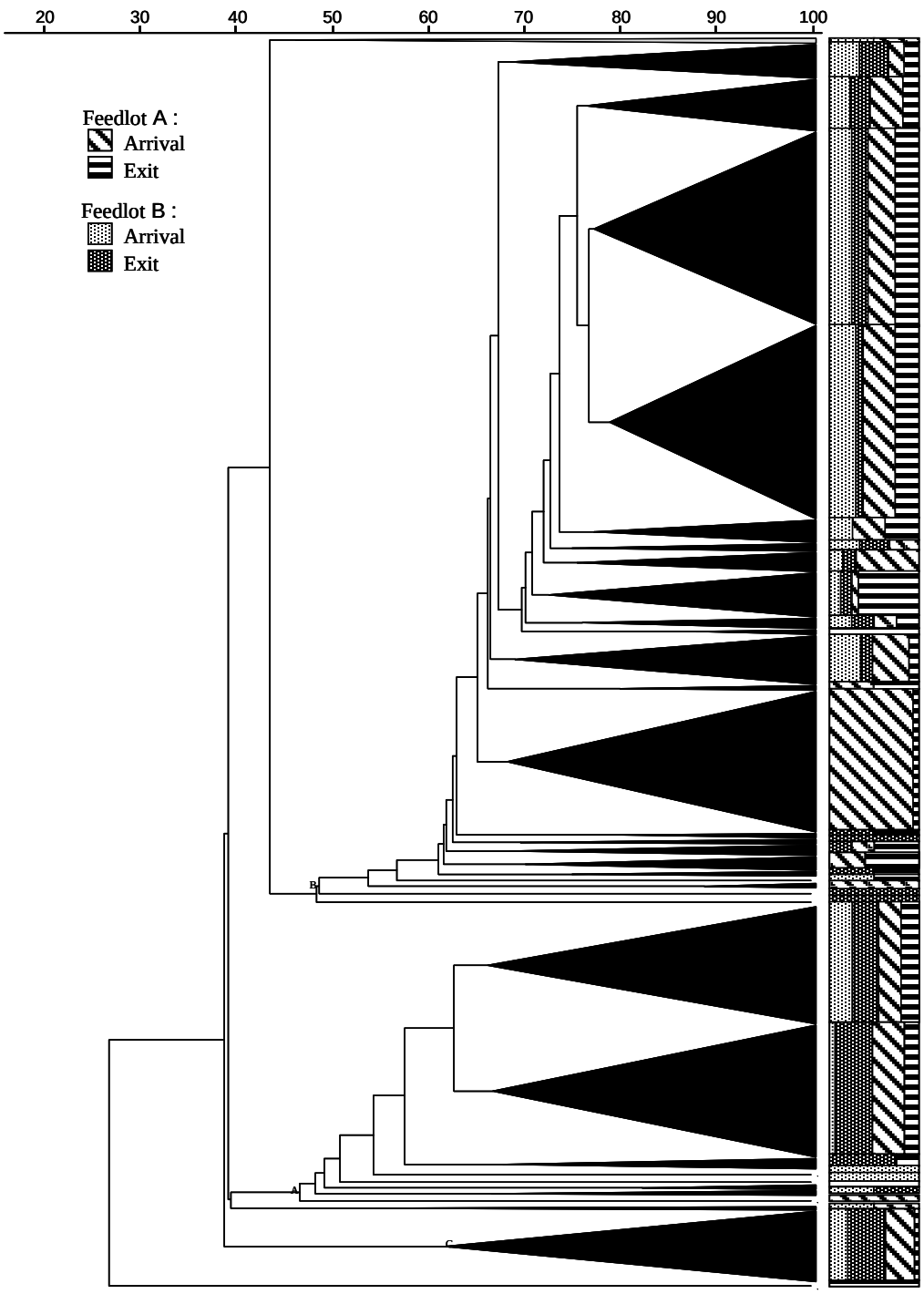


Fig. 4.1. Dendrogram based on PFGE analysis of *M. haemolytica* isolates recovered from bovine nasal swabs collected from two feedlots in southern Alberta, Canada, from 2008 to 2009. Dendrogram (shown here in simplified form) was constructed from UPGMA clustering of Dice coefficient values, optimization 1.0% and position tolerance 1.5%. Triangles represent arborizing subclusters and boxes to the right show the proportion each sampling classification contributed to each subcluster. Three main subclusters labeled A, B and C dominated the expanded form and are comprised of 97, 288 and 24 isolated respectively.

MANOVA analysis was used to create a spatial representation of the degree of separation and/or overlap of the isolates grouped within each feedlot, across each time point (Fig. 4.2). The first two discriminates accounted for 85.9% of the total discrimination. Probability values for the first two discriminates were each ≤ 0.02 and the *L* (Wilk's Lambda likelihood ratio test) for each was 0.40 and 0.69 respectively. No distinct segregation was observed between each of the groups plotted (feedlot A and B, arrival and exit). However, in general, the exit groups from both feedlots demonstrated a higher degree of spread versus the arrival isolates which tended to cluster towards the center of the plot.

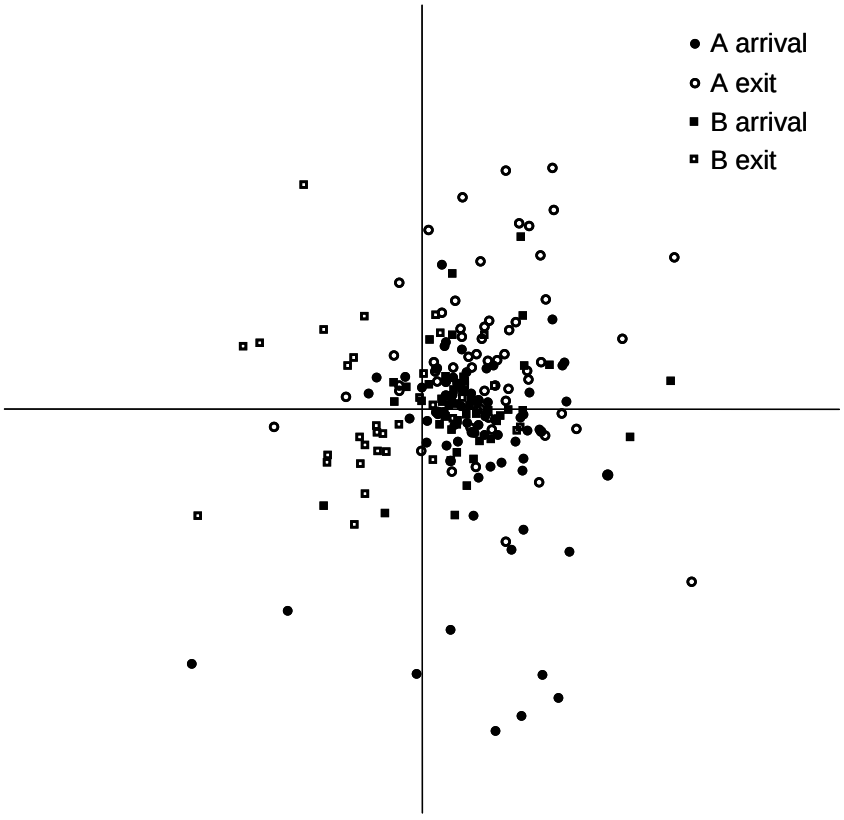


Fig. 4.2. MANOVA of PFGE fingerprints from *M. haemolytica* isolates obtained from bovine nasal swabs. Binary band-matching character tables were analyzed by MANOVA, accounting for the covariance structure. Isolates were categorized into arrival versus exit sample from two feedlots, A and B.

In twenty-four instances (12 from each feedlot) *M. haemolytica* was recovered from an animal at both arrival and exit. All other animals were positive for *M. haemolytica* at either arrival or exit, but not both. Cluster analysis of these twenty-four cases revealed modest relationships, clonal or otherwise. Of the twenty-four examined, only in three instances were the isolates collected at arrival and exit clones (Fig. 4.3).

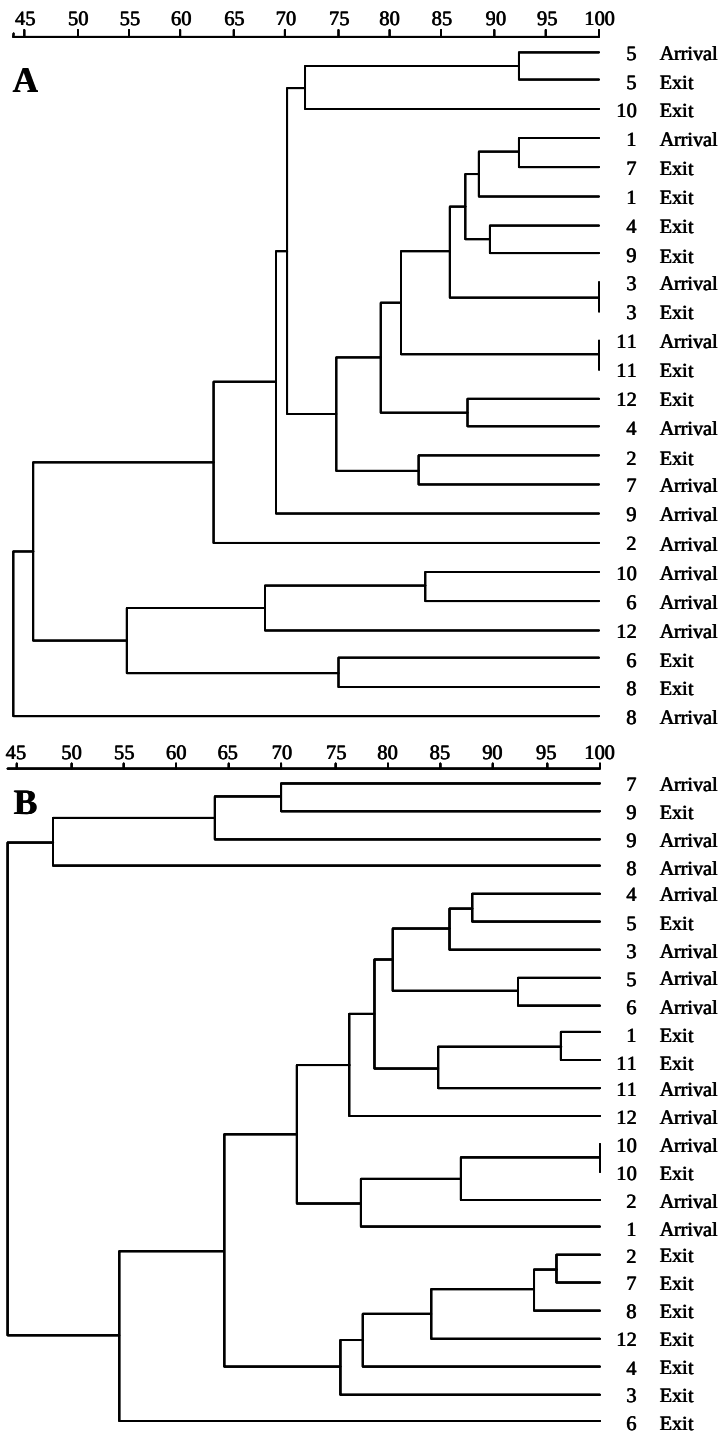


Fig. 4.3. Dendrogram of PFGE profiles from *M. haemolytica* isolates collected from corresponding animals at arrival and exit from feedlot A and feedlot B. Dendrogram created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and position tolerance settings of 1.00% and 1.5%, respectively.

Antibiogram Data

Antimicrobial resistance in *M. haemolytica* was found to be consistently low for all of the antimicrobials examined, ranging from 0.8 to 7.8% (Table 4.5). Complete susceptibility was observed for sulfamethoxazole/trimethoprim, ceftiofur, enrofloxacin, florfenicol and gentamicin. In total, 42 isolates exhibited resistance to at least one of the antimicrobials tested (Table 4.6). This represented 10.3% of the total number of *M. haemolytica* isolates collected. Resistance to a single antimicrobial was most common with resistance to oxytetracycline (*n*=16), ampicillin (*n*=10), amoxicillin/clavulanic acid (*n*=7) and tulathromycin (*n*=3) being observed. Six isolates exhibited resistance to more than one antimicrobial, 3 were resistant to ampicillin and amoxicillin/clavulanic acid, 2 were resistant to oxytetracycline and amoxicillin/clavulanic and 1 exhibited intermediate resistance to both tilmicosin and tulathromycin. There were no obvious trends in the nature of resistance in isolates obtained from different feedlots or at arrival or exit.

Antimicrobial Usage Data

Approximately 40% and 48.7% of the *M. haemolytica* isolates from feedlot A and feedlot B, respectively originated from animals administered an injectable antimicrobial agent. In almost all cases, oxytetracycline was administered; usually over two injections within 1-14 days after arrival at the feed yard. At both feedlots, ceftiofur and tulathromycin were most commonly administered subsequent to the use of oxytetracycline. Of the isolates in this study that expressed antimicrobial resistance phenotypes, less than half originated from cattle that were treated with injectable antimicrobials at the feedlot. Of those that received treatment, five showed correlation between the treatment administered and resistance phenotype.

Table 4.5

Antimicrobial resistance in *M. haemolytica* recovered from bovine nasal swabs

	% antimicrobial resistance				
	feedlot A		feedlot B		
	arrival (103)	exit (97)	arrival (80)	exit (129)	Total (409)
Amoxicillin/Clavulanic Acid	4.9	2.1	1.3	1.6	2.4
Sulfamethoxazole/trimethoprim	0.0	0.0	0.0	0.0	0.0
Ampicillin	4.9	4.1	6.3	0.8	3.7
Ceftiofur	0.0	0.0	0.0	0.0	0.0
Enrofloxacin	0.0	0.0	0.0	0.0	0.0
Florfenicol	0.0	0.0	0.0	0.0	0.0
Tulathromycin	0.0	1.0	2.5	0.8	1.0
Tilmicosin	0.0	1.0	0.0	0.0	0.2
Gentamicin	0.0	0.0	0.0	0.0	0.0
Oxytetracycline	7.8	3.1	1.3	4.7	4.4

Table 4.6

Resistance profiles, gene determinants and antimicrobial treatment records for *M. haemolytica* field isolates expressing resistance phenotypes

Isolate	Feedlot	Sampling event	AMR profile[a]	AMR genes[c]	Enrolment date	Exit date	Treatment date[d]	Treatment (mg/kg)[e]
143A	B	Arrival	AMP	n/d	3-Oct-07	18-Jan-08	n/t	n/t
239A	B	Arrival	AMP	n/d	17-Oct-07	27-Mar-08	17-Oct-07	oxy-30
							17-Oct-07	oxy-30
240A	B	Arrival	AMP	n/d	17-Oct-07	27-Mar-08	17-Oct-07	oxy-30
							17-Oct-07	oxy-30
235A	B	Arrival	TULA	n/d	17-Oct-07	27-Mar-08	17-Oct-07	oxy-30
							17-Oct-07	oxy-30
364A	B	Arrival	AMP, AMCL	n/d	25-Oct-07	no sample	n/t	n/t
369A	A	Arrival	AMP	n/d	25-Oct-07	09-May-08	16-Feb-08	ceft-1
							17-Feb-08	ceft-1
							18-Feb-08	ceft-1
							02-Nov-07	oxy-20
							25-Oct-07	oxy-30
376A	B	Arrival	AMP	n/d	25-Oct-07	03-Apr-08	18-Nov-07	tula-2.5
398A	A	Arrival	AMP, AMCL	n/d	26-Oct-07	09-May-08	02-Nov-07	oxy-20
							26-Oct-07	oxy-30
410A	A	Arrival	AMCL	n/d	28-Oct-07	08-May-08	06-Nov-07	oxy-20
							28-Oct-07	oxy-30
492A	A	Arrival	AMP, OXYT	bla_{ROB-1}	10-Nov-07	28-Mar-08	17-Nov-07	oxy-20
							10-Nov-07	oxy-30
501A	A	Arrival	AMP, OXYT	bla_{ROB-1}	10-Nov-07	14-Apr-08	17-Nov-07	oxy-20
							10-Nov-07	oxy-30
511C	B	Arrival	OXYT	*tet*(H)	15-Nov-07	29-May-08	15-Nov-07	oxy-30
553A	B	Arrival	TULA	n/d	3-Dec-07	20-Feb-08	n/t	n/t
585A	B	Exit	OXYT	*tet*(H)	29-Sep-07	3-Jan-08	n/t	n/t
611A	A	Exit	OXYT	*tet*(H)	24-Sep-07	18-Jan-08	n/t	n/t
667A	B	Exit	OXYT	n/d	10-Oct-07	12-Feb-08	n/t	n/t
894A	B	Exit	OXYT	n/d	10-Dec-07	16-Mar-08	n/t	n/t
932A	B	Exit	OXYT	*tet*(H)	15-Oct-07	13-Mar-08	15-Oct-07	oxy-30
							15-Oct-07	oxy-30
953A	A	Exit	AMP	n/d	16-Oct-07	18-Mar-08	25-Oct-07	oxy-20
							16-Oct-07	tula-2.5
956A	B	Exit	OXYT	*tet*(H)	24-Oct-07	18-Mar-08	n/t	n/t
1040A	B	Exit	AMP	n/d	17-Oct-07	27-Mar-08	17-Oct-07	oxy-30
							17-Oct-07	oxy-30
1059A	A	Exit	TILM[b], TULA[b]	*erm*(X)	11-Oct-07	28-Mar-08	19-Oct-07	oxy-20
							11-Oct-07	oxy-30
1243A	B	Exit	AMCL	n/d	25-Jan-08	15-Apr-08	25-Jan-08	oxy-20

							25-Jan-08	oxy-20
1316A	A	Exit	AMCL	n/d	31-Oct-07	18-Apr-08	06-Nov-07	oxy-20
							31-Oct-07	oxy-30
1329A	A	Arrival	AMCL	n/d	25-Apr-08	03-Jul-08	n/t	n/t
1339A	A	Arrival	AMP, AMCL	n/d	25-Apr-08	03-Jul-08	n/t	n/t
1347A	A	Arrival	AMCL	n/d	25-Apr-08	03-Jul-08	n/t	n/t
1349A	A	Arrival	OXYT	*tet*(H)	25-Apr-08	03-Jul-08	n/t	n/t
1355A	A	Arrival	OXYT	*tet*(H)	26-Apr-08	10-Jul-08	n/t	n/t
1358A	A	Arrival	OXYT	*tet*(H)	26-Apr-08	10-Jul-08	n/t	n/t
1359A	A	Arrival	OXYT	*tet*(H)	26-Apr-08	10-Jul-08	n/t	n/t
1365A	A	Arrival	OXYT	*tet*(H)	26-Apr-08	10-Jul-08	n/t	n/t
1425A	A	Exit	OXYT	*tet*(H)	21-Feb-08	07-May-08	21-Feb-08	oxy-20
1500A	A	Exit	AMP	n/d	26-Oct-07	09-May-08	02-Nov-07	oxy-20
							26-Oct-07	oxy-30
1548A	A	Arrival	OXYT	*tet*(H)	16-May-08	22-Jul-08	n/a	n/a
1719A	B	Exit	AMCL	n/d	10-Dec-07	19-Jun-08	n/a	n/a
1731A	B	Exit	TULA	n/d	1-Mar-08	19-Jun-08	n/a	n/a
1745A	A	Exit	AMP	n/d	11-Apr-08	20-Jun-08	n/a	n/a
1828A	A	Exit	AMCL	n/d	25-Apr-08	03-Jul-08	n/a	n/a
1829A	A	Exit	AMP	n/d	25-Apr-08	03-Jul-08	n/a	n/a
1886A	A	Exit	OXYT	*tet*(H)	2-May-08	07-Jul-08	n/a	n/a
5450A	B	Exit	OXYT	n/d	09-Oct-08	25-Feb-09	n/a	n/a

[a]AMP, ampicillin; AMCL, amoxicillin-clavulanic acid; OXYT, oxytetracycline; TILM, tilmmicosim; TULA, tulathromycin

[b]intermediate resistance

[c]n/d, gene not detected

[d]n/a, data not available; n/t, animal not treated

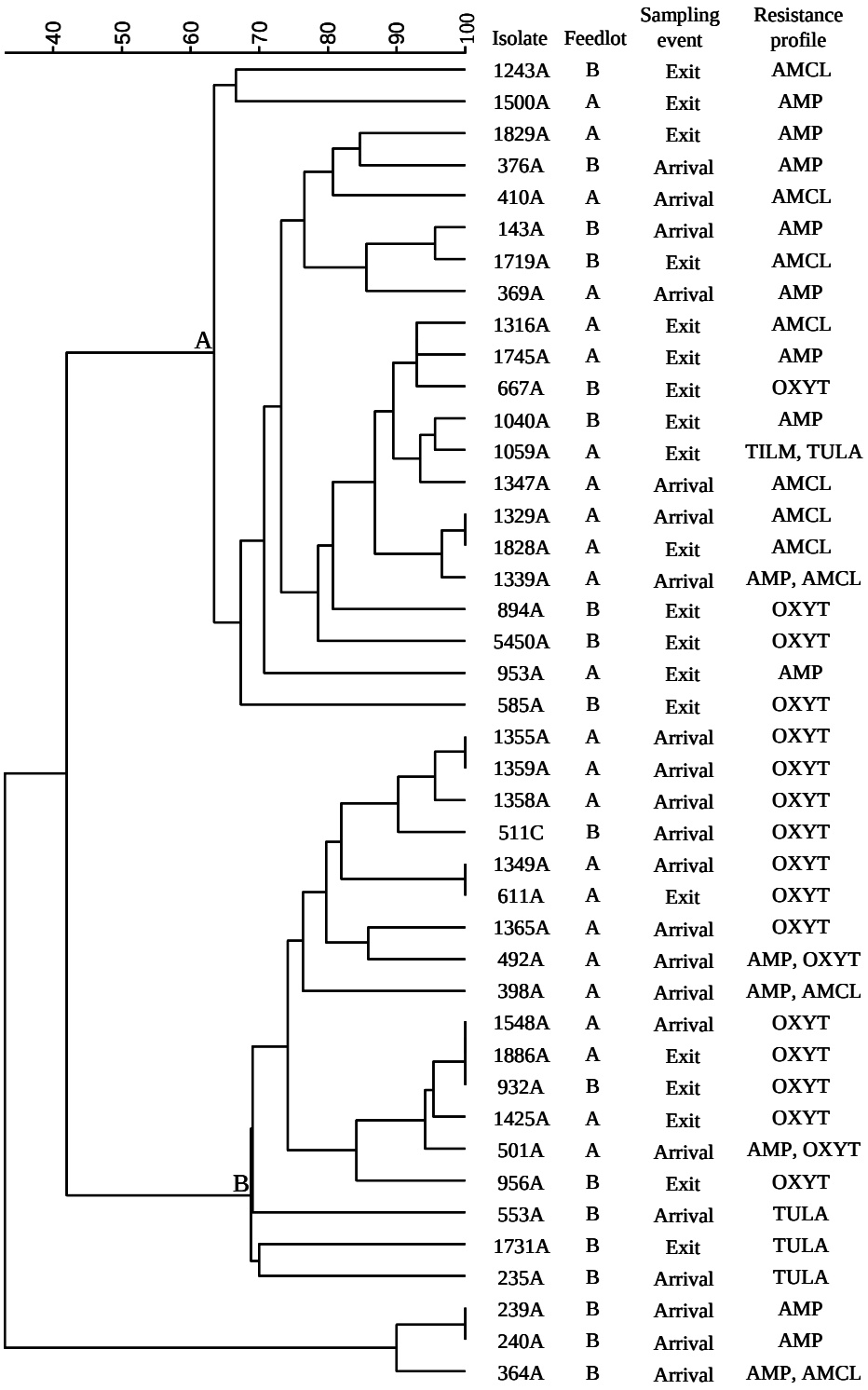
[e]oxy-03, oxytetracycline 30 mg/kg; oxy-20, oxytetracycline 20 mg/kg; ceft-1, ceftiofur 1 mg/kg; tula-2.5, tulathromycin 2.5 mg/kg

PCR Analysis

PCR analysis showed that the majority of the oxytetracycline resistant isolates harboured the *tet*H gene. The two isolates that tested negative for *tet*H were also negative for all of the *tet* genes that were screened (*tet*B, *tet*G, *tet*K, *tet*L, *tet*M). Of the four macrolide resistant isolates screened, the isolate that showed intermediate resistance for both tilmicosin and tulathromycin was the only one positive for *erm*X. The rest were negative for all of the six *erm* genes screened. None of the resistance determinants examined were detected in the amoxicillin/clavulanic acid resistant isolates and of the isolates showing ampicillin resistance only two were positive for $bla_{\text{ROB-1}}$.

PFGE Resistant Isolates

There was no apparent relationship between antimicrobial resistance, sampling location or sampling time of resistant isolates (Fig. 4.4). Resistant isolates were shown to be diverse with the two main groups (A, ampicillin and amoxicillin/clavulanic acid resistant isolates and B, oxytetracycline resistant isolates) exhibiting a 40% relatedness.



Isolate	Feedlot	Sampling event	Resistance profile
1243A	B	Exit	AMCL
1500A	A	Exit	AMP
1829A	A	Exit	AMP
376A	B	Arrival	AMP
410A	A	Arrival	AMCL
143A	B	Arrival	AMP
1719A	B	Exit	AMCL
369A	A	Arrival	AMP
1316A	A	Exit	AMCL
1745A	A	Exit	AMP
667A	B	Exit	OXYT
1040A	B	Exit	AMP
1059A	A	Exit	TILM, TULA
1347A	A	Arrival	AMCL
1329A	A	Arrival	AMCL
1828A	A	Exit	AMCL
1339A	A	Arrival	AMP, AMCL
894A	B	Exit	OXYT
5450A	B	Exit	OXYT
953A	A	Exit	AMP
585A	B	Exit	OXYT
1355A	A	Arrival	OXYT
1359A	A	Arrival	OXYT
1358A	A	Arrival	OXYT
511C	B	Arrival	OXYT
1349A	A	Arrival	OXYT
611A	A	Exit	OXYT
1365A	A	Arrival	OXYT
492A	A	Arrival	AMP, OXYT
398A	A	Arrival	AMP, AMCL
1548A	A	Arrival	OXYT
1886A	A	Exit	OXYT
932A	B	Exit	OXYT
1425A	A	Exit	OXYT
501A	A	Arrival	AMP, OXYT
956A	B	Exit	OXYT
553A	B	Arrival	TULA
1731A	B	Exit	TULA
235A	B	Arrival	TULA
239A	B	Arrival	AMP
240A	B	Arrival	AMP
364A	B	Arrival	AMP, AMCL

Fig. 4.4. Dendrogram of PFGE profiles from *M. haemolytica* isolates expressing antimicrobial resistant phenotype. Dendrogram created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and position tolerance settings of 1.00% and 1.5% respectively. Location, sampling time, antibiogram profile (also shown by group colors), detected gene determinant and available antimicrobial usage data provided.

4.4 Discussion

Longitudinal surveillance data focusing on the genetic diversity of *M. haemolytica* in feedlot cattle is largely absent from the literature. It was the intention of this study to document the prevalence and diversity of *M. haemolytica* found in feedlot cattle housed in two feed yards located in the southern region of Alberta, Canada and to investigate the effect antimicrobial use has on the subsequent development and spread of antimicrobial resistance in this important bovine pathogen.

PFGE

The data from this study revealed an impressive amount of genetic diversity in the *M. haemolytica* isolates examined both within and between feedlots. In western Canada, the average truck load arriving at a feed yard can average 60 calves sourced from as many as 20 to 30 different farms (Ribble et al., 1995c). This would account for the genetic variation observable in arrival samples, but the amount of genetic diversity observed among the exit samples is an inexplicable finding. The propensity of the *M. haemolytica* genome to be both plastic (Kodjo et al., 1999; Villard et al., 2008) and adaptable (Davies et al., 2001; Larsen et al., 2009; Rowe et al., 2001) has been previously established and may provide basis for the significant diversity observed over the duration of the sampling period but does not explain the lack of observable dissemination of genotypes over the sample period. Although previous work employing PFGE for genotypic analyses of *M. haemolytica* collected from cattle is limited, a single study conducted by Katsuda et al. (2003) did show a similar level of diversity in *M. haemolytica* serotype A1 isolates collected from cattle across locations in Japan.

There was no clear association detected between pulsotypes and sampling location in our study. On multiple occasions, clonal or strongly related isolates were recovered from both feedlots and across both sampling times, indicating a high degree of dispersion of

M. haemolytica strains within this study region. Other accounts examining *M. haemolytica* collected from multiple locals have documented a similar lack in correlation been origin and molecular subtype (Katsuda et al., 2003; Villard et al., 2006). For this particular study, some of the commonality observed between feedlots could be attributed to both feed yards from a common stocking source such as an auction mart.

It is acknowledged that a more ideal sampling point for arrival samples would have occurred around ten days after the calves arrived within the feed yard environment. This would allow the opportunity for dissemination of *M. haemolytica* to occur among animals and would coincide with the typical period where respiratory infection is observed to most frequently occur. Unfortunately this approach was not feasible for use in a study of this scale.

Out of a total of 413 samples, only in twenty- four cases occurred where *M. haemolytica* was recovered from individuals both at arrival and upon exit from the feedlots, suggesting that *M. haemolytica* may not persist in the nasal cavity of cattle throughout the feeding period or that a large number of the calves acquire this bacterium post-arrival. There is currently no data available regarding the persistence of *M. haemolytica* within feedlot cattle. However, its capacity for long term survival *in vivo* (Rowe et al., 2001) and its ability to persist in spite of prophylactic treatment in bighorn sheep (Weiser et al., 2009) has been previously documented and speaks towards its

potential for persistence. Genotypic analysis of the correlated arrival/exit cases revealed little incidence of clonality between isolates collected from each sampling point. Of the twenty- four cases identified only in three were the arrival and exit samples clones, with the remainder of arrival and exit samples exhibiting considerable diversity. These findings, in combination with the substantial number of exit cases that show no corresponding infection at arrival, imply that the feed yards themselves may be a potential source of *M. haemolytica*.

Given the animal-to-animal variation observed in pulsotypes, clonal spread of *M. haemolytica* throughout the feedlots examined appears to be limited. This finding is counter intuitive from what is expected if the feedlot environment were acting as a source of *M. haemolytica*. One potential explanation for this was touched upon above. It is possible that acquisition of *M. haemolytica* is sporadic and inconsistent, and is being derived from an environmental community. There is also the potential that some of the diversity observed is a product of divergence of *M. haemolytica*. Previous work conducted in the laboratory has documented the apparent instability of the *M. haemolytica* genome (Villard et al., 2008), but it is not known if this elasticity in the genome of *M. haemolytica* is retained *in vivo*. In our study, the duration between sampling periods (arrival to exit) was between three and six months. It is unclear if this amount of time, given the presence of *in vivo* selective pressure, would be adequate to account for the highly diverse nature of the isolates obtained. Further work that correlates the origin of incoming cattle with pulsotypes from cattle within the feed yard and those that examine the environmental and *in vivo* stability/persistence of *M.*

haemolytica over time may provide further insight into the origin of the genetic diversity observed.

Antimicrobial resistance

Currently, antimicrobial agents present the most effective tool available for control of respiratory infections in cattle (Kehrenberg et al., 2001b). As a result, the development of antimicrobial resistance in response to antimicrobial use is of concern. The results of this study reveal low levels of antimicrobial resistance in *M. haemolytica* , implying that at present the development and spread of resistance in *M. haemolytica* in response to antimicrobial use is low. Recent publications by Catry et al. (2005) and Hendricksen (2008) report similarly low levels of resistance in *M. haemolytica* and the majority of the literature reflects the same general trends in resistance phenotypes with a trend towards resistance to tetracycline (Berge et al., 2006; Catry et al., 2007; Henricks et al., 1992; Post et al., 1991; Watts et al., 1994), ampicillin (Catry et al., 2007; Hendriksen et al., 2008; Post et al., 1991; Schwarz et al., 2004; Watts et al., 1994) and sulfa drugs (Hendriksen et al., 2008; Post et al., 1991; Watts et al., 1994) with a high level of susceptibility to ceftiofur (Berge et al., 2006; Catry et al., 2007; Hendriksen et al., 2008; Schwarz et al., 2004) and florfenicol (Berge et al., 2006; Catry et al., 2007; Schwarz et al., 2004).

Scrutiny of the antimicrobial resistant pulsotypes revealed no observable link with resistance phenotype. There were very few instances of clonal spread observed either within or between feedlots and multiple occurrences of closely related isolates that exhibited dissimilar resistant phenotypes. Further, there was a lack of relationship exhibited between isolates harbouring identical resistance determinants. These findings

indicate that the resistance genes detected here are likely located on transferable elements. This is consistent with previous reports that claim most resistance genes in *M. haemolytica* are found on small plasmids or transposons (Kehrenberg et al., 2001b).

PCR

Tetracyclines have been reported to account for almost two-thirds of the antimicrobials used in prophylactic treatment (Kehrenberg et al., 2001a). This is consistent with what was reported here as the vast majority of antimicrobial usage in both feedlots consisted of the administration of oxytetracycline within 1-14 days of arrival into the feed yards. Not surprisingly, oxytetracycline resistance was the most frequently observed phenotype expressed by the resistant isolates identified. The *tet*H gene was the major allele detected from those isolates exhibiting oxytetracycline resistant phenotypes which is consistent with previous findings (Highlander, 2001). Based upon what was observed here, the lack of apparent diversity in tetracycline resistant alleles in combination with a lack of clonal spread of the resistant isolates implies that horizontal transfer may be the potential mechanism for spread of oxytetracycline resistance within each of these feedlots.

Next to oxytetracycline resistance, the most frequently observed resistant phenotypes were to ampicillin and amoxicillin/clavulanic acid. Because neither of these antimicrobials were used in either of the feedlots studied, it is suspected that these isolates may have already harboured resistance upon entry. The maintenance of resistance to both drugs from arrival through to exit was unexpected given the lack of an obvious selective pressure. The $bla_{\text{ROB-1}}$ gene was the only determinant detectable from any of the ampicillin or amoxicillin/clavulanic acid resistant isolates. It was found in two

oxytetracycline/ampicillin resistance isolates that originated from same group of cattle that arrived at the same time in one feedlot. Interestingly these isolates were not strongly related based upon PFGE pulsotype and this phenotype did not appear to persist, as it was not detected in any of the exit samples. Further genetic characterization of the resistance determinants detected here is required to comment on their rate of development and spread.

In conclusion, PFGE analysis revealed a significant amount of genetic diversity in the *M. haemolytica* isolates examined, both within and between feedlots. Clonal spread throughout the feed yards appeared to be limited. A low incidence of antimicrobial resistance was detected with the antimicrobial panel utilized here, implying that at present, the development and spread of resistance in *M. haemolytica* in response to antimicrobial use within the feedlots was low. Further studies are necessary to determine if the results reported here are representative of what is occurring in *M. haemolytica* populations in other feedlots across North America.

CHAPTER FIVE

Conclusions and Prospects

Despite the current knowledge surrounding the condition, BRD continues to be a significant problem for cattle producers in North America. In the U.S.A., BRD is considered to be the most costly disease to beef cattle (Snowder et al., 2006). In western Canada, it is reported that 10-30% of all auction market derived cattle are treated for BRD, with the overall mortality observed between two and four percent (Booker et al., 2008). Bearing in mind that *Mannheimia haemolytica* is identified as a primary bacterial agent in association with BRD (Rice et al., 2007), it is startling how little is understood about the dynamics of this pathogen within the feedlot setting. Particularly in Alberta which boasts the fifth largest cattle feeding area in North America and sources roughly 40% of the 17.3 million head comprising Canada's national cattle and calf inventories (ABP, 2009).

Antimicrobial therapies are the primary tool used for management of BRD. Over 80% of the antimicrobials licensed for use in cattle are directed against agents associated with BRD (Bowland and Shewen, 2000). Because of this, pathogens such as *M. haemolytica* are possible high-risk targets for the development and spread of antimicrobial resistance. The primary objectives of this work were to 1) characterize and document the genetic diversity of *M. haemolytica* present in two feedlots in southern Alberta and 2) to examine the potential effects antimicrobial usage within these environments might have on the development and spread of antimicrobial resistance within populations of this important veterinary pathogen.

Over the duration of this study, 2487 cattle nasal swab samples were processed and screened for the presence of *M. haemolytica.* Although recovery values ranged from 13-20%, the average prevalence from both collection sites, over both sampling times averaged 16%. Data relevant to the current prevalence of *M. haemolytica* in Canadian feedlots is largely unavailable. More information, similar in kind to what is presented here, is needed in order to help reveal the specific role *M. haemolytica* plays in the overall condition of BRD within feedlots.

PFGE analysis was used to genetically fingerprint 414 *M. haemolytica* isolates. The percentage of unique pulsotypes from the total number of isolates examined was well above 80%, showing a significant amount of diversity. What's more, this level of diversity was consistently high when examining each sampling classification (each feedlot A and B, at either arrival or exit). This finding is interesting because although it was initially suspected that the diversity between feedlots would be high, it was felt that within each location, the diversity observed would be moderate due to the effects of clonal spread. A great deal of the genetic diversity observed in arrival samples was in no doubt due to various stocking sources that are combined at market to contribute to each load of calves trucked to the feedlots. However, the rational behind the genetic diversity observed in the exit samples remains elusive.

Based upon the findings here, clonal spread of *M. haemolytica* throughout both feedlots examined was limited. On multiple occasions, clonal or strongly related isolates were recovered from both feedlots and across both sampling times, indicating a high degree of dispersion. For this study, much of the commonality observed between feedlots might be attributed to the continuing stream of influx cattle from potentially

common stocking sources or might somehow be linked to the proximity of the feed yard to one another as they were within a 50 km radius of one another. To determine if the results here are the by-product of the dynamics of *M. haemolytica* populations within the feedlot environment or the by-product of a limited geographic region of study, future work correlating the origin of incoming cattle with pulsotypes from cattle already housed within the feed yard would be beneficial. Also, collection of isolates from a more expansive and/or distant regions would serve to give a clearer picture about the overall diversity of the *M. haemolytica* populations in North America and how they might move throughout the beef production cycle.

Although PFGE is considered to be the paragon for molecular typing it has some limitations with regards to its long turnaround time and requirement for expensive, specialized equipment. As a consequence, rep-PCR methods BOX-PCR and $(GTG)_5$-PCR were applied to a subset of *M. haemolytica* isolates and *Mannheimia* spp. reference strains to examine the potential for application of rep-PCR in alternative. As was hypothesized, against rep-PCR techniques, PFGE analysis was shown to be the most appropriate method with regards to discriminatory power and repeatability. However it was also discovered that BOX-PCR may hold significant potential as a less time consuming and less costly alternative to PFGE analysis for speciation of the *Mannheimia* genera. It is hoped that the availability of fast and inexpensive methods for genetic analysis will result in a higher incidence of larger scale genotypic investigations of *M. haemolytica* and *Mannheimia* spp. Future genomic sequencing of *Mannheimia* species will hopefully allow for improved primer selection resulting in the increased value of rep-PCR methods for speciation of this genus.

Disk susceptibility testing was conducted to determine the antimicrobial resistance profiles of the *M. haemolytica* isolates studied. This method was cost effective and allowed for the efficient testing of many antimicrobial compounds simultaneously. Consistent with recent reports (Catry et al., 2005; Hendriksen et al., 2008) the results here revealed low incidence of antimicrobial resistance in the *M. haemolytica* examined. This implies that at present, the development and spread of resistance in *M. haemolytica* in response to antimicrobial use within the feedlots is low, suggesting that the use of antimicrobial therapy within the feedlot setting may present minimal risk toward the development of antimicrobial resistance. It is important to take into account that although the antimicrobials selected for examination in this study were chosen to represent those currently used for treatment, they do not encompass all the drugs utilized. It is also important to consider that antimicrobial resistance can develop in bacteria that are associated with cattle other than *M. haemolytica*, many of which can produce and transfer mobile genetic elements. Regardless, the use of antimicrobial therapy is the primary tool against respiratory disease in cattle and the findings here support their continued use. However, it is the opinion of the author that continued surveillance of antimicrobial resistance in the feedlot populations of *M. haemolytica* would be prudent.

Of the resistance phenotypes detected, the most abundant was resistance to oxytetracycline followed by ampicillin resistance and then amoxicillin/clavulanic acid resistance. A few cases of multi-drug resistance were detected but in general its occurrence was uncommon. PCR analysis for a select number of known tetracycline determinants revealed the majority of oxytetracycline resistant isolates to harbour the *tet*(H) gene. This is consistent with previous findings that report this to be the most

commonly found *tet* allele in *M. haemolytica* (Highlander, 2001). The majority of the ampicillin and amoxicillin/clavulanic acid resistant isolates screened negative for all the genes tested. Future work that focuses on further genetic characterization of resistance genes expressed, particularly through plasmid profiling, could shed light regarding the extent of development and/or of movement of these elements throughout *M.haemolytica* populations within the feedlot environment.

There is significant concern over the development of antimicrobial resistance as a result of antimicrobial use in current cattle production systems. This concern is manifested on both a production level, from producers that face increased production costs through increased morbidity and mortality of livestock, and from a public health standpoint, where the potential resides for antimicrobial resistant pathogens to infect human populations through animal contact or the food chain (Call et al., 2008). In order to address the degree of causality between current practices and the development of antimicrobial resistance, surveillance based research is required. The work conducted here is a subsidiary of a larger surveillance program designed to address issues regarding antimicrobial use and the development of antimicrobial resistance in enteric, pathogenic and zoonotic bacteria in the feedlot sector. It is hoped that the information presented here will be a stepping stone to aid in responsible decision making regarding antimicrobial use in the food production industry, the intention to help maximize antimicrobials as a resource while minimizing the threat of antimicrobial resistance as a result of their use.

Because BRD is a continuing problem in the cattle production, insight into the major causative agents of the condition will only serve toward the development of better management strategies whether that be drug therapy or otherwise. The works presented

here depicts *M. haemolytica* as a very genetically diverse pathogen with low levels of AMR against the types of antimicrobials tested and hints at some puzzles as to the dynamics of this species within the feedlot setting. The information here provides a good starting point from which to direct further investigation regarding *M. haemolytica* as it is clear that more work focusing on the epidemiology of this pathogen within the feedlot sector is needed.

REFERENCES

ABP, 2009, Alberta cattle industry statistics. Retrieved October 19, 2009 from http://albertabeef.org/consumers/new-page/-

Abdullah, K.M., Lo, R.Y., Mellors, A., 1991, Cloning, nucleotide sequence, and expression of the *Pasteurella haemolytica* A1 glycoprotease gene. J Bacteriol 173, 5597-5603.

Ackermann, M.R., Brogden, K.A., 2000, Response of the ruminant respiratory tract to *Mannheimia* (*Pasteurella*) *haemolytica*. Microbes Infect 2, 1079-1088.

Adams, O.R., Brown, W.W., Chow, T.L., Collier, J.R., Davis, R.W., Griner, L.A., Jensen, R., Pierson, R.E., Wayt, L.K., 1959, Comparison of infectious bovine rhinotracheitis, shipping fever, and calf diphtheria of cattle. J Am Vet Med Assoc 134, 85-89.

Adamu, J.Y., 2007, *Mannheimia haemolytica*: Phylogeny and genetic analysis of its major virulence factors. Israel J Vet Med 62, 6-13.

Al-Ghamdi, G.M., Ames, T.R., Baker, J.C., Walker, R., Chase, C.C., Frank, G.H., Maheswaran, S.K., 2000, Serotyping of *Mannheimia* (*Pasteurella*) *haemolytica* isolates from the upper Midwest United States. J Vet Diagn Invest 12, 576-578.

Alexander, T.W., Cook, S.R., Yanke, L.J., Booker, C.W., Morley, P.S., Read, R.R., Gow, S.P., McAllister, T.A., 2008b, A multiplex polymerase chain reaction assay for the identification of *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia ruminalis*. Vet Microbiol 130, 165-175.

Angen, O., Ahrens, P., Bisgaard, M., 2002, Phenotypic and genotypic characterization of *Mannheimia* (*Pasteurella*) *haemolytica*-like strains isolated from diseased animals in Denmark. Vet Microbiol 84, 103-114.

Angen, O., Mutters, R., Caugant, D.A., Olsen, J.E., Bisgaard, M., 1999, Taxonomic relationships of the *[Pasteurella] haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. Int J Syst Bacteriol 49 Pt 1, 67-86.

Apley, M., 2006, Bovine respiratory disease: pathogenesis, clinical signs, and treatment in lightweight calves. Vet Clin North Am Food Anim Pract 22, 399-411.

Babcock, A.H., White, B.J., Dritz, S.S., Thomson, D.U., Renter, D.G., 2008, Feedlot health and performance effects associated with the timing of respiratory disease treatment. J Anim Sci 87, 314-327

Bateman, K.G., Martin, S.W., Shewen, P.E., Menzies, P.I., 1990, An evaluation of antimicrobial therapy for undifferentiated bovine respiratory disease. Can Vet J 31, 689-696.

Berge, A.C., Sischo, W.M., Craigmill, A.L., 2006, Antimicrobial susceptibility patterns of respiratory tract pathogens from sheep and goats. J Am Vet Med Assoc 229, 1279-1281.

Berggren, K.A., Baluyut, C.S., Simonson, R.R., Bemrick, W.J., Maheswaran, S.K., 1981, Cytotoxic effects of *Pasteurella haemolytica* on bovine neutrophils. Am J Vet Res 42, 1383-1388.

Biberstein, E.L., Francis, C.K., 1968, Nucleic acid homologies between the A and T types of *Pasteurella haemolytica*. J Med Microbiol 1, 105-108.

Biberstein, E.L., Gills, M., Knight, H., 1960, Serological types of *Pasteurella haemolytica*. Cornell Vet 50, 283-300.

Bingham, D.P., Moore, R., Richards, A.B., 1990, Comparison of DNA:DNA homology and enzymatic activity between *Pasteurella haemolytica* and related species. Am J Vet Res 51, 1161-1166.

Bisgaard, M., 1984, Comparative investigations of *Pasteurella haemolytica* sensu stricto and so-called *P. haemolytica* isolated from different pathological lesions in pigs. Acta Pathol Microbiol Immunol Scand [B] 92, 201-207.

Blackalu, P., Angen, Fegan, N., Blackall, L., Mutters, R., Bisgaard, M., 2001, Characterisation of a novel *Mannheimia* sp from Australian feedlot cattle. Aust Vet J 79, 634-639.

Booker, C.W., Abutarbush, S.M., Morley, P.S., Jim, G.K., Pittman, T.J., Schunicht, O.C., Perrett, T., Wildman, B.K., Fenton, R.K., Guichon, P.T., Janzen, E.D., 2008, Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in western Canada. Can Vet J 49, 473-481.

Booker, C.W., Guichon, P.T., Jim, G.K., Schunicht, O.C., Harland, R.J., Morley, P.S., 1999, Seroepidemiology of undifferentiated fever in feedlot calves in western Canada. Can Vet J 40, 40-48.

Booker, C.W., Jim, G.K., Guichon, P.T., Schunicht, O.C., Thorlakson, B.E., Lockwood, P.W., 1997, Evaluation of florfenicol for the treatment of undifferentiated fever in feedlot calves in western Canada. Can Vet J 38, 555-560.

Bowland, S.L., Shewen, P.E., 2000, Bovine respiratory disease: commercial vaccines currently available in Canada. Can Vet J 41, 33-48.

Breider, M.A., Kumar, S., Corstvet, R.E., 1990, Bovine pulmonary endothelial cell damage mediated by *Pasteurella haemolytica* pathogenic factors. Infect Immun 58, 1671-1677.

Brogden, K.A., Adlam, C., Lehmkuhl, H.D., Cutlip, R.C., Knights, J.M., Engen, R.L., 1989, Effect of *Pasteurella haemolytica* (A1) capsular polysaccharide on sheep lung *in vivo* and on pulmonary surfactant in vitro. Am J Vet Res 50, 555-559.

Brogden, K.A., Lehmkuhl, H.D., Cutlip, R.C., 1998, *Pasteurella haemolytica* complicated respiratory infections in sheep and goats. Vet Res 29, 233-254.

Brown, G.B., Bolin, S.R., Frank, D.E., Roth, J.A., 1991, Defective function of leukocytes from cattle persistently infected with bovine viral diarrhea virus, and the influence of recombinant cytokines. Am J Vet Res 52, 381-387.

Brown, T.T., Jr., Ananaba, G., 1988, Effect of respiratory infections caused by bovine herpesvirus-1 or parainfluenza-3 virus on bovine alveolar macrophage functions. Am J Vet Res 49, 1447-1451.

Burrows, L.L., Olah-Winfield, E., Lo, R.Y., 1993, Molecular analysis of the leukotoxin determinants from *Pasteurella haemolytica* serotypes 1 to 16. Infect Immun 61, 5001-5007.

Call, D.R., Davis, M.A., Sawant, A.A., 2008, Antimicrobial resistance in beef and dairy cattle production. Anim Health Res Rev 9, 159-167.

Caprioli, A., Busani, L., Martel, J.L., Helmuth, R., 2000, Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. Int J Antimicrob Agents 14, 295-301.

Carson, C.A., Reid-Smith, R., Irwin, R.J., Martin, W.S., McEwen, S.A., 2008, Antimicrobial use on 24 beef farms in Ontario. Can J Vet Res 72, 109-118.

Catry, B., Baele, M., Opsomer, G., de Kruif, A., Decostere, A., Haesebrouck, F., 2004, tRNA-intergenic spacer PCR for the identification of *Pasteurella* and *Mannheimia* spp. Vet Microbiol 98, 251-260.

Catry, B., Dewulf, J., de Kruif, A., Vanrobaeys, M., Haesebrouck, F., Decostere, A., 2007, Accuracy of susceptibility testing of *Pasteurella multocida* and *Mannheimia haemolytica*. Microbial Drug Resist 13, 204-211.

Catry, B., Haesebrouck, F., Vliegher, S.D., Feyen, B., Vanrobaeys, M., Opsomer, G., Schwarz, S., Kruif, A.D., 2005, Variability in acquired resistance of *Pasteurella* and *Mannheimia* isolates from the nasopharynx of calves, with particular reference to different herd types. Microb Drug Resist 11, 387-394.

Catry, B., Laevens, H., Devriese, L.A., Opsomer, G., De Kruif, A., 2003, Antimicrobial resistance in livestock. J Vet Pharmacol Ther 26, 81-93.

Centers for Disease Control and Prevention. 1996. Standardized molecular subtyping of food borne bacterial pathogens by pulsed-field gel electrophoresis: a manual. National Center for Infectious Diseases, Atlanta, GA, USA (updated 2000)

Clinical and Laboratory Standards Institute, 2008. Performance standards for antimicrobial susceptibility testing, 18th informational supplement M100-S18, Wayne, PA.

Clinical and Laboratory Standards Institute, 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7. CLSI, Wayne, PA.

Chae, C.H., Gentry, M.J., Confer, A.W., Anderson, G.A., 1990, Resistance to host immune defense mechanisms afforded by capsular material of *Pasteurella haemolytica*, serotype 1. Vet Microbiol 25, 241-251.

Chaslus-Dancla, E., Lesage-Descauses, M.C., Leroy-Setrin, S., Martel, J.L., Lafont, J.P., 1995, Tetracycline resistance determinants, *Tet* B and *Tet* M, detected in *Pasteurella haemolytica* and *Pasteurella multocida* from bovine herds. J Antimicrob Chemother 36, 815-819.

Chen, J., Yu, Z., Michel, F.C., Jr., Wittum, T., Morrison, M., 2007, Development and application of real-time PCR assays for quantification of *erm* genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl Environ Microbiol 73, 4407-4416.

Chopra, I., Roberts, M., 2001, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65, 232-260

Chou, C.H., Wang, C., 2006, Genetic relatedness between *Listeria monocytogenes* isolates from seafood and humans using PFGE and REP-PCR. Int J Food Microbiol 110, 135-148.

Church, T.L., Radostits, O.M., 1981, A retrospective survey of diseases of feedlot cattle in Alberta. Can Vet J 22, 27-30.

Clinkenbeard, K.D., Clarke, C.R., Hague, C.M., Clinkenbeard, P., Srikumaran, S., Morton, R.J., 1994, *Pasteurella haemolytica* leukotoxin-induced synthesis of eicosanoids by bovine neutrophils in vitro. J Leukoc Biol 56, 644-649.

Clinkenbeard, K.D., Upton, M.L., 1991, Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. Am J Vet Res 52, 453-457.

Confer, A.W., Panciera, R.J., Mosier, D.A., 1988, Bovine pneumonic pasteurellosis: immunity to *Pasteurella haemolytica*. J Am Vet Med Assoc 193, 1308-1316.

Corbeil, L.B., Woodward, W., Ward, A.C., Mickelsen, W.D., Paisley, L., 1985, Bacterial interactions in bovine respiratory and reproductive infections. J Clin Microbiol 21, 803-807.

Corkill, J.E., Graham, R., Hart, C.A., Stubbs, S., 2000, Pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. J Clin Microbiol 38, 2791-2792.

D., Spratt, B.G., LiPuma, J.J., 2007, Using BOX-PCR to exclude a clonal outbreak of melioidosis. BMC Infect Dis 7, 68.

Cusack, P.M., McMeniman, N., Lean, I.J., 2003, The medicine and epidemiology of bovine respiratory disease in feedlots. Aust Vet J 81, 480-487.

Dabo, S.M., Confer, A., Montelongo, M., York, P., Wyckoff, J.H., 3rd, 2008, Vaccination with *Pasteurella multocida* recombinant OmpA induces strong but non-protective and deleterious Th2-type immune response in mice. Vaccine 26, 4345-4351.

Dassanayake, R.P., Shanthalingam, S., Davis, W.C., Srikumaran, S., 2007, *Mannheimia haemolytica* leukotoxin-induced cytolysis of ovine (Ovis aries) leukocytes is mediated by CD18, the beta subunit of beta2-integrins. Microb Pathog 42, 167-173.

Davies, R.L., Arkinsaw, S., Selander, R.K., 1997a, Evolutionary genetics of *Pasteurella haemolytica* isolates recovered from cattle and sheep. Infect Immun 65, 3585-3593.

Davies, R.L., Arkinsaw, S., Selander, R.K., 1997b, Genetic relationships among *Pasteurella trehalosi* isolates based on multilocus enzyme electrophoresis. Microbiology 143 (Pt 8), 2841-2849.

Davies, R.L., Baillie, S., 2003, Cytotoxic activity of *Mannheimia haemolytica* strains in relation to diversity of the leukotoxin structural gene *lkt*A. Vet Microbiol 92, 263-279.

Davies, R.L., Campbell, S., Whittam, T.S., 2002, Mosaic structure and molecular evolution of the leukotoxin operon (*lkt*CABD) in *Mannheimia (Pasteurella) haemolytica, Mannheimia glucosida*, and *Pasteurella trehalosi*. J Bacteriol 184, 266-277.

Davies, R.L., Donachie, W., 1996, Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. Microbiology 142 (Pt 7), 1895-1907.

Davies, R.L., Paster, B.J., Dewhirst, F.E., 1996, Phylogenetic relationships and diversity within the *Pasteurella haemolytica* complex based on 16S rRNA sequence comparison and outer membrane protein and lipopolysaccharide analysis. Int J Syst Bacteriol 46, 736-744.

Davies, R.L., Whittam, T.S., Selander, R.K., 2001, Sequence diversity and molecular evolution of the leukotoxin (*lkt*A) gene in bovine and ovine strains of *Mannheimia (Pasteurella) haemolytica*. J Bacteriol 183, 1394-1404.

De Ley, J., Mannheim, W., Mutters, R., Piechulla, K., Tytgat, R., Segers, P., Bisgaard, M., Frederiksen, W., Hinz, K.H., Vanhoucke, M., 1990, Inter- and intrafamilial similarities of rRNA cistrons of the *Pasteurellaceae*. Int J Syst Bacteriol 40, 126-137.

De Vuyst, L., Camu, N., De Winter, T., Vandemeulebroecke, K., Van de Perre, V., Vancanneyt, M., De Vos, P., Cleenwerck, I., 2008, Validation of the $(GTG)_5$-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. Int J Food Microbiol 125, 79-90.

Dewhirst, F.E., Paster, B.J., Olsen, I., Fraser, G.J., 1992, Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. J Bacteriol 174, 2002-2013.

Dewhirst, F.E., Paster, B.J., Olsen, I., Fraser, G.J., 1993, Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. Zentralbl Bakteriol 279, 35-44.

Donachie, W., Fraser, J., Quirie, M., Gilmour, N.J., 1984, Studies on strains of *Pasteurella haemolytica* not typeable by the indirect haemagglutination test. Res Vet Sci 37, 188-193.

Duff, G.C., Galyean, M.L., 2007, Board-invited review: recent advances in management of highly stressed, newly received feedlot cattle. J Anim Sci 85, 823-840.

Edwards, A.J., 1996, Respiratory diseases of feedlot cattle in the central USA. Bovine Pract. 30.

Encinias, A.M., Walker, D.A., Murdock, C.W., Reeves, L.A., Malcolm-Callis, K.J., Soto-Navarro, S., 2006. Effects of prophylactic administration of ceftiofur crystalline

free acid on health and performance of newly received beef calves. In: Proceedings- American Society of Animal Science, Western Section Utah State University in Logan, Utah.

Escande, F., Gerbaud, G., Martel, J.L., Courvalin, P., 1991, Resistance to trimethoprim and 2,4-diamino-6,7-diisopropyl-pteridine (0/129) in *Pasteurella haemolytica*. Vet Microbiol 26, 107-114.

Ewers, C., Lubke-Becker, A., Wieler, L.H., 2004, *Pasteurella*: insights into the virulence determinants of a heterogenous bacterial type. Berl Munch Tierarztl Wochenschr 117, 367-386.

Filion, L.G., Willson, P.J., Bielefeldt-Ohmann, H., Babiuk, L.A., Thomson, R.G., 1984, The possible role of stress in the induction of pneumonic pasteurellosis. Can J Comp Med 48, 268-274.

Foxman, B., Zhang, L., Koopman, J.S., Manning, S.D., Marrs, C.F., 2005, Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiol Perspect Innov 2, 10.

Frank, G.H., 1980, Serological groups among untypeable bovine isolates of *Pasteurella haemolytica*. J Clin Microbiol 12, 579-582.

Frank, G.H., Briggs, R.E., Loan, R.W., Purdy, C.W., Zehr, E.S., 1996, Respiratory tract disease and mucosal colonization by *Pasteurella haemolytica* in transported cattle. Am J Vet Res 57, 1317-1320.

Frank, G.H., Wessman, G.E., 1978, Rapid plate agglutination procedure for serotyping *Pasteurella haemolytica*. J Clin Microbiol 7, 142-145.

Friend, S.C., Thomson, R.G., Wilkie, B.N., 1977, Pulmonary lesions induced by *Pasteurella haemolytica* in cattle. Can J Comp Med 41, 219-223.

Gagea, M.I., Bateman, K.G., van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Shanahan, R.A., Caswell, J.L., 2006, Diseases and pathogens associated with mortality in Ontario beef feedlots. J Vet Diagn Invest 18, 18-28.

Gardner, B.A., Dolezal, H.G., Bryant, L.K., Owens, F.N., Smith, R.A., 1999, Health of finishing steers: effects on performance, carcass traits, and meat tenderness. J Anim Sci 77, 3168-3175.

Gevaert, D., 2006, The importance of *Mycoplasma bovis* in bovine respiratory disease. Tijdschr Diergeneeskd 131, 124-126.

Gevers, D., Huys, G., Swings, J., 2001, Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol Lett 205, 31-36.

Gioia, J., Qin, X., Jiang, H., Clinkenbeard, K., Lo, R., Liu, Y., Fox, G.E., Yerrapragada, S., McLeod, M.P., McNeill, T.Z., Hemphill, L., Sodergren, E., Wang, Q., Muzny, D.M., Homsi, F.J., Weinstock, G.M., Highlander, S.K., 2006, The genome sequence of *Mannheimia haemolytica* A1: insights into virulence, natural competence, and *Pasteurellaceae* phylogeny. J Bacteriol 188, 7257-7266.

Graham, W.R., 1953, The pathology of shipping fever in feedlot cattle. J Am Vet Med Assoc 123, 198-203.

Griffin, D., 1997, Economic impact associated with respiratory disease in beef cattle. Vet Clin North Am Food Anim Pract 13, 367-377.

Guenther, S., Schierack, P., Grobbel, M., Lubke-Becker, A., Wieler, L.H., Ewers, C., 2008, Real-time PCR assay for the detection of species of the genus *Mannheimia*. J Microbiol Methods 75, 75-80.

Gunawardana, G.A., Townsend, K.M., Frost, A.J., 2000, Molecular characterization of avian *Pasteurella multocida* isolates from Australia and Vietnam by REP-PCR and PFGE. Vet Microbiol 72, 97-109.

Hansen, L.M., Blanchard, P.C., Hirsh, D.C., 1996, Distribution of *tet*(H) among *Pasteurella* isolates from the United States and Canada. Antimicrob Agents Chemother 40, 1558-1560.

Hansen, L.M., McMurry, L.M., Levy, S.B., Hirsh, D.C., 1993, A new tetracycline resistance determinant, *Tet* H, from *Pasteurella multocida* specifying active efflux of tetracycline. Antimicrob Agents Chemother 37, 2699-2705.

Harland, R.J., Jim, G.K., Guichon, P.T., Townsend, H.G., Janzen, E.D., 1991, Efficacy of parenteral antibiotics for disease prophylaxis in feedlot calves. Can Vet J 32, 163-168.

Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A., Nieto, R., Woods, C., Versalovic, J., Lupski, J.R., 2005, Microbial DNA typing by automated repetitive-sequence-based PCR. J Clin Microbiol 43, 199-207.

Hendriksen, R.S., Mevius, D.J., Schroeter, A., Teale, C., Meunier, D., Butaye, P., Franco, A., Utinane, A., Amado, A., Moreno, M., Greko, C., Stark, K., Berghold, C., Myllyniemi, A.L., Wasyl, D., Sunde, M., Aarestrup, F.M., 2008, Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002-2004. Acta Vet Scand 50, 28.

Henricks, P.A., Binkhorst, G.J., Drijver, A.A., Nijkamp, F.P., 1992, *Pasteurella haemolytica* leukotoxin enhances production of leukotriene B4 and 5-

hydroxyeicosatetraenoic acid by bovine polymorphonuclear leukocytes. Infect Immun 60, 3238-3243.

Highlander, S.K., 2001, Molecular genetic analysis of virulence in *Mannheimia (pasteurella) haemolytica*. Front Biosci 6, D1128-1150.

Highlander, S.K., Fedorova, N.D., Dusek, D.M., Panciera, R., Alvarez, L.E., Rinehart, C., 2000, Inactivation of *Pasteurella* (*Mannheimia*) *haemolytica* leukotoxin causes partial attenuation of virulence in a calf challenge model. Infect Immun 68, 3916-3922.

Hoar, B.R., Jelinski, M.D., Ribble, C.S., Janzen, E.D., Johnson, J.C., 1998, A comparison of the clinical field efficacy and safety of florfenicol and tilmicosin for the treatment of undifferentiated bovine respiratory disease of cattle in western Canada. Can Vet J 39, 161-166.

Hodgins, D.C., Conlon, J.A., Shewen, P.E., 2002, Respiratory viruses and bacteria in cattle, In: Brogden, K.A., Guthmiller, J.M. (Eds.) Polymicrobial diseases. ASM Press, Washinton DC, pp. 213-230.

Hunter, P.R., Gaston, M.A., 1988, Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26, 2465-2466.

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., 2005, Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J Clin Microbiol 43, 1045-1050.

Jaworski, M.D., Hunter, D.L., Ward, A.C., 1998, Biovariants of isolates of *Pasteurella* from domestic and wild ruminants. J Vet Diagn Invest 10, 49-55.

Jericho, K.W., 1979, Update on pasteurellosis in young cattle. Can Vet J 20, 333-335.

Jeyaseelan, S., Sreevatsan, S., Maheswaran, S.K., 2002, Role of *Mannheimia haemolytica* leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis. Anim Health Res Rev 3, 69-82.

Jim, G.K., Booker, C.W., Guichon, P.T., Schunicht, O.C., Wildman, B.K., Johnson, J.C., Lockwood, P.W., 1999, A comparison of florfenicol and tilmicosin for the treatment of undifferentiated fever in feedlot calves in western Canada. Can Vet J 40, 179-184.

Johnson, J.R., O'Bryan, T.T., 2000, Improved repetitive-element PCR fingerprinting for resolving pathogenic and nonpathogenic phylogenetic groups within *Escherichia coli*. Clin Diagn Lab Immunol 7, 265-273.

Jones, F., 1921, A study of *Bacillus bovisepticus.* . J Exp Med 34, 561-577.

Katsuda, K., Kamiyama, M., Kohmoto, M., Kawashima, K., Tsunemitsu, H., Eguchi, M., 2007, Serotyping of *Mannheimia haemolytica* isolates from bovine pneumonia: 1987-2006. Vet J.

Katsuda, K., Kohmoto, M., Kawashima, K., Tsunemitsu, H., Tsuboi, T., Eguchi, M., 2003, Molecular typing of *Mannheimia (Pasteurella) haemolytica* serotype A1 isolates from cattle in Japan. Epidemiol Infect 131, 939-946.

Kehrenberg, C., Catry, B., Haesebrouck, F., de Kruif, A., Schwarz, S., 2005a, Novel spectinomycin/streptomycin resistance gene, aadA14, from *Pasteurella multocida*. Antimicrob Agents Chemother 49, 3046-3049.

Kehrenberg, C., Catry, B., Haesebrouck, F., de Kruif, A., Schwarz, S., 2005b, *tet*(L)-mediated tetracycline resistance in bovine *Mannheimia* and *Pasteurella* isolates. J Antimicrob Chemother 56, 403-406.

Kehrenberg, C., Salmon, S.A., Watts, J.L., Schwarz, S., 2001a, Tetracycline resistance genes in isolates of *Pasteurella multocida, Mannheimia haemolytica, Mannheimia glucosida* and *Mannheimia varigena* from bovine and swine respiratory disease: intergeneric spread of the tet(H) plasmid pMHT1. J Antimicrob Chemother 48, 631-640.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J.L., Chaslus-Dancla, E., Schwarz, S., 2001b, Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. Vet Res 32, 323-339.

Kehrenberg, C., Schwarz, S., 2001, Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of the genera *Pasteurella* and *Mannheimia*. FEMS Microbiol Lett 205, 283-290.

Kehrenberg, C., Schwarz, S., 2005a, dfrA20, A novel trimethoprim resistance gene from *Pasteurella multocida*. Antimicrob Agents Chemother 49, 414-417.

Kehrenberg, C., Schwarz, S., 2005b, Plasmid-borne florfenicol resistance in *Pasteurella multocida*. J Antimicrob Chemother 55, 773-775.

Kehrenberg, C., Walker, R., Wu, C., Schwarz, S., 2006, Antimicrobial resistance in members of the family *Pasteurellaceae*, In: Aarestrup, F.M. (Ed.) Antimicrobial resistance in bacteria of animal origin. ASM Press, Washinton DC, pp. 167-186.

Keiss, R.E., Will, D.H., Collier, J.R., 1964, Skin toxicity and hemodynamic properties of endotoxin derived from *Pasteurella haemolytica*. Am J Vet Res 25, 935-942.

Kelley, S.T., Cassirer, E.F., Weiser, G.C., Safaee, S., 2007, Phylogenetic diversity of *Pasteurellaceae* and horizontal gene transfer of leukotoxin in wild and domestic sheep. Infect Genet Evol 7, 13-23.

Kim, E., Aoki, T., 1996, Sequence analysis of the florfenicol resistance gene encoded in the transferable R-plasmid of a fish pathogen, *Pasteurella piscicida*. Microbiol Immunol 40, 665-669.

Kitt, T., 1885, Uber eine experimentelle, der Rinderseuche ahnliche Infektionskranheit. Sitzunbsber Ges Morphol Physiol Muenchen, 140-168.

Kodjo, A., Villard, L., Bizet, C., Martel, J.L., Sanchis, R., Borges, E., Gauthier, D., Maurin, F., Richard, Y., 1999, Pulsed-field gel electrophoresis is more efficient than ribotyping and random amplified polymorphic DNA analysis in discrimination of *Pasteurella haemolytica* strains. J Clin Microbiol 37, 380-385.

Lacroix, R.P., Duncan, J.R., Jenkins, R.P., Leitch, R.A., Perry, J.A., Richards, J.C., 1993, Structural and serological specificities of *Pasteurella haemolytica* lipopolysaccharides. Infect Immun 61, 170-181.

Lalman, D., Smith, R. 2002. *Effects of Preconditioning on Health, Performance and Prices of Weaned Calves*. In Oklahoma Cooperative Extension Service (Oklahoma State University).

Larsen, J., Pedersen, A.G., Davies, R.L., Kuhnert, P., Frey, J., Christensen, H., Bisgaard, M., Olsen, J.E., 2009, Evolution of the leukotoxin promoter in genus *Mannheimia*. BMC Evol Biol 9, 121.

Larson, R.L., 2005, Effect of cattle disease on carcass trait. J. Anim. Sci. 83, 37-47.

Larson, R.L., Tyler, J.W., 2005, Reducing calf losses in beef herds. Vet Clin North Am Food Anim Pract 21, 569-584.

Lee, C.W., Shewen, P.E., 1996, Evidence of bovine immunoglobulin G1 (IgG1) protease activity in partially purified culture supernate of *Pasteurella haemolytica* A1. Can J Vet Res 60, 127-132.

Li, J., Clinkenbeard, K.D., 1999, Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. Infect Immun 67, 2920-2927.

Lillie, L.E., 1974, The bovine respiratory disease complex. Can Vet J 15, 233-242.

Lo, R.Y., 2001, Genetic analysis of virulence factors of *Mannheimia (Pasteurella) haemolytica* A1. Veterinary microbiology 83, 23-35.

Loneragan, G.H., Thomson, D.U., Montgomery, D.L., Mason, G.L., Larson, R.L., 2005, Prevalence, outcome, and health consequences associated with persistent infection with bovine viral diarrhea virus in feedlot cattle. J Am Vet Med Assoc 226, 595-601.

Lopez, A., Thomson, R.G., Savan, M., 1976, The pulmonary clearance of *Pasteurella haemolytica* in calves infected with bovine parainfluenza-3 virus. Can J Comp Med 40, 385-391.

Maheswaran, S.K., Berggren, K.A., Simonson, R.R., Ward, G.E., Muscoplat, C.C., 1980, Kinetics of interaction and fate of *Pasteurella haemolytica* in bovine alveolar macrophages. Infect Immun 30, 254-262.

Maheswaran, S.K., Weiss, D.J., Kannan, M.S., Townsend, E.L., Reddy, K.R., Whiteley, L.O., Srikumaran, S., 1992, Effects of *Pasteurella haemolytica* A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunopathol 33, 51-68.

Malott, R.J., Lo, R.Y., 2002, Studies on the production of quorum-sensing signal molecules in *Mannheimia haemolytica* A1 and other *Pasteurellaceae* species. FEMS Microbiol Lett 206, 25-30.

Mannheim, W., 1983, Taxonomy of the family *Pasteurellaceae* Pohl 1981 as revealed by DNA:DNA hybridization. INSERM 114, 211-226.

Martin, S.W., Meek, A.H., Davis, D.G., Johnson, J.A., Curtis, R.A., 1981, Factors associated with morbidity and mortality in feedlot calves: the Bruce County beef project, year two. Can J Comp Med 45, 103-112.

Martin, S.W., Meek, A.H., Davis, D.G., Thomson, R.G., Johnson, J.A., Lopez, A., Stephens, L., Curtis, R.A., Prescott, J.F., Rosendal, S., Savan, M., Zubaidy, A.J., Bolton, M.R., 1980, Factors associated with mortality in feedlot cattle: the Bruce County Beef Cattle Project. Can J Comp Med 44, 1-10.

Masco, L., Huys, G., Gevers, D., Verbrugghen, L., Swings, J., 2003, Identification of *Bifidobacterium* species using rep-PCR fingerprinting. Syst Appl Microbiol 26, 557-563.

McVey, D.S., Loan, R.W., Purdy, C.W., Shuman, W.J., 1990, Specificity of bovine serum antibody to capsular carbohydrate antigens from *Pasteurella haemolytica*. J Clin Microbiol 28, 1151-1158.

Mohamed, R.A., Abdelsalam, E.B., 2008, A review on pneumonic pasteurellosis (respiratory mannheimiosis) with emphasis on pathogenesis, virulence mechanisms and predisposing factors Bulgarian J Vet Med 11, 139-160.

Mohapatra, B.R., Broersma, K., Mazumder, A., 2008, Differentiation of fecal *Escherichia coli* from poultry and free-living birds by (GTG)5-PCR genomic fingerprinting. Int J Med Microbiol 298, 245-252.

Mohapatra, B.R., Mazumder, A., 2008, Comparative efficacy of five different rep-PCR methods to discriminate *Escherichia coli* populations in aquatic environments. Water Sci Technol 58, 537-547.

Morck, D.W., Olson, M.E., Acres, S.D., Daoust, P.Y., Costerton, J.W., 1989, Presence of bacterial glycocalyx and fimbriae on *Pasteurella haemolytica* in feedlot cattle with pneumonic pasteurellosis. Can J Vet Res 53, 167-171.

Morck, D.W., Raybould, T.J., Acres, S.D., Babiuk, L.A., Nelligan, J., Costerton, J.W., 1987, Electron microscopic description of glycocalyx and fimbriae on the surface of *Pasteurella haemolytica*-A1. Can J Vet Res 51, 83-88.

Mraz, O., 1969, Comparative study of species *Actinobacillus lignieresii* and *Pasteurella haemolytica*. I. *Actinobacillus lignieresii* BRUMPT, 1910; emend. Zentralbl Bakteriol 209, 212-232.

Mutters, R., Bisgaard, M., Pohl, S., 1986, Taxonomic relationship of selected biogroups of *Pasteurella haemolytica* as revealed by DNA:DNA hybridizations. Acta Pathol Microbiol Immunol Scand [B] 94, 195-202.

Newson, I.E., and Cross, F., 1932, Some bipolar organisms found in pneumonia in sheep. J Am Vet Med Assoc 80, 711-719.

Nyarko, K.A., Coomber, B.L., Mellors, A., Gentry, P.A., 1998, Bovine platelet adhesion is enhanced by leukotoxin and sialoglycoprotease isolated from *Pasteurella haemolytica* A1 cultures. Vet Microbiol 61, 81-91.

Pandher, K., Confer, A.W., Murphy, G.L., 1998, Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of *Pasteurella haemolytica* serotype 1. Infect Immun 66, 5613-5619.

Post, K.W., Cole, N.A., Raleigh, R.H., 1991, *In vitro* antimicrobial susceptibility of *Pasteurella haemolytica* and *Pasteurella multocida* recovered from cattle with bovine respiratory disease complex. J Vet Diagn Invest 3, 124-126.

Pritchard, R.H., Mendez, J.K., 1990, Effects of preconditioning on pre- and post-shipment performance of feeder calves. J Anim Sci 68, 28-34.

Purdy, C.W., Raleigh, R.H., Collins, J.K., Watts, J.L., Straus, D.C., 1997, Serotyping and enzyme characterization of *Pasteurella haemolytica* and *Pasteurella multocida* isolates recovered from pneumonic lungs of stressed feeder calves. Curr Microbiol 34, 244-249.

Rehmtulla, A.J., Thomson, R.G., 1981, A review of the lesions in shipping fever of cattle. Can Vet J 22, 1-8.

Ribble, C.S., Meek, A.H., Janzen, E.D., Guichon, P.T., Jim, G.K., 1995a, Effect of time of year, weather, and the pattern of auction market sales on fatal fibrinous pneumonia (shipping fever) in calves in a large feedlot in Alberta (1985-1988). Can J Vet Res 59, 167-172.

Ribble, C.S., Meek, A.H., Jim, G.K., Guichon, P.T., 1995b, The pattern of fatal fibrinous pneumonia (shipping fever) affecting calves in a large feedlot in Alberta (1985-1988). Can Vet J 36, 753-757.

Ribble, C.S., Meek, A.H., Shewen, P.E., Guichon, P.T., Kee G.K., 1995c, Effect of pretransit mixing on fatal fibrinous pneumonia in calves. J Am Vet Med Assoc 5, 616-619.

Rice, J.A., Carrasco-Medina, L., Hodgins, D.C., Shewen, P.E., 2007, *Mannheimia haemolytica* and bovine respiratory disease. Anim Health Res Rev 8, 117-128.

Rossi, C.R., Kiesel, G.K., 1977, Susceptibility of bovine macrophage and tracheal-ring cultures to bovine viruses. Am J Vet Res 38, 1705-1708.

Rowe, H.A., Poxton, I.R., Donachie, W., 2001, Survival of *Mannheimia (Pasteurella) haemolytica* in tracheobronchial washings of sheep and cattle. Vet Microbiol 81, 305-314.

Ryff, J.F., Glenn, M.W., 1957, Possible evidence of a viral agent in shipping fever complex of cattle. J Am Vet Med Assoc 131, 469-470.

Saadati, M., Gibbs, H.A., Parton, R., Coote, J.G., 1997, Characterization of the leukotoxin produced by different strains of *Pasteurella haemolytica*. J Med Microbiol 46, 276-284.

Sanderson, M.W., Dargatz, D.A., Wagner, B.A., 2008, Risk factors for initial respiratory disease in United States' feedlots based on producer-collected daily morbidity counts. Can Vet J 49, 373-378.

Schunicht, O.C., Booker, C.W., Guichon, P.T., Jim, G.K., Wildman, B.K., Hill, B.W., Ward, T.I., Bauck, S.W., 2002, An evaluation of the relative efficacy of a new formulation of oxytetracycline for the treatment of undifferentiated fever in feedlot calves in western Canada. Can Vet J 43, 940-945.

Schunicht, O.C., Booker, C.W., Guichon, P.T., Jim, G.K., Wildman, B.K., Pittman, T.J., Perrett, T., 2007, An evaluation of the relative efficacy of tulathromycin for the treatment of undifferentiated fever in feedlot calves in Nebraska. Can Vet J 48, 600-606.

Schwarz, S., Kehrenberg, C., Salmon, S.A., Watts, J.L., 2004, *In vitro* activities of spectinomycin and comparator agents against *Pasteurella multocida* and *Mannheimia haemolytica* from respiratory tract infections of cattle. J Antimicrob Chemother 53, 379-382.

Sharma, S.A., Olchowy, T.W., Breider, M.A., 1992, Alveolar macrophage and neutrophil interactions in *Pasteurella haemolytica*-induced endothelial cell injury. J Infect Dis 165, 651-657.

Shewen, P.E., Wilkie, B.N., 1982, Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect Immun 35, 91-94.

Silbert, S., Boyken, L., Hollis, R.J., Pfaller, M.A., 2003, Improving typeability of multiple bacterial species using pulsed-field gel electrophoresis and thiourea. Diagn Microbiol Infect Dis 47, 619-621.

Smith, R.A., 1998, Impact of disease on feedlot performance: a review. J Anim Sci 76, 272-274.

Sneath, P.H., Stevens, M., 1985, A numerical taxonomic study of Actinobacillus, *Pasteurella* and Yersinia. J Gen Microbiol 131, 2711-2738.

Sneath, P.H., Stevens, M., 1990, *Actinobacillus rossii* sp. nov., *Actinobacillus seminis* sp. nov., nom. rev., *Pasteurella bettii* sp. nov., *Pasteurella lymphangitidis* sp. nov., *Pasteurella mairi* sp. nov., and *Pasteurella trehalosi* sp. nov. Int J Syst Bacteriol 40, 148-153.

Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L., 2006, Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. J Anim Sci 84, 1999-2008.

Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L., Koohmaraie, M., Dikeman, M.E., 2007, Bovine respiratory disease in feedlot cattle: phenotypic, environmental, and genetic correlations with growth, carcass, and longissimus muscle palatability traits. J Anim Sci 85, 1885-1892.

Straus, D.C., Purdy, C.W., Loan, R.W., Briggs, R.F., Frank, G.H., 1998, *In vivo* production of neuraminidase by *Pasteurella haemolytica* in market stressed cattle after natural infection. Curr Microbiol 37, 240-244.

Sutherland, D.R., Abdullah, K.M., Cyopick, P., Mellors, A., 1992, Cleavage of the cell-surface O-sialoglycoproteins CD34, CD43, CD44, and CD45 by a novel glycoprotease from *Pasteurella haemolytica*. J Immunol 148, 1458-1464.

Tefera, G., and Smola, J., 2001, *Pasteurella haemolytica* complex of *Pasteurella sensu stricto* as new genus *Mannheimia*: changes in taxonomy. Vet Med. 46, 119-124.

Tenover, F.C., Arbeit, R.D., Goering, R.V., 1997, How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect Control Hosp Epidemiol 18, 426-439.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995, Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33, 2233-2239.

Thomson, D.U., White, B.J., 2006, Backgrounding beef cattle. Vet Clin North Am Food Anim Pract 22, 373-398.

Thumbikat, P., Dileepan, T., Kannan, M.S., Maheswaran, S.K., 2005, Characterization of *Mannheimia* (*Pasteurella*) *haemolytica* leukotoxin interaction with bovine alveolar macrophage beta2 integrins. Vet Res 36, 771-786.

Vassort-Bruneau, C., Lesage-Descauses, M.C., Martel, J.L., Lafont, J.P., Chaslus-Dancla, E., 1996, CAT III chloramphenicol resistance in *Pasteurella haemolytica* and *Pasteurella multocida* isolated from calves. J Antimicrob Chemother 38, 205-213.

Villard, L., Gauthier, D., Lacheretz, A., Abadie, G., Game, Y., Maurin, F., Richard, Y., Borges, E., Kodjo, A., 2006, Serological and molecular comparison of *Mannheimia haemolytica* and *Pasteurella trehalosi* strains isolated from wild and domestic ruminants in the French Alps. Vet J 171, 545-550.

Villard, L., Gauthier, D., Maurin, F., Borges, E., Richard, Y., Abadie, G., Kodjo, A., 2008, Serotypes A1 and A2 of *Mannheimia haemolytica* are susceptible to genotypic, capsular and phenotypic variations in contrast to T3 and T4 serotypes of *Bibersteinia (Pasteurella) trehalosi*. FEMS Microbiol Lett 280, 42-49.

Vogel, G.L., Parrott, J.C., 1994, Mortality survey in feedyards: The incidence of death from digestive, respiratory and other causes in feedyards of the Great Plains. Compend. Contin. Educ. Pract. Vet. 16, 227-234.

Waggoner, J.W., Mathis, C.P., Löest, C.A., Sawyer, J.E., McCollum, F.T., 2005. Impact of preconditioning duration on feedlot performance, carcass characteristics and profitability of New Mexico ranch to rails steers. In: Proceedings- American

Society of Animal Science, Western Section, New Mexico State University, Las Cruces, NM.

Wang, Z., Clarke, C., Clinkenbeard, K., 1998, *Pasteurella haemolytica* leukotoxin-induced increase in phospholipase A2 activity in bovine neutrophils. Infect Immun 66, 1885-1890.

Watts, J.L., Yancey, R.J., Jr., Salmon, S.A., Case, C.A., 1994, A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. J Clin Microbiol 32, 725-731.

Weigel, R.M., Qiao, B., Teferedegne, B., Suh, D.K., Barber, D.A., Isaacson, R.E., White, B.A., 2004, Comparison of pulsed field gel electrophoresis and repetitive sequence polymerase chain reaction as genotyping methods for detection of genetic diversity and inferring transmission of *Salmonella*. Vet Microbiol 100, 205-217.

Weiser, G.C., Miller, D.S., Drew, M.L., Rhyan, J.C., Ward, A.C., 2009, Variation in *Pasteurella (Bibersteinia)* and *Mannheimia* spp. following transport and antibiotic treatment in free-ranging and captive Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). J Zoo Wildl Med 40, 117-125.

Welsh, R.D., Dye, L.B., Payton, M.E., Confer, A.W., 2004, Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. J Vet Diagn Invest 16, 426-431.

Whiteley, L.O., Maheswaran, S.K., Weiss, D.J., Ames, T.R., 1990, Immunohistochemical localization of *Pasteurella haemolytica* A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine *Pasteurella* pneumonia. Vet Pathol 27, 150-161.

Whiteley, L.O., Maheswaran, S.K., Weiss, D.J., Ames, T.R., Kannan, M.S., 1992, *Pasteurella haemolytica* A1 and bovine respiratory disease: pathogenesis. J Vet Intern Med 6, 11-22.

Wildman, B.K., Perrett, T., Abutarbush, S.M., Guichon, P.T., Pittman, T.J., Booker, C.W., Schunicht, O.C., Fenton, R.K., Jim, G.K., 2008, A comparison of 2 vaccination programs in feedlot calves at ultra-high risk of developing undifferentiated fever/bovine respiratory disease. Can Vet J 49, 463-472.

Wittum, T.E., Woollen, N.E., Perino, L.J., Littledike, E.T., 1996, Relationships among treatment for respiratory tract disease, pulmonary lesions evident at slaughter, and rate of weight gain in feedlot cattle. J Am Vet Med Assoc 209, 814-818.

Woldehiwet, Z., Sharma, R., 1992, Evidence of immunosuppression by bovine respiratory syncytial virus. Scand J Immunol Suppl 11, 75-80.

Wood, A.R., Lainson, F.A., Wright, F., Baird, G.D., Donachie, W., 1995, A native plasmid of *Pasteurella haemolytica* serotype A1: DNA sequence analysis and investigation of its potential as a vector. Res Vet Sci 58, 163-168.

Woolums, A.R., Loneragan, G.H., Hawkins, L.L., 2005, Baseline management practices and animal health data reported by US feedyards responding to a survey regarding acute interstitial pneumonia. Bovine Pract. 39, 116-124.

Yates, W.D., 1982, A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can J Comp Med 46, 225-263.

Yokoyama, E., Maruyama, S., Kabeya, H., Hara, S., Sata, S., Kuroki, T., Yamamoto, T., 2007, Prevalence and genetic properties of *Salmonella enterica* serovar typhimurium definitive phage type 104 isolated from *Rattus norvegicus* and *Rattus rattus* house rats in Yokohama City, Japan. Appl Environ Microbiol 73, 2624-2630.

Yoo, H.S., Rajagopal, B.S., Maheswaran, S.K., Ames, T.R., 1995, Purified *Pasteurella haemolytica leukotoxin* induces expression of inflammatory cytokines from bovine alveolar macrophages. Microb Pathog 18, 237-252.

Yoo, H.S., Rutherford, M.S., Maheswaran, S.K., Srinand, S., Ames, T.R., 1996, Induction of nitric oxide production by bovine alveolar macrophages in response to *Pasteurella haemolytica* A1. Microb Pathog 20, 361-375.

Younan, M., Fodar, L., 1995, Characterisation of a new *Pasteurella haemolytica* serotype (A17). Res Vet Sci 58, 98.

Zecchinon, L., Fett, T., Desmecht, D., 2005, How *Mannheimia haemolytica* defeats host defence through a kiss of death mechanism. Vet Res 36, 133-156.

Zhang, Y., Yakrus, M.A., Graviss, E.A., Williams-Bouyer, N., Turenne, C., Kabani, A., Wallace, R.J., Jr., 2004, Pulsed-field gel electrophoresis study of *Mycobacterium abscessus* isolates previously affected by DNA degradation. J Clin Microbiol 42, 5582-5587.