

MOLECULAR EPIDEMIOLOGY OF *PASTEURELLA*
MULTOCIDA RESPIRATORY DISEASE IN BEEF
CATTLE

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CHAPTER I.

INTRODUCTION

Bovine respiratory disease (BRD) complex is the most costly disease of beef cattle in North America.(Church and Radostits, 1981; Griffin, 1997) A variety of infectious agents have been linked to BRD, but *Mannheimia haemolytica* and *Pasteurella multocida* are generally considered the most consistent and significant agents.(Welsh et al., 2004) Yet, researchers are unable to replicate the common clinical presentation through experimental exposure to the bacterium alone.(Jericho and Langford, 1978; Yates, 1982) Additionally, these bacterial species can readily be isolated from the upper respiratory tract of normal cattle.(Allen et al., 1992; Barbour et al., 1997; Fulton et al., 2002a; Hamdy and Trapp, 1967; Hoerlein et al., 1961; Thomson et al., 1969; Yates et al., 1983) Therefore, BRD is considered a multi-factorial syndrome, with various predisposing factors being necessary to induce natural disease. Viral agents (Czuprynski et al., 2004; Martin and Bohac, 1986; Yates, 1982) and numerous other components, generally termed “stressors”, have been postulated to be among the factors impacting BRD occurrence. The intuitive nature of these suggestions is appealing, and they are widely accepted and repeated throughout the literature. However, epidemiologic investigation of these proposed factors has failed to identify steps that can consistently reduce morbidity and mortality associated with BRD. It would therefore seem prudent to examine the epidemiology of the bacterial agents

involved in BRD, to determine if variability within the bacterial population may account for some of the difficulty in predicting and mitigating disease.

Most authors consider *P. multocida* an opportunistic pathogen in cattle. It is a commensal of the upper respiratory tract and the presence or absence of *P. multocida* in the nasal pharynx does not predict lower respiratory disease.(Allen et al., 1991; Fulton et al., 2002b) However, studies in other host species have suggested that there are a limited number of strains associated with disease, with these strains clearly transmitted horizontally.(Blackall et al., 2000; Bowles et al., 2000; Davies et al., 2003; Zhao et al., 1992; Zhao et al., 1993) These strains of *P. multocida* may therefore be more pathogenic than others.(Davies et al., 2003) Similar strain differences may also exist in cattle.

A variety of molecular techniques have been employed to characterize *P. multocida* from several host species. Random amplified polymorphic DNA polymerization chain reaction (RAPD PCR) fingerprinting has been found to be effective in discriminating among isolates of *P. multocida* from rabbits(Dabo et al., 2000) and has been employed by numerous researchers for swine, poultry and rabbit isolates.(Chaslus-Dancla et al., 1996; Dabo et al., 1999a; Dabo et al., 2000; Dabo et al., 1999b; Dziva et al., 2001; Dziva et al., 2004; Shivachandra et al., 2007; Zucker et al., 1996) Due to the relative low cost and ease of conducting RAPD PCR, as well as the repeatability and effectiveness demonstrated by the above studies, it would appear to be a technique that could prove useful in epidemiologic studies of bovine respiratory pasteurellosis. Nonetheless, it has not been validated in bovine isolates of *P. multocida*.

This dissertation describes two studies employing RAPD PCR in the investigation of *P. multocida* isolated from cattle. The first study examined the diversity of *P. multocida* isolates, as determined by RAPD PCR using three different primers, as well as through examination of whole cell proteins (WCP), outer membrane proteins (OMP) and serotyping. The concordance between the various typing methods was examined, and the results of each method were compared with epidemiologic data. In the second study, the same primers were used as in the first study for RAPD PCR examination of 213 *P. multocida* isolates. These isolates were obtained from the upper respiratory tract of calves purchased from commercial salebarns and shipped to the Willard Sparks Beef Research Center. Nasal swabs were collected from all calves at time of arrival. Any calf that was diagnosed with clinical BRD was sampled again at time of treatment, as was a clinically health control calf. The results of the three RAPD PCR primers were then used to examine the diversity and epidemiology of *P. multocida* isolates obtained from the nares of healthy calves, as well as those clinically affected by BRD.

CHAPTER II.

THE EPIDEMIOLOGY OF BOVINE RESPIRATORY DISEASE:

A REVIEW OF THE LITERATURE

Bovine respiratory disease complex is the most costly disease of beef cattle in North America.(Church and Radostits, 1981; Griffin, 1997) It is also one of the most extensively studied diseases, with research that began with its description in the late 1800s continuing even today. One of the principal agents implicated in severe, often fatal bovine respiratory disease (BRD) is *Mannheimia haemolytica*. This causal association is based upon a variety of criteria, including:

- The bacterium is associated with clinical occurrences of BRD or “shipping fever”(Collier, 1968; Jensen et al., 1976; Thomson et al., 1969)
- The bacterium is isolated more frequently from sick calves than from well calves (Barbour et al., 1997; Hoerlein et al., 1961; Thomson et al., 1969)
- *M. haemolytica* is the most common bacterial isolate from BRD cases (Fulton et al., 2002a; Reggiardo, 2005)

Secondary support for associating *M. haemolytica* with BRD can also be found in vaccination (O'Connor et al., 2001) and serological (Booker et al., 1999; Martin et al., 1989) studies, although these data are less consistent and reliable in identifying the infectious agent involved.(Virtala et al., 2000)

Other bacteria have also been implicated in BRD. Most commonly cited are *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*. These organisms may be isolated alone or in conjunction with *M. haemolytica* as well as each other. Confirmation of their involvement has been demonstrated through isolation (Gagea et al., 2006; Haines et al., 2001; Janzen, 1997; Shahriar et al., 2002; Welsh et al., 2004), serologic methods (Booker et al., 1999), or reduction in morbidity and/or mortality through vaccination.(Ribble et al., 1988) While involvement of these pathogens has long been suspected, the apparent increase in prevalence may suggest new emerging patterns of pathogens (Gagea et al., 2006; Welsh et al., 2004) or may simply be the result of increased effort to detect the agents.(Shahriar et al., 2002) Efforts have been made to distinguish between the primary bacterial pathogen based upon gross necropsy and histopathological lesions.(Gagea et al., 2006; Schiefer et al., 1978; Tegtmeier et al., 1999) However, the clinical significance of this is questionable, as the clinical presentation and proper case management would not differ based upon the etiologic agent. Others have suggested that a presumptive diagnosis of involvement of certain organisms may be possible ante-mortem. For example, animals infected with *M. bovis* are described as “chronically ill and fail to thrive...lack of weight gain and failure to respond to treatment.”(Shahriar et al., 2002) Studies examining feedlot cattle with chronic, non-responsive respiratory disease corroborated this description although other organisms were also identified, either alone or in combination with *M. bovis*. (Haines et al., 2001; Shahriar et al., 2002) Animals infected with *H. somni* show little distinguishing features unless other systems are affected.(Janzen, 1997) Because of the difficulty in determining the etiology of individual cases of respiratory disease in

cattle, most research makes no distinction among the various pathogens and relies upon clinical signs to diagnose undifferentiated BRD.(Yates, 1982)

Despite overwhelming evidence that various bacterial species are at least associated with, if not the principal cause of BRD, researchers are generally unable to replicate the most common clinical presentation through experimental exposure to the bacterium alone. (Jericho and Langford, 1978; Yates, 1982) Additionally, these bacterial species can readily be isolated from the upper respiratory tract of normal cattle.(Allen et al., 1992b; Barbour et al., 1997; Fulton et al., 2002b; Hamdy and Trapp, 1967; Hoerlein et al., 1961; Thomson et al., 1969; Yates et al., 1983) Therefore, BRD is considered a multi-factorial syndrome, with various predisposing factors being necessary to induce natural disease. Viral agents are frequently cited predisposing factors which are proposed to cause direct damage to respiratory clearance mechanisms or interfere with the immune system's ability to respond to bacterial infection.(Czuprynski et al., 2004; Martin and Bohac, 1986; Yates, 1982) In addition, numerous other components, generally termed "stressors", have been postulated to play a role in BRD. These include transportation, commingling with other cattle, dust, cold, sudden and extreme changes of temperature and humidity, dehydration, hypoxia, endotoxin, cold coupled with wetness, and acute metabolic disturbances.(Irwin et al., 1979; Lillie, 1974) These are thought to alter the respiratory mucosa or hinder cattle's immune system, either directly or through the effects of endogenous agents such as cortisol, making the animals more susceptible to opportunistic infections. The intuitive nature of these suggestions is appealing, and they are widely accepted and repeated throughout the literature. However, relatively little epidemiologic

investigation has been done to confirm or refute the significance of these factors, and studies done thus far have been inconclusive. The purpose of this review is to critically assess these investigations to identify which factors have been confirmed to be significant as well as to highlight equivocal findings and gaps in research relative to BRD.

Principally, epidemiologic studies are included, although a few experimental studies are referenced. Commentary is also made on the unique challenges that make investigation of natural BRD difficult.

Proposed Predisposing Factors

Viral infections

Preceding or concurrent viral infection is perhaps the most clearly linked biological influence on BRD. Experimentally, a syndrome resembling BRD can be induced with exposure to *M. haemolytica* following infection by bovine herpesvirus-1 (BHV-1) virus. (Collier, 1968; Jericho and Langford, 1978) Similar results were obtained with endo-bronchial instillation of bovine viral diarrhea virus (BVDV) followed five days later by *M. haemolytica*.(Potgieter et al., 1984) Natural outbreaks of BRD also demonstrated these synergisms.(Booker et al., 2008b; Jensen et al., 1976) Other viruses that are commonly implicated with BRD include bovine respiratory syncytial virus (BRSV) and parainfluenza three virus (PI3V). (Cusack et al., 2003; Gagea et al., 2006) BRSV and PI3V antigens were identified in over 50% of clinically diseased lungs in a study in Mexico,(Juarez Barranco et al., 2003) while BVDV was identified in naturally affected calves in numerous studies.(Booker et al., 2008a; Booker et al., 2008b; Fulton et al., 2000; Gagea et al., 2006; Haines et al., 2001; Reggiardo, 2005; Shahriar et al., 2002) Serological data has linked BRD outbreaks to BRSV(Durham et al., 1991; Hagglund et

al., 2007; Lehmkuhl and Gough, 1977), PI3V and BVDV (Fulton et al., 2000), as well as multiple concurrent viral infections.(Martin and Bohac, 1986; Richer et al., 1988) Recent work suggested that bovine respiratory coronavirus may be related to BRD, although it has received considerably less attention than other viral agents.(Lathrop et al., 2000; Martin et al., 1998; Plummer et al., 2004; Storz et al., 2000)

Unique among these viral agents is BVDV, in that intrauterine infection can produce cattle that are persistently infected (PI). Cattle that were PI were found to be over-represented among cattle chronically ill or dying in feedlots.(Loneragan et al., 2005) In a Canadian feedlot study(Booker et al., 2008a), 61.5% (8 of 13) of PI calves died, whereas in a Kansas feedlot study (Fulton et al., 2006) 25% (22 of 86) of PI cattle died within 60 days of arrival. Both of these rates are presumably much higher than for non-PI herdmates. Unfortunately, no statistical comparisons were reported for morbidity and mortality of PI vs. non-PI cattle in either study. Persistently infected cattle also shed large quantities of the virus, potentially increasing the risk of cohorts becoming infected and being at risk for BRD. This is potentially significant, since 30% or more of pens may contain a PI calf (Booker et al., 2008a; Fulton et al., 2006) and Loneragan et al. found that exposure to PI cattle increased risk of treatment for BRD(Loneragan et al., 2005). This conclusion was supported by Stevens et al., who showed short-term exposure (13 to 18 days) to PI calves increased morbidity.(Stevens et al., 2007) Neither study identified an increased mortality risk attributable to exposure to PI cattle. However, because of the relatively low prevalence of mortality, a very large study is required to have adequate power to detect a difference in mortality attributable to treatments or exposures.(Loneragan et al., 2005) Other reports examining the effects of PI calves have

reached different conclusions. A controlled experiment also found no effect of exposure to BVDV PI calves on morbidity, mortality or performance.(Elam et al., 2008) Similarly, two epidemiologic studies found there was no difference in morbidity between pens with PI calves versus pens with no PI calves.(Booker et al., 2008a; O'Connor et al., 2003) The different conclusions among these studies may in part be due to definition of exposure- Loneragan considered cattle as exposed if they were housed in pens containing PI cattle or in pens adjacent to those containing PI cattle. Others considered only calves in pens with PI cattle as exposed. Regardless, the prevalence of PI cattle is quite low (estimates including 0.3%(Loneragan et al., 2005); 0.26%(O'Connor et al., 2003); <0.1% (Taylor et al., 1995); 0.18% (Booker et al., 2008a)and 0.4% (Fulton et al., 2006)), and thus they likely do not account for, nor contribute to, the majority of clinical disease.(Booker et al., 2008a)

Environmental factors

Shipping

Transportation of cattle is the most universally accepted non-infectious risk factor for BRD and led to the commonly used laymen's term of "shipping fever." The segmented nature of cattle production in the United States guarantees virtually all beef calves will be transported at least once in their lifetime. Therefore, most researchers have attempted to identify what component of transportation has the greatest effect on incidence of BRD. The distance and/or time in transit have been examined by several investigators, with conflicting conclusions. Ribble et al. found that the distance from point of purchase (auction barn) to destination (feedlot) had no effect on incidence of BRD.(Ribble et al., 1995d) This analysis may be confounded by effects other than transport, such as farm(s)

of origin, the auction barn of purchase and amount of commingling, among other variables. However, consistent association between “shrink” (weight loss occurring during handling and marketing) and distance traveled was interpreted by the authors to suggest such confounders had minimal significance.(Ribble et al., 1995d) In contrast, a large survey found a positive association between distance transported and morbidity.(Sanderson et al., 2008) In a controlled experimental study, calves transported 12 hours had higher morbidity levels than those transported 24 hours, whereas there was no difference between those transported 24 hours and control calves that were fasted but not transported.(Cole et al., 1988) The authors concluded that sorting, loading and early transit are likely the most stressful components of transportation, which is supported by other studies(Phillips et al., 1986; Stermer et al., 1982; Tennessen et al., 1984; Warriss et al., 1995). In one study, an association was found between disease and distance traveled, (Pinchak et al., 2004) but other factors were not considered that may impact morbidity such as weight of calf, bulls that required castration, and other confounders. Other investigators found that calves transported less than 150 miles had less morbidity than those transported 150 to 200 miles.(Schake et al., 1980) Interestingly, this was not a linear relationship; calves hauled less than 50 miles had numerically higher (although not statistically significant) incidence of clinical BRD than those transported 100-150 miles. Another trial by these same researchers found no relationship between distance transported (ranging from less than 50 to greater than 1000 miles) and mortality.(Schake et al., 1980) It was suggested in a comparison of US and Australian production systems that mode of transportation may account for differing rates of BRD. The Australian cattle industry relies more heavily on trains which have more open ventilation while the US

relies on double deck tractor trailers with more complete enclosure.(Irwin et al., 1979)
This theory was not supported in a 1982 study, wherein Canadian cattle were compared according to transport via truck or train(Martin et al., 1982). The potential effect of location of a calf within a transport trailer on BRD was examined in one study. It was theorized that proximity to exhaust may influence subsequent illness, but no difference was found in BRD incidence attributable to location in the trailer compartment.(Camp et al., 1981)

Dehydration is a frequent sequela to transportation and has been suggested as a mechanism through which transport influences disease. As mentioned above, Ribble found a positive correlation between shrink and transportation time. However, neither shrink nor transportation time were significantly correlated with BRD in several studies.(Camp et al., 1981; Cole et al., 1988; Ribble et al., 1995d) A positive association between shrink and BRD was demonstrated in one study.(Woods et al., 1973b) However, failure to account for several confounding factors precluded reaching a definitive conclusion; the most important confounder was that “preconditioned” calves had less shrink and less morbidity. Thus, it is unclear whether less disease was attributable to less shrink or to the preconditioning.

Weather

Weather conditions have long been implicated in occurrence of BRD because the highest incidence is observed in the fall.(Jensen et al., 1976; Loneragan et al., 2001; Ribble et al., 1995a) Nevertheless, one cannot conclude that the predominating weather conditions at that time are the cause of increased incidence of BRD. Fall is the traditional time for

marketing beef cattle, resulting in more at-risk calves being congregated at marketing points at that time of year. Therefore, a higher density of disease organisms is likely present at sale barns, order buyer operations and feedlots. There is also the potential for more stress upon the calves as crowding, commingling and competition for feed and water are exacerbated. Human factors have also been proposed to be involved in the seasonality of BRD. Specifically, high cattle traffic and long work hours lead to fatigue among stockyard and feedlot workers at this time of year. Additionally, potential delays in loading, unloading, transporting and processing of all calves, as well as identifying and treating sick ones, could worsen disease occurrence.(Ribble et al., 1995a) Nevertheless, higher incidence of BRD was found in the fall in a bull performance center, which would not face the same human factors and logistical issues as feedlots.(Andrews, 1976) Many authors have suggested that the sudden and extreme changes in weather conditions, rather than simply cold or inclement weather, predispose cattle to BRD. Attempts to examine this hypothesis have been modestly successful in demonstrating such a link. Ribble et al. found that BRD mortality peaked at approximately the same time as the largest decrease in mean daily ambient temperature.(Ribble et al., 1995a) However, the annual differences seen in BRD occurrence did not correspond with annual variation in weather. In that four year study, one of the two years had the most severe weather while the other year with greatest BRD risk had the mildest weather. Two other studies found opposite correlations between BRD and the maximum range in temperature within a 24 hour period; in the first, increased variation in ambient temperature corresponded with increased disease(MacVean et al., 1986), whereas in the second study increased temperature range correlated with a decrease in BRD.(Alexander et al., 1989; MacVean et al., 1986)

Recently, the relationship of temperature measurements (daily mean, minimum and range) with morbidity and mortality was examined.(Cusack et al., 2007) These researchers found that minimum temperature had a higher correlation with BRD morbidity than did temperature range or mean. There was no relationship among climate variables and mortality. Interestingly, there was a zero day lag between minimum temperature and morbidity; that is, more calves were treated on cold days. If it is assumed that BRD has an incubation period, it would seem improbable that the infection would be immediately apparent. Instead, this finding may be attributable to signs of disease becoming more notable during severe weather rather than a true increased risk.

Other environmental variables have also been investigated, including relative humidity(MacVean et al., 1986), wind speed(Cusack et al., 2007) and precipitation,(Alexander et al., 1989; Cusack et al., 2007) none of which appear to influence disease occurrence. MacVean et al. also included the effect of dust in the feedlot on respiratory morbidity.(MacVean et al., 1986) While the conclusions of that study suggest that dust particles are associated with BRD, significant challenges in data collection and analysis were described. Airborne dust was collected and separated by particle size over a period of months in 2 consecutive years. These data were then plotted parallel to morbidity data, and subjectively assessed to determine what temporal relationship may have existed. Based upon their visual appraisal, the authors determined that cattle on feed 16 to 30 days had the closest correlation between disease and presence of dust. Regression analysis was then performed, which determined that a 15-day lag time from peak exposure to peak disease yielded the closest correlation. The investigators

ultimately concluded that particles between 2.0 to 3.3 micrometers in diameter impacted BRD incidence. This conclusion was the same for each of the two years; however, the magnitude of the relationship was different, such that the data from both years could not be combined into a single equation. Other variables believed to impact BRD occurrence (age, weight, history or source of cattle) were not included in the analysis. In contrast to the implications of the study above, a controlled experiment failed to identify an impact of dust on respiratory tract clearance of pathogens in a goat model.(Purdy et al., 2003) Two limitations of this study were that the dust used had an average size much larger than that found by McVean's epidemiologic study to be associated with BRD, and the measured outcomes did not include treatment or morbidity.

Management factors

Source: Ranch vs. salebarn

While environmental factors may impact BRD incidence, it is more important to identify predisposing factors within the control of the producer. Therefore, study of these subjects has received more attention, although the results are often no more conclusive. It has been repeatedly demonstrated that newly received cattle are at greatest risk for BRD.(Alexander et al., 1989; Jensen et al., 1976; Kelly and Janzen, 1986; MacVean et al., 1986; Martin and Meek, 1986; Ribble et al., 1995b; Sanderson et al., 2008) As a result, actions taken at or around the time of receiving in the feedlot or stocker/backgrounder operation have been scrutinized. Calves purchased through a sale barn are more at risk than those arriving directly from farm sources.(Gummow and Mapham, 2000; Hansen et al., 1992; Step et al., 2008; Wilson et al., 1985) This has generally been considered to result from greater exposure to pathogens as well as

increased stress during multiple episodes of transit, marketing, commingling, etc. As such, a common recommendation is to avoid purchasing sale barn cattle and preferably buy directly from the farm or ranch of origin.(Radostits, 2001; Woods et al., 1973b) However, other factors commonly associated with sale barn calves may be responsible for the increased risk, rather than the sale barn process itself. For example, BRD incidence increases when cattle from multiple sources are commingled.(Sanderson et al., 2008) This most commonly results from buying calves from one or more sale barns and combining through an order buyer. However, commingling by other means also increases BRD risk. One example is introduction of new arrivals into a group over the course of several days to weeks, which has been shown to increase BRD incidence compared to filling the pen in a shorter period of time.(Alexander et al., 1989; Martin et al., 1982; Ribble et al., 1995c) Hence, commingling, rather than sale barn exposure, may play the greater role in BRD incidence. This idea was supported by Ribble et al.(Ribble et al., 1998) In that study, all calves were purchased through sale barns; however, some groups or pens of cattle came from fewer sources (more calves per farm) and therefore had less commingling, while other groups came from a larger number of farms, meaning there was more commingling. Incidence of BRD was higher in groups of calves put together from more sources than those created from fewer sources. Unfortunately, there was no opportunity to compare commingled versus non-commingled calves that had not gone through a sale barn. Step, et al. compared morbidity among ranch-direct calves, market calves, and commingled calves (calves from ranch and market, penned together after arrival).(Step et al., 2008) The study found commingled steers to be intermediate in respiratory disease rates (higher than ranch, but lower than market). However, it was not

reported if ranch calves had higher rates of illness due to commingling, or whether the higher rates in the commingled pens were strictly attributable to the increased disease among market calves.

Age & weight

Feedlot cattle are arbitrarily categorized as “calves” or “yearlings”, usually based upon weight and phenotype at the time of entry. Yearling cattle are reported to have lower incidence of morbidity and mortality, (Jensen and Mackey, 1979; Radostits, 2001) although no data were provided to support the claim. This assumption was considered a potential explanatory factor for variations in morbidity from year to year.(Loneragan et al., 2001) However, the relationship has not been uniformly apparent when investigated. Only one study reviewed provided the actual age of animals, and it related to bulls in a test station rather than feedlot cattle. That investigation found age to be associated with fever, a classification that was largely intended as a proxy for BRD. The youngest calf was found to be 5 times more likely to be diagnosed with fever when compared to the oldest calf, wherein there was a difference of 100 days in age.(Townsend et al., 1989) Several studies suggested that lighter-weight calves were at greater risk than heavier ones.(Bateman et al., 1990; Gummow and Mapham, 2000; Martin et al., 1989; Sanderson et al., 2008; Taylor et al., 1999) Gummow and Maphem dichotomized cattle based upon being above or below the mean weight of the group. It was determined that calves weighing less than the mean were 1.4 times more likely to develop BRD than those weighing more than the mean.(Gummow and Mapham, 2000) Bateman et al. compared the average arrival weight of calves later classified as sick to that of calves remaining healthy, and found a 7 kg difference between those groups.(Bateman et al., 1990)

Although this difference was statistically significant, it is clinically irrelevant (it reflected a 3% difference of arrival weight, a value well within the expected variability of weight of calves within a lot). Interestingly, re-analysis of the data in a later study found that weight at entry was not predictive of BRD at a group level.(Martin et al., 1990)

Sanderson et al. categorized calves as less than 250 kg, between 250 and 318 kg and greater than 318 kg. It was found that calves weighing more than 318 kg were less likely to develop BRD than calves weighing less than 250 kg (relative risk of 0.18, $p < 0.00$).

There was a trend ($p = 0.09$), although not statistically significant, for mid-range calves to also have less respiratory events than those in the lighter classification.(Sanderson et al., 2008) Other studies have contradicted these findings. In multivariate analysis, Thompson et al. found no difference in arrival weight among animals that did not suffer from BRD, those that developed subclinical BRD (never treated but had lung lesions at slaughter), and those that were treated for BRD.(Thompson et al., 2006) This study primarily utilized lighter weight calves, with mean body weight of 233.4 kg at arrival; no weight range was reported, but cattle weighing between 150 and 300 kg were eligible for inclusion in the study. It is possible that this inclusion criteria skewed the study population toward high risk calves, preventing adequate comparison to what would typically be considered yearling cattle. Indeed, age at entry was listed as ranging from 5 to 10 months.

Additionally, since the study was conducted in South Africa, cattle and/or management factors may have been significantly different from those typical for North America. Therefore, conclusions may be less applicable to US and Canadian cattle industries, although other findings (percent morbidity, percent with lung lesions, reduction in average daily gain attributable to morbidity and lung lesions) were consistent with those

detected in North American studies. Studies conducted at commercial US feedlots have also failed to find a relationship between arrival weight and BRD. The mean and standard deviation for arrival weight in one study was 335 ± 59 kg (Alexander et al., 1989). These cattle may have been too heavy to demonstrate a weight effect on disease, because the majority of calves may have been old enough to be considered yearlings. This seems unlikely, however, since the weight ranged from 217 to 530 kg; assuming the cattle were normally distributed across this weight range, such a range would seem large enough to identify weight-related effects, if they existed. Another study found no difference in arrival weight between calves later treated for BRD compared to non-treated cattle. Interestingly, cattle with lung lesions identified at slaughter actually had heavier entry weights than those without lesions. (Gardner et al., 1999) It is possible that the lesions were present at entry and did not reflect disease incidence in the feedlot. This seems unlikely, however, since the cattle with lesions had lower average daily gain in the feedlot, a finding consistent with active disease. Finally, stocker calves from the southeastern US had an association between weight and BRD in some, but not all, groups of calves examined, with lighter calves having increased incidence of BRD. (Pinchak et al., 2004)

Gender

Gender of calf has been investigated in relation to BRD, with, once again, conflicting results. In two separate studies analyzing disease from birth through feedlot, male calves were at higher risk for BRD than female calves. (Muggli-Cockett et al., 1992; Wittum and Perino, 1995) Two studies that examined only cattle after feedlot arrival also found that males were at a greater risk than females for developing respiratory disease after entering

the feedlot.(Alexander et al., 1989; Gallo and Berg, 1995) An Australian study found steers were at slightly higher mortality risk than heifers.(Cusack et al., 2007) This was reported as crude mortality and was thus not exclusive to BRD. While BRD was the greatest single risk factor for death, it is unclear what effect conditions unique to males (i.e. urolithiasis) may have had on crude mortality, and it was not reported whether BRD mortality was higher in steers. In contrast to the above studies, Loneragan's retrospective study examining records on over 21 million feedlot cattle found a higher incidence of BRD-associated mortality in heifers than in steers for the time period from 1997 to 1999. No difference was observed, however, for cattle from 1994 to 1996.(Loneragan et al., 2001) In a 1988 Canadian study, heifers were also at a greater risk of fatal BRD compared to steers.(Ribble et al., 1988) However, heifers were purchased in smaller groups and combined over a longer period of time than were steers; as previously discussed, such practice is associated with increased BRD. Therefore, extended commingling may account for the difference in respiratory disease mortality between genders in this study. Other studies have found no difference in respiratory disease between males and females, although one survey found cattle in mixed-gender pens were at higher risk than those in either exclusively steer or exclusively heifer pens.(Sanderson et al., 2008)

Covariates with gender

Numerous factors have been investigated in attempts to explain why one gender may be more commonly affected by respiratory disease. The three feedlot studies that found males at greater risk for BRD all categorized calves either as heifers and steers with no mention of bulls.(Alexander et al., 1989; Cusack et al., 2007; Gallo and Berg, 1995)

However, many calves arrive at the feedlot as bulls and must be castrated. This may explain at least part of the increased risk of BRD in steers, because calves castrated after purchase are at increased risk of BRD compared to calves castrated prior to arrival.(Berry et al., 2001; Pinchak et al., 2004) Increased morbidity in steers may also be partly attributable to the so-called “buller” syndrome, wherein a steer is repeatedly mounted and ridden by penmates. One study found “bullers” have an increased risk of BRD when compared to other steers,(Taylor et al., 1999) while another study did not find such an association.(Meyer et al., 2002) Riding behavior can also occur among heifers, in association with estrus. To prevent risk of injury and decreased weight gain, melengesterol acetate (MGA, a synthetic progesterone) is frequently fed to heifers to inhibit estrus. It has been suggested that the anti-inflammatory effects of MGA may also reduce chronicity of respiratory disease, possibly resulting in disparity of disease between heifers and steers. However, morbidity and mortality were unaffected by MGA supplementation,(Sulpizio et al., 3 A.D.) and a subsequent study found that lesions were worse in MGA supplemented heifers following experimental challenge with *M. haemolytica*.(Corrigan et al., 2007)

Castration and dehorning

As alluded to above, castration after arrival has been proposed as a risk factor for BRD. Like most other risk factors examined in relation to BRD, the association has not been consistent. Several studies regarding castration did not analyze morbidity as an outcome of interest. Instead average daily gain (ADG), or plasma concentrations of acute phase proteins or cortisol were reported(Chase, Jr. et al., 1995; Fisher et al., 2001; Pinchak et

al., 2004). Therefore, any association between castration and BRD is merely by extrapolation. Nevertheless, delayed castration has repeatedly been found to reduce ADG,(Berry et al., 2001; Brazle, 1992; Chase, Jr. et al., 1995; Faulkner et al., 1992; Fisher et al., 2001; Pinchak et al., 2004; Zwiacher et al., 1979) a result that was not influenced by analgesia during castration.(Faulkner et al., 1992) This suggests that castration is a stressful event. Indeed, castration of large bulls increased plasma cortisol concentrations.(Chase, Jr. et al., 1995) Given the immunosuppressive nature of increased cortisol levels, castration of older animals may put them at greater risk of BRD than non-castrated animals or those castrated at a younger age. This supposition was supported by several studies,(Berry et al., 2001; Brazle, 1992; Zwiacher et al., 1979) whereas others found no association or inconsistent findings between delayed castration and BRD.(Berry et al., 2001; Brazle, 1992; Faulkner et al., 1992) It is possible that failure to castrate at an early age is indicative of poor management in general, and the cumulative effects of poor management result in increased disease. Such a postulation would be difficult to assess.

Dehorning is similar to castration in that it is a painful procedure that is generally recommended to be performed early in life. Substantial research has been done regarding the immediate responses to dehorning, but few studies have evaluated longer term morbidity associated with this procedure. Martin et al. found increased BRD in groups where greater than 30% of the calves were dehorned.(Martin et al., 1982) Others found inconsistent effect of age at dehorning on weight gain,(Loxton et al., 1982) although calves dehorned in the feedlot had lower average daily gain.(Goonewardene and Hand, 1991; Hand and Goonewardene, 1989) This effect was accentuated if calves were

castrated at the same time. However, BRD morbidity was not measured in any of these latter studies.

Regardless of the negative effects that may result from dehorning and castration, if calves arrive at the feedlot with horns and/or testicles the procedures should be performed to meet current industry standards. The only questions would be how and when the measures should be performed. Most studies comparing castration methods examine surgical (knife) castration to banding. Results have not been unanimous; however, the trend has been for surgical technique to be preferred,(Berry et al., 2001; Fisher et al., 2001) with none of the reviewed studies identifying an advantage to banding. It is important to note that this statement is in regards to castration of older calves and yearlings, not pre-weaning castration. Studies examining young calves do not often measure association with BRD and thus were not reviewed for this paper. One study compared two different methods of surgical castration- emasculation versus ligation of the spermatic cord. No significant difference in morbidity was attributable to method. An advantage in ADG was found for castrating at arrival but no significant difference in morbidity was detected due to delaying the procedure one or two weeks. (Zweiacher et al., 1979)

Interventions

Processing

In most cases, castration and dehorning are done at the same time as other “processing” procedures. These procedures include administration of vaccines, growth promotant implants, anthelmintics and parenteral vitamins, as well as pregnancy examination of

heifers with abortion of those found to be pregnant. Unfortunately, these activities have received relatively little research in their relation to BRD. The metabolic effects of growth-promotant implants are well known. Phillips, et al. hypothesized that the improved protein efficiency induced by zeranol would be beneficial for stressed calves. Based upon blood glucose and urea nitrogen levels, their study supported that implantation of zeranol conserved energy reserves during transit, but morbidity was not affected.(Phillips et al., 1986) Martin found that deworming in the first two weeks after arrival had a negative impact on health.(Martin et al., 1982) However, this association may have simply been co-linear with other processing procedures, including vaccination, which were associated with increased BRD. Another study found pre-conditioned calves dewormed with thiabendazole had lower fecal egg counts and also lower disease incidence compared to calves not preconditioned and dewormed.(Woods et al., 1973b) But again, the possibility for co-linearity with other, more important factors must be considered. Two studies found no difference in BRD morbidity in calves treated with an anthelmintic compared to those not treated.(MacGregor et al., 2001; Morter et al., 1984) Similarly, comparison of anthelmintic products found no difference in BRD incidence between cattle treated with various products(Guichon et al., 2000; Schunicht et al., 2000) or detected modest differences that were deemed economically unimportant.(Jim et al., 1992) Many other studies have examined the use of deworming in stocker and feedlot cattle, but did not assess whether treatment impacts respiratory morbidity; such studies were not reviewed for this paper. Relationship of parenteral administration of vitamins A, C, D and E to BRD was examined in an Australian study. Vitamins A, D and E had no impact on disease reduction. Vitamin C, administered at the time of treatment, reduced

mortality.(Cusack et al., 2008) In another study, vitamin E and selenium administration had no effect on BRD incidence, although antibody production in response to vaccination was enhanced.(Droke and Loerch, 1989)

Only one study was found that examined the relationship between pregnancy evaluation and respiratory disease, with pregnancy examination being associated with increased morbidity.(Alexander et al., 1989) No attempt was made to explain such an association, although it could be speculated that calves that were poorly managed prior to arrival were most likely to be examined for pregnancy. Therefore, this may merely be an indicator for other factors considered to put calves at risk for disease (similar to castration). Heifers that are found to be pregnant in the feedlot are typically aborted, and it has been hypothesized that the abortion would be a stressful event that may contribute to BRD.(Edwards, 1989) While various recommendations have been made to manage such heifers to reduce stress and disease,(Edwards and Laudert, 1984; MacGregor and Falkner, 1997) few studies have examined the significance of such a relationship. One study examining open, aborted and pregnant heifers observed “no significant health problems or mortality in any of the groups,” but no data or statistical analysis was provided.(Jim et al., 1991) A model designed to assess cost of various management options assumed a 5% increase in BRD morbidity associated with aborting heifers,(Buhman et al., 2003) but this figure was based upon a study documenting immunosuppression associated with dystocia rather than documented effects of abortion on respiratory disease.

Timing of processing

It has been theorized that the added stress of the various processing procedures may contribute to morbidity. This has occasionally led to the recommendation to delay processing until cattle have acclimated to the new environment or to perform some procedures at arrival while delaying others. Empirical evidence indicates that most people in the industry do not consider processing at or shortly after arrival to be detrimental; a large percentage of feedlots employ this practice, and many of the studies reviewed (regardless of variables of interest examined) also describe prompt processing of calves. (Alexander et al., 1989; Bagley et al., 2003; Bechtol et al., 1991; Berry et al., 2001; Berry et al., 2004b; Booker et al., 1999; Buhman et al., 2000; Duff et al., 2000; Fluharty and Loerch, 1996; Frank and Duff, 2000; Gibb et al., 2000; Lofgreen, 1983; Ribble et al., 1988; Roeber et al., 2001; Wildman et al., 2008). A trend toward higher morbidity was reported in calves for whom processing was delayed when compared to calves processed at arrival. An extended period of time during which initial BRD cases occurred was also noted when processing was delayed two to three weeks. (Lofgreen et al., 1978) These trends were minor, and significance cannot be assigned because statistical analysis was not performed. Kreikemeier found no difference in morbidity or mortality but improved performance associated with processing at arrival compared to waiting three weeks. (Kreikemeier K et al., 1996) In contrast, Martin et al. found that processing calves in the first two weeks after arrival increased disease compared to waiting two to four weeks for processing. (Martin et al., 1982) Others have examined delaying some (but not all) components of processing. For example, one study administered anti-parasitic treatments and clostridial vaccines to all cattle at arrival (dehorning and castration were done, as needed, at this time as well). A modified live

viral vaccine was either given at arrival, delayed for one week, or not given at all.(Duff et al., 2000) No difference attributable to vaccine administration was noted in morbidity or mortality. However, the power of this study to detect differences due to treatment was quite low. In another study, administration of a modified live viral vaccine was delayed two weeks, whereas other processing activities (castration, deworming and vaccination with a clostridial bacterin) were performed at arrival.(Richeson et al., 2008) This resulted in increased ADG, with no difference in morbidity or mortality compared to inclusion of the modified live viral vaccine at arrival.

Vaccination

Vaccination for viral agents, and to a lesser extent bacterial pathogens, associated with BRD is widespread in the industry. Surprisingly, there is little scientific support for the practice. One of the first large epidemiologic studies regarding BRD found that vaccinating for respiratory pathogens in the feedlot was associated with increased risk of BRD.(Martin et al., 1982) In fact, it appeared to be one of the most detrimental practices employed by feedlots. In interpreting these data, it would appear feasible that calves considered at high risk for BRD were more likely to be vaccinated, thus creating a spurious association. This idea was disputed by the authors, who stated “Owners rarely vaccinate sick groups of calves and thus the increase of risk of death... is attributed to the vaccines.”(Martin et al., 1983) Even accepting the authors’ interpretations, the data for this analysis were collected from 1978 to 1980; thus it could be asserted that much has changed in vaccinology in the past three decades and findings would likely be different today. Indeed, there are numerous studies which have found a protective factor from vaccinating.(Bechtol et al., 1991; Hansen et al., 1992; Knight et al., 1972; Loan et al.,

1998; Macartney et al., 2003; MacGregor and Wray, 2004; O'Connor et al., 2001; Schunicht et al., 2003) There are similar numbers of studies in which vaccination was found to be ineffective or inconclusive.(Duff et al., 2000; Frank et al., 1996; Kiesel et al., 1972; MacGregor et al., 2003; Sanderson et al., 2008) When a rigorous critique is done of the entire body of work regarding respiratory vaccines the evidence of vaccine efficacy in real-world situations is equivocal, at best.(Mosier et al., 1989; Perino and Hunsaker, 1997) The presence of antibodies against various pathogens has been found to be protective, (Booker et al., 1999; Fulton et al., 2002b) so it would seem intuitive that immunization against those pathogens would be beneficial. But while vaccination is consistently shown to result in antibody production, vaccination induced titers are not always correlated with protection against disease.(Kiesel et al., 1972; Loan et al., 1998) The failure to identify the specific characteristics of a vaccine program that will consistently protect against disease is one of the more frustrating areas of research related to BRD.

Preconditioning

Since no combination of receiving/processing practices has been found to eliminate BRD, significant effort has been extended in identifying pre-shipment practices that would reduce disease. Such measures, typically referred to as “preconditioning,” were proposed as early as 1967.(Lalman and Smith, 2004) While variation exists in defining what should be done for calves to be considered “preconditioned”, the most common components are:

- Vaccination, most commonly for respiratory viruses and clostridial diseases; some programs include *M. haemolytica*, *P. multocida* and *H. somni* biologicals in some combination.
- Dehorning and castration, with adequate time allowed for healing prior to sale
- Weaning prior to sale, with suggested time frames varying from several days to several weeks
- Training of calves to eat from a bunk and drink from a trough

The intent is to spread the various sources of stress (weaning, surgeries, transport, feed changes) over multiple episodes rather than all at once. Additionally, vaccination should permit development of immunity prior to time of maximum stress and exposure to pathogens.

While many in the beef industry advocate adoption of a preconditioning program, disappointment is frequently expressed that the practice has failed to become widespread. This is often blamed upon a variety of reasons, yet only rarely is the validity of the idea questioned. Many reports that demonstrate positive results from preconditioning are restricted to lay publications and are not subjected to peer review. Such articles are often biased, demonstrating a desire to convince producers to adopt preconditioning practices. These studies frequently do not report on-farm morbidity that may occur during the preconditioning phase. Thus it is possible that disease is not reduced, it is just shifted from the stocker or feedlot operator to the cow-calf producer. Frequently, preconditioned calves may benefit from being older and heavier at time of selling, which, as discussed

above, may in and of itself reduce disease. Finally, some of these calves are delivered directly to the feedlot or backgrounding operation, bypassing the commingling and stress typically associated with the sale barns from which control calves may be derived. Reduction in disease therefore cannot be ascribed solely to preconditioning. To truly assess the effect of preconditioning, control calves should also bypass the sale barns, but without the weaning, vaccination and other processing activities that define preconditioning; alternatively, preconditioned calves should also be sold through the sale barn and order buyer process through which controls are obtained. The latter option has been employed in recent studies, discussed below.

Peer-reviewed field trials evaluating the effectiveness of preconditioning since at least the 1970s have been less than definitive in its benefit. Several studies found inconsistent results when multiple years are evaluated, such that morbidity was reduced in one year but not others.(Woods et al., 1973b) Other studies have demonstrated no reduction of disease attributable to preconditioning, (Kiesel et al., 1972; Pritchard and Mendez, 1990; Wieringa et al., 1976) whereas other research found reduction in disease but no overall economic advantage associated with preconditioning.(Kadel et al., 1985) A 1985 review of the research available at that time verified the equivocal findings of many studies regarding preconditioning.(Cole, 1985) On an aggregate basis, Cole found a slight reduction in disease attributable to preconditioning, although the economic benefits were deemed questionable.

Many recent studies have demonstrated more positive results attributable to preconditioning. (Hansen et al., 1992; Karren et al., 1987; Lalman and Ward, 2005; Lynch et al., 1997; Macartney et al., 2003; Roeber et al., 2001; Step et al., 2008) While these studies typically feature better design and analysis than previous research, most still have significant limitations. One major challenge in assessing these studies is a lack of uniformity in defining and implementing preconditioning. For example, the study by Lynch, et al. was more accurately a vaccination trial, as calves “were considered preconditioned if they had received both viral and *Pasteurella* vaccines 14 to 21 days prior to weaning.” In the study by Karren, et al., preconditioning included weaning in a drylot and acclimating to a concentrate ration in addition to vaccination. The Macartney, et al. and Roeber, et al. studies required calves to be vaccinated and weaned to be called preconditioned. However, the Macartney, et al. study had no requirement for “bunk breaking” or diet composition, whereas the Roeber et al. study required calves to be acclimated to feed and water troughs. Hansen et al. offered no description of what was required for calves to have been considered pre-conditioned. Such varied and vague definitions of “preconditioning” make across-study comparison difficult and results challenging to interpret. This potential confusion is further exemplified in a large survey study that found no benefit to preconditioning. (Sanderson et al., 2008) The survey nature of the study meant that the preconditioning status of the calves was defined by the feedlot managers who reported the data; therefore, the definition was likely inconsistent and may have influenced the findings.

How the calves are marketed can also impact the relevance of preconditioning studies. In one study (Karren et al., 1987), control and treatment calves came from one source and were sent directly to the feedlot. This processing eliminated much potential for variation but also reduced external validity, because most purchasers of preconditioned cattle must acquire them from multiple sources and commingle them. The studies by Macartney, et al. (Macartney et al., 2003) and Roeber et al. (Roeber et al., 2001) compared preconditioned calves to vaccinated calves and those sold through conventional sales (no vaccination or treatment requirements). Both preconditioned and vaccinated calves underwent vaccination, castration and dehorning defined time periods prior to sale; in addition, calves in the preconditioned group were weaned at least one month prior to sale. In each study, calves from the three groups were marketed through the same sale barn (on different dates). These protocols are most consistent with industry definitions and practices, and make these studies arguably the most effective in assessing preconditioning. Macartney et al. found that both treatment groups (vaccination and preconditioning) had lower incidence of morbidity compared to calves obtained through conventional auction. Indeed, the benefit attributable to preconditioning was quite large: conventionally sold calves were 4.5 times more likely to be treated than were preconditioned calves. However, the fact that those who made treatment decisions (owners) were not blinded to the origin of the cattle introduces substantial bias. Owners who purchased non-vaccinated calves may have expected more disease and therefore treated more aggressively. This concern is arguably bolstered by the absence of difference in mortality between groups. It is conceivable that the more aggressive treatment was necessary to prevent mortality in the conventional calves, but it is possible

that this group of calves were over-treated due to the owners' expectations of risk. In the Roeber, et al. study, pen riders were blinded to vaccination status, reducing bias. Results indicated that non-preconditioned calves experienced higher morbidity than either calves vaccinated and weaned 30 days prior to sale or those vaccinated but not weaned; mortality was not reported. Paradoxically, ADG was highest in non-preconditioned calves.(Roeber et al., 2001)

A recent study provided further evidence of the benefits of preconditioning, and also helped define what components are most important for success. Specifically, the authors reported that weaning alone had similar benefits to weaning and vaccination.(Step et al., 2008) Both treatments resulted in lower morbidity compared to shipping calves directly to the feedlot or purchasing calves from a salebarn. Since calves from both treatment groups were sent directly to the feedlot, it remains possible that vaccination at weaning would benefit calves marketed through more typical avenues, including special sales at salebarns.

Nutritional Factors

Receiving ration

Because the feedstuffs consumed in a feedlot are often quite different from those consumed by calves prior to feedlot entry, several studies have examined the effect that nutritional factors may have on respiratory disease. Martin et al. found that calves fed corn silage had higher morbidity than those fed hay; however, this negative result could be mitigated somewhat by adding grain to the diet.(Martin et al., 1982) In contrast, Wilson et al. found that high grain levels were associated with increased BRD.(Wilson et

al., 1985) This assessment of the impact of dietary differences may have been confounded by other variables, including the source of the cattle and time between arrival and processing. In a more controlled study, concentrates were fed at relatively high levels (72% of ration) without impacting BRD incidence, whereas higher levels (90%) increased morbidity.(Lofgreen et al., 1975) Addition of free choice hay provided no benefit. Fluharty and Loerch found no significant differences in morbidity associated with variation of feedstuffs, varying levels of concentrate (up to 85%), or percentage of protein.(Fluharty and Loerch, 1996) Their findings, however, indicated that the number of treatments required for sick calves increased as the percentage of concentrate increased. Other studies have demonstrated a trend for increasing morbidity with increasing levels of concentrate, typically becoming apparent at varying points above 50% of the diet.(Galyean et al., 1999) Berry et al. maintained a narrow range of concentrate: roughage ratio to assess the impact of varying levels of energy and starch with minimal confounding. This study failed to identify a significant association between energy and starch levels within the ration and development of disease.(Berry et al., 2004b) A comprehensive review concluded that diets with increased energy density (concentrates) can be employed for improved ADG without altering incidence of BRD.(Duff and Galyean, 2006) Crude protein (CP) content is another area frequently investigated to determine ideal levels to include in receiving rations. As mentioned above, Fluharty and Loerch found no association between CP levels and BRD. In other studies, however, CP appeared similar to concentrate percentage in that increased morbidity occurred as levels increased, with the preferred inclusion level being approximately 14%.(Galyean et al., 1999)

Various other nutritional factors have been proposed to reduce morbidity, including elevated potassium in the receiving ration, thiamine and other B-vitamins and rumen bypass protein.(Brethour and Duitsman, 1972; Hutcheson et al., 1984; Phillips et al., 1986) None of these consistently reduced disease.(Cole et al., 1979; Phillips et al., 1986) Vitamin E may be beneficial when included at increased levels in the ration (although as discussed above, injectable products may not be beneficial).(Carter et al., 2002; Galyean et al., 1999)

Micromineral status is another nutritional topic that has received attention. Researchers have studied the relationship between BRD and varying mineral levels as well as the form in which the nutrients are supplied. In one study, reduced treatment rates were noted in calves supplemented with metal-complex minerals compared to calves offered no mineral supplement or those receiving inorganic sources (sulfate-complexed).(Grotelueschen et al., 2001) However, another study found no difference when comparing organic complexes and sulfate-bound minerals in non-stressed calves.(Salyer et al., 2004) Disparity in results of such studies may be due to differences in the mineral status of the calf at the time of enrollment in the study. However, this assertion was not supported by a study which found no correlation between mineral deficiency at arrival and treatment for BRD.(Bagley et al., 2003) Other studies have examined copper, zinc, vitamin E and selenium, chromium and various combinations of these, evaluating immune function and/or calf performance (ADG, feed/gain ratio, etc.). Reviews of the interaction between nutrition and immunity concluded that the

inconsistencies in trial results preclude any definitive link between BRD and most specific nutritional factors, (Galyean et al., 1999) and “after decades of research, our ability to modify the incidence of BRD through nutritional manipulations seems limited.”(Duff and Galyean, 2006)

Increasing intake

Finally, in considering nutrition’s role in BRD, maintaining adequate feed intake may be more important than what is included in the ration. Weaned calves may not be accustomed to prepared feeds, eating from feed bunks and drinking from waterers; therefore, the use of trainer animals has been suggested to acclimate calves to these activities. Such trainers would be older cattle, acclimated to the feedlot environment and familiar with the facilities for eating and drinking. A study found that use of cull cows as trainers improved calf health in several, but not all, trials. The same investigators found that use of trainer steers was not beneficial, and led to increased disease in the trainer steers.(Loerch and Fluharty, 2000) Similarly, Gibb et al. found the presence of trainer cows had no effects on morbidity for newly received calves but did reduce ADG for calves in early feeding period.(Gibb et al., 2000)

Prophylaxis/Metaphylaxis

Parenteral antimicrobials

The involvement of *M. haemolytica* and other bacterial pathogens in BRD suggests that antimicrobials would be beneficial in controlling disease. Indeed, treatment of affected calves almost invariably involves antimicrobials. It has also been hypothesized that mass

treatment of at-risk populations could reduce disease. Such mass treatment can be accomplished in one of three ways- parenterally, in feed, or in water. Administration of parenteral products to calves that are at high risk for BRD (termed metaphylaxis) has consistently been found to reduce morbidity.(Cusack, 2004; Donkersgoed, 1992; Frank et al., 2002; Frank and Duff, 2000; Macartney et al., 2003; Step et al., 2007; Wellman and O'Connor, 2007) Products found to be successful include ceftiofur crystalline free acid, florfenicol, tilmicosin and tulathromycin, with conflicting results for oxytetracycline.(Cusack, 2004; Schunicht et al., 2002) Most studies examined administration of these products at feedlot arrival, but pre-shipment treatment was also found to be effective.(Frank and Duff, 2000; McClary and Vogel, 1999) Despite the effectiveness of metaphylaxis, cost and labeling restrictions preclude mass treatment of calves not considered at high risk for BRD.(Young, 1995) It has been found that calves that have an increased pre-transit body temperature are at greater risk of disease;(Chirase et al., 2004) therefore febrile calves may be considered prime candidates for targeted treatment without administering antimicrobials to the entire group. However, Lofgreen concluded that a treatment system based upon arrival temperatures was not reliable.(Lofgreen, 1979) Vogel et al. found selectively treating calves based upon presence of fever reduced subsequent morbidity compared to no prophylactic treatment. However, it was less effective than metaphylactic treatment of the entire group.(Vogel et al., 1998)

Oral antimicrobials

The benefits of mass administration of oral antibiotics are less certain. Martin et al. found that antimicrobials delivered in water were associated with increased mortality (no distinction was made between which antimicrobials were used).(Martin et al., 1982) This relevance of this is debatable, however, as results may have been confounded by several factors. First, cattle that had greater early morbidity were more likely to be given antimicrobials in the water. Such groups were at greater risk of having higher overall mortality, which may have been due to other factors and unrelated to administration of the antimicrobials. Second, increased mortality may have resulted in the treated calves because the number of water sources was reduced so that only those sources with antimicrobials were available. Thus, death may have been related to limited water supply and subsequent dehydration rather than to the inclusion of antimicrobials. Another hypothesis for the relationship between antimicrobials in water and mortality include decreased palatability of the water, thereby reducing intake. Alternatively, some producers may demonstrate decreased attentiveness in promptly treating sick calves when antimicrobials are used in the water, because the owner assumes that providing treatment in the water decreases the need to treat parenterally.(Martin, 1985) In the same study, administration of antimicrobials in feed reduced morbidity. This is in agreement with a study that suggested an association between chlortetracycline and sulfamethazine in feed and reduced morbidity at the time of weaning; the results appear convincing but statistical analysis was not performed.(Woods et al., 1973a) The limited evidence of efficacy of antimicrobial administration through feed and water was exemplified in a meta-analysis that found only ten well-designed, executed and analyzed studies examining use of mass medication of antimicrobials in any manner, and all ten related

solely to parenteral use.(Donkersgoed, 1992) A study reported after the meta-analysis found that chlortetracycline and sulfamethazine in the ration significantly reduced treatment and mortality in feedlot calves.(Gallo and Berg, 1995) A Kansas study reported that chlortetracycline in the feed was as effective as parenteral tilmicosin in preventing BRD.(Kreikemeier K et al., 1996) That finding was disputed in a different study, where parenteral administration of tilmicosin was preferable to inclusion of chlortetracycline in feed for reducing morbidity;(Hellwig et al., 1999) no control group was included to determine if chlortetracycline provided any benefit. A similar study also found tilmicosin to be preferable to including chlortetracycline in the feed.(Frank and Duff, 2000) Moreover, that study showed that calves receiving chlortetracycline in the feed from days five to nine had similar occurrence of BRD when compared to calves not receiving chlortetracycline.

Other Considerations

Impact of Cattle Characteristics

Disposition

A variety of other factors have been examined as to their relation to BRD. Fell, et al. assembled two groups of cattle deemed “nervous” and “calm” based upon behavioral scores under defined conditions. The study found that nervous calves were more likely to be treated for disease in the feedlot than were calm calves, although their assessment was not confined to BRD.(Fell et al., 1999)

Genetics

Heritability of BRD susceptibility appears to be low,(Muggli-Cockett et al., 1992; Snowder et al., 2005) but breed differences have been found to exist. Braunvieh cattle appear to be the most susceptible, and animals of composite breeding have greatest resistance.(Muggli-Cockett et al., 1992; Snowder et al., 2005) Other studies have reported varied findings of breed and BRD susceptibility. A Swedish bull test study reported Angus and Hereford bulls to be at higher risk of disease compared to other breeds.(Hagglund et al., 2007) However, the study was not designed to assess breed effects, and the authors cautioned about the validity of this finding. Another study found Herefords to be at higher risk, but in contrast to Hagglund et al., Durham et al. found Angus to have decreased susceptibility compared to other breeds.(Durham et al., 1991) An Australian study compared *Bos taurus* (represented by several breeds) vs. *Bos indicus* (represented by Santa Gertrudis and Santa Gertrudis cross) cattle for BRD incidence and found *Bos taurus* to be at greater risk for BRD than *Bos indicus*.(Cusack et al., 2007) Given the evolutionary origin of these types of cattle (*Bos indicus* being from hotter, drier climates), disease susceptibility could be expected to be affected by geographic location and climate factors, and may not apply universally. As BRD resistance is poorly heritable, it may be expected that hybrid vigor is beneficial in reducing respiratory disease. This was confirmed in a study that found crossbred cattle had a lower incidence.(Snowder et al., 2005) Interestingly, a report published later by the same researchers found no benefits of heterosis among composite cattle compared to purebreds.(Snowder et al., 2006)

Parental factors may play a role in BRD occurrence. One study found that calves from younger dams had higher pre-weaning incidence of BRD but lower post-weaning BRD rates.(Muggli-Cockett et al., 1992) Surprisingly, it was found that dams that appear to be resistant to BRD are more likely to have calves that are susceptible.(Snowder et al., 2005) Both of these findings may relate to strong and persisting passive immunity preventing development of active immunity. Heifers have lower antibody levels in colostrum, thus their calves would be susceptible at a younger age. Cows with greater resistance would provide longer lasting passive immunity, possibly interfering with development of acquired immunity.(Snowder et al., 2005) The importance of maternal transfer of immune factors was further bolstered by a study that found vaccination of dams for IBR and BVD prior to calving decreased BRD in pre-weaned calves(Ganaba et al., 1995) Benefits of passive immunity appear to extend beyond weaning. Calves diagnosed with failure of passive transfer (assessed with plasma protein) continued to be at higher risk for respiratory disease even in the feedlot.(Wittum and Perino, 1995)

Study Considerations

Confounders

Despite the myriad of potential factors studied and associations found, none can provide more than a minor accounting for the incidence of BRD that plagues the cattle industry. Regardless of the design of the study or factors being explored, it is common to find that one of the largest associations of BRD is related to the farm of origin, the destination operation or both.(Bagley et al., 2003; Frank et al., 1996; Fulton et al., 2002b; Macartney et al., 2003; Martin and Bohac, 1986) These ill-defined associations not only conceal

specific risk factors, they can distort interpretation of those that are identified. Examples of this were alluded to earlier, when discussing castration and pregnancy checking. Certain operations may consistently send bulls or pregnant heifers to a feedlot. These operations may also engage in unidentified management practices that impact the incidence of BRD morbidity. This may result in an apparent association, for example, between castration and BRD when one does not truly exist. Methods exist to attempt to account for potential clustering of disease due to herd effects, but they have not historically been employed in veterinary literature.(McDermott and Schukken, 1994) Even when adjustments are attempted, they can be difficult to apply and interpret. Nevertheless, when the study population is composed of animals from different sources or groupings, the potential “herd” effect should always be considered and perhaps controlled through one of a variety of techniques.(McDermott et al., 1994) One way to avoid this issue is to consider the herd or group as the unit of interest, rather than the individual animal. This approach has been advocated by Martin, et al., who noted that most management measures are implemented at the group, not individual level.(Martin et al., 1990) Such a technique would be most meaningful in comparing groups that were maintained from birth through feedlot but could potentially be used for shorter periods of time. Unfortunately, biologically plausible and perhaps real associations that are found at the animal level may not be found at the herd level. For example, *Pasteurella* species were isolated more commonly from nasal secretions of sick calves than from healthy calves,(Hoerlein et al., 1961) but morbidity was not higher in herds with a greater frequency of *Pasteurella* isolates.(Magwood et al., 1969) Additionally, caution must be exercised in drawing conclusions from ecological or group level studies. For instance,

what recommendation can be made if there is higher morbidity in a group of calves that included bulls that were castrated, but the castrated calves were not the ones that are sick?

Nature of BRD: Contagious, clustered, or random?

Another challenge frequently encountered in researching BRD is difficulty in replicating results due to large variability in outcomes. This can occur even when the same people execute the same processes using cattle from the same operation(s) in different years.(Bechtol et al., 1991; Karren et al., 1987; Muggli-Cockett et al., 1992; Snowden et al., 2005) This variability begs the question as to whether BRD is contagious, if it clusters due to risk factors, or if it occurs randomly. A small-scale study by Martin found that risk of disease is not increased by housing calves in a pen with sick cattle.(Martin et al., 1988) This may be interpreted as BRD not being contagious in the classic sense. A large, multi-year study concluded that temporal and spatial clustering effects were small.(Snowden et al., 2005) This would suggest that, not only is BRD not contagious, but that risk factors would be hard to identify or may not be important, at least in that study population. However, another study found a spike of BRD in bulls already acclimated at a test station when new bulls were introduced.(Andrews, 1976) The authors interpreted this to suggest that the newly introduced animals exposed the acclimated bulls to new pathogens, thus supporting the idea of transmissibility. A larger study found cases of respiratory disease often clustered according to transport truck (a proxy for source) and less frequently by pen.(Ribble et al., 1994) This would suggest that BRD is not a random event, and thus is suitable for epidemiologic study. Unfortunately, the investigators were unable to conclude whether contagious or non-contagious factors were more important in

determining disease occurrence. Frank et al. used a leukotoxin deficient, live attenuated *M. haemolytica* strain as an intranasal vaccine. This unique organism was subsequently cultured from non-vaccinated calves, suggesting these calves contracted it from vaccinated cattle, albeit at a low and inconsistent rate.(Frank et al., 2002) This was not the case in a similarly designed study completed at a later date, where no transmission to non-vaccinated calves occurred.(Frank et al., 2003) Further clouding the issue of transmissibility was the finding that antimicrobial resistant strains of bacteria emerged in calves treated for BRD, but there was no evidence of these strains being transmitted to other calves in the feedlot.(Allen et al., 1992b)

Molecular techniques have been developed to distinguish among both *M. haemolytica* (Katsuda et al., 2003; Murphy et al., 1993) and *P. multocida* isolates.(Dabo et al., 2000; Davies et al., 2004; Davies, 2004) These would appear to be perfect tools in assessing whether there is significant lateral transmission, but no studies have been reported to date. DeRosa et al. used ribotyping, serotyping and antimicrobial susceptibility testing on isolates from nasal and tracheal swabs. The limited number of ribotypes found was suggestive of lateral transmission, although the study was not designed to explore this possibility. Their findings also suggested nasal swab culture can be predictive of the bacterial pathogen within the lung.(DeRosa et al., 2000) This idea was supported by work reported by Godinho et al.(Godinho et al., 2007) Using post-mortem lung lavage as the gold standard, nasal swabs were found to have 100% positive predictive value for presence of *M. haemolytica* and *M. bovis* in the lung (there were too few *P. multocida* isolates for analysis). Further, random amplified polymorphic DNA polymerase chain

reaction (RAPD-PCR) found excellent correlation between lung and nasal isolates, suggesting that culture of nasal passages of sick calves may provide clues as to what strain is present in the lungs. A different conclusion was reached by Allen, et al., who found only moderate correlation between organisms isolated from nasopharyngeal swabs and those obtained by bronchoalveolar lavage(Allen et al., 1991). This conclusion was made in regards to all pathogens examined; nonetheless, good correlation was present between nasopharyngeal and lung isolation when analysis was restricted to *P. multocida*. Further investigation is warranted to determine if application of molecular techniques to isolates obtained from nasal swabs of clinical cases could provide insight into patterns of transmission of BRD pathogens.

Diagnosis of BRD

Perhaps the greatest hindrance to better elucidating the factors associated with BRD is difficulty in defining and/or identifying the disease. Post-mortem diagnosis, in the absence of prior treatment, with culture and/or histopathology is the most definitive means. It also is an objective parameter that could be more readily compared across multiple studies, years or feedlots. However, withholding treatment is unacceptable on animal welfare and economic considerations. Post-mortem examination following treatment failure has many merits, but due to selective pressures exerted by the immune system and antimicrobials, findings in such cases may not reflect etiologies involved in initiating the disease process. Necropsy is also insensitive in detecting those affected, particularly in outbreaks with low case fatality. Thus, when mortality is used as the outcome of interest most studies have relatively low power to detect treatment

differences. It could also be biased by management decisions of how to manage chronically affected cattle, which may be salvaged, euthanized or retained but provided no further treatment. Subsequently, morbidity is more commonly used as a measure of respiratory incidence than is mortality.

It has been lamented that the case definition for BRD varies greatly within the literature.(Kelly and Janzen, 1986) Some investigators have established specific criteria for classifying calves as suffering from BRD. These criteria include elevated body temperature,(Bateman et al., 1990; Berry et al., 2004b; Chirase et al., 2004; Frank and Duff, 2000) respiratory signs (nasal discharge, cough, dyspnea or tachypnea),(Andrews, 1976; Barbour et al., 1997) decreased appetite, depression, or some combination of these and other secondary signs.(Allen et al., 1991; Bechtol et al., 1991; Berry et al., 2001; Fulton et al., 2000; Gibb et al., 2000; Lofgreen, 1983) Some investigators have used a clinical scoring technique that incorporates several parameters into a semi-objective score that guides treatment decisions and increases consistency within a trial.(Allen et al., 1991; Bateman et al., 1990; Buhman et al., 2000; Step et al., 2007; Step et al., 2008) For example, one point is scored for presence of each of the previously listed clinical signs, and all animals with a score greater than 3 are treated. However, even if the same criteria are used in multiple studies, their application may vary or are used in differing combinations. For example, temperature cutoff points range from 103°F(Duff et al., 2000; Fluharty and Loerch, 1996) to 104.5°F (Booker et al., 1999) or 105°F.(Gallo and Berg, 1995) Many researchers simply accept the judgment of feedlot personnel.(Alexander et al., 1989; Buhman et al., 2000; Camp et al., 1981; Cole et al.,

1979) Thus a calf is said to suffer from BRD if it is pulled for treatment and found to have no signs referable to a system other than the respiratory tract.(Bagley et al., 2003; Cusack, 2004) This approach has been refined slightly by some researchers, who classify treated calves as having “undifferentiated fever/BRD” or “BRD with no fever” depending upon body temperature at time of treatment.(Wildman et al., 2008) It is unclear if this broad classification affords any improvement over the use of more specific criteria. The accuracy of using treatment as a proxy for morbidity is highly questionable. No correlation was found between treatment and respiratory mortality(Griffin et al., 1995; Ribble et al., 1995b), which as mentioned above, is an insensitive but specific indicator of BRD. Other researchers have found good to limited correlation between treatment and serological changes(Allen et al., 1992a; Martin et al., 1989; Martin and Bohac, 1986), poor correlation of treatment with bronchoalveolar lavage cytology(Allen et al., 1992a; Allen et al., 1992c), and no or poor correlation between treatment and lung lesions found at slaughter.(Bryant et al., 1999; Wittum et al., 1996) In fact, lung lesions are often found in nearly as many calves that were considered healthy as in those deemed ill.(Bryant et al., 1999; Gardner et al., 1999; Thompson et al., 2006; Wittum et al., 1996) Thompson et al. found calves treated for BRD were 1.5 times more likely to have lung lesions than those not treated, but 69% of those with lesions were never treated.(Thompson et al., 2006) A high prevalence of lung lesions among treated and non-treated cattle at slaughter was also noted by Buhman, et al.(Buhman et al., 2000) However, these researchers further classified lesions as either severe or minor. This led to the determination that, although there was poor diagnostic agreement between previous treatment and presence

of any lesion (minor or severe), treated calves were more likely to have severe lesions than non-treated calves.(Buhman et al., 2000)

Several studies found that treated calves have decreased ADG compared to non-treated calves.(Gardner et al., 1999; Lalman and Smith, 2004; Thompson et al., 2006; Wittum and Perino, 1995) This would seem intuitive and suggests that those treated were actually ill. But other studies have not corroborated this association.(Griffin et al., 1995; Jim et al., 1993; Martin and Bohac, 1986; Roeber et al., 2001; Wittum et al., 1996) The presence of lung lesions at slaughter were correlated with decreased ADG. (Bryant et al., 1999; Griffin et al., 1995; Thompson et al., 2006; Wittum et al., 1996) Two studies found a correlation between both treatment and lung lesions and decreased ADG, with lesions showing a stronger association.(Gardner et al., 1999; Thompson et al., 2006) These findings have led some to suggest that ADG would be a better barometer of respiratory disease than is field-diagnosis.(Martin et al., 1990; Ribble et al., 1995b) Because ADG can be impacted by things other than BRD, however, using ADG as the primary indicator of respiratory disease would open studies to many new potential confounders.

Other methods of diagnosis and/or classification of BRD are actively being researched, including ancillary testing(Coghe et al., 1999), metabolic profiling,(Aich et al., 2007; Chirase et al., 2004; Step et al., 2008) and improved field diagnostics.(Schaefer et al., 2007) Attempts to identify a single criterion as an indicator of disease have generally been disappointing. Cortisol has been frequently measured in BRD studies, but cortisol concentration does not distinguish between disease and stress, and is difficult to interpret

due to its diurnal variation and rapid changes in circulating levels.(Aich et al., 2007) Similarly, acute phase proteins have generally been unreliable for identifying cattle suffering from BRD, although haptoglobin has shown some promise.(Berry et al., 2004a; Carter et al., 2002) Multi-modal approaches appear to offer some potential for improvement in diagnosis of BRD. For example, a panel of analytes, including acute phase proteins, metabolites and microminerals was able to differentiate between stress, infection, and stress combined with infection.(Aich et al., 2007) However, it was not possible to establish a baseline value for any of the analytes that could distinguish between ‘normal’ and ‘diseased’ animals. It remains to be seen whether such approaches are viable for use in epidemiologic studies.

Conclusion

It is evident that BRD is, and will continue to be, an extremely costly condition. Although much research has been done regarding its determinants, there are only a few conclusive findings. Calves are clearly at highest risk shortly after transport/ arrival. Consistent risk factors include being purchased from sale barns and commingling with cattle from multiple sources. It is unclear whether these practices increase susceptibility to disease, increase exposure, or are proxies for other poor management decisions. It seems likely that lighter-weight calves, which are presumably younger, are at greater risk for BRD than are larger cattle, although the association has not been consistent. Persistent infection with BVDV increases BRD occurrence, but it is unclear if the presence of PI calves have a major impact on other cattle in the feedlot. Preconditioning appears to have some benefit (at least to the purchaser), but efficacy is variable. Weaning prior to sale is

perhaps the most important component of preconditioning. This is bolstered by the fact that vaccination alone appears to have limited value. The practice with the clearest benefit in reducing BRD morbidity and mortality is metaphylaxis. And yet the costs, both monetarily and in terms of potential antimicrobial overuse, preclude its inclusion as routine practice for all cattle. Other factors certainly play a role in development and prevention of BRD, but the complexity of the disease complex has made it difficult to define their role. These stressors may play a role as “necessary, but not sufficient” components, thus requiring an additive effect to manifest as disease. Identifying reasons for tremendous variability in research results may improve our ability to more accurately identify the contributions each of these factors make to disease occurrence.

It is imperative that researchers and practitioners recognize the limitations of the current knowledge, even while attempting to do all possible to reduce the impact of BRD on the beef industry. It would appear that the question warranting the greatest attention would be improved diagnosis of BRD. Until an objective, consistent criterion can be established, it is unlikely that universal findings will be identified. A second question worthy of consideration is heterogeneity within the agents associated with BRD. Given the variability in BRD morbidity and mortality that cannot be readily ascribed to host or environmental factors, it seems reasonable to investigate whether some strains within a given bacterial species are more capable of causing disease than others. Such research may also provide insight into virulence mechanisms, thus improving prevention and treatment options.

REFERENCE LIST

- Aich, P., S. Jalal, C. Czuba, G. Schatte, K. Herzog, D. J. Olson, A. R. Ross, A. A. Potter, L. A. Babiuk, and P. Griebel. 2007. Comparative approaches to the investigation of responses to stress and viral infection in cattle. *OMICS*. 11:413-434.
- Alexander, B. H., D. W. MacVean, and M. D. Salman. 1989. Risk factors for lower respiratory tract disease in a cohort of feedlot cattle. *J. Am. Vet. Med. Assoc.* 195:207-211.
- Allen, J. W., K. Viel, K. G. Bateman, E. Nagy, S. Rosendal, and P. E. Shewen. 1992a. Serological titers to bovine herpesvirus 1, bovine viral diarrhea virus, parainfluenza 3 virus, bovine respiratory syncytial virus and *Pasteurella haemolytica* in feedlot calves with respiratory disease: associations with bacteriological and pulmonary cytological variables. *Canadian Journal of Veterinary Research* 56:281-288.
- Allen, J. W., L. Viel, K. G. Bateman, and S. Rosendal. 1992b. Changes in the bacterial flora of the upper and lower respiratory tracts and bronchoalveolar lavage differential cell counts in feedlot calves treated for respiratory diseases. *Can. J. Vet. Res.* 56:177-183.
- Allen, J. W., L. Viel, K. G. Bateman, S. Rosendal, and P. E. Shewen. 1992c. Cytological findings in bronchoalveolar lavage fluid from feedlot calves: associations with pulmonary microbial flora. *Can. J. Vet. Res.* 56:122-126.
- Allen, J. W., L. Viel, K. G. Bateman, S. Rosendal, P. E. Shewen, and P. Physick-Sheard. 1991. The microbial flora of the respiratory tract in feedlot calves: associations between nasopharyngeal and bronchoalveolar lavage cultures. *Can. J. Vet. Res.* 55:341-346.
- Andrews, A. H. 1976. Factors affecting the incidence of pneumonia in growing bulls. *Vet. Rec.* 98:146-149.
- Bagley, C. V., H. R. Bingham, and J. O. Hall. 2003. The association between serum mineral and vitamin A and E concentration and respiratory disease in beef calves entering backgrounding lots. *Bovine Practitioner* 37:68-73.
- Barbour, E. K., N. H. Nabbut, S. K. Hamadeh, and H. M. Al-Nakhli. 1997. Bacterial identity and characteristics in healthy and unhealthy respiratory tracts of sheep and calves. *Vet. Res. Commun.* 21:421-430.

- Bateman, K. G., S. W. Martin, P. E. Shewen, and P. I. Menzies. 1990. An evaluation of antimicrobial therapy for undifferentiated bovine respiratory disease. *Canadian Veterinary Journal* 31:689-696.
- Bechtol, D. T., R. T. Ballinger, and A. J. Sharp. 1991. Field trial of a *Pasteurella haemolytica* toxoid administered at spring branding and in the feedlot. *Agri-Practice* 12:6.
- Berry, B. A., W. T. Choat, D. R. Gill, C. R. Krehbiel, R. A. Smith, and R. L. Ball. 2001. Effect of castration on health and performance of newly received stressed feedlot calves. *Animal Science Research Report - Agricultural Experiment Station, Oklahoma State University*.
- Berry, B. A., A. W. Confer, C. R. Krehbiel, D. R. Gill, R. A. Smith, and M. Montelongo. 2004a. Effects of dietary energy and starch concentrations for newly received feedlot calves: II. Acute-phase protein response. *J. Anim Sci.* 82:845-850.
- Berry, B. A., C. R. Krehbiel, A. W. Confer, D. R. Gill, R. A. Smith, and M. Montelongo. 2004b. Effects of dietary energy and starch concentrations for newly received feedlot calves: I. Growth performance and health. *J. Anim Sci.* 82:837-844.
- Booker, C. W., S. M. Abutarbush, P. S. Morley, P. T. Guichon, B. K. Wildman, G. K. Jim, O. C. Schunicht, T. J. Pittman, T. Perrett, J. A. Ellis, G. Appleyard, and D. M. Haines. 2008a. The effect of bovine viral diarrhea virus infections on health and performance of feedlot cattle. *Can. Vet. J.* 49:253-260.
- Booker, C. W., S. M. Abutarbush, P. S. Morley, G. K. Jim, T. J. Pittman, O. C. Schunicht, T. Perrett, B. K. Wildman, R. K. Fenton, P. T. Guichon, and E. D. Janzen. 2008b. Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in western Canada. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* 49:473-481.
- Booker, C. W., P. T. Guichon, G. K. Jim, O. C. Schunicht, R. J. Harland, and P. S. Morley. 1999. Seroepidemiology of undifferentiated fever in feedlot calves in western Canada. *Can. Vet. J.* 40:40-48.
- Brazle, F. K. 1992. Banding as a Castration Method in Relationship to its Effect on Health and Gain. *J. Anim Sci.* 70:48.
- Brethour, J. R. and W. W. Duitsman. Ascorbic acid, Aureo S-700, sodium bicarbonate and thiamine in post-weaning stress rations. 556, 35-37. 1972. Manhattan, KS, Kansas State University. 50th Roundup Report Kansas State University Bulletin.
- Bryant, L. K., L. J. Perino, D. Griffin, A. R. Doster, and T. E. Wittum. 1999. A method for recording pulmonary lesions of beef calves at slaughter, and the association of lesions with average daily gain. *Bovine Practitioner* 33:163-173.

- Buhman, M. J., L. L. Hungerford, and D. R. Smith. 2003. An economic risk assessment of the management of pregnant feedlot heifers in the USA. *Prev. Vet. Med.* 59:207-222.
- Buhman, M. J., L. J. Perino, M. L. Galyean, T. E. Wittum, T. H. Montgomery, and R. S. Swingle. 2000. Association between changes in eating and drinking behaviors and respiratory tract disease in newly arrived calves at a feedlot. *Am. J. Vet. Res.* 61:1163-1168.
- Camp, T. H., D. G. Stevens, R. A. Stermer, and J. P. Anthony. 1981. Transit factors affecting shrink, shipping fever and subsequent performance of feeder calves. *J. Anim Sci.* 52:1219-1224.
- Carter, J. N., G. L. Meredith, M. Montelongo, D. R. Gill, C. R. Krehbiel, M. E. Payton, and A. W. Confer. 2002. Relationship of vitamin E supplementation and antimicrobial treatment with acute-phase protein responses in cattle affected by naturally acquired respiratory tract disease. *Am. J. Vet. Res.* 63:1111-1117.
- Chase, C. C., Jr., R. E. Larsen, R. D. Randel, A. C. Hammond, and E. L. Adams. 1995. Plasma cortisol and white blood cell responses in different breeds of bulls: a comparison of two methods of castration. *Journal of Animal Science* 73:975-980.
- Chirase, N. K., L. W. Greene, C. W. Purdy, R. W. Loan, B. W. Auvermann, D. B. Parker, E. F. Walborg, Jr., D. E. Stevenson, Y. Xu, and J. E. Klaunig. 2004. Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. *Am. J. Vet. Res.* 65:860-864.
- Church, T. L. and O. M. Radostits. 1981. A retrospective survey of diseases of feedlot cattle in Alberta. *Can. Vet. J.* 22:27-30.
- Coghe, J., C. Uystepuyst, F. Bureau, M. L. Weerd, and P. Lekeux. 1999. Preliminary classification of the bovine respiratory complex into different levels of severity. *Bovine Practitioner* 33:85-87.
- Cole, N. A. 1985. Preconditioning calves for the feedlot. *Veterinary Clinics of North America, Food Animal Practice* 1:401-411.
- Cole, N. A., T. H. Camp, L. D. Rowe, Jr., D. G. Stevens, and D. P. Hutcheson. 1988. Effect of transport on feeder calves. *Am. J. Vet. Res.* 49:178-183.
- Cole, N. A., J. B. McLaren, and M. R. Irwin. 1979. Influence of pretransit feeding regimen and posttransit B-vitamin supplementation on stressed feeder steers. *J. Anim Sci.* 49:310-317.
- Collier, J. R. 1968. Pasteurellae in Bovine Respiratory Disease. *J. Am. Vet. Med. Assoc.* 152:824-828.

- Corrigan, M. E., J. S. Drouillard, M. F. Spire, D. A. Mosier, J. E. Minton, J. J. Higgins, E. R. Loe, B. E. Depenbusch, and J. T. Fox. 2007. Effects of melengestrol acetate on the inflammatory response in heifers challenged with *Mannheimia haemolytica*. *J. Anim Sci.* 85:1770-1779.
- Cusack, P. M. 2004. Effect of mass medication with antibiotics at feedlot entry on the health and growth rate of cattle destined for the Australian domestic market. *Aust. Vet. J.* 82:154-156.
- Cusack, P. M., N. McMeniman, and I. J. Lean. 2003. The medicine and epidemiology of bovine respiratory disease in feedlots. *Aust. Vet. J.* 81:480-487.
- Cusack, P. M., N. P. McMeniman, and I. J. Lean. 2007. Feedlot entry characteristics and climate: their relationship with cattle growth rate, bovine respiratory disease and mortality. *Aust. Vet. J.* 85:311-316.
- Cusack, P. M., N. P. McMeniman, and I. J. Lean. 2008. Effects of injectable vitamins A, D, E and C on the health and growth rate of feedlot cattle destined for the Australian domestic market. *Aust. Vet. J.* 86:81-87.
- Czuprynski, C. J., F. Leite, M. Sylte, C. Kuckleburg, R. Schultz, T. Inzana, E. Behling-Kelly, and L. Corbeil. 2004. Complexities of the pathogenesis of *Mannheimia haemolytica* and *Haemophilus somnus* infections: challenges and potential opportunities for prevention? *Anim Health Res. Rev.* 5:277-282.
- Dabo, S., A. Confer, and Y. Lu. 2000. Single primer polymerase chain reaction fingerprinting for *Pasteurella multocida* isolates from laboratory rabbits. *American Journal of Veterinary Research* 61.
- Davies, R. L. 2004. Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology* 150:4199-4210.
- Davies, R. L., R. MacCorquodale, and S. Reilly. 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Vet. Microbiol.* 99:145-158.
- DeRosa, D. C., G. D. Mechor, J. J. Staats, M. M. Chengappa, and T. R. Shryock. 2000. Comparison of *Pasteurella* spp. simultaneously isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. *J. Clin. Microbiol.* 38:327-332.
- Donkersgoed, J. v. 1992. Meta-analysis of field trials of antimicrobial mass medication for prophylaxis of bovine respiratory disease in feedlot cattle. *Canadian Veterinary Journal* 33:786-795.

- Droke, E. A. and S. C. Loerch. 1989. Effects of parenteral selenium and vitamin E on performance, health and humoral immune response of steers new to the feedlot environment. *J. Anim Sci.* 67:1350-1359.
- Duff, G. C. and M. L. Galyean. 2006. BOARD-INVITED REVIEW: Recent advances in management of highly stressed, newly received feedlot cattle. *J. Anim Sci.*
- Duff, G. C., K. J. Malcolm-Callis, D. A. Walker, M. W. Wiseman, M. L. Galyean, and L. J. Perino. 2000. Effects of intranasal versus intramuscular modified live vaccines and vaccine timing on health and performance by newly received beef cattle. *Bovine Practitioner* 34:66-71.
- Durham, P. J., L. E. Hassard, and D. J. Van. 1991. Serological studies of infectious bovine rhinotracheitis, parainfluenza 3, bovine viral diarrhea, and bovine respiratory syncytial viruses in calves following entry to a bull test station. *Can. Vet. J.* 32:427-429.
- Edwards, A. J. 1989. The effect of stressors like ruman overload and induced abortion on BRD in feedlot cattle. *Agri-Practice* 10:10-15.
- Edwards, A. J. and S. B. Laudert. 1984. Economic evaluation of the use of feedlot abortifacients. *Bovine Practitioner* 148-150.
- Elam, N. A., D. U. Thomson, and J. F. Gleghorn. 2008. Effects of long- or short-term exposure to a calf identified as persistently infected with bovine viral diarrhea virus on feedlot performance of freshly weaned, transport-stressed beef heifers. *J. Anim Sci.* 86:1917-1924.
- Faulkner, D. B., T. Eurell, W. J. Tranquilli, R. S. Ott, M. W. Ohl, G. F. Cmarik, and G. Zinn. 1992. Performance and health of weanling bulls after butorphanol and xylazine administration at castration. *J. Anim Sci.* 70:2970-2974.
- Fell, L. R., I. G. Colditz, K. H. Walker, and D. L. Watson. 1999. Associations between temperament, performance and immune function in cattle entering a commercial feedlot. *Australian Journal of Experimental Agriculture* 39:795-802.
- Fisher, A. D., T. W. Knight, G. P. Cosgrove, A. F. Death, C. B. Anderson, D. M. Duganzich, and L. R. Matthews. 2001. Effects of surgical or banding castration on stress responses and behaviour of bulls. *Aust. Vet. J.* 79:279-284.
- Fluharty, F. L. and S. C. Loerch. 1996. Effects of dietary energy source and level on performance of newly arrived feedlot calves. *J. Anim Sci.* 74:504-513.
- Frank, G. H., R. E. Briggs, G. C. Duff, and H. S. Hurd. 2003. Effect of intranasal exposure to leukotoxin-deficient *Mannheimia haemolytica* at the time of arrival at the feedyard on subsequent isolation of *M haemolytica* from nasal secretions of calves. *Am. J. Vet. Res.* 64:580-585.

- Frank, G. H., R. E. Briggs, G. C. Duff, R. W. Loan, and C. W. Purdy. 2002. Effects of vaccination prior to transit and administration of florfenicol at time of arrival in a feedlot on the health of transported calves and detection of *Mannheimia haemolytica* in nasal secretions. *Am. J. Vet. Res.* 63:251-256.
- Frank, G. H., R. E. Briggs, R. W. Loan, C. W. Purdy, and E. S. Zehr. 1996. Respiratory tract disease and mucosal colonization by *Pasteurella haemolytica* in transported cattle. *Am. J. Vet. Res.* 57:1317-1320.
- Frank, G. H. and G. C. Duff. 2000. Effects of tilmicosin phosphate, administered prior to transport or at time of arrival, and feeding of chlortetracycline, after arrival in a feedlot, on *Mannheimia haemolytica* in nasal secretions of transported steers. *Am. J. Vet. Res.* 61:1479-1483.
- Fulton, R. W., B. J. Cook, D. L. Step, A. W. Confer, J. T. Saliki, M. E. Payton, L. J. Burge, R. D. Welsh, and K. S. Blood. 2002a. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. *Can. J. Vet. Res.* 66:173-180.
- Fulton, R. W., B. J. Cook, D. L. Step, A. W. Confer, J. T. Saliki, M. E. Payton, L. J. Burge, R. D. Welsh, and K. S. Blood. 2002b. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. *Can. J. Vet. Res.* 66:173-180.
- Fulton, R. W., B. Hessman, B. J. Johnson, J. F. Ridpath, J. T. Saliki, L. J. Burge, D. Sjeklocha, A. W. Confer, R. A. Funk, and M. E. Payton. 2006. Evaluation of diagnostic tests used for detection of bovine viral diarrhea virus and prevalence of subtypes 1a, 1b, and 2a in persistently infected cattle entering a feedlot. *J. Am. Vet. Med. Assoc.* 228:578-584.
- Fulton, R. W., C. W. Purdy, A. W. Confer, J. T. Saliki, R. W. Loan, R. E. Briggs, and L. J. Burge. 2000. Bovine viral diarrhea viral infections in feeder calves with respiratory disease: interactions with *Pasteurella* spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. *Can. J. Vet. Res.* 64:151-159.
- Gagea, M. I., K. G. Bateman, D. T. van, B. J. McEwen, S. Carman, M. Archambault, R. A. Shanahan, and J. L. Caswell. 2006. Diseases and pathogens associated with mortality in Ontario beef feedlots. *J. Vet. Diagn. Invest* 18:18-28.
- Gallo, G. F. and J. L. Berg. 1995. Efficacy of a feed-additive antibacterial combination for improving feedlot cattle performance and health. *Can. Vet. J.* 36:223-229.
- Galyean, M. L., L. J. Perino, and G. C. Duff. 1999. Interaction of cattle health/immunity and nutrition. *J. Anim Sci.* 77:1120-1134.
- Ganaba, R., D. Belanger, S. Dea, and M. Bigras-Poulin. 1995. A seroepidemiological study of the importance in cow-calf pairs of respiratory and enteric viruses in beef operations from northwestern Quebec. *Can. J. Vet. Res.* 59:26-33.

- Gardner, B. A., H. G. Dolezal, L. K. Bryant, F. N. Owens, and R. A. Smith. 1999. Health of finishing steers: effects on performance, carcass traits, and meat tenderness. *J. Anim Sci.* 77:3168-3175.
- Gibb, D. J., K. S. Schwartzkopf-Genswein, J. M. Stookey, J. J. McKinnon, D. L. Godson, R. D. Wiedmeier, and T. A. McAllister. 2000. Effect of a trainer cow on health, behavior, and performance of newly weaned beef calves. *J. Anim Sci.* 78:1716-1725.
- Godinho, K. S., P. Sarasola, E. Renoult, N. Tilt, S. Keane, G. D. Windsor, T. G. Rowan, and S. J. Sunderland. 2007. Use of deep nasopharyngeal swabs as a predictive diagnostic method for natural respiratory infections in calves. *Vet. Rec.* 160:22-25.
- Goonewardene, L. A. and R. K. Hand. 1991. Studies on dehorning steers in Alberta feedlots. *Canadian Journal of Animal Science* 71:1249-1252.
- Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. *Vet. Clin. North Am. Food Anim Pract.* 13:367-377.
- Griffin, D., L. Perino, and T. E. Wittum. Feedlot respiratory disease: cost, value of preventives and intervention. Proceedings of the Twenty Seventh Annual Convention American Association of Bovine Practitioners, Pittsburgh, Pennsylvania, USA, September 22-25, 1994. Williams, E. I. 157-160. 1995.
- Grotelueschen, D. M., A. Wohlers, C. Dewey, I. G. Rush, W. E. Braselton, D. Hamar, A. B. Johnson, and J. H. Pollreis. 2001. Effect of pasture trace mineral supplementation on liver mineral levels and feedlot morbidity and mortality. *Bovine Practitioner* 35:73-84.
- Guichon, P. T., G. K. Jim, C. W. Booker, O. C. Schunicht, B. K. Wildman, and J. R. Brown. 2000. Relative cost-effectiveness of treatment of feedlot calves with ivermectin versus treatment with a combination of fenbendazole, permethrin, and fenthion. *J. Am. Vet. Med. Assoc.* 216:1965-1969.
- Gummow, B. and P. H. Mapham. 2000. A stochastic partial-budget analysis of an experimental *Pasteurella haemolytica* feedlot vaccine trial. *Prev. Vet. Med.* 43:29-42.
- Hagglund, S., M. Hjort, D. A. Graham, P. Ohagen, M. Tornquist, and S. Alenius. 2007. A six-year study on respiratory viral infections in a bull testing facility. *Vet. J.* 173:585-593.
- Haines, D. M., K. M. Martin, E. G. Clark, G. K. Jim, and E. D. Janzen. 2001. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can. Vet. J.* 42:857-860.

- Hamdy, A. H. and A. L. Trapp. 1967. Investigation of nasal microflora of feedlot calves before and after weaning. *Am. J. Vet. Res.* 28:1019-1025.
- Hand, R. K. and L. A. Goonewardene. 1989. The effects of dehorning and/or castrating feedlot cattle. *Proceedings, Western Section, American Society of Animal Science and Western Branch Canadian Society of Animal Science* 40:88-90.
- Hansen, D. E., R. Syvrud, and D. Armstrong. 1992. Effectiveness of A Bovine Respiratory Syncytial Virus-Vaccine in Reducing the Risk of Respiratory-Disease. *Agri-Practice* 13:19-22.
- Hellwig, D., B. Kegley, and S. Silzell. 1999. A comparison of mass antibiotic treatments on arrival with regards to morbidity, mortality, and performance in Arkansas stocker cattle. *Research Series - Arkansas Agricultural Experiment Station* 96-97.
- Hoerlein, A. B., S. P. Saxena, and M. E. Mansfield. 1961. Studies on shipping fever of cattle. II. Prevalence of *Pasteurella* species in nasal secretions from normal calves and calves with shipping fever. *Am. J. Vet. Res.* 22:470-472.
- Hutcheson, D. P., N. A. Cole, and J. B. McLaren. 1984. Effects of pretransit diets and post-transit potassium levels for feeder calves. *J. Anim Sci.* 58:700-707.
- Irwin, M. R., S. McConnell, J. D. Coleman, and G. E. Wilcox. 1979. Bovine respiratory disease complex: a comparison of potential predisposing and etiologic factors in Australia and the United States. *J. Am. Vet. Med. Assoc.* 175:1095-1099.
- Janzen, E. 1997. The role of *Haemophilus somnus* in bovine respiratory disease (BRD). *Bovine Practitioner*.
- Jensen, R. and D. Mackey. 1979. *Diseases of Feedlot Cattle*. 3 ed. Lea & Febiger, Philadelphia.
- Jensen, R., R. E. Pierson, P. M. Braddy, D. A. Saari, L. H. Lauerman, J. J. England, H. Keyvanfar, J. R. Collier, D. P. Horton, A. E. McChesney, A. Benitez, and R. M. Christie. 1976. Shipping fever pneumonia in yearling feedlot cattle. *J. Am. Vet. Med. Assoc.* 169:500-506.
- Jericho, K. W. and E. V. Langford. 1978. Pneumonia in calves produced with aerosols of bovine herpesvirus 1 and *Pasteurella haemolytica*. *Can. J. Comp Med.* 42:269-277.
- Jim, G. K., C. W. Booker, and P. T. Guichon. 1992. Comparison of a combination of oxfendazole and fenthion versus ivermectin in feedlot calves. *Canadian Veterinary Journal* 33:599-604.
- Jim, G. K., C. W. Booker, C. S. Ribble, P. T. Guichon, and B. E. Thorlakson. 1993. A field investigation of the economic impact of respiratory disease in feedlot calves. *Canadian Veterinary Journal* 34:668-673.

- Jim, G. K., C. S. Ribble, P. T. Guichon, and B. E. Thorlakson. 1991. The relative economics of feeding open, aborted, and pregnant feedlot heifers. *Canadian Veterinary Journal* 32:613-617.
- Juarez Barranco, F., F. J. Trigo Tavera, G. Chavez Gris, and R. E. Vargas Garcia. 2003. Viral participation in respiratory disease in feedlot cattle, as identified by immunohistochemistry. *Veterinaria Mexico* 34:1-12.
- Kadel, W. L., M. M. Chengappa, and C. E. Herren. 1985. Field-trial evaluation of a *Pasteurella* vaccine in preconditioned and nonpreconditioned lightweight calves. *Am. J. Vet. Res.* 46:1944-1948.
- Karren, D. B., J. A. Basarab, and T. L. Church. 1987. The growth and economic performance of preconditioned calves and their dams on the farm and of calves in the feedlot. *Canadian Journal of Animal Science* 67:327-336.
- Katsuda, K., M. Kohmoto, K. Kawashima, H. Tsunemitsu, T. Tsuboi, and M. Eguchi. 2003. Molecular typing of *Mannheimia (Pasteurella) haemolytica* serotype A1 isolates from cattle in Japan. *Epidemiol. Infect.* 131:939-946.
- Kelly, A. P. and E. D. Janzen. 1986. A review of morbidity and mortality rates and disease occurrence in North American feedlot cattle. *Canadian Veterinary Journal* 27:496-500.
- Kiesel, G. K., C. R. Rossi, H. D. Alexander, and G. G. Brooks. 1972. A study of weaning, acclimatization, and limited vaccination on the incidence of respiratory disease complex in cattle. *Cornell Vet.* 62:454-463.
- Knight, A. P., R. E. Pierson, A. B. Hoerlein, J. H. Collier, D. P. Horton, and J. B. Pruett. 1972. Effect of vaccination time on morbidity, mortality, and weight gains of feeder calves. *J. Am. Vet. Med. Assoc.* 161:45-48.
- Kreikemeier K, Stokka G, and Marston T. Influence of delayed processing and mass medication with either chlortetracycline (CTC) or tilmicosin phosphate (Micotil) on health and growth of highly stressed calves. 773, 23-27. 1996. Manhattan, KS, Kansas State University. Southwest Research Extension Center Progress Report.
- Lalman, D. and R. Smith. Effects of Preconditioning on Health, Performance and Prices of Weaned Calves. F-3529. 2004. Stillwater, Ok, Oklahoma Cooperative Extension Service.
- Lalman, D. and C. Ward. 2005. Effects of preconditioning on health, performance and prices of weaned calves. Proceedings of the Thirty-Eighth Annual Convention, American Association of Bovine Practitioners, Salt Lake City, Utah, USA, 24-24 September, 2005.

- Lathrop, S. L., T. E. Wittum, S. C. Loerch, L. J. Perino, and L. J. Saif. 2000. Antibody titers against bovine coronavirus and shedding of the virus via the respiratory tract in feedlot cattle. *Am. J. Vet. Res.* 61:1057-1061.
- Lehmkuhl, H. D. and P. M. Gough. 1977. Investigation of causative agents of bovine respiratory tract disease in a beef cow-calf herd with an early weaning program. *Am. J. Vet. Res.* 38:1717-1720.
- Lillie, L. E. 1974. The bovine respiratory disease complex. *Can. Vet. J.* 15:233-242.
- Loan, R. W., H. Tung, and J. B. Payne. 1998. Comparative efficacy and duration of immunity of commercial *Pasteurella haemolytica* vaccines. *Bovine Practitioner* 32:18-21.
- Loerch, S. C. and F. L. Fluharty. 2000. Use of trainer animals to improve performance and health of newly arrived feedlot calves. *J. Anim Sci.* 78:539-545.
- Lofgreen, G. P. The relationship of arrival temperature and subsequent sickness in newly received southern calves. 12, 1-4. 1979. Clayton, NM, New Mexico State University Clayton Livestock Research Center. Clayton Livestock Research Center Progress Report.
- Ref Type: Report
- Lofgreen, G. P. 1983. Mass medication in reducing shipping fever-bovine respiratory disease complex in highly stressed calves. *J. Anim Sci.* 56:529-536.
- Lofgreen, G. P., D. G. Addis, J. R. Dunbar, and J. G. Clark. 1978. Time of processing calves subjected to marketing and shipping stress. *Journal of Animal Science* 47:1324-1328.
- Lofgreen, G. P., J. R. Dunbar, D. G. Addis, and J. G. Clark. 1975. Energy level in starting rations for calves subjected to marketing and shipping stress. *Journal of Animal Science* 41:1256-1265.
- Loneragan, G. H., D. A. Dargatz, P. S. Morley, and M. A. Smith. 2001. Trends in mortality ratios among cattle in US feedlots. *J. Am. Vet. Med. Assoc.* 219:1122-1127.
- Loneragan, G. H., D. U. Thomson, D. L. Montgomery, G. L. Mason, and R. L. Larson. 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.
- Loxton, I. D., M. A. Toleman, and A. E. Holmes. 1982. The effect of dehorning Brahman crossbred animals of four age groups on subsequent bodyweight gain. *Aust. Vet. J.* 58:191-193.

- Lynch, J. M., P. L. Houghton, L. R. Corah, and G. L. Stokka. Economic Impact of Preweaning Vaccinations on Health and Performance of Weaned Feeder Cattle. Johnson, M. A. 783, 23-25. 1997. Manhattan, Kansas, Kansas State University. Cattlemen's Day Report of Progress.
- Macartney, J. E., K. G. Bateman, and C. S. Ribble. 2003. Health performance of feeder calves sold at conventional auctions versus special auctions of vaccinated or conditioned calves in Ontario. *J. Am. Vet. Med. Assoc.* 223:677-683.
- MacGregor, D. S., D. R. Yoder, and R. S. Rew. 2001. Impact of doramectin treatment at the time of feedlot entry on the productivity of yearling steers with natural nematode infections. *Am. J. Vet. Res.* 62:622-624.
- MacGregor, S. and T. R. Falkner. 1997. Managing pregnant heifers in the feedlot. *Compendium on Continuing Education for the Practicing Veterinarian* 19:1389-1391, 1395, 1407.
- MacGregor, S., D. Smith, L. J. Perino, and B. D. Hunsaker. 2003. An evaluation of the effectiveness of a commercial *Mannheimia (Pasteurella) haemolytica* vaccine in a commercial feedlot. *Bovine Practitioner* 37:78-82.
- MacGregor, S. and M. I. Wray. 2004. The effect of bovine respiratory syncytial virus vaccination on health, feedlot performance and carcass characteristics of feeder cattle. *Bovine Practitioner* 38:162-170.
- MacVean, D. W., D. K. Franzen, T. J. Keefe, and B. W. Bennett. 1986. Airborne particle concentration and meteorologic conditions associated with pneumonia incidence in feedlot cattle. *Am. J. Vet. Res.* 47:2676-2682.
- Magwood, S. E., D. A. Barnum, and R. G. Thomson. 1969. Nasal bacterial flora of calves in healthy and in pneumonia-prone herds. *Can. J. Comp Med.* 33:237-243.
- Martin, S. W. 1985. A mail survey of the efficacy of prophylactic medication in feed and/or water of feedlot calves. *Can. J. Comp Med.* 49:15-20.
- Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, and J. E. Bohac. 1989. The frequency, distribution and effects of antibodies, to seven putative respiratory pathogens, on respiratory disease and weight gain in feedlot calves on Ontario. *Canadian Journal of Veterinary Research* 53:355-362.
- Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. *Can. J. Vet. Res.* 54:337-342.

- Martin, S. W. and J. G. Bohac. 1986. The association between serological titers in infectious bovine rhinotracheitis virus, bovine virus diarrhea virus, parainfluenza-3 virus, respiratory syncytial virus and treatment for respiratory disease in Ontario feedlot calves. *Can. J. Vet. Res.* 50:351-358.
- Martin, S. W., G. Darlington, K. Bateman, and J. Holt. 1988. Undifferentiated bovine respiratory disease (shipping fever): Is it communicable? *Preventive Veterinary Medicine* 6:27-35.
- Martin, S. W., J. D. Holt, and A. H. Meek. The effect of risk factors, as determined by logistic regression, on health of beef feedlot cattle. Third International Symposium on Veterinary Epidemiology and Economics, Arlington, Virginia, USA, 6-10 September 1982. 68-75. 1983.
- Martin, S. W. and A. H. Meek. 1986. A path model of factors influencing morbidity and mortality in Ontario feedlot calves. *Can. J. Vet. Res.* 50:15-22.
- Martin, S. W., A. H. Meek, D. G. Davis, J. A. Johnson, and R. A. Curtis. 1982. Factors associated with mortality and treatment costs in feedlot calves: the Bruce County Beef Project, years 1978, 1979, 1980. *Can. J. Comp Med.* 46:341-349.
- Martin, S. W., E. Nagy, P. E. Shewen, and R. J. Harland. 1998. The association of titers to bovine coronavirus with treatment for bovine respiratory disease and weight gain in feedlot calves. *Can. J. Vet. Res.* 62:257-261.
- McClary, D. and G. Vogel. 1999. Effect of timing of tilmicosin metaphylaxis on control of bovine respiratory disease and performance in feeder cattle. *Bovine Practitioner* 33:155-161.
- McDermott, J. J. and Y. H. Schukken. 1994. A review of methods used to adjust for cluster effects in explanatory epidemiological studies of animal populations. *Preventive Veterinary Medicine* 18:155-173.
- McDermott, J. J., Y. H. Schukken, and M. M. Shoukri. 1994. Study design and analytic methods for data collected from clusters of animals. *Preventive Veterinary Medicine* 18:175-191.
- Meyer, B. D., M. D. Apley, and P. M. Imerman. 2002. Comparison of serum steroidal hormone concentrations in buller steers, riders, and uninterested penmates. Investigation of sickness, body weight, feed bunk status, and implant condition during buller occurrence. *Bovine Practitioner* 36:27-32.
- Morter, R. L., K. R. Kazacos, and H. E. Amstutz. 1984. Comparative clinical efficacy of fenbendazole and thiabendazole in feedlot heifers [17th Ann. Conf., Amer. Ass. of Bovine Practitioners, 27-30 Nov. 1984, Des Moines, Iowa.]. *Bovine Practitioner* 213-215.

- Mosier, D. A., A. W. Confer, and R. J. Panciera. 1989. The evolution of vaccines for bovine pneumonic pasteurellosis. *Res. Vet. Sci.* 47:1-10.
- Muggli-Cockett, N. E., L. V. Cundiff, and K. E. Gregory. 1992. Genetic analysis of bovine respiratory disease in beef calves during the first year of life. *J. Anim Sci.* 70:2013-2019.
- Murphy, G. L., L. C. Robinson, and G. E. Burrows. 1993. Restriction endonuclease analysis and ribotyping differentiate *Pasteurella haemolytica* serotype A1 isolates from cattle within a feedlot. *J. Clin. Microbiol.* 31:2303-2308.
- O'Connor, A., S. W. Martin, E. Nagy, P. Menzies, and R. Harland. 2001. The relationship between the occurrence of undifferentiated bovine respiratory disease and titer changes to bovine coronavirus and bovine viral diarrhea virus in 3 Ontario feedlots. *Can. J. Vet. Res.* 65:137-142.
- O'Connor, A. M., S. D. Sorden, and M. D. Apley. The Association Between Persistently Bovine Viral Diarrhea Virus-Infected Calves and Pen Levels of Morbidity and Mortality in an Iowa Feedlot. Smith, R. A. Proceedings of the Thirty-Sixth Annual Conference, American Association of Bovine Practitioners, Columbus, Ohio, USA, 18-20 September, 2003 , 152. 2003. 9-18-2003.
- Perino, L. J. and B. D. Hunsaker. 1997. A Review of Bovine Respiratory Disease Vaccine Field Efficacy. *Bovine Practitioner* 31:59-66.
- Phillips, W. A., J. B. McLaren, and N. A. Cole. 1986. The effect of a preassembly zeranol implant and post-transit diet on the health performance and metabolic profile of feeder calves. *J. Anim Sci.* 62:27-36.
- Pinchak, W. E., D. R. Tolleson, M. McCloy, L. J. Hunt, R. J. Gill, R. J. Ansley, and S. J. Bevers. 2004. Morbidity effects on productivity and profitability of stocker cattle grazing in the Southern Plains. *J. Anim Sci.* 82:2773-2779.
- Plummer, P. J., B. W. Rohrbach, R. A. Daugherty, R. A. Daugherty, K. V. Thomas, R. P. Wilkes, F. E. Duggan, and M. A. Kennedy. 2004. Effect of intranasal vaccination against bovine enteric coronavirus on the occurrence of respiratory tract disease in a commercial backgrounding feedlot. *J. Am. Vet. Med. Assoc.* 225:726-731.
- Potgieter, L. N., M. D. McCracken, F. M. Hopkins, R. D. Walker, and J. S. Guy. 1984. Experimental production of bovine respiratory tract disease with bovine viral diarrhea virus. *Am. J. Vet. Res.* 45:1582-1585.
- Pritchard, R. H. and J. K. Mendez. 1990. Effects of preconditioning on pre- and post-shipment performance of feeder calves. *J. Anim Sci.* 68:28-34.
- Purdy, C. W., D. C. Straus, N. Chirase, J. R. Ayers, and M. D. Hoover. 2003. Effects of aerosolized dust in goats on lung clearance of *Pasteurella* and *Mannheimia* species. *Curr. Microbiol.* 46:174-179.

- Radostits, O. M. 2001. Herd health: food animal production medicine. 3rd ed ed. Saunders, Philadelphia.
- Reggiardo, C. Role of Virus in Shipping Fever of Feedlot Cattle. Case Studies and Diagnostic Considerations. 22nd Annual Proceedings Amer.Assn.Veterinary Diagnosticians , 315-320. 2005.
- Ribble, C. S., G. K. Jim, and E. D. Janzen. 1988. Efficacy of immunization of feedlot calves with a commercial *Haemophilus somnus* bacterin. Can. J. Vet. Res. 52:191-198.
- Ribble, C. S., A. H. Meek, E. D. Janzen, P. T. Guichon, and G. K. Jim. 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., A. H. Meek, G. K. Jim, and P. T. Guichon. 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., A. H. Meek, P. E. Shewen, P. T. Guichon, and G. K. Jim. 1995c. Effect of pretransit mixing on fatal fibrinous pneumonia in calves. J. Am. Vet. Med. Assoc. 207:616-619.
- Ribble, C. S., A. H. Meek, P. E. Shewen, G. K. Jim, and P. T. Guichon. 1995d. Effect of transportation on fatal fibrinous pneumonia and shrinkage in calves arriving at a large feedlot. J. Am. Vet. Med. Assoc. 207:612-615.
- Ribble, C. S., A. H. Meek, M. M. Shoukri, P. T. Guichon, and G. K. Jim. Risk factors associated with fatal fibrinous pneumonia (shipping fever) in feedlot calves. Proceedings of the Thirty-First Annual Conference American Association of Bovine Practitioners, Spokane, Washington, USA, 24-26 September, 1998. Williams, E. I. 104-109. 1998.
- Ribble, C. S., M. M. Shoukri, A. H. Meek, and S. W. Martin. 1994. Clustering of fatal fibrinous pneumonia (shipping fever) in feedlot calves within transport truck and feedlot pen groups. Preventive Veterinary Medicine 21:251-261.
- Richer, L., P. Marois, and L. Lamontagne. 1988. Association of Bovine Viral Diarrhea Virus with Multiple Viral Infections in Bovine Respiratory Disease Outbreaks. Can. Vet. J. 29:713-717.
- Richeson, J. T., P. A. Beck, M. S. Gadberry, S. A. Gunter, T. W. Hess, D. S. Hubbell, III, and C. Jones. 2008. Effects of on-arrival versus delayed modified live virus vaccination on health, performance, and serum infectious bovine rhinotracheitis titers of newly received beef calves. J. Anim Sci. 86:999-1005.

- Roeber, D., N. Speer, J. Gentry, J. Tatum, C. Smith, J. Whittier, G. Jones, K. Belk, and G. Smith. 2001. Feeder cattle health management: effects on morbidity rates, feedlot performance, carcass characteristics, and beef palatability. *The Professional Animal Scientist* 17.
- Salyer, G. B., M. L. Galyean, P. J. Defoor, G. A. Nunnery, C. H. Parsons, and J. D. Rivera. 2004. Effects of copper and zinc source on performance and humoral immune response of newly received, lightweight beef heifers. *J. Anim Sci.* 82:2467-2473.
- Sanderson, M. W., D. A. Dargatz, and B. A. Wagner. 2008. Risk factors for initial respiratory disease in United States' feedlots based on producer-collected daily morbidity counts. *Can. Vet. J.* 49:373-378.
- Schaefer, A. L., N. J. Cook, J. S. Church, J. Basarab, B. Perry, C. Miller, and A. K. Tong. 2007. The use of infrared thermography as an early indicator of bovine respiratory disease complex in calves. *Res. Vet. Sci.* 83:376-384.
- Schake, L. M., W. W. Webster, and R. C. Conway, Jr. Effect of origin and shipping distance on feedlot performance of cattle. *Beef Cattle Research in Texas.* 90-92. 1980.
- Schiefer, B., G. E. Ward, and R. E. Moffatt. 1978. Correlation of microbiological and histological findings in bovine fibrinous pneumonia. *Vet. Pathol.* 15:313-321.
- Schunicht, O. C., C. W. Booker, G. K. Jim, P. T. Guichon, B. K. Wildman, and B. W. Hill. 2003. Comparison of a multivalent viral vaccine program versus a univalent viral vaccine program on animal health, feedlot performance, and carcass characteristics of feedlot calves. *Can. Vet. J.* 44:43-50.
- Schunicht, O. C., P. T. Guichon, C. W. Booker, G. K. Jim, B. K. Wildman, B. W. Hill, T. I. Ward, S. W. Bauck, and J. A. Jacobsen. 2002. A comparison of prophylactic efficacy of tilmicosin and a new formulation of oxytetracycline in feedlot calves. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* 43:355-362.
- Schunicht, O. C., P. T. Guichon, C. W. Booker, G. K. Jim, B. K. Wildman, T. I. Ward, S. W. Bauck, and S. J. Gross. 2000. Comparative cost-effectiveness of ivermectin versus topical organophosphate in feedlot yearlings. *Can. Vet. J.* 41:220-224.
- Shahriar, F. M., E. G. Clark, E. Janzen, K. West, and G. Wobeser. 2002. Coinfection with bovine viral diarrhea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. *Can. Vet. J.* 43:863-868.
- Snowder, G. D., L. D. Van Vleck, L. V. Cundiff, and G. L. Bennett. 2005. Influence of breed, heterozygosity, and disease incidence on estimates of variance components of respiratory disease in preweaned beef calves. *J. Anim Sci.* 83:1247-1261.

- Snowder, G. D., L. D. Van Vleck, L. V. Cundiff, and G. L. Bennett. 2006. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J. Anim Sci.* 84:1999-2008.
- Step, D. L., T. Engelken, C. Romano, B. Holland, C. Krehbiel, J. C. Johnson, W. L. Bryson, C. M. Tucker, and E. J. Robb. 2007. Evaluation of three antimicrobial regimens used as metaphylaxis in stocker calves at high risk of developing bovine respiratory disease. *Vet. Ther.* 8:136-147.
- Step, D. L., C. R. Krehbiel, H. A. DePra, J. J. Cranston, R. W. Fulton, J. G. Kirkpatrick, D. R. Gill, M. E. Payton, M. A. Montelongo, and A. W. Confer. 2008. Effects of commingling beef calves from different sources and weaning protocols during a forty-two-day receiving period on performance and bovine respiratory disease. *J. Anim Sci.* 86:3146-3158.
- Stermer, R. A., T. H. Camp, and D. G. Stevens. 1982. Feeder cattle stress during handling and transportation. *Transactions of the ASAE (American Society of Agricultural Engineers)* 25:246-249.
- Stevens, E., D. Thomson, G. Loneragan, and N. Lindberg. 2007. Effects of short-term exposure of feeder cattle to calves persistently infected with bovine viral diarrhea virus. *Bovine Practitioner* 41.
- Storz, J., X. Lin, C. W. Purdy, V. N. Chouljenko, K. G. Kousoulas, F. M. Enright, W. C. Gilmore, R. E. Briggs, and R. W. Loan. 2000. Coronavirus and *Pasteurella* infections in bovine shipping fever pneumonia and Evans' criteria for causation. *J. Clin. Microbiol.* 38:3291-3298.
- Sulpizio, M. J., J. S. Drouillard, T. J. Kessen, R. E. Loe, Montgomery S.P., J. N. Pike, and J. J. Sindt. Effects of MGA in Receiving Diets on Health, Performance, and Carcass Characteristics. *Cattlemen's Day. Cattlemen's Day 2003*, 203-206. 3 A.D. Manhattan, KS, Kansas State University Research and Extension.
- Taylor, L. F., C. W. Booker, G. K. Jim, and P. T. Guichon. 1999. Sickness, mortality and the buller steer syndrome in a western Canadian feedlot. *Bovine Practitioner* 33:80-84.
- Taylor, L. F., D. J. Van, E. J. Dubovi, R. J. Harland, J. V. van den Hurk, C. S. Ribble, and E. D. Janzen. 1995. The prevalence of bovine viral diarrhea virus infection in a population of feedlot calves in western Canada. *Can. J. Vet. Res.* 59:87-93.
- Tegtmeier, C., A. Uttenthal, N. F. Friis, N. E. Jensen, and H. E. Jensen. 1999. Pathological and microbiological studies on pneumonic lungs from Danish calves. *Zentralbl. Veterinarmed. B* 46:693-700.
- Tennessen, T., M. A. Price, and R. T. Berg. 1984. Comparative responses of bulls and steers to transportation. *Canadian Journal of Animal Science* 64:333-338.

- Thompson, P. N., A. Stone, and W. A. Schultheiss. 2006. Use of treatment records and lung lesion scoring to estimate the effect of respiratory disease on growth during early and late finishing periods in South African feedlot cattle. *J. Anim Sci.* 84:488-498.
- Thomson, R. G., M. L. Benson, and M. Savan. 1969. Pneumonic pasteurellosis of cattle: microbiology and immunology. *Can. J. Comp Med.* 33:194-206.
- Townsend, H. G., A. H. Meek, T. G. Lesnick, and E. D. Janzen. 1989. Factors associated with average daily gain, fever and lameness in beef bulls at the Saskatchewan Central Feed Test Station. *Can. J. Vet. Res.* 53:349-354.
- Virtala, A. M. K., Y. T. Gröhn, G. D. Mechor, H. N. Erb, and E. J. Dubovi. 2000. Association of seroconversion with isolation of agents in transtracheal wash fluids collected from pneumonic calves less than three months of age. *Bovine Practitioner* 34:77-80.
- Vogel, G. J., S. B. Laudert, A. Zimmermann, C. A. Guthrie, G. D. Mechor, and G. M. Moore. 1998. Effects of tilmicosin on acute undifferentiated respiratory tract disease in newly arrived feedlot cattle. *Journal of the American Veterinary Medical Association* 212:1919.
- Warriss, P. D., S. N. Brown, T. G. Knowles, S. C. Kestin, J. E. Edwards, S. K. Dolan, and A. J. Phillips. 1995. Effects on cattle of transport by road for up to 15 hours. *Vet. Rec.* 136:319-323.
- Wellman, N. G. and A. M. O'Connor. 2007. Meta-analysis of treatment of cattle with bovine respiratory disease with tulathromycin. *J. Vet. Pharmacol. Ther.* 30:234-241.
- Welsh, R. D., L. B. Dye, M. E. Payton, and A. W. Confer. 2004. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. *J. Vet. Diagn. Invest* 16:426-431.
- Wieringa, F. L., R. A. Curtis, and R. A. Willoughby. 1976. The influence of preconditioning on plasma corticosteroid levels, rectal temperatures and the incidence of shipping fever in beef calves. *Can. Vet. J.* 17:280-286.
- Wildman, B. K., T. Perrett, S. M. Abutarbush, P. T. Guichon, T. J. Pittman, C. W. Booker, O. C. Schunicht, R. K. Fenton, and G. K. Jim. 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.
- Wilson, S. H., T. L. Church, and S. D. Acres. 1985. The influence of feedlot management on an outbreak of bovine respiratory disease. *Canadian Veterinary Journal* 26:335-341.

- Wittum, T. E. and L. J. Perino. 1995. Passive immune status at postpartum hour 24 and long-term health and performance of calves. *Am. J. Vet. Res.* 56:1149-1154.
- Wittum, T. E., N. E. Woollen, L. J. Perino, and E. T. Littledike. 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.
- Woods, G. T., M. E. Mansfield, and G. F. Cmarik. 1973a. Effect of certain biologic and antibacterial agents on development of acute respiratory tract disease in weaned beef calves. *J. Am. Vet. Med. Assoc.* 162:974-978.
- Woods, G. T., M. E. Mansfield, and R. J. Webb. 1973b. A three year comparison of acute respiratory disease, shrink and weight gain in preconditioned and non-preconditioned Illinois beef calves sold at the same auction and mixed in a feedlot. *Can. J. Comp Med.* 37:249-255.
- Yates, W. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Canadian Journal of Comparative Medicine* 46.
- Yates, W., B. Kingscote, J. Bradley, and D. Mitchell. 1983. The relationship of serology and nasal microbiology to pulmonary lesions in feedlot cattle. *Canadian Journal of Comparative Medicine* 47.
- Young, C. 1995. Antimicrobial metaphylaxis for undifferentiated bovine respiratory disease. *Compendium on Continuing Education for the Practicing Veterinarian* 17:133-142.
- Zweiacher, E. R., R. M. Durham, B. D. Boren, and C. T. Gaskins. 1979. Effects of method and time of castration of feeder calves. *Journal of Animal Science* 49:5-9.

CHAPTER III.

MOLECULAR CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATES FROM FATAL CASES OF BOVINE RESPIRATORY DISEASE

Bovine respiratory disease (BRD) complex is the most costly disease of beef cattle in North America.(Church and Radostits, 1981; Griffin, 1997) *Mannheimia haemolytica* is the most common bacterial isolate from BRD cases (Fulton et al., 2002; Reggiardo, 2005), but other species frequently implicated include *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma* species. (Gagea et al., 2006; Haines et al., 2001; Janzen, 1997; Shahriar et al., 2002; Welsh et al., 2004) Recent work has suggested that the proportion of fatal cases of respiratory disease in feedlot cattle attributable to *P. multocida* appears to be increasing.(Lundeen, 2008; Welsh et al., 2004) Potential causes for this shift include changes in virulence among the pathogens, efficacy of antimicrobial agents, and changes in identification and/or management of sick cattle.(Welsh et al., 2004) Despite this possible shift, little research has been done to examine the role of *P. multocida* in BRD. In contrast, *P. multocida* isolates from other species (including swine, poultry and rabbits) have been relatively well characterized.(Bowles et al., 2000; Dabo et al., 1999a; Dabo et al., 2000; Davies et al., 2003a; Davies et al., 2003b; Dziva et al., 2004; Shivachandra et al., 2007; Zhao et al., 1993; Zucker et al., 1996)

P. multocida is a commensal of the upper respiratory tract of cattle and the presence or absence of *P. multocida* in the nasal pharynx does not predict lower respiratory

disease.(Allen et al., 1991; Fulton et al., 2002) Thus, most authors consider it to be an opportunistic pathogen, believing that it can cause disease only in immunocompromised cattle. However, studies in swine have suggested that there are a limited number of strains associated with disease, with these strains clearly transmitted horizontally.(Blackall et al., 2000; Bowles et al., 2000; Davies et al., 2003a; Zhao et al., 1992; Zhao et al., 1993) These strains of *P. multocida* may therefore be more pathogenic than others.(Davies et al., 2003a) Similar strain differences may also exist in cattle and could correlate with unidentified virulence factors. This contention is supported by the finding that multi-locus sequence analysis, outer membrane protein characterization, ribotyping and serogrouping all found limited diversity among isolates of *P. multocida* from bovine pneumonia, even when those isolates were from diverse geographic regions.(Davies et al., 2004; Davies, 2004) Ribotyping detected moderate variation in isolates obtained from lungs compared to those from nasal swabs,(Dabo et al., 1999b) which could be consistent with differences existing between commensal and pathogenic isolates. However, no background information was available for these isolates, and it is likely that they were obtained from numerous cattle. In contrast, DeRosa (DeRosa et al., 2000) compared *P. multocida* isolates obtained from both the lower (via trans-tracheal washes) and upper (via nasopharyngeal swabs) respiratory tract of sick calves. They found only a single ribotype and concluded that this method is not sensitive enough to discern relatedness. However, it is possible that all isolates in DeRosa's population were clonal in origin.

A variety of molecular techniques have been employed to characterize *P. multocida* from several host species. This not only makes it difficult to compare across studies but also limits the usefulness of some of the investigations, as many of the techniques employed have been found to be inadequate for epidemiologic purposes.(Blackall et al., 1998; Davies, 2004; Dziva et al., 2004) Polymerization chain reaction (PCR) fingerprinting has been found to be effective in discriminating among isolates of *P. multocida* from rabbits(Dabo et al., 2000) and has been employed by numerous researchers for swine, poultry and rabbit isolates.(Chaslus-Dancla et al., 1996; Dabo et al., 1999a; Dabo et al., 2000; Dabo et al., 1999b; Dziva et al., 2001; Dziva et al., 2004; Shivachandra et al., 2007; Zucker et al., 1996) Due to the relative low cost and ease of conducting PCR fingerprinting, as well as the repeatability and effectiveness demonstrated by the above studies, it would appear to be a technique that could prove useful in epidemiologic studies of bovine respiratory pasteurellosis. Nonetheless, it has not been validated in bovine isolates of *P. multocida*.

The purpose of this study was to compare the effectiveness of PCR fingerprinting at typing *P. multocida* isolates from cases of bovine pneumonia to more traditional approaches, including characterization of whole cell protein (WCP) profiles, outer membrane protein (OMP) profiles and serotyping (collectively, WCP, OMP and serotyping will be referred to as antigenic or phenotypic methods). We hypothesized that PCR fingerprinting using microsatellite and minisatellite primers would permit repeatable discernment among isolates of *P. multocida* associated with BRD. Given the multi-factorial nature of BRD and the heterogeneity of cattle shipped to feedlots, we further

hypothesized that *P. multocida* isolates associated with BRD would consist of a diverse population of strains. This would be consistent with the widely held view of *P. multocida* as an opportunistic pathogen.

To test these hypotheses, we assessed discriminatory power and concordance of microsatellite and minisatellite PCR to classical protein and serotyping characterization techniques. The information obtained was then used to examine the diversity of isolates to determine if disease was primarily attributable to a limited number of strains. Finally, the groupings produced by the various techniques were compared to epidemiologic evidence to identify which approach(es) would be most useful for future epidemiologic investigations.

Materials and Methods

Cattle

All calves at a commercial feedlot between March 2002 and March 2003 that died from fatal fibrinous pleuropneumonia were necropsied. Tissues were submitted to the Oklahoma Animal Disease Diagnostic Laboratory for bacteriologic, virologic, and histopathologic examination. Data available for these cattle included date of arrival at the feedlot, whether the animal was treated, and date of death.

Bacterial strains and growth conditions

Following routine bacteriologic culture and identification, all isolates were stored at -80° C in 30% (v/v) glycerol in brain heart infusion broth (BHIB; Oxoid). Forty three *P. multocida* isolates were recovered from -80° C stock cultures and were streaked onto

blood agar [brain heart infusion agar containing 5% (v/v) defibrinated sheep's blood] for aerobic overnight incubation at 37° C.

DNA isolation and PCR analyses

Overnight seed cultures were used to inoculate BHI broth which was grown to mid-logarithmic phase (OD₆₀₀ of 0.5-0.6) at 37° C with shaking. Approximately four 1 ml aliquots from each isolate were centrifuged at 13,500 x g for 5 minutes to pellet cells. The DNA was extracted using a commercial kit^a according to the manufacturer's instructions. The DNA was rehydrated with 10mM tris HCl (pH 8.0) and stored at -20° C until used for PCR.

Prior to PCR, all DNA samples were quantified spectrophotometrically^b and diluted in 10 mM Tris to a final concentration of approximately 20 ng/μl. Each sample was examined on a 1% agarose gel to confirm quality and purity of genomic DNA. Three primers were used for PCR, two that detect minisatellite DNA sequences (M13 core: 5' GAGGGTGGCGTTCT-3' and modified M13 core: 5' GAGGGTGGNGGNTCT-3') and one that detects microsatellite sequences ((GTG)₅). The oligonucleotide primers were obtained from a commercial source.^c A unique master mix and time- temperature PCR protocol was made for each primer and optimized for maximum discernment of banding patterns and reproducibility. Each master mix was made as a 2x solution, then diluted with sterile water for a final volume of 100 μl per reaction. Final component concentrations were 1x commercial PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2mM of each dNTP, 1μM primer, 2.5u Taq and 40ng template DNA. Magnesium

concentration was 3.5mM for M13 core, 3mM for modified M13 core, and 2.5mM for (GTG)₅. All protocols initiated with a 3 minute denaturation at 95°C for 3 minutes, followed by 30 amplification cycles and concluding with a 7 minute extension at 72°C. Each amplification cycle included an initial 30 second denaturation step at 95° followed by a 58 second annealing stage and then a 70 second extension stage at 72°C. Annealing temperature varied for each primer: 44°C for core M13, 43°C for modified core M13 and 42°C for (GTG)₅.

PCR products were subjected to horizontal electrophoresis at 65 volts in 1.5% agarose gel made in TAE with incorporation of 0.5µg/ml of ethidium bromide. Gels were de-stained in ddH₂O for 2 to 6 hours following electrophoresis. Digital photographs were taken of each gel under UV light and images were imported into commercial software.^d The software was used to crop the image, subtract background and filter artifacts from the image. Lanes were framed and software was used to detect bands. Sensitivity was adjusted for each gel and each lane was manually curated as needed to ensure consistency across gels. All positions were identified where at least one band was present in any lane. Each sample was examined for presence/absence of a band (regardless of intensity) at this location. This was recorded as binary data (band present=1, absent=0). Most samples were analyzed using multiple gels. In these cases, each gel was examined for confirmation that banding patterns were identical for a given sample on all gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) of WCP and OMP

Five ml of overnight growth in BHI broth were used for WCP collection. Cells were collected through centrifugation (13,500 x g for 5 minutes), resuspended in a small volume of PBS, mixed with an equal volume of 2x SDS-bromophenol sample buffer and boiled for 5 minutes. Forty μ l of combined solution per lane was used in SDS-PAGE.

The OMP were extracted following envelope preparation via sonication and centrifugation to separate non-lysed cells. The sarcosyl insoluble method, which is based on the differential solubility of cytoplasmic and outer membranes in N-lauroyl sarcosine, was used.(Dabo et al., 1997) Briefly, this involved disruption of cells followed by cell envelope isolation through solubilization in a HEPES-sucrose solution subjected to centrifugation (5,850xg, 4°C for 20 minutes). The supernatant was then ultracentrifuged (280,000xg, 4°C for 70 minutes) to pellet membranes, which were washed by 3 additional ultracentrifugation cycles in ddH₂O. The envelope material was then incubated in N-lauroyl sarcosine for 45 minutes and subjected to ultracentrifugation (280,000xg, 4°C for 70 minutes). The remaining pellet was again washed and ultracentrifuged 3 times in ddH₂O. The outer membrane proteins were then re-suspended in a minimum amount of ddH₂O and stored at -80° C. Protein concentration was determined by the modified Lowry technique, using a commercial kit.^e Samples were adjusted to a final protein concentration of 2 μ g/ μ l and mixed with an equal volume of 2x SDS-bromophenol sample buffer and boiled for 5 minutes. Five μ g of each sample was then used for SDS-PAGE.

Whole cell and outer membrane proteins were analyzed by SDS-PAGE (4.5% stacking, 12.5% separating gel) using the discontinuous buffer system of Laemmli.(Laemmli,

1970) For visualization of protein bands, gels were stained with Coomassie Brilliant Blue (Fisher Scientific, Fair Lawn, NJ).

Whole cell and OMP gels were assessed visually. For OMP preparations, OMP A and OMP H were confirmed by heat modifiability, and used as primary criterion for grouping, as described previously.(Davies et al., 2004) Samples deemed similar were re-run together to permit comparison on the same gel.

Serotyping

Somatic antigen serotyping was performed by USDA NVSL, using agar gel diffusion of chicken antisera against the 16 serotypes, as previously described.(Heddleston et al.,)

Data interpretation and statistical analysis

Samples for which all results were not available were excluded from all analyses. This resulted in inclusion of 41 isolates. For PCR primers, commercial software^f was used to calculate Dice's coefficient of similarity (S) and the resulting distance (D, where $D=1-S$) for all pairwise comparisons of binary data. The software was then used to create an unweighted pair group method using arithmetic averages (UPGMA) dendrogram from the distance coefficients. This resulted in a unique dendrogram for each primer. Dendrograms were used to create groupings based upon a 92.5% cluster-difference cutoff value.

The groups resulting from visual assessment of OMP and WCP profiles, as well as the serotype results and PCR dendrograms (Figure 1-4) were used to assess the effectiveness of the various typing approaches. This was done by use of Simpson's index of diversity (Simpson, 1949) to calculate the discrimination index (D, the probability that two unrelated strains randomly selected from the test population would fall into different typing groups) (Table 1-1). A D value ≥ 0.9 has been deemed as necessary for confidence in typing results. (Hunter and Gaston, 1988) Because no typing method achieved this threshold, results obtained from genotypic techniques (PCR) were combined in 2- and 3-way cross-tabulations. This sub-divided most large groups into multiple smaller types, increasing discrimination power. Phenotypic characterization techniques were similarly combined. Confidence intervals were calculated (Grundmann et al., 2001) for each value of D permitting objective comparison between the discriminatory power of various typing methods (Table 1-1). Adjusted Rand and Wallace coefficients were calculated as described previously (Miragaia et al., 2008) to measure agreement between typing methods (Tables 1-2 and 1-3). The adjusted Rand coefficient calculation assesses the overall concordance of two methods while taking into account that those partitions could arise by chance alone. The Wallace coefficient calculates the ability to predict the results generated from a dataset by a typing method, given the results from the same dataset analyzed by a second typing method. Simpson's index and confidence intervals and similarity coefficients were calculated by software available online.[§] Simpson's index of diversity estimates the discriminatory ability of typing systems (i.e., how effective is a typing method at distinguishing between unrelated strains).

Groupings produced by RAPD PCR, WCP, OMP and serotyping were compared to available epidemiologic data for cattle from which samples were collected. Data available included date of arrival (28 of 41 samples), number of times treated for BRD (41 samples), number of days from onset of disease until death (41 samples) and other bacteria isolated from the calf (41 samples). These variables were converted to categorical or ordinal data: date of arrival was classified into 1 month periods; days of illness was categorized as acute (>6 days), subacute (7-20 days) and chronic (≥ 21 days); number of treatments were classified into 3 categories (no treatment prior to death, 1 or 2 treatments, and 3 or more); categories for concurrently isolated bacteria included none, normal flora, *M. haemolytica*, *H. somni*, *A. pyogenes* and multiple types of *Pasteurella*. Commercial software^f was used to create contingency tables and calculate contingency coefficients between each grouping and each epidemiologic variable. Association was considered significant at $p < 0.05$.

Results

All three primers successfully amplified variable DNA fragments from all 41 isolates (100% typability). These products varied with altered PCR substituents and conditions but were repeatable under identical conditions. A fourth primer, (GACA)₄, which had been used previously for rabbit and swine isolates, failed to yield reproducible results and was therefore not included in analysis and interpretation. Complexity of banding ranged from a low of 12 bands (M13 core) to high of 18 bands ((GTG)₅ and Modified core M13). Whole cell protein and OMP profiles also yielded 100% typability. Whole cell protein

electrophoresis resulted in very complex banding patterns with 25 to 30 bands being individually identifiable between 25 and 150 kiloDaltons (kDa). Proteins smaller than 20 kDa were smeared and therefore not used in analysis. OMP gels were less complex, featuring 10 to 15 bands varying from 29 to 115 kDa. Two samples were non-typeable using serotyping (95% typability). Eighteen samples belonged to only serotype 3. The remainder of the isolates cross-reacted with serum indicative of multiple serotypes with 14 belonging to both serotypes 3 and 4, six samples belonging to 3, 4 and 7, and one sample belonging to 4 and 7.

Both the M13 core and the modified M13 core mini-satellites yielded 13 groups each while the (GTG)₅ micro-satellite yielded 9 groups. The largest group generated by the M13 core primer included 13 isolates (32%), the largest group from modified core contained 14 (34%), and the largest group from (GTG)₅ primer contained 14 isolates (34%). Serotyping resulted in only 5 groups, with the largest group containing 18 samples (44%). The WCP produced 14 groups (largest group containing 12 samples or 29%) and OMP had 12 groups (largest containing 8 samples or 20%).

When considered alone, the characterization techniques produced a D value ranging from 0.68 (serotyping) to 0.89 (WCP and OMP) (Table 1-1). As expected, combinations of results produced more groups and improved D-value. All two-way combinations yielded a D>0.9, with 20 to 28 groups. Combining the results from all three primers resulted in 34 groups and a D of 0.99 (95% CI 0.98-1.00). Combining results from all 3 antigenic techniques produced 31 groups and a D value of 0.98 (95% CI of 0.95-1.00). The

discriminatory index, D, was typically improved by recognition of additional groups but was also dependent upon the size of the groups created (Table 1-1). For example, WCP and OMP both produced a D=0.89, despite creating differing numbers of groups (14 and 12, respectively). This is because WCP resulted in one very large group (12 samples), while OMP produced fewer groups of more equal size.

The adjusted Rand coefficient showed poor correlation between the various genotypic and phenotypic characterization approaches (Table 1-2). Comparing the results of the 3-way cross-tabulation results from RAPD PCR with those of the 3-way cross-tabulation of WCP-OMP-serotyping yielded an adjusted Rand value of 0.203. The highest correlation was found between the combined results of M13-(GTG)₅ and OMP-WCP (0.284). The highest concordance between individual genotypic and phenotypic approaches was (GTG)₅-WCP at 0.249. Of the individual primers, (GTG)₅ had the best overall correlation with the phenotypic techniques while the modified core primer had the worst. Of the antigenic approaches, OMP had the best overall correlation with the PCR approaches, but many of these were <0.10. There was also poor correlation between results from various primers (no 2-way comparison yielded a coefficient >0.20) as well as between the antigenic techniques (no 2-way comparison yielded a coefficient >0.31). As expected, highest correlation values were found when comparing a method to a conjugation of itself with another method; even in these circumstances correlation values rarely exceeded 0.6, with the highest being 0.73 (WCP-OMP and 3-way antigenic cross-tabulations).

The Wallace coefficient showed better correlation between the various genotypic and phenotypic characterization approaches than did the adjusted Rand, although values were still typically low (Table 1-3). By definition of the Wallace coefficient, conjugated results are perfectly predictive of the results of their constituents (i.e., (GTG)₅-M13 is 100% predictive of the results for both (GTG)₅ and M13); thus, these will not be discussed. The ability of the 3-way cross-tabulated RAPD PCR results to predict results of 3-way cross-tabulated antigenic approach was estimated at 0.375. In contrast, the 3-way antigenic results had a 0.15 Wallace value for predicting the results of 3-way RAPD PCR. Of the individual primers, (GTG)₅ had the best ability to predict phenotypic results. Similarly, the phenotypic techniques were most predictive of results from the (GTG)₅ primer, including a 0.700 predictive value from the 3-way antigenic cross-tabulation results. The modified core M13 primer again showed the poorest overall correlation, being ineffective in predicting results of the protein techniques and poorly predicted by them. Combined results of M13 core and (GTG)₅ yielded increased Wallace values, as did the 3-way cross-tabulated PCR results. The OMP groupings were better able to predict results of the PCR primers than either WCP or serotyping. Similarly, OMP was predicted more accurately by PCR results. The combination of WCP-OMP results were more effective than either alone, with fair to good predictive ability for results from M13, (GTG)₅ and M13-(GTG)₅. This includes the highest predictive value found between any genetic and phenotypic approach (0.706 predictability of (GTG)₅, given results from WCP-OMP). However, predictive value of results from 3-way cross-tabulated RAPD PCR analysis by WCP-OMP was poor (0.088). Serotyping was least effective at predicting PCR

classification, but was the most predictable protein approach. This is due to the small number of divisions that were created by serotyping.

Contingency coefficient analysis found there was no grouping method with a significant association with numbers of treatment. Duration of illness was associated with serotyping results ($p < 0.031$). Other bacteria concurrently isolated was associated with OMP and WCP-OMP classification results ($p < 0.021$ and $p < 0.05$, respectively). Month of arrival was associated with modified core M13 primer results ($p < 0.039$).

Discussion

Numerous studies have employed RAPD PCR to characterize *P. multocida*. (Chaslus-Dancla et al., 1996; Dabo et al., 1999a; Dabo et al., 2000; Dabo et al., 1999b; Dziva et al., 2001; Dziva et al., 2004; Shivachandra et al., 2007; Zucker et al., 1996) Most of these required perfect agreement for isolates to be grouped together. Such an approach precludes quantitative assessment of the relationship between isolates, potentially concealing relationships between strains. This is particularly important when using multiple primers that result in varying numbers of bands. For example, 1 band difference between 2 isolates is more significant if only 8 bands are generated than if 16 are produced. Use of the similarity index permits consistency in establishing these relationships without requiring 100% similarity. The hypervariability of the target regions for micro-satellite and mini-satellite primers increases the likelihood of relatively rapid change in banding patterns, justifying a threshold lower than 100%. A 92.5% combined cluster similarity coefficient was chosen as a relatively stringent initial criterion. Such

stringency would serve to increase the apparent discriminatory capability (Simpson D-value) of this approach but would also increase the confidence that can be placed in epidemiologic relationships detected, if any. Computer programs^d can also quantify intensity of banding and take this information into consideration in calculating comparisons. While use of this approach has been advocated for PCR techniques (Carrico et al., 2005), the appropriateness and utility of this has not been proven for RAPD PCR. In the absence of real-time data, PCR is generally not considered reliable for quantification purposes.(Jung et al., 2000; Kubista et al., 2006) It is impossible to determine what factor(s) contribute to variation in banding intensity (concentration of template, fidelity of annealing and degradation of product by Taq's 5'-3' exonuclease activity). Thus, it was decided to restrict analysis to consideration of similarity in presence or absence of bands.

The use of OMP for characterization of *P. multocida* was reported previously,(Arora et al., 2007; Dabo et al., 1999a; Dabo et al., 1997; Davies et al., 2003a; Davies et al., 2003b; Davies et al., 2003c; Davies et al., 2004; Davies, 2004) as has WCP, to a lesser extent.(Dabo et al., 1999a; Ireland et al., 1991; Nawaz et al., 2006) Interpretive criteria described for protein profiles is more vague and subjective than that reported for RAPD. We sought to apply methods similar to those reported previously for OMP analysis; namely, focusing primarily upon the heat-modifiable OMP A and H with other bands being a secondary criterion. Nonetheless, our method yielded greater discrimination than reported by others. Our results produced 12 groups out of 41 isolates for a D value of 0.89. This compared with 5 groups from 35 isolates,(Davies et al., 2003c) 10 groups from

158 isolates,(Davies et al., 2003a) 13 groups out of 153 isolates,(Davies et al., 2004) 19 groups from 100 isolates,(Davies et al., 2003b) and 10 groups from 50 isolates (resulting in a $D=0.81$).(Dabo et al., 1999a) This may represent truly increased diversity or simply be due to variation in interpretation. Diversity discerned by WCP profiling appears similar to the result of Dabo et al.(Dabo et al., 1999a) who found 10 types and 1 sub-type from 50 isolates for a $D=0.88$, compared with 14 groups from 41 isolates for a $D=0.88$ in our study. In contrast, Nawaz et al.(Nawaz et al., 2006) found no significant differences among 8 isolates. However, all of these were from hemorrhagic septicemia-associated isolates, which may demonstrate less diversity than those associated with BRD.

It was necessary to combine results from multiple primers to meet the previously established Simpson's index of diversity criterion of $D \geq 0.9$. Nonetheless, the resulting D values demonstrated that RAPD PCR using micro-satellites and mini-satellites was adequate for discriminating between strains of *P. multocida* from respiratory disease in cattle. It should be noted that Simpson's approach requires making 2 assumptions: 1) That samples are randomly selected from the population of interest; and 2) All groups are equally represented in the population. The validity of these assumptions in this study is questionable. Collection of samples was restricted to fatal cases of respiratory disease in a limited geographical region over a 12 month period. They were not randomly selected from the entire range of *P. multocida* population associated with beef cattle respiratory disease, and likely do not represent that population. Secondly, it is not clear if all possible groups are expected to be represented in fatal disease cases, or if certain strains may be over-represented as a result of increased pathogenicity. However,

violation of these assumptions in this manner would be expected to decrease the discriminatory capability; thus, the fact RAPD PCR was still adequate to discern differences amongst these strains speaks to its robust capabilities. Similar results were obtained by phenotypic characterization techniques as well. Considering the limited information provided by serotyping and the additional labor involved in collecting, processing and analyzing WCP and OMP, it would appear that RAPD PCR is a preferred method.

The low correlation found by adjusted Rand and Wallace coefficients between genotypic and phenotypic approaches suggests that these approaches provide different information. The adjusted Rand coefficient calculation assesses the overall concordance of two methods while taking into account that those partitions could arise by chance alone. Thus, in the current study the low concordance states that genotypic and phenotypic characterization approaches identified different relationships among the isolates. It has been suggested^g that the adjusted Rand coefficient can be used to determine cut-off values for dendrograms where it maximizes the congruence between the method used to create the dendrogram and any other method. Thus, subsequent analyses were done using both more and less stringent similarity values from the UPGMA dendrograms of PCR results (0.95 and 0.90). As expected, the more stringent criteria resulted in more groups with fewer samples per group thus increasing the D value, while reducing stringency had the opposite effect (Table 1-4). The impact on adjusted Rand and Wallace coefficients is less consistent (data not shown). While some values improved with decreased stringency, overall outcomes were improved by increasing stringency (which resulted in more groups

with fewer samples per group). When stringency was reduced, the adjusted Rand coefficient showed improved concordance within primers. It also improved correlation between some genotypic-phenotypic groupings. However, concordance of more genotypic-phenotypic groupings (including all 3-way RAPD PCR results with various antigenic groupings) was decreased. In contrast, when stringency was increased (which created more groups with fewer samples per group), there was a mixed but overall positive effect on concordance between results obtained from the PCR primers. There was also mixed but overall positive effect on concordance between genotypic and phenotypic results. This was particularly the case for WCP and OMP, which are more reliable for epidemiologic purposes than is serotyping. Nonetheless, compared to the original cutoff value, increased stringency reduced concordance of cross-tabulation results from RAPD PCR with all phenotypic approaches. Variation in stringency had a predictable effect on Wallace coefficients. By reducing stringency of the PCR approach and thus lowering the number of groups, there was an improved ability to use phenotypic results to predict PCR groupings. In contrast, increasing the number of groups by increasing stringency improved the ability to use PCR results to predict antigenic groupings. Nonetheless, overall Wallace values were improved more by use of less stringent PCR cutoff values. Higher and lower cutoff points (0.975 and 0.875) were also examined; neither of these offered an advantage over other cutoff points.

In spite of the improvement in correlation between antigenic and phenotypic groupings achieved by increasing stringency, it is still apparent that the two approaches provide different information. It is impossible to determine which one would be more reliable in

epidemiologic investigation. The best way to assess this would be through comparison with epidemiologic data on the cattle from which the isolates were obtained.

Unfortunately, few significant associations were identified between groupings and epidemiologic data and those that were found were not consistent. The association of serotype and duration of illness was likely spurious, as previous studies have shown serotyping to be of no epidemiologic value.(Blackall et al., 1998; Wilson et al., 1992)

The relevance of OMP and WCP-OMP with isolation of other bacteria is also questionable; it is likely that presence of other bacteria is influenced by multiple factors, including previous treatment and duration of disease, which may be more important than strain of *P. multocida* present. The association of month of arrival at the feedlot and modified M13 core primer could potentially be meaningful evidence of temporal clustering of strains. Ironically, the results for that primer showed poorest correlation with all phenotypic techniques.

The epidemiologic analysis was greatly hindered by tremendous dispersion of data points, not only among typing schemes but also within epidemiologic data. For example, many of the types created by 2-way grouping methods contained a single sample. From the host-related data, arrival dates were available for 28 of the 41 calves from which the samples were obtained. These 28 head arrived on 20 different dates, with most dates being represented by a single calf. Similarly, the 41 calves were housed in 35 different pens. We attempted to overcome some of this dispersion by grouping dates into categorical variables; duration of illness was similarly categorized. However, the validity

of the groupings used in this approach is uncertain. There was no means to overcome dispersion of data for pens of origin.

The samples examined in this study demonstrated significant diversity. The number of groups resulting from OMP analysis was greater than reported by others.(Dabo et al., 1999a; Davies et al., 2003a; Davies et al., 2003b; Davies et al., 2003c; Davies et al., 2004) Other techniques found at least 5 groups (serotyping) and up to 34 groups (3-way cross-tabulation of RAPD PCR results). The application of less stringent cutoff points could reduce these numbers in many cases, but no specific criteria have been validated as most effective. Indeed, adjusted Rand correlation coefficient suggests that more stringent similarity values may be justified. Most other studies have examined *P. multocida* from swine, poultry or rabbits. Thus, the increased diversity of our study may reflect diversity of *P. multocida* isolates in cattle in general, or be reflective of these isolates in particular. One factor that may have served to increase diversity among these isolates is that many of the cases were chronically ill prior to death. Given the selective pressure exerted by the host's response, it is impossible to determine what relationship, if any, may exist between the pathogen recovered at time of death and those which incited the disease. Similarly, many of the calves had received antimicrobial treatment prior to death, which is another selective pressure. Other potential contributors to bacterial diversity include the diversity of calves arriving at commercial feedlots, including genetic variation, source, vaccination and treatment history, and commingling and marketing exposure. Given the limited information available regarding the epidemiology of *P. multocida* in cattle, it is impossible to determine whether the diversity in disease-associated strains seen in this

study reflects the diversity of commensal strains found in the upper respiratory tract of healthy cattle. Further epidemiologic investigation is needed to assess this relationship and elucidate whether there exists evidence of increased virulence amongst *P. multocida* isolated from cattle.

Given the limited usefulness of serotyping and previous use of OMP and WCP for epidemiologic purposes, it would seem most beneficial to seek a PCR method that coincides more closely with WCP, OMP and WCP-OMP results. It would therefore appear that best results may be obtained by use of (GTG)₅ and M13 primers (and a combination of both, if necessary). Nonetheless, considering the modified M13 core primer generated the only meaningful association found between epidemiologic data and classification method it would seem prudent to include this primer, as well. A similarity cutoff of approximately 95% offered maximum concordance with phenotypic methods. This is significantly higher than those chosen by others using RAPD.(Shivachandra et al., 2007; Shivachandra et al., 2008) However, the investigators did not report the effect of varying similarity cutoffs, nor explain why their criteria were selected. Adopting an 80% similarity cutoff in the current study, as Shivachandra employed, would have resulted in very few groups and a greatly reduced discriminatory index. Future studies would benefit from the opportunity to utilize results from all 3 PCR primers, with varying cutoff criteria, for comparison with more complete epidemiologic data.

In conclusion, this study validates RAPD PCR using micro-satellite and mini-satellite primers as being a repeatable and reliable means of discriminating between *P. multocida*

isolates obtained from cattle. Isolates obtained from fatal cases of BRD in calves in a commercial feedlot demonstrated significant diversity, potentially supporting the hypothesis that *P. multocida* is a strictly opportunistic pathogen in cattle. However, additional work is needed to characterize the diversity of commensal strains found in healthy cattle. It is possible that this population would offer even greater diversity, suggesting that some strains possess virulence factors that influence the occurrence of disease. Moreover, larger numbers of isolates are needed with more complete epidemiologic data to examine the possibility of horizontal transmission and temporal and spatial clustering.

Footnotes

^a Promega Wizard Genomic DNA Purification Kit, Promega Corp., Madison, WI

^b Nano Drop Spectrophotometer, ND-1000, Thermo FisherScientific, Waltham, MA

^c Integrated DNA Technologies, Coralville, IA

^d Quantity One, Biorad, Hercules, CA

^e RC DC Protein Assay, Bio-Rad Laboratories, Hercules, CA

^f SPSS 16.0 for Windows, SPSS Inc., Chicago, IL

^g <http://www.comparingpartitions.info>

REFERENCE LIST

- Allen, J. W., L. Viel, K. G. Bateman, S. Rosendal, P. E. Shewen, and P. Physick-Sheard. 1991. The microbial flora of the respiratory tract in feedlot calves: associations between nasopharyngeal and bronchoalveolar lavage cultures. *Can. J. Vet. Res.* 55:341-346.
- Arora, A. K., S. K. Jand, K. S. Sandhu, and M. S. Oberoi. 2007. Outer membrane protein profiles of *Pasteurella multocida* isolates of bovine origin. *Indian Journal of Animal Sciences* 77:663-666.
- Blackall, P. J., N. Fegan, G. T. Chew, and D. J. Hampson. 1998. Population structure and diversity of avian isolates of *Pasteurella multocida* from Australia. *Microbiology* 144 (Pt 2):279-289.
- Blackall, P. J., N. Fegan, J. L. Pahoff, G. J. Storie, G. B. McIntosh, R. D. Cameron, D. O'Boyle, A. J. Frost, M. R. Bara, G. Marr, and J. Holder. 2000. The molecular epidemiology of four outbreaks of porcine pasteurellosis. *Vet. Microbiol.* 72:111-120.
- Bowles, R. E., J. L. Pahoff, B. N. Smith, and P. J. Blackall. 2000. Ribotype diversity of porcine *Pasteurella multocida* from Australia. *Aust. Vet. J.* 78:630-635.
- Carrico, J. A., F. R. Pinto, C. Simas, S. Nunes, N. G. Sousa, N. Frazao, L. H. de, and J. S. Almeida. 2005. Assessment of band-based similarity coefficients for automatic type and subtype classification of microbial isolates analyzed by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 43:5483-5490.
- Chalus-Dancla, E., M. Lesage-Descauses, S. Leroy-Setrin, J. Martel, P. Coudert, and J. Lafont. 1996. Validation of random amplified polymorphic DNA assays by ribotyping as tools for epidemiological surveys of *Pasteurella* from animals. *Veterinary Microbiology* 52.
- Church, T. L. and O. M. Radostits. 1981. A retrospective survey of diseases of feedlot cattle in Alberta. *Can. Vet. J.* 22:27-30.
- Dabo, S., A. Confer, and Y. Lu. 2000. Single primer polymerase chain reaction fingerprinting for *Pasteurella multocida* isolates from laboratory rabbits. *American Journal of Veterinary Research* 61.

- Dabo, S., A. Confer, M. Montelongo, and Y. Lu. 1999a. Characterization of rabbit *Pasteurella multocida* isolates by use of whole-cell, outer-membrane, and polymerase chain reaction typing. *Laboratory Animal Science* 49.
- Dabo, S. M., A. W. Confer, and G. L. Murphy. 1997. Outer membrane proteins of bovine *Pasteurella multocida* serogroup A isolates. *Vet. Microbiol.* 54:167-183.
- Dabo, S. M., B. M. Debey, M. Montelongo, and A. W. Confer. 1999b. Genomic DNA restriction site heterogeneity in bovine *Pasteurella multocida* serogroup A isolates detected with an rRNA probe. *J. Med. Microbiol.* 48:279-286.
- Davies, R., R. MacCorquodale, S. Baillie, and B. Caffrey. 2003a. Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *Journal of Medical Microbiology* 52.
- Davies, R., R. MacCorquodale, and B. Caffrey. 2003b. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Veterinary Microbiology* 91.
- Davies, R. L. 2004. Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology* 150:4199-4210.
- Davies, R. L., R. MacCorquodale, and S. Reilly. 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Vet. Microbiol.* 99:145-158.
- Davies, R. L., P. J. Watson, and B. Caffrey. 2003c. Comparative analyses of *Pasteurella multocida* strains associated with the ovine respiratory and vaginal tracts. *Vet. Rec.* 152:7-10.
- DeRosa, D. C., G. D. Mechor, J. J. Staats, M. M. Chengappa, and T. R. Shryock. 2000. Comparison of *Pasteurella* spp. simultaneously isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. *J. Clin. Microbiol.* 38:327-332.
- Dziva, F., H. Christensen, J. E. Olsen, and K. Mohan. 2001. Random amplification of polymorphic DNA and phenotypic typing of Zimbabwean isolates of *Pasteurella multocida*. *Vet. Microbiol.* 82:361-372.
- Dziva, F., H. Christensen, L. A. van Leengoed, K. Mohan, and J. E. Olsen. 2004. Differentiation of *Pasteurella multocida* isolates from cases of atrophic rhinitis in pigs from Zimbabwe by RAPD and ribotyping. *Vet. Microbiol.* 102:117-122.
- Fulton, R. W., B. J. Cook, D. L. Step, A. W. Confer, J. T. Saliki, M. E. Payton, L. J. Burge, R. D. Welsh, and K. S. Blood. 2002. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. *Can. J. Vet. Res.* 66:173-180.

- Gagea, M. I., K. G. Bateman, D. T. van, B. J. McEwen, S. Carman, M. Archambault, R. A. Shanahan, and J. L. Caswell. 2006. Diseases and pathogens associated with mortality in Ontario beef feedlots. *J. Vet. Diagn. Invest* 18:18-28.
- Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. *Vet. Clin. North Am. Food Anim Pract.* 13:367-377.
- Grundmann, H., S. Hori, and G. Tanner. 2001. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. *J. Clin. Microbiol.* 39:4190-4192.
- Haines, D. M., K. M. Martin, E. G. Clark, G. K. Jim, and E. D. Janzen. 2001. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can. Vet. J.* 42:857-860.
- Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. Fowl Cholera: Gel Diffusion Precipitin Test for Serotyping *Pasteurella multocida* from Avian Species.
- Hunter, P. R. and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26:2465-2466.
- Ireland, L., B. Adler, and A. R. Milner. 1991. Proteins and Antigens of *Pasteurella multocida* Serotype-1 from Fowl Cholera. *Veterinary Microbiology* 27:175-185.
- Janzen, E. 1997. The role of *Haemophilus somnus* in bovine respiratory disease (BRD). *Bovine Practitioner*.
- Jung, R., K. Soondrum, and M. Neumaier. 2000. Quantitative PCR. *Clin. Chem. Lab Med.* 38:833-836.
- Kubista, M., J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, R. Sindelka, R. Sjogback, B. Sjogreen, L. Strombom, A. Stahlberg, and N. Zoric. 2006. The real-time polymerase chain reaction. *Mol. Aspects Med.* 27:95-125.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680-685.
- Lundeen, T. 2008. *Pasteurella* as cause of BRDC on the rise. *Feedstuffs* 80.
- Miragaia, M., J. A. Carrico, J. C. Thomas, I. Couto, M. C. Enright, and L. H. de. 2008. Comparison of molecular typing methods for characterization of *Staphylococcus epidermidis*: proposal for clone definition. *J. Clin. Microbiol.* 46:118-129.
- Nawaz, I., R. Munir, U. Farooq, R. Kausar, and A. Khanum. 2006. Whole cell protein profiling of *Pasteurella multocida* field isolates in Pakistan. *Pakistan Veterinary Journal* 26.

- Reggiardo, C. Role of Virus in Shipping Fever of Feedlot Cattle. Case Studies and Diagnostic Considerations. 22nd Annual Proceedings Amer. Assn. Veterinary Diagnosticians , 315-320. 2005.
- Shahriar, F. M., E. G. Clark, E. Janzen, K. West, and G. Wobeser. 2002. Coinfection with bovine viral diarrhea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. Can. Vet. J. 43:863-868.
- Shivachandra, S. B., A. A. Kumar, and P. Chaudhuri. 2007. Differentiation of avian *Pasteurella multocida* strains by single-primer PCR. Vet. Res. Commun. 31:941-949.
- Shivachandra, S. B., A. A. Kumar, and P. Chaudhuri. 2008. Molecular characterization of avian strains of *Pasteurella multocida* serogroup-A:1 based on amplification of repetitive regions by PCR. Comp Immunol. Microbiol. Infect. Dis. 31:47-62.
- Simpson, E. H. 1949. Measurement of species diversity. Nature (London) 163:688.
- Welsh, R. D., L. B. Dye, M. E. Payton, and A. W. Confer. 2004. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. J. Vet. Diagn. Invest 16:426-431.
- Wilson, M. A., R. B. Rimler, and L. J. Hoffman. 1992. Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multocida* isolates. J. Clin. Microbiol. 30:1518-1524.
- Zhao, G., C. Pijoan, and M. P. Murtaugh. 1993. Epidemiology of *Pasteurella multocida* in a farrow-to-finish swine herd. Can. J. Vet. Res. 57:136-138.
- Zhao, G., C. Pijoan, M. P. Murtaugh, and T. W. Molitor. 1992. Use of restriction endonuclease analysis and ribotyping to study epidemiology of *Pasteurella multocida* in closed swine herds. Infect. Immun. 60:1401-1405.
- Zucker, B., M. Kruger, and F. Horsch. 1996. Differentiation of *Pasteurella multocida* subspecies *multocida* isolates from the respiratory system of pigs by using polymerase chain reaction fingerprinting technique. Journal of Veterinary Medicine. Series B 43.

Characterization Method	# of groups	Simpson's Index of Diversity	C.I. (95%)	# of samples in largest group
M13	13	86.71	(80.19-93.23)	12
CM	13	86.1	(77.84-94.35)	14
GTG	9	80	(73.10-86.90)	14
M13-CM	27	96.34	(93.08-99.61)	7
M13-GTG	23	95.61	(92.94-98.28)	5 (2 groups)
CM-GTG	23	95.73	(93.14-98.32)	6
3-way PCR	34	99.02	(98.08-99.97)	3
WCP	14	88.66	(82.16-95.16)	12
OMP	12	89.39	(85.95-92.83)	8
Serotyping	5	68.29	(60.31-76.27)	18
WCP-OMP	28	95.85	(91.79-99.91)	8
WCP-Serotyping	24	93.41	(87.84-98.99)	10
OMP-Serotyping	20	95.24	(92.75-97.74)	6
3-way Antigen	31	97.56	(94.94-100.18)	6

Table 1-1: Comparison of number of groups formed by characterization technique. Results of various characterization techniques with the respective Simpson's Index of Diversity value, 95% confidence interval, and the number of samples in the largest group produced by this technique.

M13														
CM	0.1541													
GTG	0.1238	0.105												
M13-CM	0.3971	0.3808	0.022											
M13-GTG	0.461	0.0428	0.3103	0.2109										
CM-GTG	0.0497	0.4327	0.3027	0.2152	0.1903									
3-way PCR	0.1208	0.115	0.0758	0.412	0.3533	0.362								
WCP	0.1764	0.0118	0.2493	0.0275	0.1476	0.0338	0.0422							
OMP	0.1439	0.0894	0.2368	0.069	0.1765	0.0748	0.0675	0.3012						
Sero.	0.0363	0.0123	0.0688	0.0036	-0.003	0.0066	0.0111	0.1667	0.0782					
WCP-OMP	0.1714	-0.0105	0.1866	0.0571	0.2837	0.0469	0.1291	0.5054	0.5342	0.0677				
WCP-Sero.	0.0918	0.0064	0.165	0.0506	0.0851	0.0639	0.0812	0.7106	0.2203	0.2636	0.4253			
OMP-Sero.	0.07	0.0222	0.1515	0.0476	0.1198	0.0944	0.1133	0.253	0.5923	0.1942	0.527	0.3968		
3-way Antigen	0.0864	0.0034	0.1136	0.0935	0.152	0.0805	0.2032	0.327	0.348	0.1022	0.7325	0.5236	0.6672	
	M13	CM	GTG	M13- CM	M13- GTG	CM- GTG	3-way PCR	WCP	OMP	Sero.	WCP- OMP	WCP- Sero.	OMP- Sero.	3-way Antigen

Table 1-2: Adjusted Rand correlation coefficients

The values reflect overall concordance of two methods, taking into account those agreements that could arise by chance alone.

	M13	CM	Con. GTG	M13-CM	M13-GTG	CM-GTG	3-way PCR	WCP	OMP	Sero.	WCP-OMP	WCP-Sero.	OMP-Sero.	3-way Antigen
M13		0.2752	0.3303	0.2752	0.3303	0.0734	0.0734	0.2569	0.2202	0.3670	0.1468	0.1284	0.0917	0.0734
CM	0.2632		0.3070	0.2632	0.0702	0.3070	0.0702	0.1228	0.1754	0.3333	0.0351	0.0702	0.0614	0.0263
Con. GTG	0.2195	0.2134		0.0488	0.2195	0.2134	0.0488	0.2805	0.2622	0.3841	0.1463	0.1646	0.1341	0.0854
M13-CM	1.0000	1.0000	0.2667		0.2667	0.2667	0.2667	0.1667	0.2333	0.3333	0.1000	0.1333	0.1000	0.1000
M13-GTG	1.0000	0.2222	1.0000	0.2222		0.2222	0.2222	0.3611	0.3889	0.3056	0.3056	0.1667	0.1667	0.1389
CM-GTG	0.2286	1.0000	1.0000	0.2286	0.2286		0.2286	0.1714	0.2286	0.3429	0.0857	0.1429	0.1429	0.0857
3-way PCR	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		0.3750	0.5000	0.5000	0.3750	0.3750	0.3750	0.3750
WCP	0.3011	0.1505	0.4946	0.0538	0.1398	0.0645	0.0323		0.3656	0.5806	0.3656	0.5806	0.2151	0.2151
OMP	0.2759	0.2299	0.4943	0.0805	0.1609	0.0920	0.0460	0.3908		0.4483	0.3908	0.2299	0.4483	0.2299
Sero.	0.1538	0.1462	0.2423	0.0385	0.0423	0.0462	0.0154	0.2077	0.1500		0.0769	0.2077	0.1500	0.0769
WCP-OMP	0.4706	0.1176	0.7059	0.0882	0.3235	0.0882	0.0882	1.0000	1.0000	0.5882		0.5882	0.5882	0.5882
WCP-Sero.	0.2593	0.1481	0.5000	0.0741	0.1111	0.0926	0.0556	1.0000	0.3704	1.0000	0.3704		0.3704	0.3704
OMP-Sero.	0.2564	0.1795	0.5641	0.0769	0.1538	0.1282	0.0769	0.5128	1.0000	1.0000	0.5128	0.5128		0.5128
3-way Antigen	0.4000	0.1500	0.7000	0.1500	0.2500	0.1500	0.1500	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

Table 1-3: Wallace coefficients

The values reflect directional agreement of groupings formed by two methods- the number in each cell reflects the ability of the row technique to predict results from the column technique.

Simpson's Index of Diversity values and 95% confidence interval produced from increased stringency (95% combined cluster similarity)

Simpson's Index of Diversity values and 95% confidence interval produced from decreased stringency (90% combined cluster similarity)

Characterization method	# of groups	Simpson's Index of Diversity	C.I. (95%)
M13	16	90.98	(86.59-95.36)
CM	17	88.05	(80.39-95.70)
GTG	13	84.63	(77.87-91.40)
M13-CM	29	97.2	(94.58-99.81)
M13-GTG	31	97.8	(95.73-99.88)
CM-GTG	27	96.71	(94.09-99.32)
3-way PCR	36	99.27	(98.36-100.17)
WCP	14	88.66	(82.16-95.16)
OMP	12	89.39	(85.95-92.83)
Serotyping	5	68.29	(60.31-76.27)
WCP-OMP	28	95.85	(91.79-99.91)
WCP-Serotyping	24	93.41	(87.84-98.99)
OMP-Serotyping	20	95.24	(92.75-97.74)
3-way Antigen	31	97.56	(94.94-100.18)

Characterization method	# of groups	Simpson's Index of Diversity	C.I. (95%)
M13	10	83.05	(76.96-89.14)
CM	10	78.05	(66.83-89.26)
GTG	7	79.39	(73.03-85.75)
M13-CM	20	92.44	(87.73-97.14)
M13-GTG	18	93.78	(91.00-96.56)
CM-GTG	18	92.2	(87.77-96.62)
3-way PCR	27	97.56	(96.00-99.12)
WCP	14	88.66	(82.16-95.16)
OMP	12	89.39	(85.95-92.83)
Serotyping	5	68.29	(60.31-76.27)
WCP-OMP	28	95.85	(91.79-99.91)
WCP-Serotyping	24	93.41	(87.84-98.99)
OMP-Serotyping	20	95.24	(92.75-97.74)
3-way Antigen	31	97.56	(94.94-100.18)

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Table 1-4: Simpson's Index of Diversity values for varying stringency
 Simpson's Index of Diversity values for increased (left) and decreased (right) stringency requirements, based upon UPGMA dendrogram. Note: because the sensitivity analysis was performed only on the primer results, no change is seen in values for phenotypic methods.

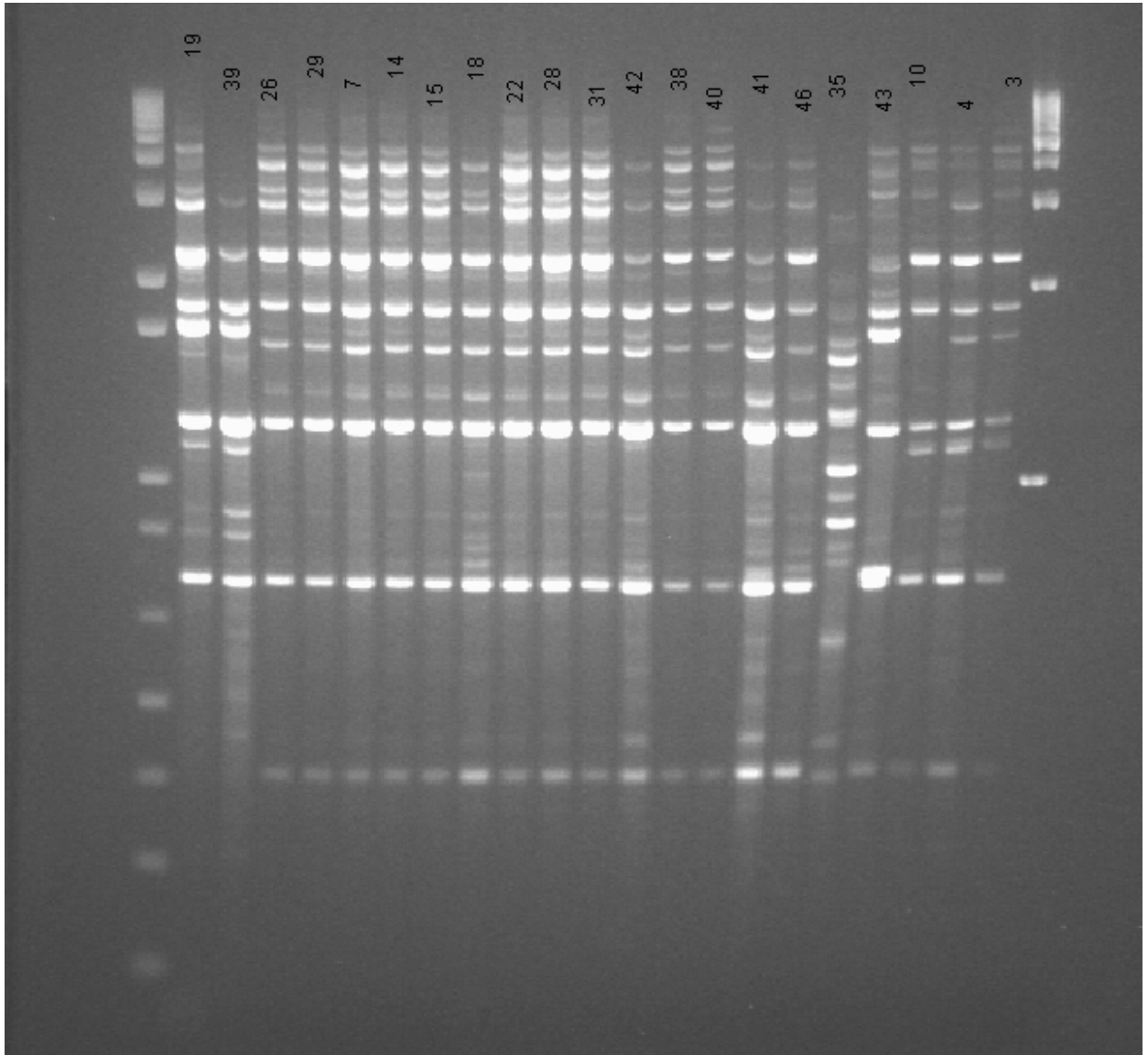


Figure 1-1: Picture of PCR gel GTG 08-31 #1.

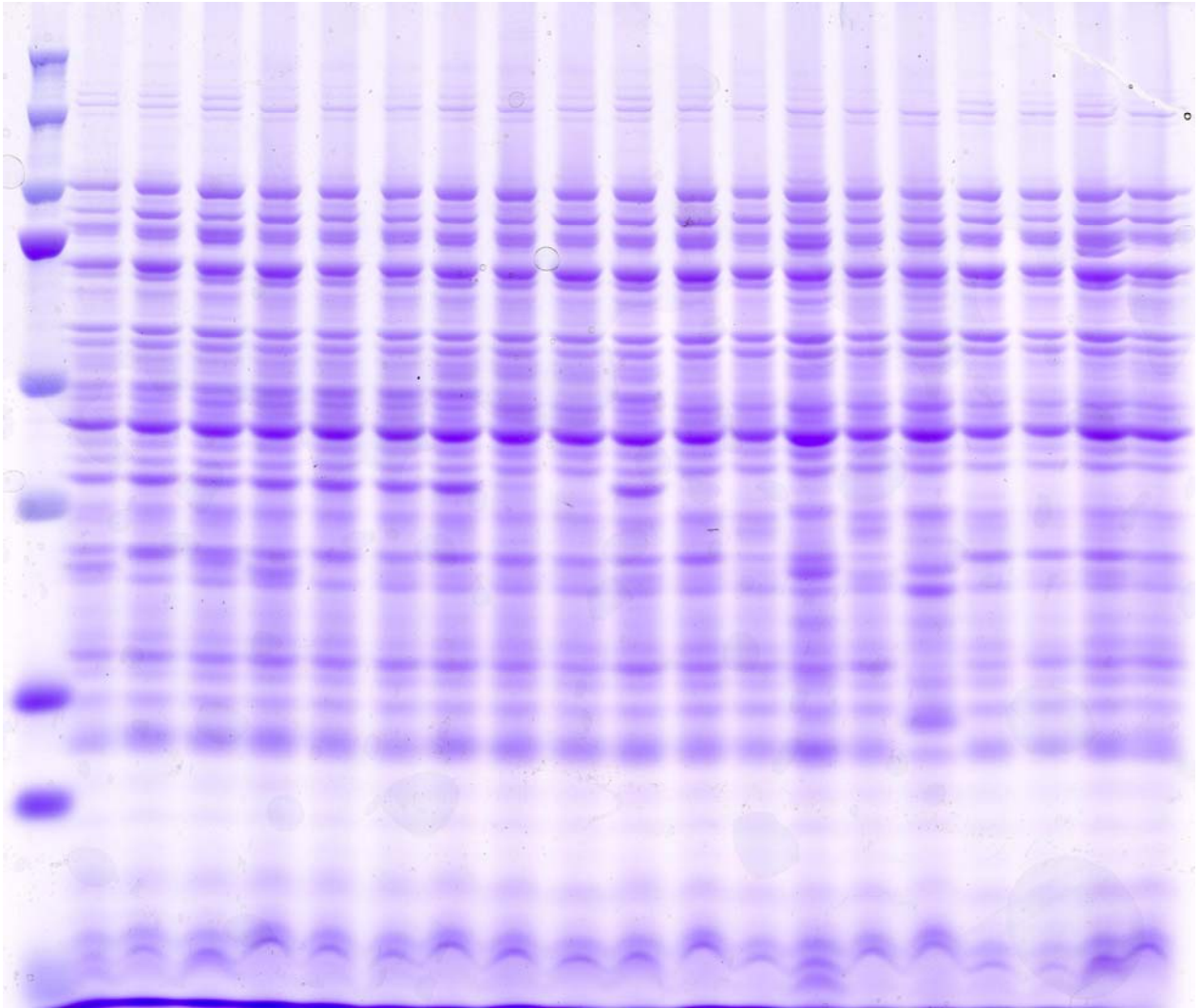


Figure 1-2: Picture of SDS-PAGE of whole cell proteins, gel 10-4 #1.

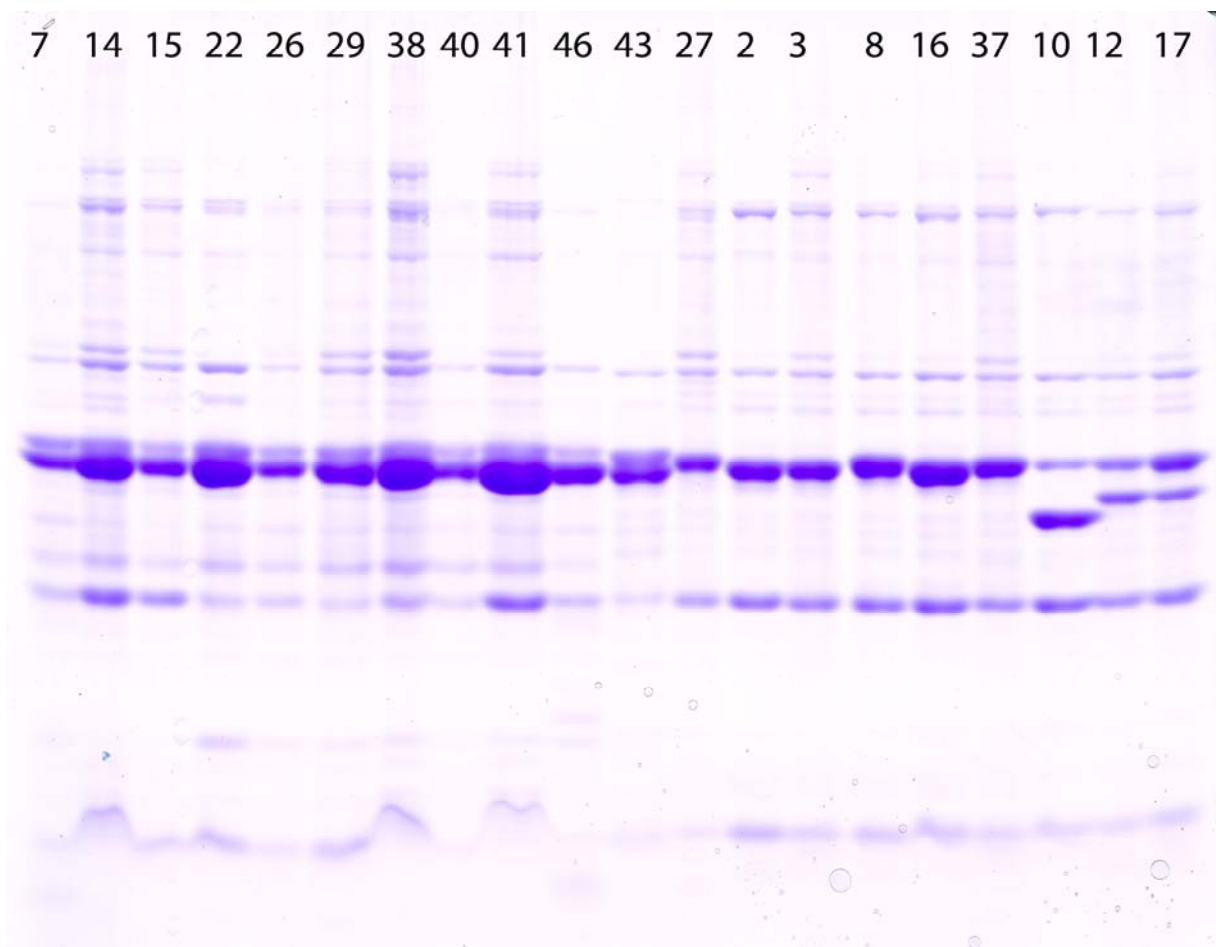


Figure 1-3: Picture of SDS-PAGE of outer membrane proteins, gel 4-3 #7-17.

Dendrogram produced by (GTG)₅ primer
 Dendrogram using Average Linkage (Between Groups)

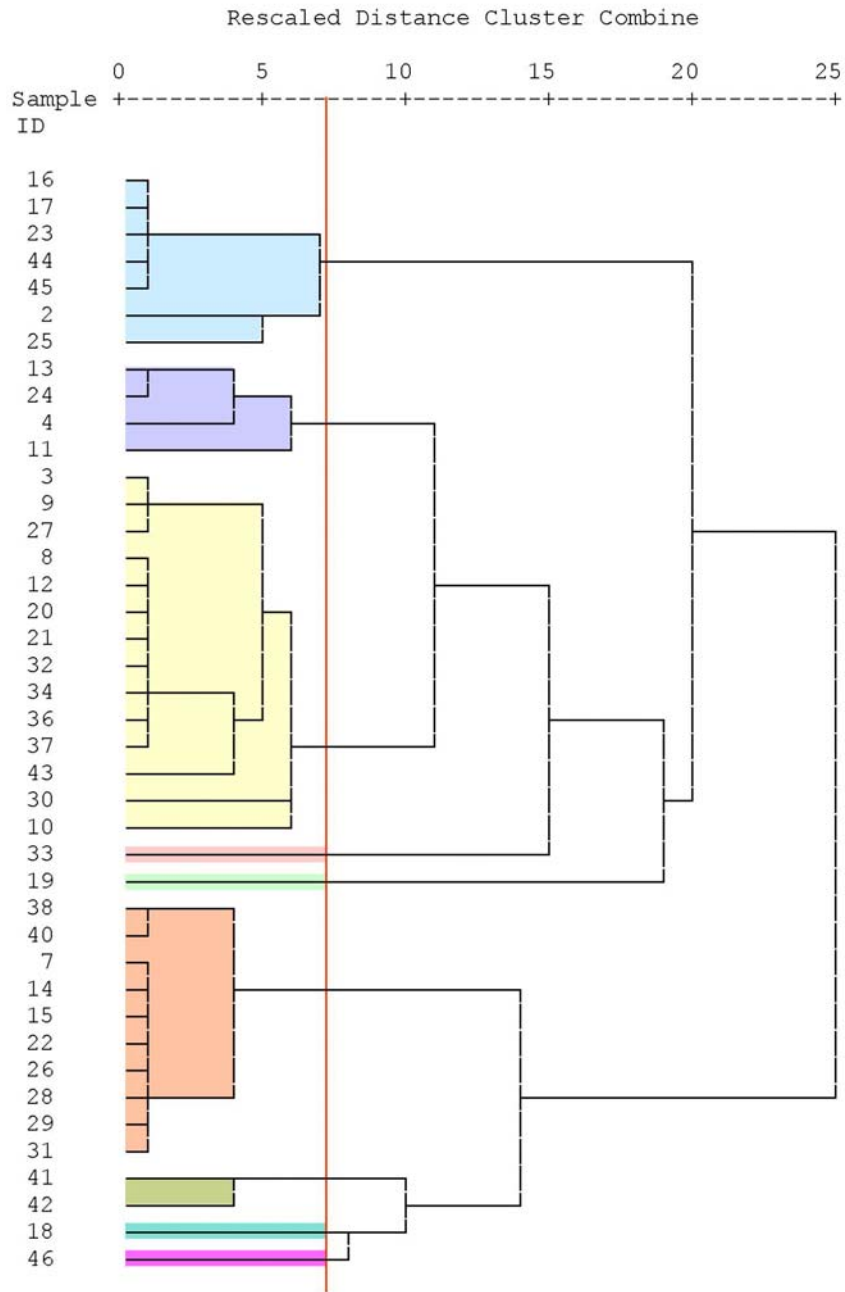


Figure 1-4: Dendrogram produced by results of (GTG)₅ primer
 Figure was generated by SPSS using the UPGMA approach. Dashed line indicates 92.5% combined cluster similarity. Colors indicate groupings established by 92.5% cutoff: light blue is group B; purple is group C; yellow is group A; light pink is group H; light green is group G; orange is group D; lime is group F; cyan is group E; bright pink is group I.

CHAPTER IV.

MOLECULAR CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATES

FROM NASAL PASSAGES OF HEALTHY AND BRD-AFFECTED CATTLE

Bovine respiratory disease complex is the most costly disease of beef cattle in North America.(Church and Radostits, 1981; Griffin, 1997) *Mannheimia haemolytica* is the most common bacterial isolate from BRD cases (Fulton et al., 2002; Reggiardo, 2005), but other species frequently implicated include *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma* species. (Gagea et al., 2006; Haines et al., 2001; Janzen, 1997; Shahriar et al., 2002; Welsh et al., 2004) Recent work has suggested that the proportion of fatal cases of respiratory disease in feedlot cattle attributable to *P. multocida* appears to be increasing.(Lundeen, 2008; Welsh et al., 2004) Potential causes for this shift include changes in virulence among the pathogens, efficacy of antimicrobial agents, and changes in identification and/or management of sick cattle.(Welsh et al., 2004) Despite this possible shift, little research has been done to examine the role of *P. multocida* in BRD. In contrast, *P. multocida* isolates from other species (including swine, poultry and rabbits) have been relatively well characterized.(Bowles et al., 2000; Dabo et al., 1999a; Dabo et al., 2000; Davies et al., 2003a; Davies et al., 2003b; Dziva et al., 2004; Shivachandra et al., 2007; Zhao et al., 1993; Zucker et al., 1996) *P. multocida* is a commensal of the upper respiratory tract of cattle and the presence or absence of *P. multocida* in the nasal pharynx does not predict lower respiratory disease.(Allen et al., 1991; Fulton et al., 2002) Thus, most authors consider it to be an

opportunistic pathogen, believing that it can cause disease only in immunocompromised cattle. However, studies in swine have suggested that there are a limited number of strains associated with disease, with these strains clearly transmitted horizontally.(Blackall et al., 2000; Bowles et al., 2000; Davies et al., 2003a; Zhao et al., 1992; Zhao et al., 1993) These strains of *P. multocida* may therefore be more pathogenic than others.(Davies et al., 2003a) Similar strain differences may also exist in cattle, and could correlate with unidentified virulence factors. Research done on cattle isolates thus far have found conflicting results. This contention is supported by the finding that multi-locus sequence analysis, outer membrane protein characterization, ribotyping and serogrouping all found limited diversity among isolates of *P. multocida* from bovine pneumonia, even when those isolates were from diverse geographic regions.(Davies et al., 2004; Davies, 2004) Ribotyping found moderate variation in isolates obtained from lungs compared to those from nasal swabs,(Dabo et al., 1999b) which could be consistent with differences existing between commensal and pathogenic isolates. However, no background information was available for these isolates, and it is likely that they were obtained from numerous cattle. In contrast, DeRosa (DeRosa et al., 2000) compared *P. multocida* isolates obtained from both the lower (via trans-tracheal washes) and upper (via nasopharyngeal swabs) respiratory tract of sick calves. They found only a single ribotype and concluded that this method is not sensitive enough to discern relatedness. However, it is possible that all isolates in DeRosa's population were clonally related. Recent work found significant diversity in *P. multocida* isolates obtained from fatal cases of respiratory disease.(Taylor et al., 2008) However, many factors identified in that study may have led to those isolates not being representative of strains that initiate disease.

Moreover, it is uncertain how the apparent diversity in those isolates compares with the diversity found in commensal populations. To determine if strains associated with disease are distinct from those typically found in the upper respiratory tract of healthy cattle, a survey of commensals is needed.

The purpose of this study was to examine the diversity of *P. multocida* isolates obtained from nasal swabs of calves at time of arrival at a research feedlot and to compare these to isolates collected from clinically ill calves as well as from healthy cohorts throughout the feeding. Given the multi-factorial nature of BRD and the heterogeneity of cattle shipped to feedlots, we hypothesized that *P. multocida* isolates found in the nasal passage of calves at arrival would consist of a diverse population of strains. We further hypothesized that disease occurrence during the feeding period would bear no apparent relationship with the presence of *P. multocida* in the nares at time of arrival, or the strain present. This would be consistent with the premise that any strain of *P. multocida* is capable of causing disease, supporting the widely held view that this bacterial species is an opportunistic pathogen.

Materials and Methods

Cattle

395 steer and bull calves (mean initial body weight of 218.6 ± 22.4 kg) were obtained through order buyers from two auction markets in central Oklahoma between September 12th and 23rd, 2005. They were delivered to the Oklahoma State University Willard Sparks Beef Research Center feedlot and allowed to rest approximately 12 hours after arrival before undergoing initial weighing, individual identification, and sample

collection, including collection of nasal swabs from all calves. This entailed use of sterile cotton swabs inserted deep into one nares of each animal. Swabs were then placed in a holder containing charcoal-free transport media and transported to the Oklahoma Animal Disease Diagnostic Laboratory, where they were used to inoculate aerobic and *Mycoplasma* cultures. Bacterial species were confirmed through standard laboratory techniques. Cattle were processed approximately 36 (loads 1-3) or 72 (load 4) hours after arrival. This included administering a viral respiratory vaccine,^a clostridial bacterin/toxoid,^b and anthelmintic.^c Castration and dehorning were also done at this time, as necessary. Cattle were blocked by weight and assigned into 25 open air, dirt pens. Calves were monitored by trained personnel throughout the feeding period and identified as potentially suffering from BRD based upon a clinical scoring technique (Table 2-1). When a calf was suspected to be clinically ill, the calf and a clinically healthy cohort (used as a control) were removed from the pen and taken to the treatment area. Calves used for control sampling were from the same arrival and pen as treated calves and had never been treated for respiratory disease prior to use as a control. Rectal temperature was measured for both suspect and control calves, and suspect calves with rectal temperature greater than 40°C were treated. Treatment was administered according to typical feedlot protocol, and consisted of tilmicosin^d for first treatment, enrofloxacin^e for second treatment, and ceftiofur hydrochloride^f for third or later treatments. Each product was administered at label dose. Nasal swabs were collected and processed from treated and control calves in the same manner as at time of arrival. Records were maintained throughout the study period documenting for each animal: sale barn and date of purchase; arrival group at the feedlot; arrival weight; whether a bull or steer at arrival; pen number;

date and reason for all samplings (at arrival, for clinical signs consistent with BRD or as a clinically healthy control); when treated and what antimicrobial was used; and date of death.

Bacterial strains and growth conditions

After initial identification by standard microbiological methods, bacterial isolates were stored at -80°C in 30% (v/v) glycerol in brain heart infusion broth (BHIB; Oxoid). All *P. multocida* isolates that could be identified were taken from -80°C stock cultures and streaked onto blood agar [brain heart infusion agar containing 5% (v/v) defibrinated sheep's blood] and incubated aerobically overnight at 37°C . Growth was assumed to be *P. multocida* if colonies possessed typical morphology and characteristics. In the case of contaminated growth, if a colony that appeared consistent with *P. multocida* was identified it was re-plated to obtain purity. If no such colony could be found the sample was discarded.

DNA isolation and PCR analyses

DNA was harvested by using overnight seed cultures to inoculate BHI broth which was grown to mid-logarithmic phase at 37°C with shaking. Approximately four 1 ml aliquots from each isolate were centrifuged at $13,500 \times g$ for 5 minutes to pellet cells. Cells were resuspended in lysis solution and combined for processing with a commercial kit[§] according to the manufacturer's instructions. The DNA was rehydrated with 10 mM tris-EDTA (pH 8.0) and stored at -20°C until used for PCR.

Prior to PCR, all DNA samples were quantified spectrophotometrically^h and diluted in 10 mM Tris-EDTA to a final concentration of approximately 20 ng/μl. Each sample was examined on a 1% agarose gel to confirm quality and purity of genomic DNA. Five sets of primers were used for PCR (Table 2-2). The first two sets of primers identified sequences previously shown to be unique to *P. multocida*. Targets for these primers were Pm0762 and Pm1231, transcriptional regulator genes with no apparent homology to published DNA sequences.(Liu et al., 2004) The products of these reactions were examined on agarose gels. Only those samples that demonstrated distinct bands consistent with the intended products of these primers (single band of appropriate size) were subjected to polymerization with the remaining three primers. The remaining three primers are considered random amplified polymorphic DNA PCR (RAPD-PCR) primers, and include two that detect minisatellite DNA sequences and one that detects microsatellite sequences. All oligonucleotide primers were obtained from a commercial source.ⁱ A unique master mix and time- temperature PCR protocol was made for each primer and optimized for maximum discernment of banding patterns and reproducibility. Each master mix was made as a 2x solution, then diluted with sterile water for a final volume of 100 μl per reaction. Final component concentrations were 1x commercial PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2mM of each dNTP, 1μM primer, 2.5u Taq and 80ng template DNA. Magnesium concentration was 3.5mM for M13 core, 3mM for modified M13 core, and 2.5mM for (GTG)₅. All protocols initiated with a 3 minute denaturation at 95°C for 3 minutes, followed by 30 amplification cycles and concluding with a 7 minute extension at 72°C. Each amplification cycle included an initial 30 second denaturation step at 95° followed by a 58 second annealing stage and then a 70 second

extension stage at 72°C. Annealing temperature varied for each primer: 44°C for core M13, 43°C for modified core M13 and 42°C for GTG.

PCR products were subjected to horizontal electrophoresis at 65 volts in 1.5% agarose gel made in TAE with incorporation of 0.5µg/ml of ethidium bromide. Gels were de-stained in ddH₂O for 2 to 6 hours following electrophoresis. Digital photographs were taken of each gel under UV light and images were imported into commercial software.^j For RAPD PCR gels, the software was also used to crop the image, subtract background and filter artifacts from the image. Lanes were framed and software was used to detect bands. Sensitivity was adjusted for each gel and each lane was manually curated as needed to ensure consistency across gels. All positions were identified where at least one band was present in any lane. Each sample was examined for presence/absence of a band (regardless of intensity) at this location. This was recorded as binary data (band present=1, absent=0).

Data interpretation and statistical analysis

Commercial software^k was used to calculate Dice's coefficient of similarity (S) and the resulting distance (D, where $D=1-S$) for all pair-wise comparisons of binary data obtained from RAPD PCR gels. The software was then used to create an unweighted pair group method using arithmetic averages (UPGMA) dendrogram from the distance coefficients. This resulted in a unique dendrogram for each primer. Dendrograms were used to create groupings based upon a 92.5% cluster-difference cutoff value (Figure 2-2). The groupings obtained from dendrograms of the three RAPD primers were combined in 2-

and 3-way cross-tabulations. This sub-divided most large groups into multiple smaller types, increasing discrimination power. Commercial software^k was used to generate descriptive statistics and to calculate contingency coefficients comparing RAPD PCR grouping results to epidemiologic data for cattle from which samples were collected.

Results

Approximately 800 samples were collected between September 13th and September 30th, 2005. A total of 257 samples yielded a *P. multocida* isolate (Figure 1-3). One hundred ninety five calves had at least one sample positive for *P. multocida*. Five hundred twenty three samples were collected on weigh days (either at time of arrival or 14 days after arrival). From these, 100 *P. multocida* samples were obtained from 99 different calves. One hundred twenty six samples were collected from 124 different calves used as control; these yielded 80 *P. multocida* samples from 78 different calves. One hundred fifty eight treatments were administered to 135 calves. *P. multocida* samples were obtained at the time of treatment 77 times, from 69 different calves. Nearly half of the cattle from which a *P. multocida* sample was obtained (112 of 257) had at least one sample that was negative for *P. multocida* prior to having a positive sample collected.

DNA was collected and subjected to RAPD PCR for 215 *P. multocida* isolates, from 168 head of cattle. Two DNA samples did not produce the expected product from the confirmatory primers, and were excluded from all analysis. The remaining 213 samples included:

- 111 samples that were the only *P. multocida* sample recovered from each calf
- 87 that were isolated from calves with two samples positive for *P. multocida*
- 15 samples from five calves with three *P. multocida* isolates each

The number of isolates obtained from each collection point included:

- 86 at arrival or time of re-weigh
- 67 at time of treatment
- 60 from control calves

There were 41 calves that had more than one sample typed. Thirty six calves had both of two samples typed and 5 calves had three isolates for each calf typed. Eighty nine samples were from 78 calves that were negative for *P. multocida* on the first sample collected from them (typically at arrival/processing), while 124 samples were from 89 calves for whom the first sample was positive. Sixty eight of the cattle from whom these samples were collected were eventually treated; 99 were not. Calves positive for *P. multocida* on nasal swabs taken at or before treatment were at increased risk of being treated for BRD compared to calves negative for the bacterium (RR=1.36, 95% CI 1.03, 1.80), while calves that had multiple samples positive for *P. multocida* were more likely to be treated than those with a single positive culture (RR=1.46, 95% CI 1.04, 2.06). Moreover, *P. multocida* was more likely to be isolated at time of treatment than from samples obtained at non-treatment collections (arrival/weigh and control) (RR=2.03, 95% CI 1.54-2.67).

All RAPD PCR primers successfully typed all 213 isolates. However, for all three primers, a large majority of the samples fell into a single dominant strain type (Table 2-3). The M13 core primer analysis produced four strain types, with 200 samples contained within type A, 11 in type B, and one each in C and D. The modified core M13 produced six strain types, with 194 in type D, 10 in type B, six in type C and one each in types A, E and F. The (GTG)₅ microsatellite produced five strain types, with C containing 203 samples, B containing seven, and one sample in each of groups A, D and E. Cross-tabulation produced greater discrimination (more groups, with fewer samples per group); however, a single strain was still dominant in all cases (Table 2-4). For the combined results of M13 and the modified M13 core, eight groups were created, with the largest one representing 186 samples. The modified core*(GTG)₅ also produced eight groups, again with 186 samples in the largest group. The combined M13*(GTG)₅ produced six groups, with 192 samples in the largest type. A three-way cross-tabulation of all primers yielded 10 types, with 178 samples in the largest group.

Contingency coefficient analysis found no association between any RAPD PCR strain type and reason for sample collection (treatment, control or arrival/weight). There was also no association between results of any primer or cross-tabulation and whether or not the calf was ever treated. However, results from several typing methods were correlated with arrival group and pen (Table 2-5). Specifically, results from the (GTG)₅ microsatellite were correlated with both arrival group and pen. Cross-tabulation results from M13*(GTG)₅ and Modified core*(GTG)₅, as well as the results of the 3-way cross-tabulation were associated with arrival group, and M13*(GTG)₅ was associated with pen

Discussion

Conflicting results have been reported regarding the usefulness of nasal swabs for diagnosing pathogens involved in BRD. Some studies have suggested that identifying *P. multocida* in the nares of a clinically ill calf suggests involvement of the bacterium in the lower tract. For example, Allen, et al. found clinically ill cattle from which *P. multocida* was isolated from nasopharyngeal swabs were likely to also have the bacterium recovered from the lung via bronchoalveolar lavage.(Allen et al., 1991). No molecular characterization was done to determine the relatedness of the isolates from the nares and lung. Additional evidence was provided by DeRosa et al., who used ribotyping, serotyping and antimicrobial susceptibility testing on isolates from nasal and tracheal swabs of cattle suffering from BRD. Their findings also suggested nasal swab culture can be predictive of the bacterial pathogen within the lung.(DeRosa et al., 2000) Finally, Godinho et al. found 100% positive predictive value of nasal swabs for retrieval of *M. haemolytica* and *M. bovis* from post-mortem lung lavage (there were too few *P. multocida* isolates for analysis).(Godinho et al., 2007) Further, RAPD-PCR found excellent correlation between lung and nasal isolates, suggesting that culture of nasal passages of sick calves may provide clues as to what strain is present in the lungs.(Godinho et al., 2007)

We were unable to obtain samples from lower respiratory tract of the calves examined in this study; however, the association between presence of *P. multocida* and treatment suggest the upper respiratory tract samples were representative of lower tract

colonization. This applied not only to samples collected at time of treatment, but also prior to being diagnosed ill. This is in contrast to a previous report, where presence or absence of *P. multocida* in the nasal pharynx at processing did not predict lower respiratory disease during the feeding period.(Fulton et al., 2002) Reasons for this difference are unclear, although Fulton, et al. only acquired samples at arrival and when treated; perhaps the increased sampling employed in our study permitted identification of *P. multocida* colonization that occurred after arrival but prior to disease onset. Indeed, nearly half of the *P. multocida* isolates were obtained after a previously negative sample. Of these 112, 69 were eventually treated. *P. multocida* was also detected more frequently in our study than in the study by Fulton et al. In our study, 195/395 (49.4%) calves had a positive *P. multocida* isolate, including 98/395 (24.8%) at arrival and 69/135 (51.1%) treated calves; Fulton et al. reported only 35/417 (8.4%) to have *P. multocida* at arrival and 4/107 (3.7%) treated calves. It is unclear whether this is attributable to different prevalence, different sensitivity in detection, or some other cause.

The groups produced by (GTG)₅ shared the most significant associations with epidemiologic data (Tables 2-5 and 2-6), both alone (six significant associations, including analysis conducted on the complete data set and various sub-sets) and in cross-tabulations (nine 2-way combinations and three 3-way). Core M13 primer was next, with seven 2-way cross-tabulations and three 3-way associations. This is consistent with other work using these primers with *P. multocida* isolates from cattle, which found (GTG)₅ and core M13 results to have the greatest concordance with other characterization techniques(Taylor et al., 2008).

Contingency analysis found arrival group and pen to be associated with the results of a number of RAPD PCR primer groupings (Table 2-5). This supports the validity of this analytical approach as being capable of identifying epidemiologically related isolates. Further refinement of data helped strengthen some of the conclusions. For example, in examining only cattle that had a negative culture for *P. multocida* prior to the first isolation, results of the (GTG)₅ primer showed a stronger association with pen (Table 2-6). This is consistent with horizontal transmission after arrival. In another refinement, samples were reduced to include only one typed sample from each calf (always including the first sample isolated). In this reduced data set, groupings produced by the modified core M13 primer became significantly associated with arrival, as did the groupings produced by cross-tabulation of core M13*modified core M13 (Table 2-6). Previously shown associations remained significant. This is consistent with the primers successfully detecting strains common to calves from each source group; this homogeneity within a source was weakened as calves acquired different strains after arrival at the feedlot.

There were 41 calves that had more than one sample typed (36 had two isolates typed and 5 had three). This permitted 51 pair-wise comparisons between two isolates obtained from the same calf. Twenty seven calves had no difference in the multiple strains isolated at different time whereas 14 animals had 2 or more different strains isolated at different times (13 had 2 different strains, 1 had 3 different strains). Thus, 15 comparisons had at least one difference in grouping among results from the 3 primers. This rate of disagreement in pair-wise comparisons (29.4%, 95% confidence interval 28.6-30.2%)

was significantly greater than the variation observed amongst all samples, where only 12.5% of pair-wise comparisons found two samples classified differently. Of the 15 pairs of samples with differences, ten pairs had a different result for only one primer, four differed in groupings produced by two of the primers, and one pair had different results from all three primers. There was no difference in percent morbidity based upon whether a calf had a single strain cultured multiple times, or different strains detected at different times. There was also no discernable trend in disease occurrence associated with a specific change, i.e., calves switching from a dominant strain to an uncommon strain were no more likely to get sick than calves switching from an uncommon strain to a dominant one. This would support the widely held view that *P. multocida* is an opportunistic pathogen, and no strain-associated virulence factors exist.

The absence of association between any RAPD PCR grouping method and apparent ability to cause disease also appears to support the opportunistic pathogen hypothesis. Alternatively, it is also possible that no particularly virulent strains were circulating in this group of cattle. While morbidity was typical of industry standards (34%), mortality was extremely low (only one BRD-related death occurred). Additional research examining isolates obtained during severe outbreaks of BRD is warranted to further clarify the presence or absence of strains with increased virulence. A final possible explanation for failure to identify variations in virulence is that nasal isolates may not be representative of invasive strains that are contributing to lower respiratory tract disease. Previous research has suggested that nasal swabs are beneficial in determining what pathogens are present in the lungs.(Allen et al., 1991; DeRosa et al., 2000; Godinho et al.,

2007) However, Allen et al. did not attempt to characterize the isolates beyond species, DeRosa used techniques (antibiogram and ribotyping) that appear to lack adequate sensitivity for molecular epidemiology, and Godinho obtained too few *P. multocida* isolates for meaningful analysis. Thus, additional work is needed to compare *P. multocida* isolates obtained from both the upper and lower airways at time of clinical illness to determine if isolates obtained from nasal swabs are indicative of agents causing clinical BRD.

Relatively little diversity was found among the 213 isolates of *P. multocida* examined in this study. This is in contrast to other work involving these primers and *P. multocida*, where a high level of diversity was found among isolates obtained from fatal cases of BRD.(Taylor et al., 2008) A number of reasons may exist for this discrepancy. Most of the cases used in the previous study were chronic and had been treated with antimicrobials. The isolates collected at death had been under significant selective pressure by host immune response and treatment, and may not reflect strain(s) present at initiation of the disease. In contrast, many of the isolates in this study were collected prior to any known administration of antimicrobials. Samples used by Taylor et al. were also collected over the course of a year, in a commercial feedlot receiving cattle from a large number of sources, further contributing to diversity in bacterial isolates. The current study utilized cattle acquired at two large auction markets, and were purchased on one of four days. Source data did not extend beyond the point of purchase, so it is impossible to determine how many source and intermediary herds were represented by the 395 head. It is possible that the limited diversity was attributable to the calves originating from very

few source herds. Indeed, contingency analysis confirmed the association of RAPD PCR results with arrival group. However, if the cattle were representative of the majority of cattle purchased by order buyers at these sale barns, it would be expected they originated from at least 12 to 20 herds.¹ It is impossible to estimate how much transmission could have occurred between arrival at the sale barn and collection of initial samples at the feedlot. One of the four groups was purchased and held at a private property for nearly a week prior to delivery to the feedlot; however, others were delivered directly from the sale. Calves were permitted very limited time to mingle at the feedlot prior to sample collection, making widespread transmission at that time unlikely. It is also possible that relatively little diversity exists in all *P. multocida* strains populating the upper respiratory tract of cattle. Investigation of cattle from a larger number of sources would be required to confirm or refute such a possibility.

Conclusion

This study offers further support for the use of RAPD PCR in epidemiologic investigation of *P. multocida* associated with BRD. The primers used were able to type all isolates and resulted in groupings that corresponded with logical epidemiologic associations. The limited diversity observed in these isolates is not indicative of lack of sensitivity of the approach, as the same primers demonstrated satisfactory discriminatory power previously. (Taylor et al., 2008) Rather, it suggests either a limited diversity among nasal isolates in general, or in this population in particular. Further research is required to distinguish between these possibilities. The findings of this study support the hypothesis that *P. multocida* is an opportunistic pathogen of cattle. No relationship was detected

between strain identified at various sampling times and likelihood of being treated, suggesting most or all strains can cause disease in a compromised host. However, this conclusion should be confirmed in relation to a more severe BRD outbreak. Perhaps most importantly, additional research is required to confirm nasal isolates are clonally related to those found in the lungs. If nasal isolates are an unreliable predictor of strains associated with disease, the findings of this study would not negate the possibility that some strains of *P. multocida* are more virulent than others.

Footnotes

^aBovishield Gold 5, Pfizer Animal Health, Exton, PA

^bUltrachoice 7, Pfizer Animal Health, Kalamazoo, MI

^cIvomec Plus, Merial, Duluth, GA

^dMicotil, Elanco Animal Health, Indianapolis, IN

^eBaytril 100, Bayer Animal Health, Shawnee Mission, KS

^fExcenel RTU, Pfizer Animal Health, Kalamazoo, MI

^gPromega Wizard Genomic DNA Purification Kit, Promega Corp., Madison, WI

^hNano Drop Spectrophotometer, ND-1000, Thermo FisherScientific, Waltham, MA

ⁱIntegrated DNA Technologies, Coralville, IA

^jQuantity One, Biorad, Hercules, CA

^kSPSS 16.0 for Windows, SPSS Inc., Chicago, IL

^lPersonal communication, Jerry Alexander USDA AMS.

REFERENCE LIST

- Allen, J. W., L. Viel, K. G. Bateman, S. Rosendal, P. E. Shewen, and P. Physick-Sheard. 1991. The microbial flora of the respiratory tract in feedlot calves: associations between nasopharyngeal and bronchoalveolar lavage cultures. *Can. J. Vet. Res.* 55:341-346.
- Blackall, P. J., N. Fegan, J. L. Pahoff, G. J. Storie, G. B. McIntosh, R. D. Cameron, D. O'Boyle, A. J. Frost, M. R. Bara, G. Marr, and J. Holder. 2000. The molecular epidemiology of four outbreaks of porcine pasteurellosis. *Vet. Microbiol.* 72:111-120.
- Bowles, R. E., J. L. Pahoff, B. N. Smith, and P. J. Blackall. 2000. Ribotype diversity of porcine *Pasteurella multocida* from Australia. *Aust. Vet. J.* 78:630-635.
- Church, T. L. and O. M. Radostits. 1981. A retrospective survey of diseases of feedlot cattle in Alberta. *Can. Vet. J.* 22:27-30.
- Dabo, S., A. Confer, and Y. Lu. 2000. Single primer polymerase chain reaction fingerprinting for *Pasteurella multocida* isolates from laboratory rabbits. *American Journal of Veterinary Research* 61.
- Dabo, S., A. Confer, M. Montelongo, and Y. Lu. 1999a. Characterization of rabbit *Pasteurella multocida* isolates by use of whole-cell, outer-membrane, and polymerase chain reaction typing. *Laboratory Animal Science* 49.
- Dabo, S. M., B. M. Debey, M. Montelongo, and A. W. Confer. 1999b. Genomic DNA restriction site heterogeneity in bovine *Pasteurella multocida* serogroup A isolates detected with an rRNA probe. *J. Med. Microbiol.* 48:279-286.
- Davies, R., R. MacCorquodale, S. Baillie, and B. Caffrey. 2003a. Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *Journal of Medical Microbiology* 52.
- Davies, R., R. MacCorquodale, and B. Caffrey. 2003b. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Veterinary Microbiology* 91.
- Davies, R. L. 2004. Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology* 150:4199-4210.

- Davies, R. L., R. MacCorquodale, and S. Reilly. 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Vet. Microbiol.* 99:145-158.
- DeRosa, D. C., G. D. Mechor, J. J. Staats, M. M. Chengappa, and T. R. Shryock. 2000. Comparison of *Pasteurella* spp. simultaneously isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. *J. Clin. Microbiol.* 38:327-332.
- Dziva, F., H. Christensen, L. A. van Leengoed, K. Mohan, and J. E. Olsen. 2004. Differentiation of *Pasteurella multocida* isolates from cases of atrophic rhinitis in pigs from Zimbabwe by RAPD and ribotyping. *Vet. Microbiol.* 102:117-122.
- Fulton, R. W., B. J. Cook, D. L. Step, A. W. Confer, J. T. Saliki, M. E. Payton, L. J. Burge, R. D. Welsh, and K. S. Blood. 2002. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. *Can. J. Vet. Res.* 66:173-180.
- Gagea, M. I., K. G. Bateman, D. T. van, B. J. McEwen, S. Carman, M. Archambault, R. A. Shanahan, and J. L. Caswell. 2006. Diseases and pathogens associated with mortality in Ontario beef feedlots. *J. Vet. Diagn. Invest* 18:18-28.
- Godinho, K. S., P. Sarasola, E. Renoult, N. Tilt, S. Keane, G. D. Windsor, T. G. Rowan, and S. J. Sunderland. 2007. Use of deep nasopharyngeal swabs as a predictive diagnostic method for natural respiratory infections in calves. *Vet. Rec.* 160:22-25.
- Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. *Vet. Clin. North Am. Food Anim Pract.* 13:367-377.
- Haines, D. M., K. M. Martin, E. G. Clark, G. K. Jim, and E. D. Janzen. 2001. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhoea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can. Vet. J.* 42:857-860.
- Janzen, E. 1997. The role of *Haemophilus somnus* in bovine respiratory disease (BRD). *Bovine Practitioner.*
- Liu, D., M. L. Lawrence, and F. W. Austin. 2004. Specific PCR identification of *Pasteurella multocida* based on putative transcriptional regulator genes. *J. Microbiol. Methods* 58:263-267.
- Lundeen, T. 2008. *Pasteurella* as cause of BRDC on the rise. *Feedstuffs* 80.
- Reggiardo, C. Role of Virus in Shipping Fever of Feedlot Cattle. Case Studies and Diagnostic Considerations. 22nd Annual Proceedings Amer. Assn. Veterinary Diagnosticians , 315-320. 2005.

- Shahriar, F. M., E. G. Clark, E. Janzen, K. West, and G. Wobeser. 2002. Coinfection with bovine viral diarrhoea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. *Can. Vet. J.* 43:863-868.
- Shivachandra, S. B., A. A. Kumar, and P. Chaudhuri. 2007. Differentiation of avian *Pasteurella multocida* strains by single-primer PCR. *Vet. Res. Commun.* 31:941-949.
- Taylor, J. D., R. W. Fulton, S. Dabo, T. W. Lehenbauer, and A. Confer. 2008. Molecular characterization of *Pasteurella multocida* isolates from fatal cases of bovine respiratory disease.
- Welsh, R. D., L. B. Dye, M. E. Payton, and A. W. Confer. 2004. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. *J. Vet. Diagn. Invest* 16:426-431.
- Zhao, G., C. Pijoan, and M. P. Murtaugh. 1993. Epidemiology of *Pasteurella multocida* in a farrow-to-finish swine herd. *Can. J. Vet. Res.* 57:136-138.
- Zhao, G., C. Pijoan, M. P. Murtaugh, and T. W. Molitor. 1992. Use of restriction endonuclease analysis and ribotyping to study epidemiology of *Pasteurella multocida* in closed swine herds. *Infect. Immun.* 60:1401-1405.
- Zucker, B., M. Kruger, and F. Horsch. 1996. Differentiation of *Pasteurella multocida* subspecies *multocida* isolates from the respiratory system of pigs by using polymerase chain reaction fingerprinting technique. *Journal of Veterinary Medicine. Series B* 43.

System Monitored	Signs Observed
Depression	Attitude, head carriage, posture, eyes (glazed or sunken), ability/willingness to rise and ambulate.
Appetite	Interest in feed, willingness to eat, amount eaten, pace of consumption, rumen fill, obvious weight loss.
Respiratory system	Breathing character and effort, auditory breath sounds, extension of head and neck.
Temperature	Taken and recorded in the morning, after being deemed a candidate for treatment.

A score is assigned for each calf based upon evaluation of the first three categories. 0 is assigned for normal, 1 for mildly abnormal, 2 for moderate, 3 for severe and 4 for moribund. The following protocol is then followed.

First Pull:

1. Severity score mild or moderate (score 1 or 2)
2. Temperature of 104°F or higher
3. If meets above:
 - a. Treat with antimicrobial A according to label
 - b. Record on health card
4. In not \geq 104°F, return to pen – record on health card
5. If treated, re-evaluate at 7 days

Second Pull:

1. At least 72 hours since treated (with the first pull treatment protocol)
2. Severity score mild or moderate (score 1 or 2)
3. Temperature 104°F or higher
4. If meets above:
 - a. Treat with antimicrobial B (may require 2nd dose in 24 to 48 hours, depending on product)
 - b. Record each treatment on health card
5. In not \geq 104°F, return to pen – record on health card
6. If treated, re-evaluate at 48 hours

Third Pull:

1. At least 48 hours since treated (with the second pull treatment protocol)
2. Severity score mild or moderate (score 1 or 2)
3. Temperature 104°F or higher
4. If meets above:
 - a. Treat with antimicrobial C (may require additional treatments on subsequent days, depending on product)
 - b. Record each treatment on health card
5. If not \geq 104°F, return to pen – record on health card

*If severity score is a “3 or 4” (severe or moribund); treat regardless of temperature. This should be a rare occasion!

All products are to be administered following Beef Quality Assurance Guidelines

Table 2-1: DART clinical scoring system and treatment protocol used for BRD cases

List of PCR Primers Employed		
Primer	Primer ID	Sequence
Pm0762-f	Forward primer for UlaR ^a	5'-ttg tgc agt tcc gca aat aa-3'
Pm0762-r	Reverse primer for UlaR ^a	5'-ttc acc tgc aac agc aag ac-3'
Pm1231-f	Forward primer for gntR_2 ^b	5'-aga aag cac atg acc aaa gg-3'
Pm1231-r	Reverse primer for gntR_2 ^b	5'-gca gct act cgc aga agg tt-3'
M13 core	Mini-satellite	5'-gag ggt ggc ggt tct-3'
Modified M13 core	Degenerate mini-satellite	5'-gag ggt ggn ggn tct-3'
(GTG) ₅	Micro-satellite	5'-gtg gtg gtg gtg gtg-3'

^a Transcriptional repressor protein (DeoR family)

^b Transcriptional repressor protein (GntR family)

Table 2-2: Sequences of PCR primers used

Pm 0762 and Pm 1231 are genes unique to *P. multocida*, and were used to confirm the DNA used for RAPD PCR was from *P. multocida* isolates. Core M13, modified core M13 and (GTG)₅ were used to generate banding patterns used for classifying isolates.

RAPD PCR Primer Results					
Core M13		Modified Core M13		(GTG) ₅	
Strain type	# of isolates	Strain type	# of isolates	Strain type	# of isolates
A	200	A	1	A	1
B	11	B	10	B	7
C	1	C	6	C	203
D	1	D	194	D	1
		E	1	E	1
		F	1		

Table 2-3: Classification results for RAPD PCR primers
Each primer has a listing of groups created and number of isolates contained in each group.

Cross-Tabulations							
M13 & CM		M13& GTG		CM & GTG		3-way	
Strain type	# of isolates	Strain type	# of isolates	Strain type	# of isolates	Strain type	# of isolates
AD	186	AC	192	DC	186	ABC	10
AB	10	BC	11	BC	10	ACC	4
BD	8	AB	7	DB	7	ADB	7
AC	4	DA	1	CC	6	ADC	178
BC	2	AD	1	AA	1	ADD	1
DA	1	CE	1	FE	1	BCC	2
CF	1			DD	1	BDC	8
BE	1			EC	1	BEC	1
						CFE	1
						DAA	1

Table 2-4: Classification results for RAPD PCR primer cross-tabulations

Cross-tabulation tables were created by using results from two or three primers to increase discriminatory ability.

RAPD PCR associations with all typed samples		
Primer or cross-tabulation	Epidemiologic variable association	p-value
GTG	Arrival	0.000
M13*GTG	Arrival	0.000
Modified M13*GTG	Arrival	0.000
Three-way	Arrival	0.000
GTG	Pen	0.010
M13*GTG	Pen	0.011

Table 2-5: Significant associations identified between RAPD PCR results and epidemiologic data
 Listing of all associations identified by contingency coefficient analysis as meeting the $\alpha=0.05$ level of significance. This includes analysis of the complete data set.

RAPD PCR associations with samples from calves whose first sample was negative for <i>P. multocida</i>		
Primer or cross-tabulation	Epidemiologic variable association	p-value
GTG	Arrival	0.000
M13*GTG	Arrival	0.000
Modified M13*GTG	Arrival	0.000
Three-way	Arrival	0.000
GTG	Pen	0.003
M13*GTG	Pen	0.030

RAPD PCR associations with the 1st sample to be typed from each calf		
Primer or cross-tabulation	Epidemiologic variable association	p-value
Modified M13	Arrival	0.027
M13*Modified M13	Arrival	0.050
GTG	Arrival	0.000
Modified core*GTG	Arrival	0.000
M13*GTG	Arrival	0.000
Three-way	Arrival	0.000
GTG	Pen	0.002
M13*GTG	Pen	0.004

Table 2-6: Significant associations identified between RAPD PCR results and sub-sets of epidemiologic data
 Listing of all associations identified by contingency coefficient analysis as meeting the $\alpha=0.05$ level of significance. This includes analysis of examined sub-sets of typed samples.

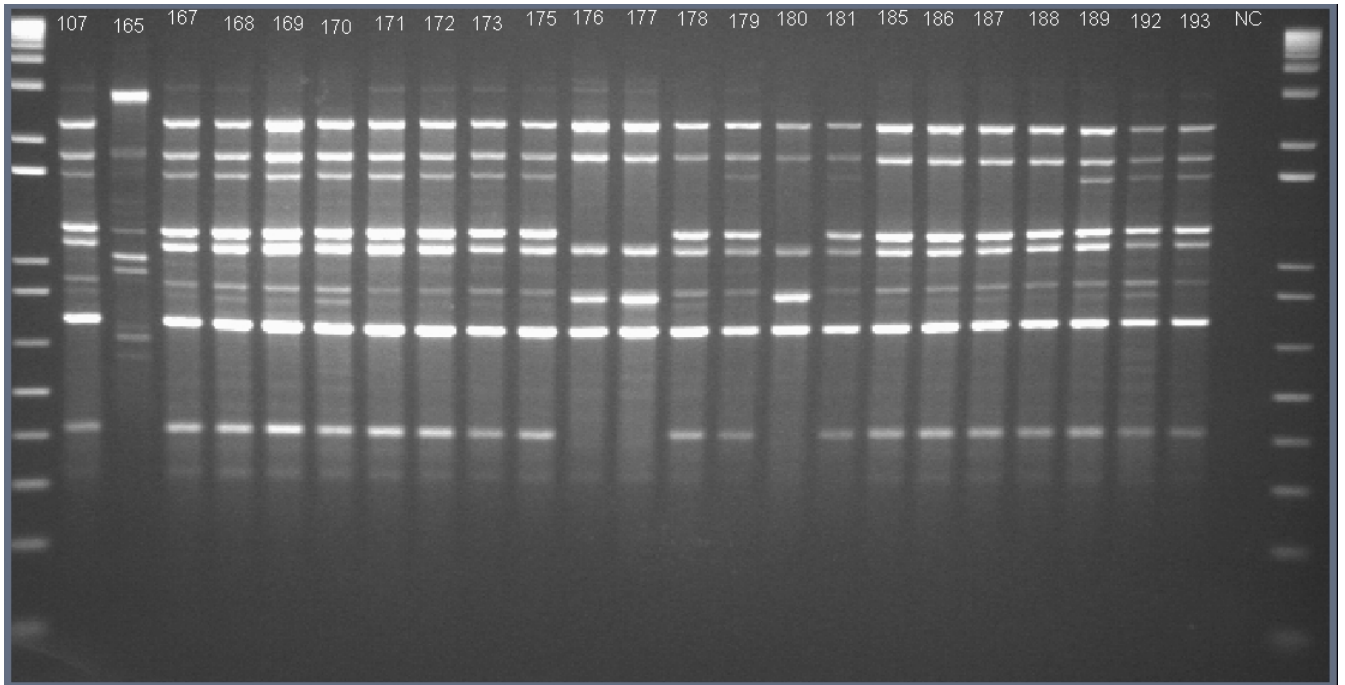


Figure 2-1: Picture of RAPD PCR primer gel, 7-30, GTG #107, #165-193

Dendrogram produced by modified core M13 RAPD PCR primer

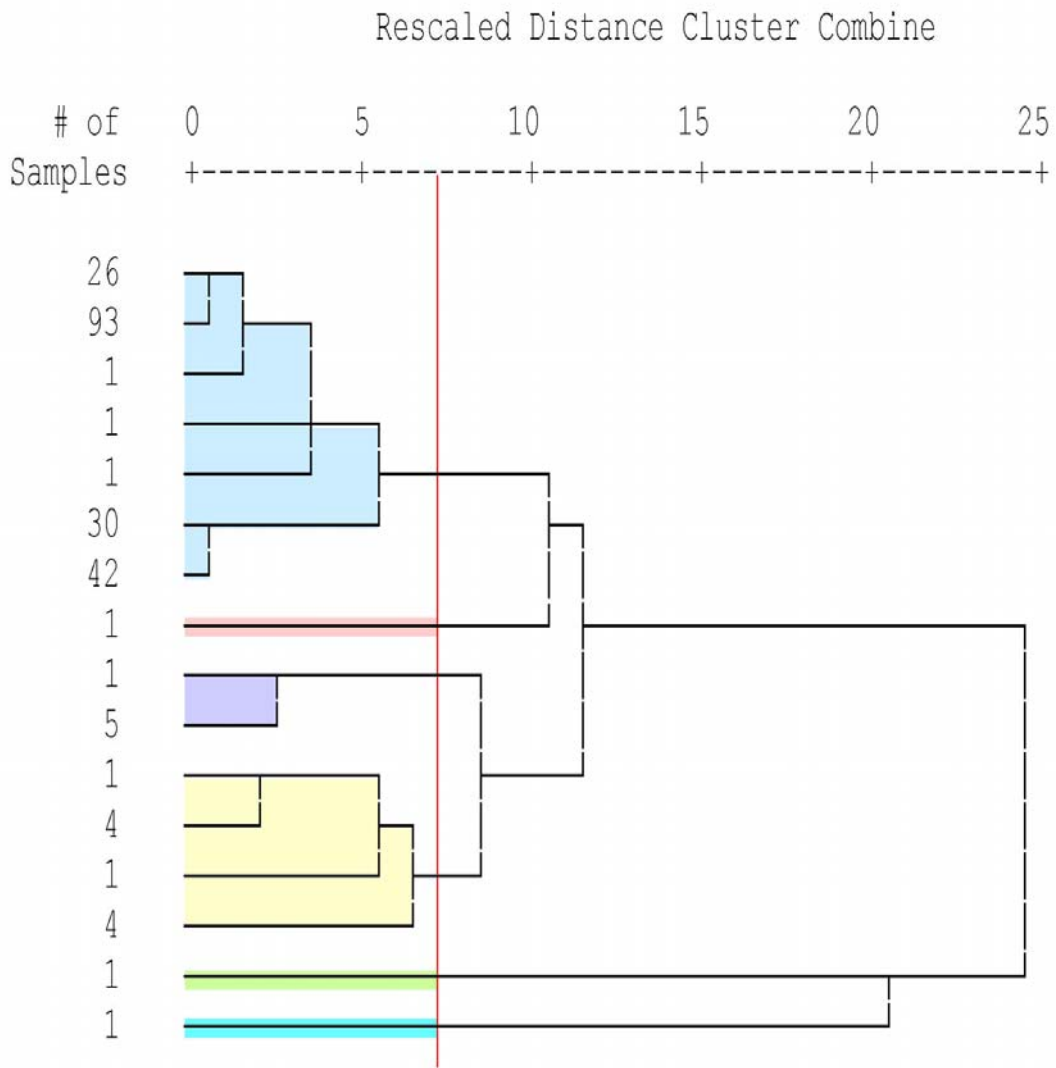


Figure 2-2: Dendrogram produced from RAPD PCR gel, using modified core M13 primer

Figure was generated by SPSS using the UPGMA approach. Dashed line indicates 92.5% combined cluster similarity. The numbers to the left are the number of samples composing each branch. Colors indicate groupings using the 92.5% cutoff: light blue is group D; pink is group E; purple is group C; yellow is group B; light green is group A; cyan is group F.

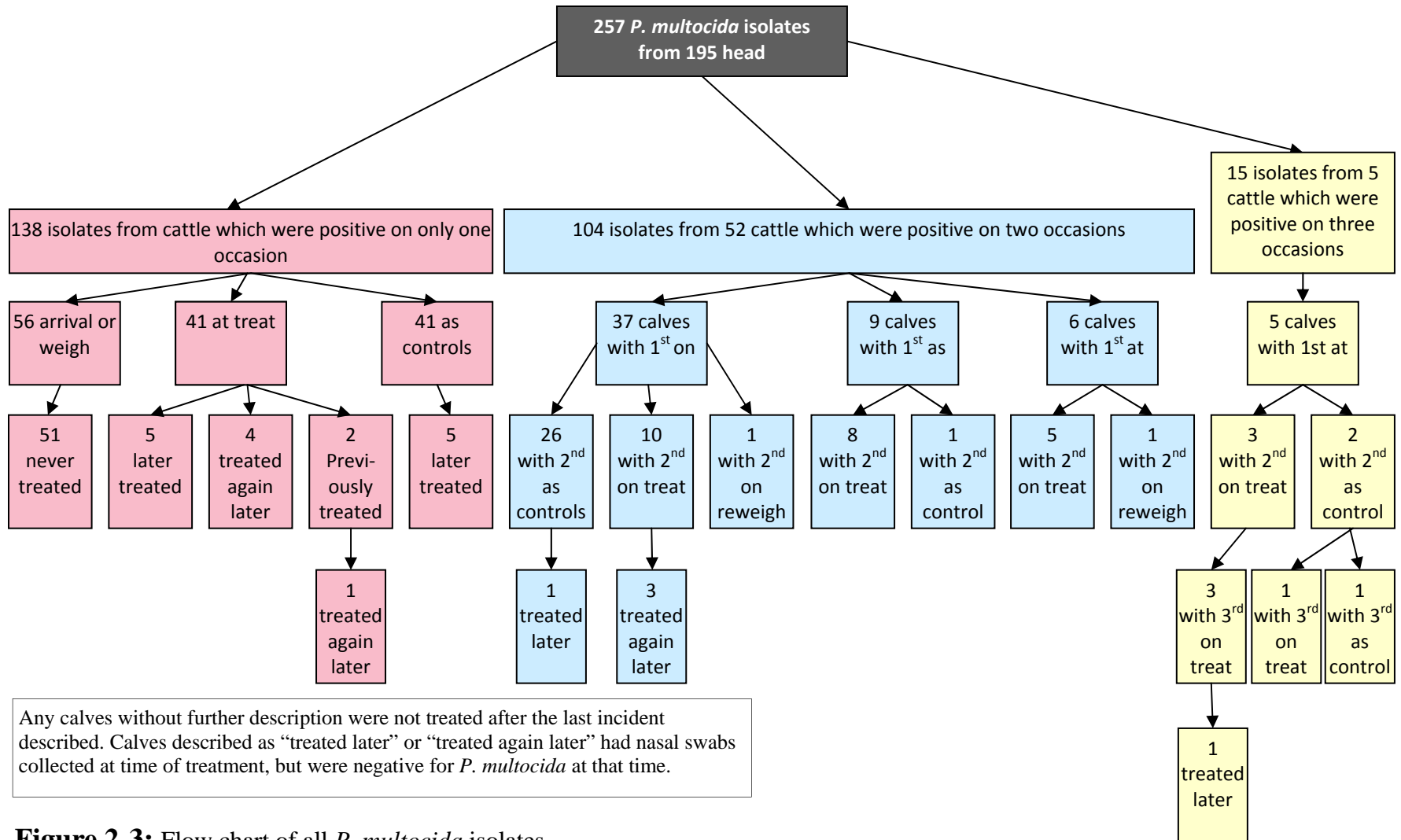


Figure 2-3: Flow chart of all *P. multocida* isolates

The chart details what sampling time each isolate was collected, as well as information about the calf from which the sample was collected (whether *P. multocida* samples were collected at other times, was the calf treated, etc.).

VITA

Jared D. Taylor

Candidate for the Degree of

Doctor of Philosophy

Dissertation: MOLECULAR EPIDEMIOLOGY OF *PASTEURELLA MULTOCIDA*
RESPIRATORY DISEASE IN BEEF CATTLE

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Data: I was born and raised in West Virginia, graduating from Nicholas County High School in 1992. I have been married to Wendy (Guilliams) Taylor for over 10 years, and have 3 children, Alex, Beth and Adam.

Education: I received my Doctor in Veterinary Medicine from Virginia Polytechnic Institute and State University in May 2002. I then earned a Masters in Public Health from University of Iowa in December 2004 before joining Oklahoma State University in 2005. I completed the requirements for the Doctor of Philosophy in Veterinary Biomedical Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2008.

Experience: I was a practicing veterinarian in Monett, Missouri for a year and a half prior to joining the Center for Food Security and Public Health at Iowa State University in January 2004. I left Iowa in 2005 to come to Oklahoma State to pursue my PhD. During my time at Oklahoma State University Center for Veterinary Health Sciences I also completed a residency in food animal medicine and surgery, enabling me to become board certified in veterinary internal medicine (large animal).

Professional Memberships: American College of Veterinary Internal Medicine; American Veterinary Medical Association; American Association of Bovine Practitioners; licensed to practice veterinary medicine in Missouri (inactive) and Oklahoma (faculty).

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Date of Degree: December, 2008

Institution: Oklahoma State University

Location: Stillwater, Oklahoma

Title of Study: MOLECULAR EPIDEMIOLOGY OF *PASTEURELLA MULTOCIDA* RESPIRATORY DISEASE IN BEEF CATTLE

Pages in Study: 130

Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Two studies were performed to investigate the epidemiology of *P. multocida* isolated from the respiratory tracts of cattle. The first study used 41 *P. multocida* isolates obtained from fatal cases of bovine respiratory disease (BRD). These isolates were subjected to random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) fingerprinting using three different primers, as well as characterized through examination of whole cell proteins (WCP), outer membrane proteins (OMP) and serotyping. Simpson's index of diversity was used to assess the ability of the various typing methods to discriminate between strains. All typing methods were able to discern differences amongst the isolates, but cross-tabulation of typing results was needed to attain adequate discriminatory ability. Adjusted Rand and Wallace correlation coefficients found poor concordance between the groupings produced by the various typing techniques.

In the second study, the same primers were used as in the first study for RAPD PCR examination of 213 *P. multocida* isolates. These isolates were obtained from the upper respiratory tract of calves purchased from commercial salebarns and shipped to the Willard Sparks Beef Research Center. Nasal swabs were collected from all calves at time of arrival. Any calf that was diagnosed with clinical BRD was sampled again at time of treatment, as was a clinically health control calf. Little diversity was observed among the isolates, and no association was detected between typing results and treatment. However, significant associations were detected between typing results and arrival group and pen. These findings support the traditional hypothesis that *P. multocida* is an opportunistic pathogen, but provide evidence of horizontal transmission from colonized to naïve cattle. Further research is warranted to confirm that strains obtained from the nares are indicative of strains associated with lower respiratory tract disease.

ADVISER'S APPROVAL: Dr. Anthony W. Confer
