

**QUALIFICATION AND QUANTIFICATION OF BACTERIAL PATHOGEN LOAD IN  
ACUTE BOVINE RESPIRATORY DISEASE CASES**

by

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## Abstract

One hundred ninety four steers, bulls, and heifers weighing 182-318 kg were purchased at an Arkansas sale barn and shipped 12 hours to a northern Kansas feedlot. There was no previous history of treatment and the cattle had been delivered to the sale barn within the 24 hour period prior to the sale. The objectives of the study were to evaluate (1) bacterial pathogen isolates in different locations in the respiratory tract, (2) pathogen load in clinically ill and clinically normal calves, and (3) compare histological damage that may be a result of clinical disease. Fifteen calves were identified with signs of acute bovine respiratory disease (BRD) based on clinical score and a minimum rectal temperature of 40° C. An additional 5 calves with no clinical signs and rectal temperatures < 40° C were selected as controls. Cattle were humanely euthanized following recording of antemortem clinical observations. At postmortem, samples for microbiologic and histologic (hematoxylin and eosin stain) analysis were collected from grossly normal and/or consolidated tissue in each lung lobe. Samples were also collected from the tonsils and trachea. Quantification of the BRD pathogens per gram were determined for each positive site and then converted to total counts for each animal. Total colony forming units (CFU) of pathogens in the entire lung for cattle with identified pathogens ranged from  $2 \times 10^7$  –  $2 \times 10^8$  CFU for *Pasturella multocida* and  $9 \times 10^6$  –  $9 \times 10^8$  CFU for *Mannheimia haemolytica*. Total visual estimated percent consolidation ranged from 0.0% to 45.0% of the total lung. Isolated pathogens from the upper and lower respiratory tract were compared and showed to have no significant agreement. Histology scores of 0-4 were assigned to the tissue samples and compared to the quantified BRD pathogens to test a possible association between the pathologic process and the total agents in that tissue sample. A significant difference in bacterial counts between histology scores of zero or 1 and a histology score of 4 was observed.

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## **Dedication**

This thesis is dedicated to my father Walter Roof, my mother Toni Roof, my brother Levi Roof, and last, but not least, my sister Emily Roof. I have been blessed to have them as a part of my life. Without their patience, love, understanding, and confidence this work would not have been possible. I am appreciative of and thankful for their support and encouragement to pursue my aspirations of a Master's degree. I would also like to dedicate this work to my close friends and classmates for their support and feedback. Their contributions helped my completion of this task.

# CHAPTER 1 - General Introduction

## Introduction

Despite the use of management interventions, antibiotics and vaccines, one of the most financially important obstacles in the United States (US) feedlot industry is the occurrence of bovine respiratory disease (BRD) complex (Babcock, *et al*, 2009; Ellis, 2001; Gardner, *et al*, 1999; Smith 1998). The economic impact of bovine respiratory disease (BRD) is much greater in lightweight, high-risk, calves than in yearlings (Lechtenberg, *et al*, 1998; Loneragan, *et al*, 2001). It is estimated that BRD annually costs the industry almost \$1 billion in losses and \$3 billion in treatment and prevention (Griffin, 1997; Snowden, *et al*, 2007). This cost estimate is measured by the sum of treatment cost, mortality, reduced animal performance, decreased carcass quality, and increased labor (Jim, 2009). However, much debate still surrounds the attempts to calculate and average financial losses as a result of BRD complex (Johnson, 1985; Schneider, *et al*, 2010; Thompson, *et al*, 2006). Recently it has been noted that animals with gross evidence of lung disease, at slaughter had a lower average daily gain than those that did not (Gardner, *et al*, 1999). Fulton, *et al*, showed in 2002 that animals treated one or more times for clinical signs of BRD had a reduced daily gain, as well as a lower returned net value ranging from <\$40.64 - \$291.93 at slaughter.

BRD is thought to occur due to poor insufficient immune response to BRD etiological agents which may have lasting effects for the entire life of the animal. (Blowey and Weaver, 2003; Gardner, *et al*, 1999). In a fifteen year study conducted by Snowden, *et al* (2006), the annual incidence of BRD was shown to vary year to year and group to group within a range of 5%-44% of group populations in the feedlot. This disease is typically qualified as bacterial

bronchopneumonia and is the primary cause of morbidity and mortality in feedlot cattle (Smith, 1998; Step, *et al*, 2008; Ribble, *et al*, 1995).

A survey conducted by the United States Department of Agriculture's National Animal Health Monitoring System (NAHMS) revealed that overall, 14.4% of cattle developed BRD while in the feedyard. This cumulative incidence rate is almost five times that of the next reported disease, acute interstitial pneumonia (AIP). It was also estimated that the typical cost associated with treating one sick animal ranges from \$11.09 to \$16.26, excluding labor, veterinary services, or indirect costs (NAHMS, 2001). Most likely the actual cost per head is much greater. A great deal of time, advanced investigation techniques, funding, and effort is directed to understanding the etiology, identification of clinical signs, treating, and preventing BRD in cattle (Fulton, 2009b; Gagea, *et al*, 2006; Panciera and Corstvet, 2010; Smith, 1996). Even with all this, the proportion of cattle that do not make it to slaughter weight because of BRD appears to be increasing (Loneragan, *et al*, 2001; Seliem, 2005; Storz, *et al*, 2000; Smith, 1996; USDA, 2000).

It is critical for the cattle industry to reduce BRD occurrence. The significance of healthy animals is more important than ever with the ever-increasing concern of antimicrobial resistance (both for public health and animal health), rising cost of production, and consumer demand (Latsuda, *et al*, 2009). Improving our comprehension of the pathogenesis of BRD complex, and the etiologic agents involved, is vital for developing appropriate preventive and treatment strategies (Aich, *et al*, 2009; Booker, *et al*, 2008; Ellis, 2001; Hanzlicek, *et al*, 2010). Therefore, exploring the quantification of bovine respiratory disease pathogens and their potential to develop resistance to current antimicrobials should ultimately aid cattle producers in increasing cattle health (McVey, 2009).

## **Thesis Organization**

This thesis is arranged with an abstract, general introduction, literature review, two individual papers, and an implication for further research summary. Each individual paper is composed of an abstract, introduction, materials and methods, results, and discussion. References cited in the literature review and the two papers are located at the end of each section, after the tables and figures. All reference citations follow the format set by the Journal of Veterinary Microbiology to which these papers will be submitted.

## **CHAPTER 2 - Literature Review**

### **Diagnosis**

Cattle of all ages and production systems can be affected by BRD. The United States beef cattle industry has evolved into a system that often exposes cattle to weaning, transportation, auction markets, and introduction to feedyards all within a short time frame, with each stressor contributing to the incidence of “shipping fever” (Dabo, *et al*, 2008). Clinical BRD commonly occurs within the first 1-6 weeks after feedyard arrival and displays seasonal occurrence that peaks in the fall and winter (Ribble, 1995a; Ribble, 1995b). The main clinical signs of BRD are both subjective and qualitative. Early detection depends on the experience level of the observer, the level of importance the clinician gives to each disease sign, and the extent of the pathogenesis of the disease process (Eitam, *et al*, 2010; Zecchinon, *et al*, 2005). The severity of the clinical signs among those who develop illness can vary substantially (Eitam, *et al*, 2010; Snowden, *et al*, 2006; Weary, *et al*, 2009; Zecchinon, *et al*, 2005). This said, there are a substantial proportion of cattle with BRD in a feedyard that are not detected, which is estimated

as 29% to 68% due to failure to exhibit traditional clinical signs (Gardner, *et al*, 1999; Reeve-Johnson, 2001; Thompson, *et al*, 2006; Weary, *et al*, 2009; Wittum, *et al*, 1996).

Combinations of different signs and observations are often considered for diagnosis, these include, but are not limited to, increased respiratory rate, abnormal breathing, decreased rumen fill, observed anorexia, serous nasal discharge, ocular discharge, depression, and a rectal temperature  $\geq 40^{\circ}\text{C}$  ( $104^{\circ}\text{F}$ ) (Apley, 2006; Lechtenberg, *et al*, 1998; Schneider, *et al*, 2010; Step, *et al*, 2008). Unfortunately these clinical signs are not pathognomonic for BRD and over-emphasis on a subset of these signs may lead to over or under-treating of cases (Apley; 2006). In order for the pathogenesis of BRD to be better understood, clinical trials where animals can be monitored closely with the ability to gather both antemortem and postmortem data should be conducted.

## **Pathogens**

Bovine respiratory disease (BRD), pneumonia, or “shipping fever” in feedlot cattle is considered to be the result of an intricate multifactorial etiology (Dabo, *et al*, 2008; Griffin, 1996; Watts, *et al*, 1994). This etiology includes several bacterial and viral pathogens contribute to BRD; however a viral component is not essential (Confer, 2009; Lechtenberg, *et al*, 1998). These pathogens alone are not considered to be capable of causing significant disease in cattle without a concurrent complex of additional factors (Dabo, *et al*, 2008; Griffin, 1996; Step, *et al*, 2008; Yates, *et al*, 1983; Cusack, *et al*, 2003). These bacterial pathogens are normally present as commensal organisms, in the upper respiratory tract of healthy animals (Cusack, *et al*, 2003; Eitam, *et al*, 2010). It appears that these infectious agents shift to pathogenic status when there is a compromise of the innate immune system and pulmonary defense mechanisms are overcome in the animal (Schneider, *et al*, 2010). This compromise is often due to a variety of stressors

including comingling, sub-optimal environment, respirable dust, transportation and handling, and weaning, that are critical for enhancement of pathogen proliferation in the lower respiratory system (Eitam, *et al*, 2010; Griffin, 2010; Snowden, *et al*, 2006; Step, *et al*, 2008). It is worth noting that stress does not have a clear definition (Grandin, 1997; Kelley, 1980). This is due to the inability to accurately measure stress, or the effect of stress on animals (Grandin, 1997; Kelley, 1980). However, it is agreed that stressors include physical and psychological events that are unpleasant to cattle (Grandin, 1997; Jacobson and Cook, 1998). Therefore, BRD develops as a result of exposure of susceptible animals to respiratory pathogens, at a time when the animals are undergoing stress; thereby lessening their ability to respond to exposure and allowing colonization of bacterial pathogens in the lower respiratory tract (Confer, 2009; Baskerville, 1981; Lechtenberg, *et al*, 1998). A synergistic effect is considered to occur between the viral and bacterial pathogens, which contributes greatly to the etiology of this complex (Snowden, *et al*, 2006; Yates, 1982).

The specific roles of each of the infectious pathogens and the stressors involved with BRD in a feedyard are still not fully understood (Aich, *et al*, 2009). Likely this is due to the general acknowledgment of the synergism of the pathogens with stressors and animal susceptibility. This complex includes many known and unknown factors that make the exact research reproduction of the BRD complex process highly variable and likely improbable.

### **Bacterial pathogens**

It is widely recognized that *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are the bacterial pathogens involved in BRD complex (Apley, 2006; Fulton, 2009a). Bacteria are not considered primary pathogens of BRD complex in healthy unstressed cattle (Confer, 2009; Yates, *et al*, 1983). It is believed that BRD develops

when stressful conditions favor viral respiratory tract infections that can become further complicated by bacterial infections, however not necessarily in that order (Schneider, *et al*, 2010; Storz, *et al*, 2000). It is also recognized that these bacterial pathogens are ubiquitous in cattle populations, commensally inhabiting the upper respiratory tract (Cusack, *et al*, 2003; Eitam, *et al*, 2010; Hanzlicek, *et al*, 2010). However, following stress, such as environmental factors, and/or viral infection, these commensal organisms can be inhaled into the lungs where they proliferate (Baskerville, 1981; Blowey and Weaver, 2003; Cusack, *et al*, 2003; Eitam, *et al*, 2010; Woldehiwet, 1990). Virulence factors associated with these organisms can cause severe inflammatory and immune responses leading to tissue damage and lesions in the lungs (Confer, 2009). At this point, these agents are considered highly infectious, but are not generally considered contagious (Griffin, 2010). Often it is difficult to clinically differentiate bovine pneumonia associated with specific bacterial pathogens (Aich, *et al*, 2009). In 2006, Gagea, *et al*, showed that the isolation of more than one type of bacterial pathogen commonly occurs in pneumonic cattle. In addition, it has been observed that both *Mannheimia haemolytica* and *Pasteurella multocida* have been isolated in the airways of both clinically affected and healthy cattle (Cusack, *et al*, 2003).

### ***Mannheimia haemolytica***

*Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) is considered to be one of the most important and common etiological agents acting as an opportunistic pathogen in bovine respiratory disease (Ames, *et al*, 1985; Latsuda, *et al*, 2009; Whiteley, *et al*, 1992). It is a Gram negative, facultative anaerobic bacterium with coccobacilli morphology (Ackermann and Brogden, 2000; Jaramillo-Arango, *et al*, 2007). It is believed that cattle acquire this pathogen early in life through environmental contact with other cattle; after which the pathogen is

considered to be a common inhabitant of the nasal mucosa and tonsils (Confer, 2009). *Mannheimia haemolytica* consists of 12 capsular serotypes (A1, A2, A5-A9, A12, A13, A14, A16, and A17) with serotype A1 and A6 being most predominately recorded in bovine pneumonia cases (Fulton, 2009b; Griffin, 2010). Virulence factors include adhesion proteins, fimbria, neuraminidase, leukotoxin (LKT), outer membrane proteins (OMP), capsular polysaccharides, endotoxin lipopolysaccharides (LPS), and protein adhesions (Fulton, 2009b; Seliem, 2005; Zecchinon, *et al*, 2005). The host response to the presence of these virulence factors is pulmonary inflammation with alveolar and vascular damage (Ackermann and Brogden, 2000; Whiteley, *et al*, 1992). This pathogen is associated with acute pleuropneumonia which is commonly seen in the first few weeks of cattle on feed (Confer, 2009; Ribble, 1995a; Ribble, 1995b). *Mannheimia haemolytica* resides in the nasopharynx and tonsils, with the tonsils accepted as the most significant source (Rice, *et al*, 2007).

*Mannheimia haemolytica* has many factors that facilitate virulence, including a capsule that provides both adhesin and antiphagocytic properties against neutrophils (Confer, 2009; Griffin, 2010). In addition, the pathogenicity of this agent is furthered by its endotoxic LPS and LKT that interact with inflammatory cytokines. These complexes work through coagulation cascades, leading to osmotic swelling, membrane pore formation, leukocyte apoptosis, cytolysis of macrophage and neutrophils, and necrosis (Corrigan, *et al*, 2007; Cusack, *et al*, 2003; Narayanan, *et al*, 2002, Sun, 1999; Zecchinon, *et al*, 2005). *Mannheimia haemolytica* produces a ruminant specific leukotoxin that severely impairs immune response by inhibiting phagocytosis and killing macrophages (Cusack, *et al*, 2003; Ellis, 2001). *Mannheimia haemolytica* diseased cattle often have acute cranioventral fibrinous to fibrinopurulent pleuropneumonia with whole



lobules demonstrating hemorrhage, coagulation necrosis, and distended edematous interlobular septa (Ames, *et al*, 1985; Confer, 2009; Narayanan, *et al*, 2002).

### ***Pasteurella multocida***

*Pasteurella multocida* is a Gram negative, facultative anaerobe with coccobacillus morphology and is a common nasopharyngeal commensal pathogen of cattle (Catry, *et al*, 2008; Harper, *et al*, 2006; Jaramillo-Arango, *et al*, 2007). This pathogen is more commonly isolated in respiratory disease affecting younger cattle, especially in enzootic neonatal calf pneumonia (Griffin, 2010). It has 5 capsular serogroups (A-F) and has 16 somatic serotypes that are lipopolysaccharide (LPS)-based (Harper, *et al*, 2006). Serotype A:3 is the most commonly isolated *Pasteurella multocida* from cattle with respiratory disease followed by D:3 at a much lower isolation rate (Dabo, *et al*, 2008; Griffin, 2010). The virulence factors are less characterized than those associated with *Mannheimia haemolytica*, but probably include OMP, neuraminidase, siderophores, capsular polysaccharides, LPS, and protein adhesions (Confer, 2009; Dabo, *et al*, 2008, Seliem, 2005). Similar to *Mannheimia haemolytica*, *Pasteurella multocida* has a capsule that provides adhesin and antiphagocytic properties, allowing resistance to phagocytosis and interference with the complement mediated bacteriolysis (Cusack, *et al*, 2003; Mosier, 1997; Dabo, *et al*, 2008). The LPS induces endotoxic shock due to its stimulation of inflammatory cytokines (Confer, 2009; Harper, *et al*, 2006). *Pasteurella multocida* diseased cattle often have acute to subacute cranioventral bronchopneumonia lung lesions with associated fibrinopurulent pleuritis and edematous interlobular septa in moderate cases (Confer, 2009; Dabo, *et al*, 2008).

## *Histophilus somni*

*Histophilus somni* (formerly *Haemophilus somnus*) is a Gram negative, nonencapsulated bacterium that is a commensal inhabitant of the nasopharyngeal regions but is considered to preferentially colonize the lower respiratory tract (LRT) (Confer, 2009; Griffin, 2010).

*Histophilus somni* has been isolated more often from the LRT than from the nasal mucosa, indicating a potential preference for the LRT (Corbeil, 2008). This pathogen has been reported to be isolated in 15% to 50% of newly received, clinically healthy cattle (Griffin, 2010). However, this pathogen has not been reported in fatal cases of BRD as commonly in the United States as it has been in Canada, but is often associated with subacute to chronic infections (Griffin, 1996; Donkersgoed, *et al*, 1990; Ellis, 2001). The virulence factors are similar to both *Mannheimia haemolytica* and *Pasteurella multocida* with *Histophilus somni* containing OMP, lipooligosaccharide (LOS), histamine, structural exopolysaccharides, and Ig protease (that resist compliment-mediated killing), all of which contribute to pathogenesis of disease (Cusack, *et al*, 2003; Griffin, 2010). However, this agent appears to be missing a capsule, pili, and other structures that could potentially enhance virulence (Yang, 1998). The LOS seems to be an important virulence factor that allows the infectious agent to induce cell apoptosis and aids in inhibition of phagocytosis, as well as, causes vasculitis and necrosis of host tissues (Confer, 2009; Mosier, 1997; Yang, 1998.). *Histophilus somni* diseased cattle can demonstrate similar lesions to *Mannheimia haemolytica* and are seen in the form of fibrinopurulent bronchopneumonia, fibrinous pericarditis, thromboembolic meningoencephalitis (TME), myocardial infarcts and polyarthritis (Confer, 2009; Donkersgoed, *et al*, 1990; Mosier, 1997; Griffin, 2010).

## *Mycoplasma bovis*

*Mycoplasma bovis* is a mollicute, facultative anaerobe that lacks a cell wall and consists of a tri-layered cell membrane (Casewell and Archambault, 2008; Griffin, 2010; White, *et al*, 2010). The role of *Mycoplasma bovis* as a primary respiratory pathogen in the pathogenesis of BRD complex is not as clear as with the previously mentioned pathogens, but this agent is consistent in its involvement with arthritis and chronic respiratory disease (Apley, 2006; Griffin, 2010; Rosendal and Martin, 1986; White, *et al*, 2010). The virulence factors of this pathogen include 5 variable surface lipoproteins (VspA, VspB, VspC, VspF, and VspO) that function as adhesins; which allow for the degradation of immune responses (Casewell and Archambault, 2008; Griffin, 2010). Some strains of *Mycoplasma bovis* have the ability to produce hydrogen peroxide which provides oxygen free radicals causing peroxidation of host tissue and aiding to the degradation of the pulmonary tissues (Confer, 2009). It should be noted that while a polysaccharide toxin has been described, the existence as well as the function of the toxin is still unclear (Casewell and Archambault, 2008; Confer, 2009; White, *et al*, 2010). As with *Histophilus somni*, isolation of the bacterium occurs more often in the LRT than in the nasal mucosa (Casewell and Archambault, 2008; Griffin, 2010). It is proposed that a synergistic effect has been observed with *Mycoplasma bovis* and *Mannheimia haemolytica* in severe disease cases (Griffin, 2010). *Mycoplasma bovis* infected cattle often have enzootic pneumonia, otitis, or chronic pneumonia complicated with arthritis (Confer, 2009; Griffin, 2010). A common lesion associated with *Mycoplasma bovis* infections is cranioventral caseonecrotic bronchopneumonia (Gagea, *et al*, 2006).

## **Viral Pathogens**

There have been many viral agents connected to BRD, these include Parainfluenzavirus-3 (PI-3), Bovine Herpesvirus-1 (BHV-1), Bovine Viral Diarrhea Virus (BVDV), and Bovine Respiratory Syncytial Virus (BRSV) (Fulton, 2009a; Hodgins, *et al* 2002). Each has a variety of distinguishing characteristics, with virulence factors varying within and across the pathogens that can cause acute BRD without interaction with other pathogens (Hodgins, *et al* 2002; Snowden, 2009). Fulton, 2000b, found that concurrent infections of these viral agents occur. These agents are often associated with ciliated epithelial damage due to local infection and replication (Griffin, 1996). This damage allows receding of cellular function and, if left unchecked, can have a negative impact on the innate bacterial clearance mechanism (Griffin, 1996; Griffin, 2010). This causes a lysis of respiratory cells that leads to inflammation, compromising the immune system, thereby creating opportunity for secondary infection by other pathogens (Mussli-Cockett, *et al*, 1992; Ryan, *et al*, 1993). It is understood that infections with these viruses in a feedyard setting can produce BRD complex (Fulton, 2009a). However, exposure to these viral agents has been documented to only cause disease in some individuals but not in all animals, indicating a select circumstance for the virus to be successful (Eitam, *et al*, 2010).

### **Parainfluenzavirus-3: (PI-3)**

Parainfluenzavirus-3 is an enveloped, single-stranded RNA paramyxovirus that is considered to be wide-spread viral agent, associated with both acute and chronic pneumonia. It may be a severe pathogen in young cattle (Griffin, 1996). However, there is debate to the extent and significance of this agent as a respiratory pathogen in general cattle populations (Ellis, 2001). This virus replicates in alveolar type 2 cells and epithelial cells of both the upper and lower respiratory tract (Cusack, *et al*, 2003; Griffin, 1996). However, damage occurs primarily in

the lower respiratory tract where decreased inflation, as well as consolidation of the anterior portion of the lung lobes is observed (Cusack, *et al*, 2003). This virus affects many types of cells in the respiratory tract which can impair the mucociliary clearance and the phagocytosis of inhaled particles, ranging from other bacterial organisms to intruding particles. Viral replication in the epithelial cells of the lower respiratory tract causes bronchitis, bronchiolitis and alveolitis (Cusack, *et al*, 2003; Ellis, 2001). In the acute stage of PI-3 virus infection, there is proliferation within and necrosis of bronchiolar epithelial cells with subsequent widespread destruction of cilia and of ciliated cells in small bronchi and bronchioli. PI-3 also infects alveolar macrophages (Cusack, *et al*, 2003; Griffin, 1996).

### **Bovine Herpesvirus-1: (BHV-1)**

Bovine Herpesvirus-1, a large enveloped single-stranded RNA alpha herpesvirus, was first isolated in 1956, it has several subtypes, but subtypes 1 and 2a, are considered to be the etiological agents responsible for Infectious Bovine Rhinotracheitis (IBR) (Ellis, 2009; Hodgins, *et al*, 2002). Though this virus is associated with several disorders, such as encephalitis, abortions, reproductive tract disease, mastitis, and tracheitis, it is considered to be a respiratory pathogen (Fulton, 2009b; Griffin, 1996; Hodgins, *et al* 2002). BHV-1 infection causes immunosuppression that has shown to increase susceptibility to secondary bacterial infections resulting in severe pneumonia (Cusack, *et al*, 2003). This immunosuppression may reduce neutrophil migration, polymorphonuclear leukocyte (PMN) adhesion, cell-mediated cytotoxicity, mitogen responses of peripheral blood lymphocytes, and some functional activities of alveolar macrophages (Cusack, *et al*, 2003; Warren, *et al*, 1996). BHV-1 replicates in the mucosal cells and in other cell types of the submucosa and connective tissue peripheral to the tracheal rings (Cusack, *et al*, 2003). This can lead to the destruction of the epithelium of the upper respiratory

tract with cessation of ciliary activity resulting in the loss of function of the mucociliary escalator in conjunction with intense inflammation of the trachea (Cusack, *et al*, 2003; Yates, 1982).

Though the mechanisms of virulence have been reported, they are still not fully understood (Ellis, 2009). The lesions and signs associated with BHV-1 range from serous, hyperemic and edematous membranes, through mucopurulent exudates with focal necrosis, to pseudomembranous inflammation in severe cases (Cusack, *et al*, 2003). Secondarily bronchopneumonia may also occur due to inhalation of infectious tracheal exudates and failure to clear particulate fomites and bacteria from the lungs (Cusack, *et al*, 2003).

### **Bovine Viral Diarrhea Virus: (BVDV)**

Bovine Viral Diarrhea Virus is an enveloped single-strand RNA pestivirus with considerable antigenic variability (Baker, 1987; Fulton, 2000a; Potgieter, 1997). This virus was first recognized in the US in 1946 and it is considered to cause several disorders including, congenital birth defects, acute diarrhea, enteritis, mucosal disease, and reproductive syndromes (Baker, 1987; Deregt and Loewen, 1995; Fulton, 2009c). The role of BVDV in the pathogenesis of BRD has been subjected to much conjecture due to a lack of evidence implicating it as a primary BRD pathogen, despite years of research (Cusack, *et al*, 2003; Hodgins, *et al* 2002; Potgieter, 1997). Due to the immunosuppressive nature of this virus, it is still considered a factor in the development of respiratory disease (Fulton, 2009b; Potgieter, *et al*, 1984; Ryan, *et al*, 1993). This virus shows tropism for rapidly reproducing and dividing cells, such as lymphocytes, mononuclear phagocytes, and epithelial cells where it disseminates systemically and continues to replicate in the lymphoid tissues (Fulton, 2009b). This virus can cause a fetus of pregnant heifers/cows to become persistently infected (PI), which will allow this animal to shed the virus to pen mates through the remainder of its life (Fulton, 2000b; Fulton, 2009c).

Bovine Viral Diarrhea Virus may aid colonization of the lungs by other pathogens due to the mentioned adverse effects on the immune status of the animal, with marked decrease in leukocyte and alveolar macrophage function (Cusack, *et al*, 2003; Fulton, 2000a; Fulton, 2000b). Infection with BVDV is commonly seen in combination with other pathogens, and the combined disease syndrome is often considered more severe when BVDV is present alone (Fulton, 2000a; Fulton, 2000b; Yates, 1982). Bovine Viral Diarrhea Virus is broken down into cytopathic and noncytopathic biotypes, with the later being considered the most common, as well as in to type 1 and type 2 genotypes (Fulton, 2009c; Deregt and Loewen, 1995; Pellerin *et al*, 1994; Potgieter, 1997; Richey, 2004). The occurrence of type 2 strains was emphasized around 2000 when most BVDV vaccines contained only type 1 isolates; most vaccines now contain both strains (Fulton, 2009b, Fulton, 2000a; Richey, 2004). The immunosuppressive effect of a BVDV infection appears to be mediated by initial hyperplasia of all lymphoid organs, often within 10 days of systemic infection, followed by lymphoid reduction (Cusack, *et al*, 2003).

### **Bovine Respiratory Syncytial Virus: (BRSV)**

Bovine Respiratory Syncytial Virus, first described in the US in the 1970s, is considered to be a common respiratory pathogen of newly weaned cattle (Fulton, 2009b; Griffin, 1996; Stott, *et al*, 1980). It is an enveloped single-stranded RNA pneumovirus that is characterized as pleomorphic and filamentous (Gershwin, 2008; Ellis, 2009). The pulmonary destruction associated with infection with this virus, as with other major viral respiratory disease agents, BHV-1 and PI-3, is the direct result of the host's response to the virus which leads to the subjugation of the ciliated respiratory epithelium and infection of alveolar macrophages (Cusack, *et al*, 2003; Gershwin, 2008). However, unlike BHV-1, BRSV infection occurs in the epithelial cells of the terminal airways of the animal and conducts replication within the alveolar

macrophages forming nucleocapsids which allows viral particles to bud through the apical membrane (Ellis, 2009). The tissue damage associated with this virus results in interference with pulmonary clearance, thereby predisposing cattle to secondary bacterial pulmonary infection (Cusack, *et al*, 2003). Lesions in morbid cattle are presented as bronchiolitis and interstitial pneumonia, with emphysematous bulla in severe cases (Gershwin, 2008). The pathogenesis of this virus is still not fully understood (Gershwin, 2008). However, it is thought that BRSV may specifically enhance *Histophilus somni* infections, potentially due to immunomodulation. In a 2008 study conducted by Gershwin, calves with both pathogens exhibited a more severe infection than with either pathogen alone. This indicates that synergism of these pathogens may significantly increase the magnitude of the damage caused to the host compared to invasion of one offending agent.

### **Antimicrobials**

Antimicrobials are classified as having either bactericidal or bacteriostatic mechanisms and are an important tool used in the treatment of bacterial diseases in food animals (Gaurdabassi and Courvalin, 2006; Morley, *et al*, 2005). Antimicrobials are typically used as prophylactic and metaphylactic measures with some agents being used to allow full expression of growth potential in beef cattle (Schwarz and Chaslus-Dancla, 2001; Zecchinon, *et al*, 2005). A common difficulty facing food animal veterinarians is the selection of the proper antimicrobials for treating animals in a timely fashion. Due to the time lag for analyzing causative isolates from clinically ill animals in diagnostic labs, veterinarians are required to start therapy immediately to prevent continued loss or degradation of more animals (Woolums, *et al*, 2004; Zecchinon, *et al*, 2005). There have been reports of increasing antimicrobial resistance in BRD respiratory pathogens, which may have an effect on treatment outcome (Morley, *et al*, 2005; Watts, *et al*, 1994).



The incidence for resistance appears to be increasing with the *Pasteurella sp.* (Post, *et al*, 1991; Shoo, 1989). Chang in 1976 showed, using a Kerby-Bauer procedure, that 79.1% and 96.1% of *Pasteurella multocida* and *Mannheimia haemolytica* isolates, respectively, were resistant to at least one of the following, streptomycin, penicillin, tetracycline, and chloramphenicol. This is indicative of BRD pathogens showing resistant mechanisms to earlier antimicrobials. Watts, *et al*, 1994 showed in a four year study that *Mannheimia haemolytica* and *Pasteurella multocida* are showing an increased resistance to more recently developed antimicrobials as well. It is important to mention that most commensal pathogenic flora are exposed to varying amounts of antimicrobial drug at some point during treatment of disease, even if they are not considered the pathogenic agent (Morley, *et al*, 2005). Though resistance in these “bystander bacteria” is not considered to be important, these pathogens could serve as reservoirs of resistance genes and cause increased difficulty in treatment of disease in other hosts (Morley, *et al*, 2005).

### **Antimicrobial Resistance Mechanisms**

Studies have shown that diseases caused by antimicrobial resistant bacteria are more difficult to treat and often result in increased morbidity and mortality (Morley, *et al*, 2005). Antimicrobial resistance is considered a complex phenomenon that has been around as long as antimicrobials have been available (Morley, *et al*, 2005). This decrease in bacterial susceptibility is due to the pathogen’s ability to survive and propagate through development of mechanisms to aid in survival (Apley, *et al*, 1998; Morley, *et al*, 2005; Requera, 1988). Antimicrobial resistance has been defined in many ways, but is commonly evaluated related to *in vitro* and *in vivo* components (Guardabassi and Courvalin, 2006). *In vitro* resistance is defined based on laboratory concentrations which may or may not be well correlated to clinical outcome. *In vivo*

resistance is related to lack of clinical response, but may or may not be primarily due to the interaction or lack of interaction of the antimicrobial with the pathogen. This lack of clinical response may be due to many factors, including location of the infection, drug dosage, administration method, tissue distribution, and immune status of the treated animal. Resistance is quantifiable by determining the minimum inhibitory concentration (MIC) for a pathogen with a given drug and is considered to occur when higher concentrations of an antimicrobial than are clinically achievable are needed to inhibit the growth of a pathogen (Apley, 1997; Blondeau, 2009; Schwarz and Chaslus-Dancla, 2001).

Antibiotic resistance varies from pathogen to pathogen but is considered to occur in two ways, through acquisition of genetic elements, and intrinsic structural characteristics which preclude interaction with the antimicrobial (Guardabassi and Courvalin, 2006; Schwarz and Chaslus-Dancla, 2001). Acquisition of genetic elements is considered the most feared due to its potential world health impact. Genetic mutations may occur spontaneously; if the mutation does not decrease the environmental fitness of the organism, then this resistance may provide an advantage in the presence of antimicrobial pressure. Additionally, chromosomal recombination, transformation and transduction of foreign DNA, as well as horizontal obtainment of resistance pathogen genes can contribute to spread of these resistant organisms (Apley, *et al*, 1998; Martinez, 2000; Morley, *et al*, 2005; Prescott and Baggot, 1985). These acquired traits allow drug modification, drug inactivation, decreased cell wall permeability, active efflux of drugs, and failure to metabolize a drug to its active form (Morley, *et al*, 2005; Schwarz and Chaslus-Dancla, 2001). The production of enzymes is considered to be the most clinically relevant antibiotic resistance mechanism exhibited (Reguera, *et al*, 1988; Davies, 1997). Schwarz and Chaslus-Dancla, 2001, present an excellent review of the individual genes that are considered to be

involved in resistance as well as the veterinary antimicrobials and the pathogens in which resistance is occurring. Additionally, Schwarz and Chaslus-Dancla, 2001, illustrate the several forms of resistance mechanisms, including chemical modification, hydrolysis, active export via specific exporters, active export via specific transport proteins, active export via multiple drug transporters, protection of the target site, chemical modification of the target site, replacement of a sensitive target structure by a resistant target, and lastly the mutational modification of the target site.

Morley, *et al*, 2005, asserts that tetracycline and macrolides are connected to multiple mechanisms of resistance; this exhibition is thought to contribute to the lack of efficient methods to prevail over resistance to these antimicrobials. In addition, for a single mutation encoding for resistance to multiple antimicrobials, multiple resistance genes may be contained in one mobile genetic element; selection pressure from one antimicrobial may now select for resistance to multiple antimicrobials with unrelated mechanisms of action.

There is much debate amongst scientists and the public in regards to antimicrobial resistance. This controversy involves everything from how microbes are becoming resistant to antimicrobials; whether from spontaneous mutation or from our protocols of antimicrobial use, to the potential impact on public health. It is also thought that continued exposure of pathogen populations to antimicrobials or other factors such as disinfectants, after the bacteria obtain their antimicrobial resistance, can provide selection pressure that provides an advantage to resistant bacteria, thereby increasing the prevalence of resistant bacteria in the bacteria populations (Apley, *et al*, 1998; Morley, *et al*, 2005; Davies, 1997). Food animals are considered to contribute to MIC shifts of some antimicrobials for zoonotic organisms (Apley, *et al*, 1998). Exposure to these agents are considered to allow for selective pressure for naturally existing

resistant clones to contribute to the bacteria population, multiplying in the favorable environment of antimicrobial exposure, thereby decreasing effective use of our antibiotics (Apley, *et al*, 1998; Apley, 2009). It is important to note that contact with antimicrobial drugs is not believed to cause bacteria to actively mutate or develop new types of resistance; because, genetic mutation is a normal process that occurs during bacterial replication (Apley, *et al*, 1998; Martinez, 2000; Morley, *et al*, 2005; Prescott and Baggot, 1985). It is also thought that resistant subpopulations exist within in the drug susceptible populations (Jumbe, *et al*, 2002). The probability of this depends on the total pathogen load on the animal and the mutational frequency to resist the drug (Jumbe, *et al*, 2002).

### **Mutation Rate or Frequency**

Due to short generation intervals of pathogens and the large number of bacteria being produced during replication, mutation of bacterial organisms is seen as a common event (Morley, *et al*, 2005; Schwarz and Chaslus-Dancla, 2001). Mutation rate is defined by Martinez, 2000, as the frequency at which detectable mutants arise in a bacterial population; moreover, there appears to be multiple (different) mutation rates within a population making mutation rate a complex event. However, that publication further explains that the possibility of predicting the emergence of an effective mutation is confounded by many complex variables and makes it unlikely to predict by simple experimental procedure (Martinez and Baquero, 2000). The mutational frequency for resistance mechanisms attributable to mutations is considered to be in the range of  $10^{-6}$  to  $10^{-8}$  CFUs (Jumbe, *et al*, 2002). Lipsitch and Levin, 1997, have developed several models for the ascent of resistance, they assume that when the total bacterial population burden exceeds the inverse of mutational frequency to resistance by at least one order of magnitude; there is a high probability that a resistant subpopulation will be present in the total

population at base line, before any selective pressure is even applied. Published work appears to have established that mutation processes in bacterial populations are not static events (Jumbe, *et al*, 2002; Lipsitch and Levin, 1997; Martinez, 2000; Schwarz and Chaslus-Dancla, 2001).

### **Inoculum Effect of Bacterial Pathogens**

Inoculum effect is described as the increase in MIC and Minimum Bactericidal Concentration (MBC) of antimicrobial drugs with the increase in the size of the pathogen population (Brook, 1989; Davey and Barza, 1987). William Kirby first described the inoculum effect in 1945 with in vitro penicillin activity against *Staphylococci aureus*. Since this time many studies have been conducted to assess the effect of colony forming units (CFU) on the MIC in many different stains and species of pathogens (Bulirra, 2009; Martinez and Baquero, 2000). High CFUs are associated with increased mortality and decreased antibiotic effects in animal models (Bulirra, 2009). High bacterial load infections provide an increased probability for the emergence of resistance due to the higher likelihood of having at least one resistant bacterium within the total population (Bulirra, 2009; Requera, 1988). Knowing the total pathogen load or level of BRD pathogens, and their concentrations in localized areas of infection, are important when considering the potential effect of inoculum size on antibiotic activity. Pathogens associated with other clinical infections have shown a level at which they cause variation in antibiotic activity (Bulirra, 2009).

### **Testing**

The standard testing of veterinary antimicrobial susceptibility has been determined by the Clinical and Laboratory Standards Institute (CLSI). This institute determines the breakpoints of antimicrobials based on the Minimum Inhibitory Concentration (MIC) values for classification as susceptible, intermediate, and resistant. MIC is the lowest concentration of an antimicrobial

agent that prevents visible growth of a microorganism in an agar or broth solution susceptibility test (Blondeau, 2009). Both serial dilution interpretive breakpoints and disk diffusion interpretive zone diameters are approved for determining approved veterinary breakpoints (Apley, 2010). It should be noted that a susceptible testing result does not guarantee successful treatment outcome of the specific animal from which the pathogen isolate was collected. Instead the result indicates that the animal is in a population of the animal-drug regimen-pathogen relationship with a characteristic relationship between the probabilities of the different possible clinical outcomes (Apley, 2010). CLSI publication M37-A3 and publication M-31-A3 offer a complete and more precise explanation of the approved testing procedures as well as interpretive criteria, clear definitions of breakpoints, discussion of the relationship between serial dilution and disk diffusion breakpoints.

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# **CHAPTER 3 - Qualification and Quantification of Bacterial Pathogen Load in Acute Bovine Respiratory Disease Cases**

## **Abstract**

One hundred ninety four steers, bulls, and heifers weighing 182-318 kg were purchased at an Arkansas sale barn and shipped 12 hours to a northern Kansas feedlot the following day. There was no previous history of treatment and the cattle had been delivered to the sale barn within the 24 hour period prior to the sale. The objectives of the study were to evaluate (1) bacterial pathogen isolates in different locations in the respiratory tract, and (2) pathogen load in clinically ill and clinically normal calves. Fifteen calves were identified with signs of acute bovine respiratory disease (BRD) based on clinical score and a minimum rectal temperature of 40° C. An additional 5 calves with no clinical signs and rectal temperatures < 40° C were selected as controls. Cattle were humanely euthanized following recording of antemortem clinical observations.

At postmortem, samples for microbiologic analysis were collected from grossly normal and/or consolidated tissue in each lung lobe. Samples were also collected from the tonsils and trachea. Quantification of the BRD pathogens per gram were determined for each positive site and then converted to total counts for each animal. Total colony forming units (CFU) of pathogens in lungs of cattle with identified pathogens ranged from  $2 \times 10^7$  –  $2 \times 10^8$  CFU for *Pasturella multocida* and  $9 \times 10^6$  –  $9 \times 10^8$  CFU for *Mannheimia haemolytica*. Total visual estimated percent consolidation ranged from 0.0% to 45.0% of the lungs. Pathogens isolated from the upper and lower respiratory tract were compared and found to have varying degrees of agreement.

## Introduction

Bovine Respiratory Disease (BRD) is a critical factor in animal welfare and sustainability of cattle production (Babcock, *et al*, 2009; Ellis, 2001; Gardner, *et al*, 1999). Pathogens commonly associated with BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. (Confer, 2009; Fulton, *et al*, 2009; Eitam, *et al*, 2010). These pathogens are ubiquitous in cattle populations, commonly colonizing the nasopharynx and providing a source for lung inhalation and disease following stress, transportation, or viral infection. (Chen, *et al*, 2003; Confer, 2009; Cusack, *et al*, 2003). The virulence factors of these pathogens can generate severe inflammatory and immune response by the animal, leading to tissue damage and lesions of the lung lobes (Confer, 2009). It is common procedure in the cattle industry to treat BRD affected cattle with antimicrobials to reduce infection and prevent further damage by the pathogens. The timing of this therapeutic treatment in relation to onset of the infectious process is tied with the performance and health of these cattle (Babcock, *et al*, 2009).

One of the possible sequelae to antimicrobial therapy is the selection for, and the subsequent proliferation of resistant pathogens. A key consideration when a resistant pathogen is detected in the diagnostic laboratory is whether the pathogen is likely to have been a spontaneous mutation in the animal, which was selected for during therapy, or if it was most likely a component of the pathogen population at the start of therapy. This distinction has huge implications as whether diagnostic laboratory susceptibility data detects true trends in resistant clone dissemination or just the immediate effects of mutant selection during therapy in the individual animal. To provide data for consideration of this question, this study was designed to allow estimation of total colony forming units (CFU) of respiratory pathogens in acute bovine respiratory disease

(BRD) cases. These populations could then be considered, in light of anticipated mutation rates, to assess the probability of selection for spontaneous mutations during therapy.

Control calves were selected to determine base-line respiratory tract bacterial populations in apparently healthy calves in the same population as the calves clinically diagnosed as meeting BRD criteria. The primary objective of this study was to isolate and quantify bacterial pathogens in the lungs of cattle meeting clinical criteria for acute BRD and also in negative controls from the same population. Secondly, evaluation of the consistency of bacterial pathogen isolates from upper respiratory tract (tonsils and trachea) and lower respiratory tract (lung lobes) was also conducted.

## **Materials and Methods**

### ***Animals***

A total of one hundred ninety four steers, bulls, and heifers weighing 182-318 kg were purchased in December, 2009 at an Arkansas sale barn on a Friday and shipped 12 hours to a northern Kansas feedlot on Saturday. All animals were considered to be in good condition with no apparent injuries or disease at the time of arrival at the feed yard. The study population did not receive antimicrobials for control of BRD either at the sale barn or at the feedlot. Animals were placed in an outdoor, dirt-surfaced, dry-lot pen of approximately 44,850 square feet at a commercial feedlot located in Northern Kansas. The pen contained in-line concrete feed bunks across the entire width of the pen allowing approximately 12 inches of bunk space per calf. Pens contained automatic, electrically heated water tanks with water being provided ad libitum throughout the study. Animals received an arrival ration during the selection period for the study. All cattle were processed as a group within 48 hours of arrival, receiving a modified-live 5-way

viral vaccine<sup>1</sup> and a pour-on dewormer<sup>2</sup>. No experimental treatments were administered to the cattle. No specific randomization was indicated for this study. Calves were enrolled as they met the case definition for a BRD case with representative negative controls selected concurrently with the diseased animals. Each calf in the study pen was identified with a uniquely numbered ear tag. Throughout the live phase of this study ambient temperatures typically ranged from -13 to -11 °C.

### ***Antemortem Data Collection***

Clinically diseased study calves were identified as showing signs of naturally occurring BRD during routine pen observations. Calves with a clinical score of 1 to 3 (0-4 scale, Table 1.1), were examined for evidence of another disease and rectal temperature was determined. The thermometer in the study was calibrated using a factory-calibrated mercury thermometer and was found to be accurate at the lower and upper ends of the expected temperature range. Calves meeting clinical score criteria and with a minimum rectal temperature of 40° C (104° F) were admitted to the study as clinically diseased animals. An exclusion criterion was included in the study if insufficient calves (at least three per day) were not identified; in this case candidates were not enrolled but instead were treated as first time BRD cases and excluded from the study. Only animals with no previous history of treatment at the feeding facility were eligible for study admission. Enrollment continued until 15 calves diagnosed with BRD were assigned to the study. An additional 5 calves with clinical scores of 0 and rectal temperatures < 40° C were selected as controls and also enrolled in the study. These calves were used to determine base-line respiratory tract bacterial populations in apparently healthy calves penned with calves meeting the case definition for BRD.

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<sup>1</sup> Bovi-Shield Gold, Pfizer Animal Health

<sup>2</sup> Ivermectin pour-on for cattle, Aspen Veterinary Resources



### *Postmortem Data Collection*

Postmortem examination was conducted and a complete necropsy was performed. The entire respiratory tract was excised and transported to a laboratory facility for further examination, dissection, and sample collection. At the time of lung sample examination, the lungs were cleaned with tap water, blotted dry with paper towels, and photographed. A gross visual estimate was recorded for the percent diseased tissue in each lobe, which was then converted to an estimate of total lung diseased tissue by the following equation (Fajt, *et al*, 2003). Total percentage lung consolidation = (0.053 x cranial segment of left cranial lobe %) + (0.049 x caudal segment of left cranial lobe %) + (0.319 x left caudal lobe %) + (0.043 x accessory lobe %) + (0.352 x right caudal lobe %) + (0.061 x right middle lobe %) + (0.060 x caudal segment of right cranial lobe %) + (0.063 x cranial segment of right cranial lobe %). Note that this equation separates the right cranial lobe into cranial and caudal segments. In contrast, all other data collection and analysis treated the right cranial lobe as one lobe.

Samples for microbiological analysis were collected from visually determined grossly normal and/or consolidated tissue in each of the seven lung lobe areas for each animal. Weights were recorded for each lobe or portion of lobe when areas were designated as both grossly normal and grossly diseased. If all of a lobe was either grossly normal or grossly diseased, then only one sample was taken and bacteriological counts were attributed to the entire lobe. Additionally, histological samples were taken from loci adjacent to the microbial lung samples and submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL) for analysis, where histological scores were applied (Table 1.2). Samples for microbiological analysis were also collected from the palatine tonsils and trachea of each animal. Since no test article was administered to study calves, the carcasses were disposed via rendering.

### ***Sample Collection and Analysis***

Samples for microbiological analysis were stored in *Whirl-Pak*® Bags and placed in styrofoam coolers, covered with ice, and transported by vehicle to the University of Nebraska Veterinary Diagnostic Center where samples were stored at -80° C until analysis was conducted.

### ***Microbiological Analysis***

An estimate of colony forming units (CFUs) for each of the lung samples was determined for any pathogen that was cultured. The result was an estimate of CFUs for visually normal and visually abnormal areas within each lobe. An estimate for number of bacteria per lobe or lobe sub-portion was calculated by multiplying the CFU per gram of tissue by the weight of the lobe which that sample represented. These measures were used to determine the total estimated CFU for each pathogen per each lobe.

$$\text{Total CFU for entire lung} = (\sum (\text{CFU per gross normal lung lobe/segment}^*)) \\ + \sum (\text{CFU per consolidated lung lobe/segment}^*))$$

\*CFU per lung lobe/segment = WT x (CFUC x DF)); Where WT= weight of lung lobe segment (normal/consolidated), CFUC = colon forming unit count, and DF = dilution factor of 20,000.

All microbiological procedures were performed at the University of Nebraska Veterinary Diagnostic Center. Target pathogens for isolation and culture quantification were *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. In addition, polymerase chain reaction (PCR) was performed to identify Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD), or Bovine Respiratory Syncytial Virus (BRSV) in the samples. DNA Fingerprinting of *Mannheimia haemolytica* and *Pasteurella multocida* isolates

from tonsil, trachea, and lung from each quantitative culture locus was conducted. Bacteria were tested using the BioLog© GENII metabolic fingerprinting system<sup>3</sup>, TREK© Sensititre system with BoPo plates<sup>4</sup>, and by ERIC-PCR fingerprinting of genomic DNA<sup>5</sup>; adapted from Rafiee, *et al*, 2000. Antimicrobial susceptibility testing of pathogen isolates was conducted using extended range dilution methods as described by the Clinical and Laboratory Standards Institute (CLSI, 2008).

### ***Statistical Analysis***

Data for statistical analysis were entered in a software package (JMP 8.0.2, SAS Institute Inc copyright 2009; Cary, NC) for calculation and manipulation. One-way analysis of variance (ANOVA) was conducted on the results. Data were log transformed for this analysis. Pearson's chi-squared test was used to test for association between pathogen presence in the trachea or tonsil as compared to the lung. Sensitivity and specificity were calculated for the association and comparison of the upper and lower respiratory tract respectively. Tracheal and tonsil results were converted to binary format (1, positive; 0, negative) prior to analysis. An alpha level of 0.05 was observed throughout the study for evaluating statistical differences.

## **Results**

### ***Individual Animal Observations – Study Enrollment***

The day after arrival was designated study day 1. During study days 1 and 2 (December 13 and 14, 2009) no calves were enrolled. A total of one and two calves were examined and met the criteria for study inclusion on study days one and two respectively, but a minimum of three eligible calves were required to justify the inclusion of a negative control, so these calves were

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<sup>3</sup> Biolog, Inc., Hayward, CA

<sup>4</sup> TREK Diagnostic Systems, Cleveland, OH

<sup>5</sup> Bio-Rad Laboratories, Philadelphia, PA (Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction)

not enrolled and were treated according to feedlot protocol. An additional calf was identified by the feedlot crew during processing on study day 2 as being ill. It was treated according to feedlot protocol. A heifer calf was observed with a swollen vulva and was also treated with an antibiotic. Neither of these calves was eligible for the study.

On study day 3, nine calves were identified as having signs of clinical BRD. Four met study entry criteria by having a confirmatory rectal temperature of  $\geq 40.0^{\circ}\text{C}$  or better. A heifer calf with no observed signs of BRD and a clinical depression score of “0” was examined as a potential control. This calf’s rectal temperature was  $< 40.0^{\circ}\text{C}$  thus making it eligible as a control.

On study day 4 (December 16, 2009), 11 calves were identified with clinical depression scores between 1 and 3. Seven of these met study inclusion criteria with a rectal temperature  $\geq 40.0^{\circ}\text{C}$  ( $104.0^{\circ}\text{F}$ ). Two additional calves with no signs of BRD were examined as potential controls. They met control inclusion criteria with a rectal temperature  $< 40.0^{\circ}\text{C}$  and were enrolled as controls. In addition calf number 21 was found dead in the study pen following postmortem examination of enrolled calves. This calf was added to the study although no antemortem data could be collected. A postmortem examination was conducted and revealed this calf had an advanced BRD episode. Lung photographs were taken and tissue samples collected for histopathology and microbiologic culturing, but this animal was not included in analysis of the acute cases.

On study day 5, six calves were identified with clinical depression scores of 1 to 3. Four met rectal temperature requirements and were entered into the study. Several calves were examined as control candidates with two meeting control enrollment criteria thus concluding study

enrollment. All remaining calves were treated for control of bovine respiratory disease with the standard feedlot protocol.

### ***Individual Animal Culture***

*Mannheimia haemolytica* and *Pasteurella multocida* were the only BRD pathogens isolated from tissue samples collected from study calves. No *Histophilus somni* or *Mycoplasma bovis* were detected by culture in any animal. All PCR examination for common respiratory viruses including Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD), and Bovine Respiratory Syncytial Virus (BRSV) were negative. Bacteriological culture results are grouped by clinically diseased and clinically normal calves in Table 2.

Review of individual data for diseased calves (Table 2) demonstrates that on the first day of enrollment only one of four qualifying calves had a lung pathogen isolated. On the second day, six of seven qualifying calves had at least one bacterial pathogen isolated from their lungs. By the third day of enrollment, all four diseased calves were positive for lung pathogens. Values for the respiratory disease mortality calf (#21) are also included in Table 2, but are not included in the statistical analysis. This calf was the only animal with a total lung CFU count exceeding  $1 \times 10^9$ , which was exceeded for both isolated bacterial pathogens. Calves with a positive culture had organisms isolated from at least one segment (tonsil, trachea, or lung lobes) of the respiratory tract. *Mannheimia haemolytica* (n=5) or *Pasteurella multocida* (n=2) was isolated as a sole pathogen from a segment of the respiratory tract in seven diseased calves. Thirteen of the diseased calves had isolates from multiple sites within the respiratory tract and of those twelve calves had at least one pathogen isolated from lungs. Two diseased calves had no pathogen isolated from any segment of the respiratory tract. All of the *Mannheimia haemolytica* and

*Pasteurella multocida* isolates isolated from lungs were demonstrated to be clonal based on the similarity of biochemical fingerprints (Table 3), and ERIC-PCR (Figure 1).

Antimicrobial susceptibility results are reported in Table 4. Animals numbered 18 and 21 (the respiratory mortality) have susceptibility profiles that are noticeably different from the other isolates, with animal number 21 displaying the most extensive resistance profile. Antimicrobial susceptibility testing results showed that the *Mannheimia haemolytica* and *Pasteurella multocida* isolates from the lung lobes were almost completely pansusceptible.

### ***Total Culture***

*Mannheimia haemolytica* had the largest total CFU count/calf in this study with a range of  $9 \times 10^6$  through  $9 \times 10^8$  CFUs per affected calf. *Pasteurella multocida* had a CFU/calf count range of  $2 \times 10^7$  through  $2 \times 10^8$  CFUs per affected calf. Adding the total CFUs together in animals with both pathogens resulted in a total CFU range for all pathogens in a study animal from  $9 \times 10^4$  to  $9.2 \times 10^8$  CFU/calf (Table 5). It is interesting to note that mean CFU for the three non-diseased controls with positive lung cultures was  $5 \times 10^7$  compared to mean total CFU of  $2 \times 10^8$  for the 11 diseased calves with positive lung cultures.

Figure 2 illustrates the distribution of total CFU/lungs for *Mannheimia haemolytica* ( $9 \times 10^6$  -  $9 \times 10^8$  CFUs/lungs), *Pasteurella multocida* ( $2 \times 10^7$  -  $2 \times 10^8$  CFUs/lungs), and total CFU/lungs including all pathogens in each animal ( $9 \times 10^4$  -  $9.2 \times 10^8$  CFUs/lungs). The relationship between classifying a lung area as grossly diseased and the isolation of bacteria by lung lobe is displayed in Table 6. For each lung area, the percent reported as grossly diseased and the percent with bacteria isolated from that area are reported together. To be included in this table, an animal must have had at least one area of grossly diseased lung.

### ***Relationship Between Upper and Lower Respiratory Tract Pathogen Isolation***

*Mannheimia haemolytica* was isolated from at least one segment of the respiratory tract (lung, tonsil, or trachea) in 11 of 15 clinical BRD cases. The organism was isolated from the lungs in nine diseased calves. In those cases, *Mannheimia haemolytica* was isolated from the tonsils in seven cases and from the trachea in four. Results for sensitivity/specificity for ability of tonsillar culture results and tracheal results to reflect results of lung cultures are presented in Table 7, Table 8, and Table 9 respectively.

If the positive lung cultures are considered a definitive diagnosis for *Mannheimia haemolytica* pneumonia and therefore are considered the actual positives (gold standard), the sensitivity of a positive tonsil culture for *Mannheimia haemolytica* was 7 of 9 or 0.78 (lower 95% CI=0.40; upper 95% CI=0.97), while sensitivity for positive tracheal isolates to predict a positive lung isolate was 0.44 (lower 95% CI=0.14; upper 95% CI=0.79).

Lungs of six diseased calves were negative for *Mannheimia haemolytica*. In five of those cases the organism was also not isolated from the tonsils. Specificity for a negative tonsil sample to predict absence of the organism in the lung was 0.83 (lower 95% CI=0.36; upper 95% CI=1.00). Four of the six cases with negative *Mannheimia haemolytica* lung cultures were also negative on their tracheal samples for a specificity for negative tracheal isolate to predict a negative lung culture of 0.67 (lower 95% CI=0.22; upper 95% CI=0.96).

*Mannheimia haemolytica* was isolated from at least one segment of the respiratory tract in four of five control calves. *Mannheimia haemolytica* was isolated from the lungs in three of these calves. The organism was identified from the tonsil in two calves and from the trachea in one. One control calf was negative for *Mannheimia haemolytica* in the lungs but positive on the

tonsillar sample. The only control calf with *Mannheimia haemolytica* isolated from the trachea also had the organism isolated in the lung.

*Pasteurella multocida* was isolated from a segment of the respiratory tract in eight of the 15 diseased calves. As described for *Mannheimia haemolytica* cases, calves with a positive *Pasteurella multocida* lung isolate and signs of BRD including elevated body temperature (>40.0°C) were considered actual positives. The organism was isolated from the lung in seven calves; two of these calves had *Pasteurella multocida* isolated from tonsillar samples and three had positive tracheal samples, resulting in a sensitivity of 0.29 (lower 95% CI=0.04; upper 95% CI=0.71), and 0.43 (lower 95% CI=0.09; upper 95% CI=0.82) of tonsillar and tracheal samples, respectively, to identify the presence of *Pasteurella multocida* in the lungs.

Lung samples from eight of the 15 clinically diseased calves were negative for *Pasteurella multocida*. Specificity for a negative tonsillar sample to predict the absence of *Pasteurella multocida* from the lung was 0.88 (lower 95% CI=0.47; upper 95% CI=1.00), while specificity for a negative tracheal sample was 1.0 (lower 95% CI=0.63; upper 95% CI=1.00).

In control calves, *Pasteurella multocida* was isolated from at least one segment of the respiratory tract in four of five cases. *Pasteurella multocida* was isolated from the lungs in two calves but neither had a positive tonsil sample. Two calves with negative lung samples were positive for *Pasteurella multocida* in the tonsil samples. Only one calf, who was negative for both lung and tonsil samples had agreement between the two sites for *Pasteurella multocida*. Of two calves positive for *Pasteurella multocida* on lung samples, one was also positive on the tracheal sample. All three calves negative for *Pasteurella multocida* on their lung samples were also negative on their tracheal samples.



## *Discussion*

Bacterial BRD is considered a secondary infection that follows viral infection or stress factors that impede the animal's immune system. Initiating viruses include Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD), or Bovine Respiratory Syncytial Virus (BRSV) (Storz, *et al*, 2000). In this study, no viruses were detected in the study animals.

To the authors knowledge, this study is the first standardized study to qualify, quantify, and evaluate the consistency of bacterial pathogen isolates from various locations throughout the upper and lower respiratory tract in calves experiencing acute BRD.

Comparing microbiological results from upper respiratory tract (tonsil or trachea) cultures to lower respiratory tract (lung) cultures, it appeared the tonsillar samples (sensitivity 0.78, specificity 0.83) were a better predictor of the presence or absence of *Mannheimia haemolytica* in the lungs compared to results from tracheal samples (sensitivity 0.44, specificity 0.67). Contrarily, neither the tonsil nor the trachea appeared to be a good predictor of a positive isolation of *Pasteurella multocida* from the lung (sensitivity 0.29, and 0.43 for tonsillar and lung samples respectively), however the number of cases for making the comparisons are quite small and the results perhaps misleading due to the small number of observations.

A key question related to interpretation of shifts toward higher MICs observed in diagnostic laboratory antimicrobial susceptibility testing trends is whether changes in minimal inhibitory concentration (MIC) distributions are due to spontaneous mutations within the treated animals during therapy with subsequent selection by antimicrobial pressure, or whether increasing MICs are due to an increased prevalence of resistant clones in the population. One consideration in this debate is whether the bacterial population in the diseased animals is sufficient for spontaneous mutations to antimicrobial resistance to occur.

In 2003, Chen *et al*, showed that the gene DNA adenine methylase (Dam) regulates the expression of virulence activity in *Pasteurella multocida*, and when cloned and put under the control of a promoter caused a significant increase in spontaneous mutation rate.. This indicates that under favorable conditions mutation rates can occur due to already present genes; however these conditions are influenced by the strength of a mutator gene, the number of mutations required for adaption, competition with other present strains, environmental consistency, and bacterial population size (Denamur and Matic, 2006; Boe, *et al*, 2000). Any bacterial population is considered to have a subpopulation of mismatched repair mutants due to spontaneous mutations, as demonstrated in a mutation frequency of less than  $3 \times 10^{-5}$  in *Esherichia coli* strains not subjected to selective pressures (Mao, *et al*, 1997). It should be noted that spontaneous mutations include both deleterious and beneficial effects on the fitness of the bacterium, though they are considered to occur at different rates (Denamur and Matic, 2006). Within *Esherichia coli* K-12, deleterious mutations occur at a rate of  $2-8 \times 10^{-4}$  while beneficial mutations are estimated to occur at a rate of  $2 \times 10^{-9}$  per cell generation (Imhof and Schlotterer, 2001; Boe, *et al*, 2000)

Hypermutable bacteria have been recorded to develop spontaneous resistance, such as with *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, *Esherichia coli*, *Haemophilus influenza*, and *Neisseria meningitides* (Henderson-Begg, *et al*, 2010) indicating a possibility that mutation could occur in other pathogens types, such as *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*. Since the 1980's, *Escherichia coli* strains have been shown to be resistant against extended spectrum antimicrobials; a mutation frequency of  $\geq 4 \times 10^{-8}$  was found in a study conducted in a hospital in Madrid, Spain; this is thought to be a result of a combination of already present hypermutable colonies and intensity of antimicrobial selection (Baquero, *et al*,

2005). Lipsitch and Levin (1997), have developed several models for the ascent of resistance. Their hypothesis is that when the total bacterial population burden exceeds the inverse of mutational frequency to resistance by at least one order of magnitude there is a high probability that a resistant subpopulation will be present in the total population prior to any selection pressure.

A spontaneous mutation rate of 1 in  $1 \times 10^9$  has been stated by other researchers for various bacteria/antimicrobial combinations. Taking this population threshold into consideration, along with the total bacterial populations estimated for acute BRD cases in this study, it is reasonable to hypothesize that spontaneous mutations occurring during therapy of individual acute cases of BRD are not the primary driver of changes in MIC distributions. The more likely explanation is the dissemination of resistant clones or mobile resistance elements in the population. This hypothesis is also supported by the observed multi-antimicrobial resistant (MAR) isolates in a diagnostic laboratory survey (Zhao, *et al*, 2007). In their survey of *Salmonella* isolates, obtained from state veterinary laboratories, 82% of isolates were resistant to at least one antimicrobial, and 70% were resistant to three or more antimicrobials. The overall rate of resistance was 77% for isolates of bovine origin. For these resistant patterns to be due to mutations, concurrent spontaneous mutations would have had to occur in individual animals.

This study does not address the potential for spontaneous mutations because study animals were not allowed to incubate pathogens beyond the first day of detection, nor was an antimicrobial selection pressure applied to potentially increase the CFU of a resistant mutant in relation to susceptible isolates. The only animal exceeding  $1 \times 10^9$  CFU for pathogens was the BRD mortality which occurred in the pen without treatment (#21), which had a lung pathogen load that exceeded  $1 \times 10^9$  for both *Mannheimia haemolytica* and *Pasteurella multocida*.

In a BRD quantification study conducted at the University of Nebraska utilizing chronic and fatal BRD cases, *Mannheimia haemolytica* isolate counts ranged from  $1 \times 10^3$  to  $>8 \times 10^8$  CFU/g (McVey, *et al*, 2007). *Pasteurella multocida* isolate counts ranged from  $1 \times 10^3$  to  $3 \times 10^5$  CFU/g, and *Histophilus somni* isolate counts ranged from  $1.4 \times 10^2$  to  $3.7 \times 10^5$  CFU/g. These data show counts observed in the current study are high but are constant with other BRD affected animals. In addition, McVey showed with antimicrobial sensitivity testing that  $>95\%$  of the BRD isolates were sensitive to most commonly used antimicrobials; but were consistently resistant against ampicillin, tetracyclines, and erythromycin. These data, in conjunction with the study reported here, suggest that the pathogen loads are at a threshold that could allow for spontaneous mutation against antimicrobials. Moreover, results from this study emphasize the need for early detection and treatment of infections when they are below the pathogen population level that supports spontaneous mutations which could encode for antimicrobial resistance.

Although numbers were insufficient for statistical evaluation, these data suggest that a clinical score  $\leq 2$  fails to provide discernment for the presence or absence of a respiratory pathogen in the conditions of this study. However all four animals with a clinical score of 3 had pathogens isolated from their lungs. Furthermore it appeared that during the very early phase of a clinical disease outbreak, the ability to isolate pulmonary pathogens may be limited as only one of four calves meeting the case definition for a clinical BRD case had pathogens isolated from the lungs on day 3, the first day cattle were entered on to the study. Perhaps during the early stages of the outbreak, CFU numbers were too low to consistently get a positive culture.

The study was also not adequately powered to allow statistical evaluation between rectal temperature and presence or absence of lung pathogens. When evaluating the data for trends for consideration in the design of future studies, and in consideration for the bitterly cold weather

conditions of this study, it could be noted that 2 of the 3 animals with elevated clinical scores but with rectal temperatures below 40.6°C (approximately 105° F) had no pathogens isolated from their lungs. For the 12 animals with elevated clinical scores and rectal temperatures  $\geq 40.6^\circ\text{C}$ , 2 of the 12 animals had no pathogens isolated from their lungs.

Other authors have stated that the patent appearance of clinical signs does not necessarily represent the underlying pathological progress of disease that may, or may not, be occurring in the animal (Reeve-Johnson, 2001; Eitam, *et al*, 2010). This concept is further illustrated in a pending publication (refer to Roof, *et al*, 2011). BRD clinical signs are subjective and some animals, as many as 29% to 68%, may never express the traditional signs, thereby going undetected (Eitam, *et al*, 2010; Gardner, *et al*, 1999; Reeve-Johnson, 2001; Thompson, *et al*, 2006; Wittum, *et al*, 1996). These studies, combined with the data reported here, suggest that lower clinical scores (1-2 on a 0-4 scale) and traditional rectal temperature criteria may not be reliable in predicting presence or absence of bacterial pathogens in acute BRD.

## Figures and Tables

Table 1.1: Depression Score Definitions

Depression Score	Clinical Signs
0	Normal, no signs of depression
1	Slower than pen mates but still perks up when approached and does not appear weak, actively follows your movements with a raised head.
2	Stands with head lowered, will perk up when approached but will return to depressed stance, moves slowly and falls towards back of group, may display signs of weakness such as incoordination
3	Obviously very weak, difficulty in moving with group, raises head only when approached closely
4	Moribund, unable to rise

Table 1.2: Histology Score Definitions

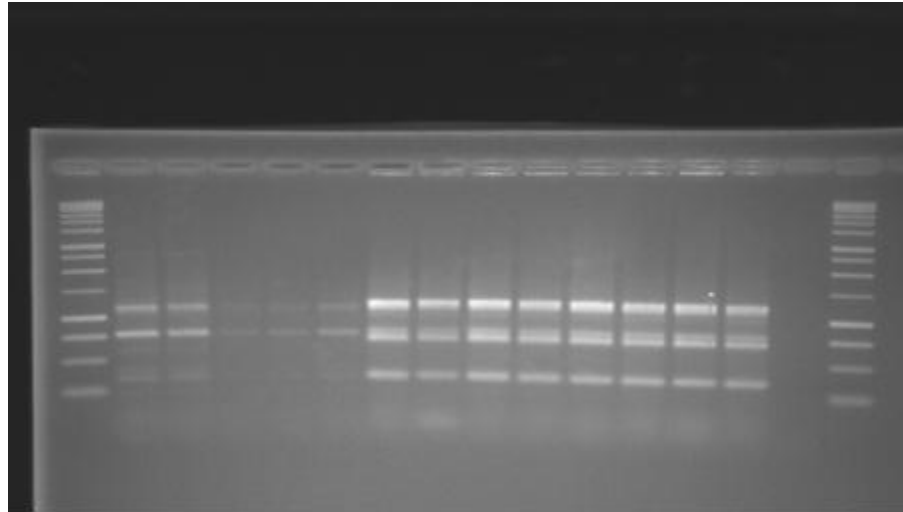
Score	Definition
0	Normal
1	Focal or multifocal pneumonia
2	Single focally extensive pneumonia
3	Multifocally extensive pneumonia
4	Diffuse pneumonia in one or both the sections

Table 2: Individual Animal Summary Data for Lung, Trachea, and Tonsil Culture Results

Clinical Description							Lung					Trachea		Tonsil		
Clinical Status	Animal #	Study Day	Clinical Score	Rectal Temperature (°C)	# Grossly diseased lobes	Total % Grossly Disease	M. haem	P. mult	Total CFU M. haem	Total CFU P. mult	Total CFU/ Lung	M. haem	P. mult	M. haem	P. mult	
Acutely Diseased	1	3	2	40.4	0	0.0			0	0	0	X				
	2	3	2	42.1	7	9.1		X	0	1.3 x10 <sup>8</sup>	1.3 x10 <sup>8</sup>		X		X	
	3	3	2	40.7	1	0.2			0	0	0					
	5	3	2	40.0	0	0.0			0	0	0					
	6	4	1	40.8	3	17.6	X		7.2x10 <sup>8</sup>	0	7.2 x10 <sup>8</sup>	X			X	
	7	4	2	40.1	2	1.7	X		2.0 x10 <sup>8</sup>	0	2.0 x10 <sup>8</sup>				X	
	8	4	1	41.3	1	1.3			0	0	0	X			X	
	9	4	1	41.3	4	5.8	X	X	1.7 x10 <sup>7</sup>	1.0 x10 <sup>8</sup>	1.2 x10 <sup>8</sup>				X	
	10	4	1	40.6	3	15.5	X	X	1.4 x10 <sup>7</sup>	1.7 x10 <sup>8</sup>	1.8 x10 <sup>8</sup>	X			X	
	11	4	2	40.8	1	4.9	X		9.4 x10 <sup>6</sup>	0	9.4 x10 <sup>6</sup>				X	
	12	4	3	41.6	7	34.8	X	X	9.3 x10 <sup>8</sup>	2.0 x10 <sup>7</sup>	9.5 x10 <sup>8</sup>	X	X			
	15	5	3	40.6	3	6.5	X	X	1.2 x10 <sup>7</sup>	7.6 x10 <sup>7</sup>	8.8 x10 <sup>7</sup>	X			X	
	16	5	3	41.4	3	5.2	X	X	3.8 x10 <sup>8</sup>	8.4 x10 <sup>7</sup>	4.6 x10 <sup>8</sup>				X	
	17	5	3	41.5	6	29.6	X		7.6 x10 <sup>7</sup>	0	7.6 x10 <sup>7</sup>				X	
	18	5	2	41.3	1	0.3		X	0	5.1 x10 <sup>7</sup>	5.1 x10 <sup>7</sup>		X		X	
	Acutely Diseased Means									1.6 x10 <sup>8</sup>	4.2 x10 <sup>7</sup>	2.0 x10 <sup>8</sup>				
	Control	4	3	0	39.4	0	0.0			0	0	0				X
		13	4	0	39.2	0	0.0	X	X	5.7 x10 <sup>7</sup>	3.6 x10 <sup>7</sup>	9.3 x10 <sup>7</sup>		X		
14		4	0	39.1	0	0.0	X	X	8.9 x10 <sup>7</sup>	3.9 x10 <sup>7</sup>	1.3 x10 <sup>8</sup>				X	
19		5	0	39.1	0	0.0	X	X	2.0 x10 <sup>7</sup>	0	2.0 x10 <sup>7</sup>	X			X	
20		5	0	39	0	0.0			0	0	0				X	
Control Means									3.3 x10 <sup>7</sup>	1.5 x10 <sup>7</sup>	5.0 x10 <sup>7</sup>					
Untreated Mortality	21	4	N/A	N/A	6	45.0	X	X	2.6 x10 <sup>9</sup>	2.8 x10 <sup>9</sup>	5.0 x10 <sup>9</sup>				X	

(The accessory lung lobe weight was not recorded for animal #21; an estimated weight of 100 g was used for calculations)

Figure 1: ERIC-PCR Fingerprinting of Isolates Tested for Antimicrobial Sensitivity



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16  
**1, 16** – DNA Ladder **2 – 6** – *Pasteurella multocida*  
**7 – 14** *Mannheimia haemolytica* **15** – Extraction Control, No DNA

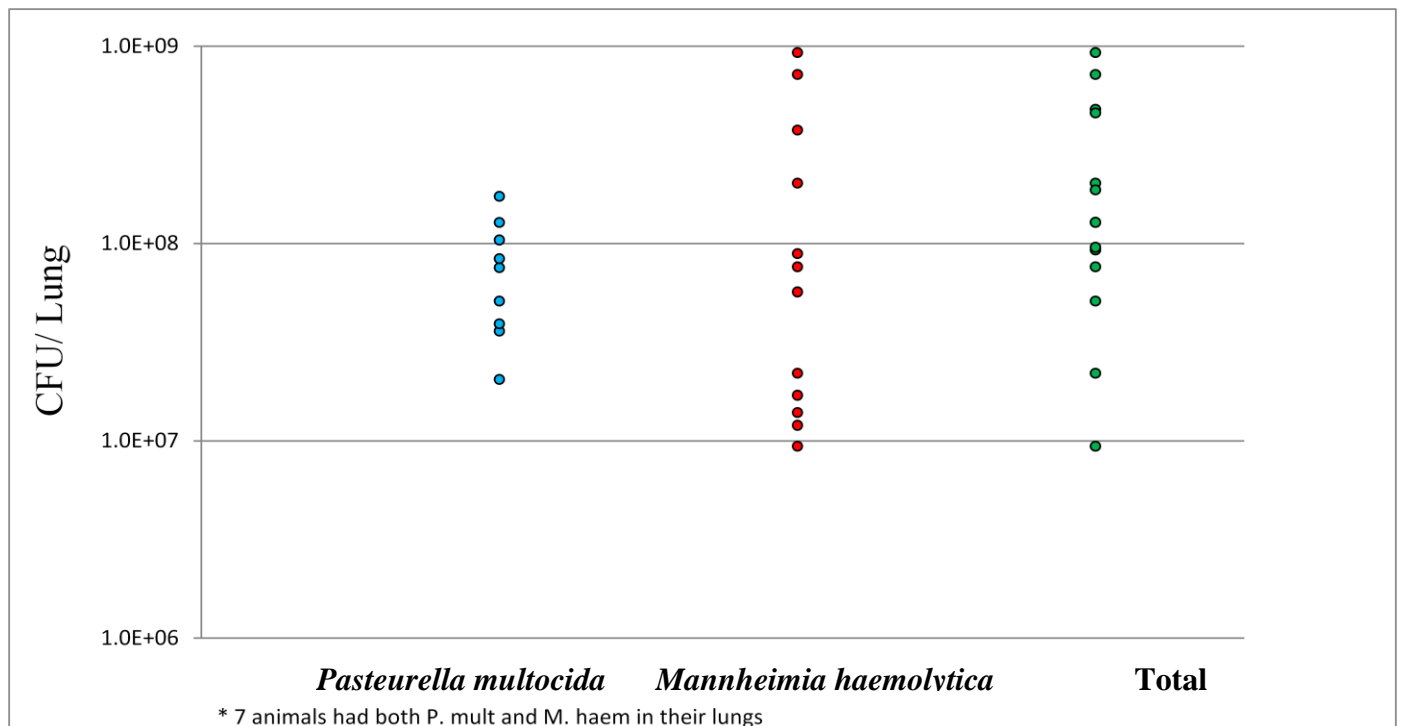


Figure 2: Illustrates the distribution of total CFU/lung for *Mannheimia haemolytica*, *Pasteurella multocida*, and total combined CFU/lung.



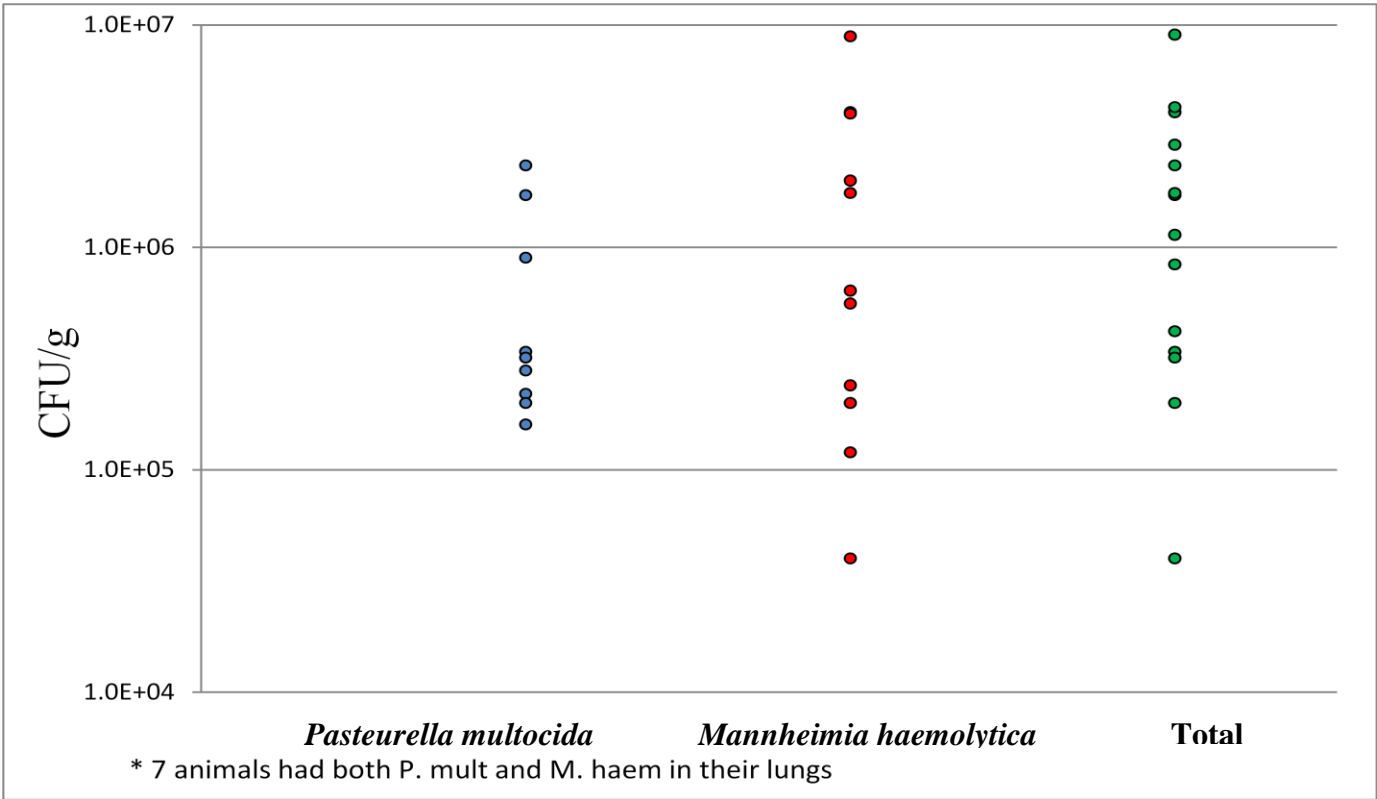


Figure 3: Illustrates the distribution of total CFU/g for *Mannheimia haemolytica*, *Pasteurella multocida*, and total combined CFU/g.

Table 3: Clonality of *Mannheimia haemolytica* Within Individual Animal Isolates

Animal ID Number	Location Specific Lung Lobes*/Tonsil/Trachea	Clone Types
6	ACC, RCR, RM, RCA, LC, CDLC	1
7	ACC, LC, CDLC	1
10	Tonsil	1/2
	Trachea, ACC	1
11	Tonsil, ACC	1
12	RCR, RM, RCA, CRLC	1
	Trachea	1/2
19	Tonsil	1/3
	RM	1
21	Tonsil	1/2
	CRLC, LC, CDLC	1

Clone type 1 – Serotype 1, hemolytic, mucoid, identical AST phenotypes  
Clone type 2 – Serotype 2, hemolytic / Clone type 3 – untypable, non-hemolytic

\* RCR, Cranial segment of the right cranial lobe; RM, Right middle lobe; RCA, Right Caudal lobe; ACC, Accessory lobe; LC, Left caudal lobe; CRLC, Cranial segment of the left cranial lobe; CDLC, Caudal segment of the left cranial lobe.

Table 4: Count of Susceptible, Intermediate, Resistant Results

ID	Organism	Susceptible	Intermediate	Resistant	Intermediate Resistance	
					to Antimicrobial (MIC)	Resistance to Antimicrobial* (MIC)
2	<i>Pasteurella multocida</i>	25	0	2	None	Clind (>16) & Tyl (16)
6	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.5)	Clind (>16) & Tyl (>32)
7	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.25)	Clind (16) & Tyl (>32)
9	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.5)	Clind (16) & Tyl (>32)
9	<i>Pasteurella multocida</i>	23	1	3	Amik (32)	Clind (16), Neo (16), & Tyl (32)
12	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.25)	Clind (16) & Tyl (>32)
15	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.25)	Clind (16) & Tyl (>32)
15	<i>Pasteurella multocida</i>	19	1	7	Tetra (8)	Clind (>16), Kana (>64), Neo (>32), Oxy (8), Spect (>64), Strept (>64), Sulpha (>256), & Tyl (16)
16	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.5)	Clind (8) & Tyl (>32)
17	<i>Mannheimia haemolytica</i>	24	1	2	Pen (0.25)	Clind (8) & Tyl (>32)
18	<i>Pasteurella multocida</i>	18	2	8	Tetra (8) & Trim (1)	Clind (>16), Kana (>64), Neo (>32), Oxy (>8), Spect (>64), Strept (>64), Sulpha (>256), & Tyl (16)
21	<i>Mannheimia haemolytica</i>	14	4	10	Chlor (8), Enro (0.5), Pen (0.25), & Tilm (16),	Clind (8), Dano (0.5), Kana (>64), Nali (>32), Oxy (>8), Spect (>64), Strept (>64), Tetra (32), Tula (>64), & Tyl (>32)
21	<i>Pasteurella multocida</i>	23	1	3	Tetra (8)	Clind (16), Oxy (>8), & Tyl (32)

\*Antimicrobial Tested - Amikacin (Amik), Amoxicillin/Clavulanic Acid (Amox), Ampicillin (Amp), Cefoxitin (Cefo), Ceftiofur (Ceftio), Ceftriaxone (Ceftt), Chloramphenicol (Chlora), Chlortetracycline (Chlortet), Ciprofloxacin (Cipro), Clindamycin (Clind), Danofloxacin (Dano), Enrofloxacin (Enro), Florfenicol (Florf), Kanamycin (Kana), Nalidixic Acid (Nali), Neomycin (Neo), Oxytetracycline (Oxy), Penicillin (Peni), Spectinomycin (Spect), Streptomycin (Strept), Sulfisoxazole (Sulfi), Sulphadimethoxime (Sulph), Tetracycline (Tetra), Tiamulin (Tia), Tilmicosin (Tilm), Trimethoprim/Sulfamethoxazole (Tri), Tulathromycin (Tula), Tylosin (Tyl)

Table 5: Range of Pathogen Total CFU in Lung

Pathogen N=20	Low	High	Mean
M. haem (n=12)	9.0x10 <sup>6</sup>	9.0x10 <sup>8</sup>	1.0x10 <sup>8</sup>
P. mult (n=9)	2.0x10 <sup>7</sup>	2.0x10 <sup>8</sup>	4.0x10 <sup>7</sup>
Total (n=14)	9.0x10 <sup>6</sup>	9.2x10 <sup>8</sup>	2.0x10 <sup>8</sup>

\*7 animals had both pathogens in their lungs

Table 6: The Relationship between Classifying a Lung Area as Grossly Diseased and Isolation of Bacteria by Lung Lobe.

Lung Area	(%)	Bacterial Isolation (N=13 Animals)*
Cranial Segment of the Right Cranial Lobe (RCR)	10 (76.9)	7
Right Middle Lobe (RM)	5 (38.5)	4
Right Caudal Lobe (RCA)	4 (30.8)	2
Accessory Lobe (ACC)	6 (46.2)	6
Cranial Segment of Left Cranial Lobe (CRLC)	5 (38.5)	4
Caudal Segment of the Left Cranial Lobe (CDLC)	8 (61.5)	7
Left Caudal Lobe (LC)	5 (38.5)	5

\* There were 13 animals with grossly diseased tissue in at least one lobe. Bacterial isolation numbers are a subset of the number with grossly diseased tissue in that lobe.

Table 7: Sensitivity and Specificity of Tonsillar and Tracheal Samples to Detect the Presence or Absence of BRD Pathogen in the Lung.

<b>Enrolled Study Animals (n=20)</b>				
	<i>Mannheimia haemolytica</i>		<i>Pasteurella multocida</i>	
	Sensitivity	Specificity	Sensitivity	Specificity
Tonsil	0.78	0.83	0.29	0.88
Trachea	0.44	0.67	0.43	1.0

Table 8: Comparison Pathogen Isolates and Tonsil.

<i>Mannheimia haemolytica</i>				<i>Pasteurella multocida</i>			
		Lower CI	Upper CI			Lower CI	Upper CI
Sens.	0.78	0.399906	0.971855	Sens.	0.29	0.036693	0.70958
Spec.	0.83	0.358765	0.995789	Spec.	0.88	0.47349	0.99684
A. Prev	53.30%			A. Prev	20.00%		
True Prev	60.00%			True Prev	46.70%		
Likelihood Ratio +	4.666667			Likelihood Ratio +	2.285714		
Likelihood Ratio -	0.266667			Likelihood Ratio -	0.816327		

Table 9: Comparison Pathogen Isolates and Trachea.

<i>Mannheimia haemolytica</i>				<i>Pasteurella multocida</i>			
		Lower CI	Upper CI			Lower CI	Upper CI
Sens.	0.44	0.137	0.788	Sens.	0.43	0.099	0.8159
Spec.	0.67	0.2228	0.9567	Spec.	1	0.6306	1
A. Prev	40.00%			A. Prev	20.00%		
True Prev	60.00%			True Prev	46.70%		
Likelihood Ratio +	1.333333			Likelihood Ratio +	0		
Likelihood Ratio -	0.833333			Likelihood Ratio -	0.571429		

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# **CHAPTER 4 - Correlation of Histopathological Scores to Bacterial Presence and Status in Acute Bovine Respiratory Disease Cases**

## **Abstract**

One hundred ninety four steers, bulls, and heifers weighing 182-318 kg were purchased at an Arkansas sale barn and shipped 12 hours to a northern Kansas feedlot within 24 hours of purchase. There was no previous history of treatment and the cattle had been delivered to the sale barn within a 24 hour period prior to the sale. Objectives of the study were to evaluate pathogen load in clinically ill and clinically normal calves and compare histological scoring of the corresponding lesions. Fifteen calves were identified with signs of acute bovine respiratory disease (BRD) based on clinical score and minimum rectal temperature of 40° C. An additional 5 calves with no clinical signs and rectal temperatures < 40° C were selected as controls. Cattle were humanely euthanized following recording of antemortem clinical observations.

At postmortem examination, samples for hematoxylin and eosin stain analysis were collected from grossly normal and/or consolidated tissue in each lung lobe. Quantification of the BRD pathogens per gram of lung tissue were performed for each positive site and then converted to total counts for each animal. Total colony forming units (CFU) of pathogens in the entire lung for cattle with identified pathogens ranged from  $2 \times 10^7$  –  $9 \times 10^8$  CFU for *Pasturella multocida* and *Mannheimia haemolytica*. Histology scores of 0-4 were assigned to the tissue samples and compared to the quantified BRD pathogens to evaluate association between the pathologic process and the total agents in that tissue sample. A significant difference in bacterial counts between histology scores of zero or 1 and a histology score of 4 was observed.

## Introduction

Acute Bovine Respiratory Disease (BRD) is a prevailing disease in the cattle industry affecting both the welfare of cattle and cost of production. The cost of BRD is estimated to exceed \$3 billion annually in the beef production system (Babcock, *et al*, 2009; Ellis, 2001; Griffin, 1997; Loneragan, *et al*, 2001; Snowden, *et al*, 2007; NAHMS, 2001). Most management strategies involve control of the etiological agents responsible for the pathogenesis of BRD (Booker, *et al*, 2008). The commonly associated BRD agents include, but may not be limited to, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* (Confer, 2009; Fulton, *et al*, 2009; Eitam, *et al*, 2010). Many reported studies have examined the presence of lesions and infectious agents in BRD feedlot cases (Booker, *et al*, 2008; Fulton, *et al*, 2009; Gagea, *et al*, 2006).

Over the years, many diagnostic tools, including PCR, ELISA, serology, and pathogen culture have been used to aid in the identification and understanding of the associated etiological agents (Step, *et al*, 2008; Fulton, *et al*, 2000). Better comprehension of the microbiological agents associated with the pathogenesis of acute BRD is important in the development of treatment protocols and diagnosis of the disease (Apley, 2006; Caswell and Archambault, 2008; Hanzlicek, *et al*, 2010). To better understand the pathological process of acute BRD, this study selected animals that had natural occurring disease cases based on depression score and febrile evaluation. The pulmonary tissues of these selected animals were then systemically examined by a combination of bacterial culture and hematoxylin and eosin (H&E) staining. The aim of the study was to determine the relationship between the pathogen population and the pathologic process in cattle experiencing acute bovine respiratory disease.

## Materials and Methods

### *Animals*

One hundred calves weighing 182-318 kg were purchased in December of 2009 from an Arkansas sale barn and transported to a Kansas feedlot, where ambient temperatures ranged from -13 to -11° C during the study. The study animals did not receive any antimicrobial treatments at arrival and were considered to be in good health. They were allowed *ad libitum* access to water and were fed an arrival ration during the enrollment of study animals. Only animals with no previous history of treatment at the feeding facility were included in the study.

### *Data Collection*

During daily observation, calves with a visual clinical score of 1 to 3 on a 0-4 scale, with 0 being normal and 4 being unable to rise, were further examined for evidence of disease (Table 1). Calves meeting clinical score criteria and with a minimum rectal temperature of 40° C (104° F) were enrolled in the study as clinically diseased animals. Enrollment continued until 15 calves diagnosed with BRD were assigned to the study. Five additional calves with clinical scores of 0 and rectal temperatures < 40° C were selected as controls and also admitted to the study. No treatment was administered to the animals. Details on study conduct and the relationship between clinical scores and bacterial culture results are published elsewhere (Chapter 3)<sup>1</sup>.

Within four hours of enrollment animals were euthanized by captive bolt, followed by a complete necropsy where the lungs, trachea, and tonsils were excised. Lungs were examined and samples for microbiological and histological analysis were retrieved from tissue identified as grossly normal/diseased, dependant on the presence of each category in each lung lobe.

Microbiological samples were submitted to the University of Nebraska Veterinary Diagnostic

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<sup>1</sup> Roof, *et al*, 2011 Qualification and quantification of bacterial Pathogen load in acute bovine respiratory disease cases.

Center for quantification and analysis of BRD pathogen isolates. Histology samples were placed in 10% buffered formalin and submitted to the Kansas State Veterinary Diagnostic Laboratory.

### ***Histological Analysis***

Respiratory tract histopathology samples were collected immediately adjacent to the area from which the microbiological samples were collected. All tissues were processed, paraffin embedded, and stained with hematoxylin and eosin. Samples were identified by animal number as well as location. Histology scores (Table 2) were assigned to the samples using previously reported criteria (Booker, *et al.* 2008).

### ***Statistical Analysis***

Data for statistical analysis were entered in a software package (JMP 8.0.2, SAS Institute Inc copyright 2009; Cary, NC) for calculation and manipulation. One-way analysis of variance (ANOVA) was conducted for histology and bacteriology results. Data were log transformed for this analysis. The Tukey-Kramer HSD comparison was used to test the association between histological scores and bacterial CFU's. An alpha level of 0.05 was specified throughout the study for evaluating statistical differences.

## **Results**

The relationship between histology score and pathogen CFU/g of tissue is presented in Table 3. Lung tissue with a histology score of 0 or 1 was demonstrated to be statistically different in bacterial count from pulmonary tissue with a histology score of 4. Bacterial counts in tissues with histology scores of 2 or 3 were not significantly different from the other scores. Figure 1 provides a visual distribution of the histology scores as they correlate with the quantified CFU/g of cultured isolated pathogen. There is a semi-multifocal distribution of data points, with some points having an elevated CFU/g regardless of histology score. These few data points still

allowed for a statistical difference to be observed on opposite ends of the histology scoring scale. Table 3 shows the CFU/g of lung tissue by histology score. Values in a column with different superscripts are significantly different ( $P \leq 0.05$ ). A histology score of 0 demonstrated a range of  $2 \times 10^4$  through  $2 \times 10^6$  CFU/g in this study, while a histological score of 4 demonstrated a range of  $6 \times 10^4$  through  $2 \times 10^6$  CFU/g. The statistical results for the combined CFU/g in lung tissue by histology score are illustrated in Figure 1. The “combined” results entail pooling results from both *Mannheimia haemolytica* and *Pasteurella multocida* isolates for a total CFU/g count when both were present.

The relationship between classification of a lung area as grossly diseased, the assigned histological score, and isolation of bacteria by lung lobe is displayed in Table 4. To be included in this table, an animal must have had at least one area of grossly diseased lung tissue. Of the 15 clinically diseased calves identified in the study, 13 had at least one lobe with an area classified as grossly diseased. Of these thirteen animals, ten had a right cranial lobe lesion with a histology score of 1-4 (with zero being normal); seventy percent of these 10 animals had bacteria isolated from the diseased tissue. Eight animals had a left cranial lobe lesion which was assigned a 1-4 histological score; eighty-eight percent of these lesions were positive for bacterial isolation. Six animals had an accessory lobe lesion, with 100% of these confirmed by histological and bacteriological confirmation. The confirmation of disease via histology and bacterial isolation of the remaining caudally placed lung lobes are reported in Table 4.

## **Discussion**

This observational study allowed for the development of naturally occurring, acute bovine respiratory disease in recently received feedlot cattle. In this field outbreak, there is an apparent trend linking day of the outbreak with probability of pathogen isolation from the lung

lesions. Animals began enrollment on day three, where only one of four animals identified as having clinical disease had a pathogen isolated from its lungs. On day four of enrollment, six of the seven animals identified as having clinical disease had a pathogen isolated from the lungs. On study day 5, the last day of enrollment, all four of the animals identified as having clinical disease had pathogens isolated from their pulmonary tissue. This suggests that the further into the BRD outbreak, the more likely the animals were to have had pathogen counts sufficient for isolation from their lungs at the time they would have been typically treated for acute respiratory disease.

When looking at CFU/g of lung tissue as compared to histology scoring, it was shown that a score of 4 was significantly different from scores of 0-1. This indicates that the presence of pneumonia suggests a pathogen being present when an elevated score is assigned. It was observed that the more severe the histology scores the greater the CFU/g of pathogen present. However there is a small degree of difference in the other scores and the CFU/g isolated. Therefore, a score in the middle of the scale was a poor indicator of presence or absence of CFU/g of pathogen in the pulmonary tissue. The severity of pneumonia is thought to affect the growth of pathogens in the lungs of the cattle in this study. Additionally a score of either 0 or 1 proved to be significantly different from a score of 4. Indicating that there is a lower number of CFU/g isolated at lower histology scores. It is worth noting that some samples with a low histology score provided CFU/g at a level similar to samples with a histology score of 4. This gradual variation could be better differentiated or solidified with a greater study population in future research.

The use of clinical depression scoring systems is believed to correlate with the severity of disease in BRD cases and the visualization of gross lesions, though there is a lack of literature



describing this (White and Renter, 2009). In our study, five animals received a clinical depression score of 0, with three of them having pathogens isolated from their lungs and one of these animals had CFU/g  $\geq$  to animals that received a greater clinical depression score. Two animals received a clinical depression score of 2, but neither animal had any pathogens isolated and had 0% grossly visible consolidation calculated. One animal also received a clinical depression score of 2, had no pathogen isolated but had a calculated gross consolidation of 0.2%.

Two animals in this study received a clinical depression score of 3 and had 29.6% and 34.8% gross consolidation calculated, respectively. These two animals fit the hypothesized correlation of an elevated clinical depression score to visualization of severe gross lesions. However, the other two other animals that received a clinical depression score of 3 had a calculated gross consolidation of 5.2% and 6.5% respectively; Therefore, a lack of correlation of clinical depression score to gross lesions was observed in this study. Note the formula and calculation of these gross consolidation percentages are pending publication (refer to Roof, *et al*, 2011). Gardner, *et al*, 1999, showed that not all animals that exhibited clinical signs of BRD, including depression, and elevated temperature, have lung lesions. As seen in Figure 2, and supported by Reeve-Johnson, 2001, the appearance of clinical signs in an animal suspected of acute BRD does not necessarily indicate severity or even presence of the underlying pathological processes that was thought to correspond to gross lesions. However this could be better determined with a larger study population.

Even with the available range of viral and bacterial agents that are associated with acute BRD complex, the animals in this study only had two bacteria types, *Mannheimia haemolytica* and *Pasteurella multocida*, isolated from their lungs and no viral agents. It was hypothesized that a histology score of 0 would correlate with no gross pulmonary lesions and have zero

pathogens isolated from the pulmonary tissue. However this trial showed that those tissue samples with a histology score of 0 had pathogens, of varying levels, isolated from 65.0% of those lung samples. A histology score of 1 had pathogens isolated from 62.5% of those samples. A histology score of 2 had pathogens isolated from 40.0% of those samples. A histology score of 3 had pathogens isolated from 81.8% of those samples. A histology score of 4 had pathogens isolated from 72.7% of those samples. Table 5 provides the corresponding confidence intervals for the histology scores and their isolated pathogens. Figure 3 further illustrates the histology scores and the percent of samples with pathogens isolated. This shows that lower histological scores had isolation of some infectious pathogens, but not at the same level as discussed above and observed in Table 3. Despite the presence or absence of gross consolidated lesions, the presence of pneumonia on H&E staining is an indicator of elevated CFU/g of BRD pathogens, when a high histology score (4) is assigned, but not a predictor of isolation of BRD pathogens alone. Meaning that regardless of histological score or severity of pneumonia, every score type provided the isolation of BRD pathogens in this study.

To the authors' knowledge this study is the first to attempt to correlate histological scores of pulmonary tissues from animals exhibiting acute bovine respiratory disease with the quantified pathogen load and estimated gross consolidation of lesions from adjacent sampling loci. However, the general extrapolation of these results is limited due to the population used, samples collected, and methods used to identify the histological samples. Therefore, to obtain a better understanding of the potential usefulness of histological scores and the correlation to the pathogenesis of BRD, as well as BRD bacterial pathogen populations, further research should be conducted with this disease.

## Figures and Tables

Table 1: Depression Score Definitions

Depression Score	Clinical Signs
0	Normal, no signs of depression
1	Slower than pen mates but still perks up when approached and does not appear weak, actively follows your movements with a raised head.
2	Stands with head lowered, will perk up when approached but will return to depressed stance, moves slowly and falls towards back of group, may display signs of weakness such as incoordination
3	Obviously very weak, difficulty in moving with group, raises head only when approached closely
4	Moribund, unable to rise

Table 2: Histology Score Definitions

Score	Definition
0	Normal
1	Focal or multifocal pneumonia
2	Single focally extensive pneumonia
3	Multifocally extensive pneumonia
4	Diffuse pneumonia in one or both of the sections

Table 3: Range of Pathogen CFU/g in Lung Lobes Positive for Pathogen Growth  
by Histology Score

Histology Score (N=20)	Low bacterial count (CFU/g)	High bacterial count (CFU/g)	Mean bacterial count (CFU/g)
0 (n=20)	2.0E+04	2.0E+06	1.11E+05 <sup>B</sup>
1 (n=8)	2.0E+04	2.0E+06	7.26E+04 <sup>B</sup>
2 (n=5)	8.0E+04	8.0E+04	8.00E+04 <sup>A,B</sup>
3 (n=11)	4.0E+04	1.5E+06	2.24E+05 <sup>A,B</sup>
4 (n=11)	6.0E+04	2.0E+06	4.36E+05 <sup>A</sup>

\* Values in a column with the same superscript are not significantly different ( $P \leq 0.05$ )

Table 4: The Relationship between Classifying a Lung Area as Grossly Diseased, Histological Classification as Diseased, and Isolation of Bacteria by Lung Lobe.

Lung Area	(%)	Histology Score 1-4 (N=13)*	Bacterial Isolation (N=13)*
Cranial Segment of the Right Cranial Lobe (RCR)	10 (76.9)	10	7
Right Middle Lobe (RM)	5 (38.5)	5	4
Right Caudal Lobe (RCA)	4 (30.8)	4	2
Accessory Lobe (ACC)	6 (46.2)	6	6
Cranial Segment of Left Cranial Lobe (CRLC)	5 (38.5)	6	4
Caudal Segment of the Left Cranial Lobe (CDLC)	8 (61.5)	8	7
Left Caudal Lobe (LC)	5 (38.5)	4	5

\* There were 13 animals with grossly diseased tissue in at least one lobe. The histology score and bacterial isolation numbers are a subset of the number with grossly diseased tissue in that lobe.

Figure 1: Study Population Distribution of CFU/g by Histology Score

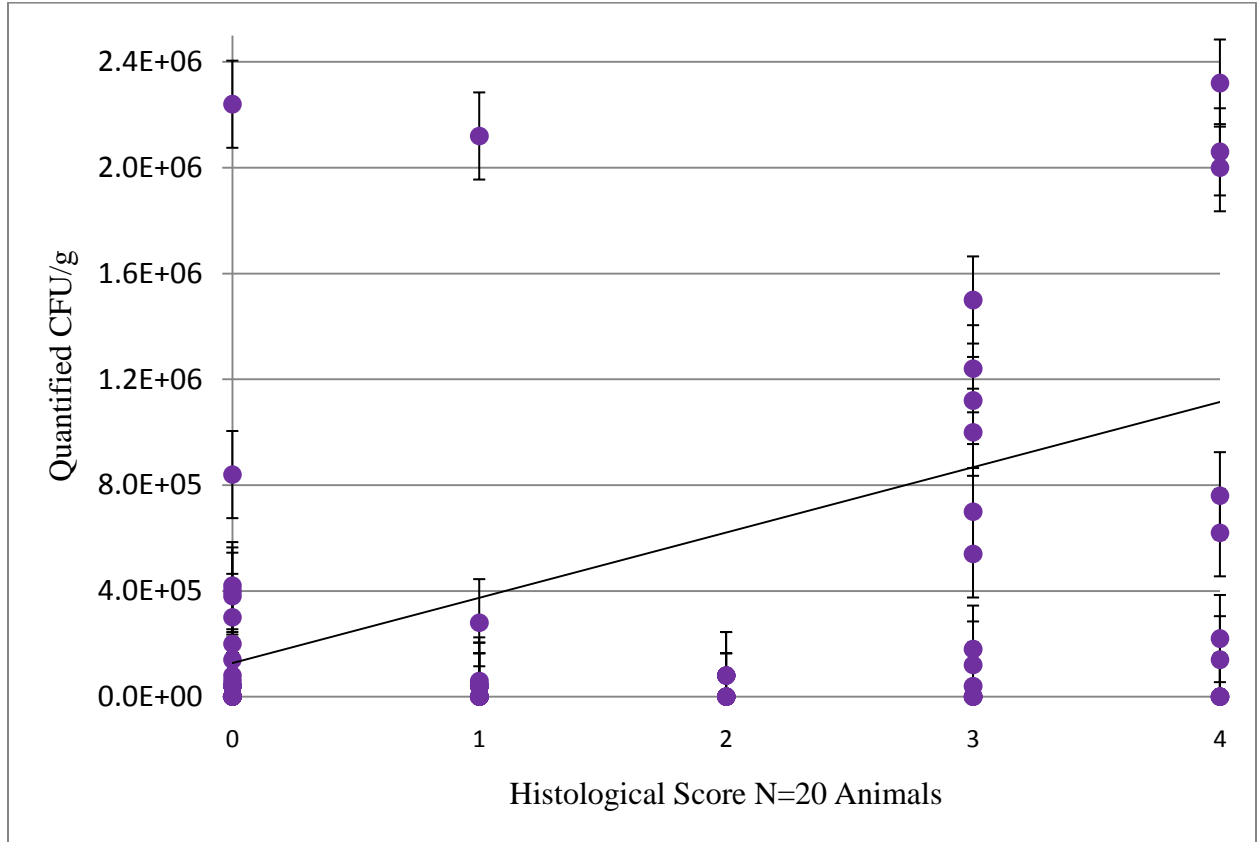


Figure 2: Clinical Depression Score as it Compares to the Estimated Gross Consolidation of Pulmonary Tissue

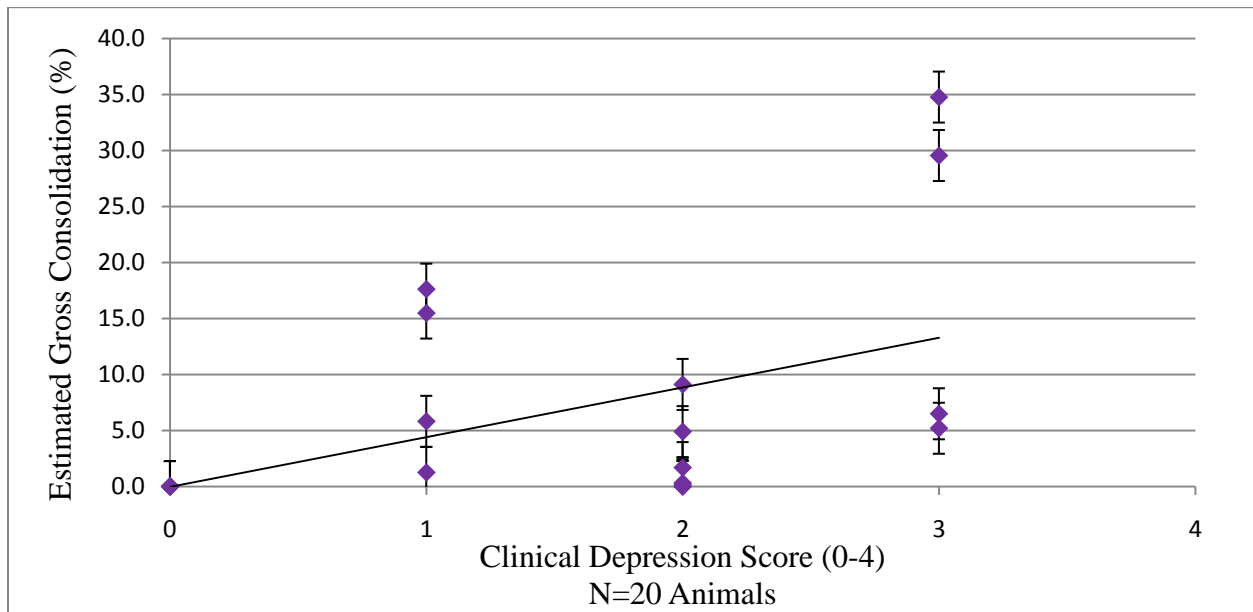
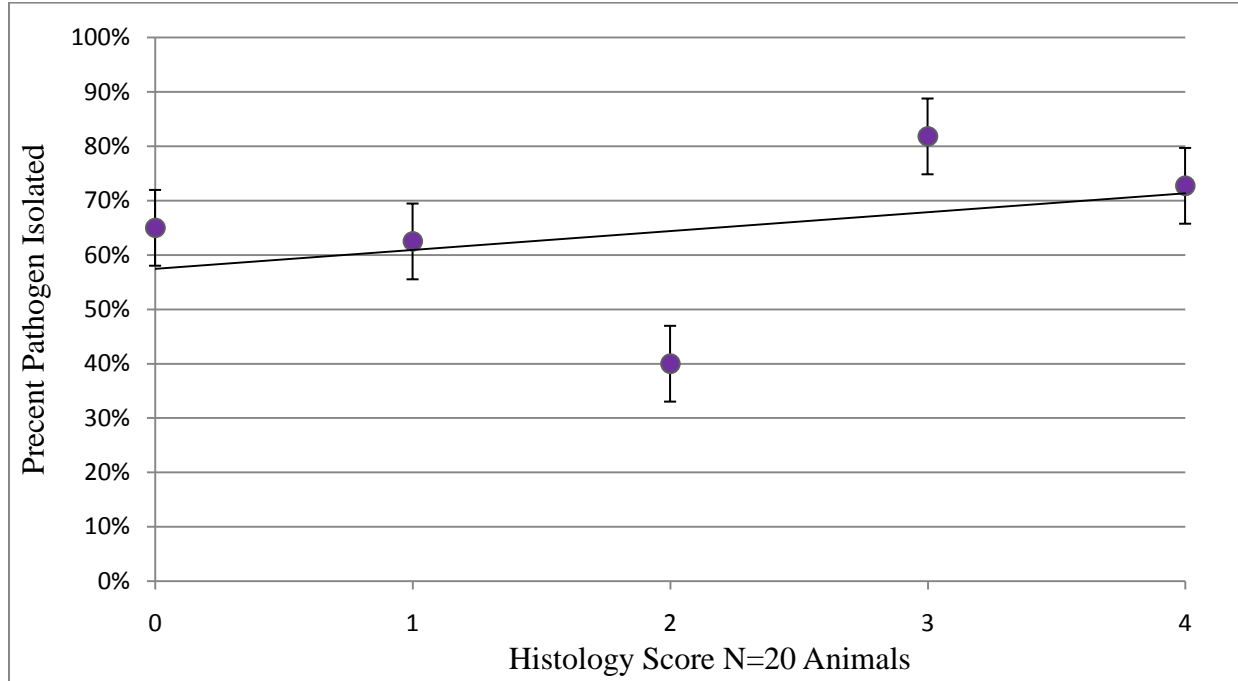


Table 5: Confidence Interval of Histology Score with Pathogen Isolated

Histology Score N=20	Percentage With Pathogen Isolated	Lower C.I.	Upper C.I.
0 (n=20)	65.0%	0.41	0.85
1 (n=8)	62.5%	0.24	0.91
2 (n=5)	40.0%	0.05	0.85
3 (n=11)	81.8%	0.48	0.98
4 (n=11)	72.7%	0.39	0.94

Figure 3: Study Population Pathogen Isolation Percentage Reported by Histology Score





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## **CHAPTER 5 - Implications for Further Research**

In the cattle industry there is a significant economic impact associated with bovine respiratory disease (BRD). Therefore, there is an increased interest by both veterinarians and cattle producers in curbing this impact on the cattle industry. This includes the early identification of diseased cattle, metaphylactic and prophylactic treatments of animals, and decreasing the potential for antimicrobial resistance to occur. Therefore it is important to consider total colony forming units (CFU) of respiratory pathogens in acute bovine respiratory disease (BRD) cases due to the potential of these populations providing conditions suitable for antimicrobial resistant mutation rates that could impact the efficacy of therapy.

Studies, such as the one described in this thesis, are necessary to better understand the probability of selection for spontaneous mutations during therapy of BRD cases. The data used in this research consisted of antemortem and postmortem observations combined with quantification and qualification of respiratory pathogens from cattle in a feedlot setting. The objective of this research was to quantify the pathogen load and observe the correlations to histological scoring, gross consolidation of pulmonary tissue, as well as agreement of tonsillar and tracheal pathogen isolation as it compares to the lower respiratory tract, and lastly antimicrobial susceptibility.

From the research conducted and described in Chapters 3 and 4 a few points for further research can be made regarding the selection and identification of cattle with clinical signs of bovine respiratory disease, the research design, and methodology of the tissues selected for analysis.

Suggestions for further research include, continuing to evaluate opportunities for increases in consistency and predictability of selecting animals that are truly experiencing acute

BRD. Also, the quantified pathogen load in animals with acute BRD could be a very useful tool in developing strategies for avoiding antimicrobial resistance selection during therapy of BRD. The study reported here had a few animals that approached the hypothesized total bacterial load threshold for spontaneous resistance populations, but this may be different in additional studies with different cattle and pathogen populations.

The results of the tonsillar and tracheal isolates compared to the lower respiratory tract analysis were shown to be largely inconclusive in this research. This portion of the study should be replicated with greater power to accept or reject them as supplemental methods for diagnosing and treating BRD cattle. Additionally, the use of nasal swabs and transtracheal washes could be beneficial in the analysis of this data, thereby providing multiple data points for each animal as opposed to the few collected upon euthanasia. Including this data could potentially provide a useful tool that could benefit the therapeutic outcome in BRD cases.

Lastly, the analysis of the presence of lung lesions at necropsy revealed that they are not uniformly correlated with the clinical disease process of BRD or consistent with the quantified pathogens isolated from the diseased lungs. These results would likely be more conclusive with a larger BRD study population.

The results of this study indicate that there is still much to be learned about BRD and its pathogenic impact on feedlot cattle. More questions than answers were gathered from this research and it is strongly believed that this study should be reproduced on a larger scale to further explore the potential impact of the pathogen load on antimicrobial resistance development in cattle being treated for bovine respiratory disease.