Pathogenicity of Bibersteinia trehalosi in bovine calves

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Pathogenicity of *Bibersteinia trehalosi* in bovine calves

by

Christy Hanthorn

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Preventive Medicine

Program of Study Committee:
Grant Alan Dewell, Major Professor
Paul Joseph Plummer
Vickie Lou Cooper

Iowa State University
Ames, Iowa
2014

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ABSTRACT

Introduction - *Bibersteinia trehalosi* has been associated with respiratory disease in ruminants particularly in wild and domestic sheep. Recently, there has been an increased interest in *B. trehalosi* isolates obtained from diagnostic samples from bovine respiratory disease cases. This study evaluated the role of *B. trehalosi* in the bovine respiratory disease complex using an intra-tracheal inoculation model in calves. This study also evaluated the potential of haptoglobin-matrix metalloproteinase 9 (Hp-MMP 9) serum concentrations as a useful diagnostic tool in calves affected with *B. trehalosi* and *M. haemolytica* associated respiratory disease.

Materials and Methods – Thirty-five cross-bred dairy steers were inoculated intra-tracheally with either leukotoxin negative or leukotoxin positive *B. trehalosi*, *Mannheimia haemolytica*, a combination of leukotoxin negative *B. trehalosi* and *M. haemolytica*, or negative control. Physical examinations were conducted and serum samples were collected throughout the study. Calves were euthanized and necropsies performed on day 10 of the study.

Results - No significant differences were found between the groups of calves challenged with *B. trehalosi* alone or in conjunction with *M. haemolytica* and the negative control calves with respect to percent lung involvement, rectal temperature, respiratory or depression score, or serum Hp or Hp-MMP 9 concentration in this study. *M. haemolytica* inoculated calves, but not *B. trehalosi* inoculated calves had increased lung involvement compared to control calves. *B. trehalosi* was only cultured once from the lungs of inoculated calves at necropsy. Increases in serum Hp and Hp-MMP 9 concentrations for the *M. haemolytica* group became significantly different from other groups on day 7. The
leukotoxin positive *B. trehalosi* group demonstrated increased serum Hp-MMP 9 concentrations on days 3-10 compared to pre-inoculation concentrations.

**Conclusions** - Based on these findings *B. trehalosi* may not be a primary pathogen of respiratory disease in cattle. Culture of *B. trehalosi* from diagnostic submissions should not be immediately identified as a primary cause of respiratory disease. Serum Hp-MMP 9 concentration is a useful diagnostic tool for detecting early pulmonary inflammation in calves challenged with *B. trehalosi* and *M. haemolytica*. Serum Hp-MMP 9 may also be a useful tool in detecting subclinical pulmonary inflammation in challenged calves.
CHAPTER 1
INTRODUCTION

The bovine respiratory disease complex (BRDC) is bronchopneumonia of polymicrobial and multifactorial etiology. This complex is a major cause of morbidity and mortality among cattle, particularly cattle in confined feeding operations [1, 2]. Diagnosis with BRDC is associated with subsequent negative health, welfare and performance. Because of the cost of treatment and the subsequent performance loss, BRDC is of major economic importance to the North American beef industry [3-5]. Many viral and bacterial pathogens as well as environmental conditions have been identified as components of or sufficient causes of BRDC [6]. Historically, in the United States *Bibersteinia trehalosi* has not been included as a pathogenic cause of BRDC for several reasons. Until 2012, very few case reports had been published associating *Bibersteinia trehalosi* with disease in cattle. The great majority of the reports were published in Europe. *Bibersteinia trehalosi*’s potential role in BRDC in North America was not given much consideration [7-11]. Cortese et al. published a series of case reports in 2012 describing peracute to fatal pneumonia in cattle caused by *Bibersteinia trehalosi* in the United States [12]. The purpose of this thesis was to determine whether or not *Bibersteinia trehalosi* should be included as a component of BRDC.
Thesis Organization

Chapter 2 is a literature review of the *Bibersteinia trehalosi* organism, its disease presentations in sheep and cattle, and its comparison to the *Mannhaemia haemolytica* organism.

Chapter 3 is a manuscript that has been published in BioMed Central Veterinary Research. It describes a pilot study that was designed to evaluate the pathogenicity of *B. trehalosi* in respiratory disease among calves using field strains of *B. trehalosi*. The study design and results were used to determine if *Bibersteinia trehalosi* is capable of causing respiratory disease in dairy cross calves in an experimental setting [13].

Chapter 4 is a manuscript that has been submitted to BioMed Central Veterinary Research (submitted 11 July 2014). This manuscript assesses and describes the diagnostic potential of haptoglobin-matrix metalloproteinase 9 (Hp-MMP 9) complexes as an antemortem assessment of respiratory disease in cattle infected with *Bibersteinia trehalosi* or *Mannhaemia haemolytica* [14].

Chapter 5 summarizes conclusions from research described in chapters 3 and 4. Suggestions for directions of future research related to *Bibersteinia trehalosi* in cattle are also discussed.

References


Background

*Bibersteinia trehalosi* is and has historically been an important pathogen of sheep, primarily as the cause of septicemia in 4-10 month old lambs. It has also been associated with pneumonia [1, 2]. Reports have been published in which the organism was isolated from other ruminant species including cattle, goats, and bison [2-5]. *Bibersteinia trehalosi* is currently the most common member of the Pasteurellaceae family to be cultured from the tonsils of commercially reared American bison [1].

Bacterial Characteristics

*Bibersteinia trehalosi* is a small, non-motile, gram negative rod that exhibits slight pleomorphism. The organism can be seen occurring singly, in pairs, or in short chains depending on growth stage [1, 6].

When grown on bovine blood agar, colonies are round, regular, greyish or yellowish in color with a smooth, shiny surface that graduates to semi-transparent at the periphery. Some isolates exhibit a weak margin of β-hemolysis [1, 6]. All 14 bovine isolates used in a classification study failed to show hemolytic activity [1]. Colonies are about 2 millimeters in diameter after 24 hours of aerobic incubation at 37°C. Growth occurs between 25 and 40°C, with an optimum temperature of 37°C. Isolates have been demonstrated to be CAMP-positive (Christie Atkins Munch-Petersen) and have weak
growth on MacConkey agar. Cultures produce both oxidase and phosphatase, but not catalase, urease, or indole. Acid is produced without gas from glucose, cellobiose, raffinose, aesculin, amygdalin, arbutin, gentibiose, dulcitol, galactose, mannose, trehalose, and salicin, but not from glycerol or myo-inositol [1, 6]. The organisms’ ability to ferment trehalose has historically been the characteristic differentiating Bibersteinia trehalosi from other members of the Pasteurellaceae family.

Young log phase cultures show good encapsulation of the organisms. Older cultures show little cell associated capsular material. The predominantly smooth lipopolysaccharide (LPS) of Bibersteinia trehalosi has been shown to have a negative effect on lung function in sheep following direct instillation into the lung. Initially a marked decrease in circulating leukocyte count was observed followed by mild leukocytosis. Lung lavage showed increased cell counts with most being neutrophils. LPS invoked inflammation, edema, hyperemia, and hemorrhage in the lung. These effects are similar to those reported for experimental acute pneumonia and are more marked for smooth LPS than rough LPS. LPS complexes with the sheep’s lung surfactant potentiating its damaging effect [6].

Some strains of Bibersteinia trehalosi have been demonstrated to produce leukotoxin. Acutely growing rather than stationary phase organisms are required for leukotoxin production. The leukotoxin is specific for alveolar macrophages and circulating blood lymphocytes, neutrophils, and cultured blood mononuclear cells from cattle, sheep, and goats. It has little or no effect on cells from swine, horses, or humans. Leukotoxin significantly reduces the production of oxidative metabolites. The alveolar macrophages from calves appear to be more susceptible to the effects of the leukotoxin than those of
adult cattle. Neutrophils also appear to be more sensitive than either alveolar or mammary macrophages [6].

The ability of Pasteurellaceae isolates to produce beta hemolysis on culture media has been extensively studied. Attempts have been made to correlate this ability to leukotoxin production and pathogenicity. Conflicting results have been produced from studies [7-9] designed to determine whether beta hemolysis should be used as an index for pathogenicity in adult sheep, in lambs, or not at all. In the Miller et al. 2013 study described below only 9 of the 16 Bibersteinia trehalosi strains were leukotoxin positive on PCR. None of the clade strains were leukotoxin positive despite reportedly showing beta hemolysis in culture [9]. The ability of Bibersteinia trehalosi strains to produce leukotoxin rather than beta hemolysis in culture has been determined to be the most important virulence factor of the organism [10].

**Taxonomic Classification**

Literature describing Bibersteinia trehalosi is complicated by the constantly evolving nomenclature of the organism as it undergoes increasing differentiation from other members of the Pasteurellaceae family. The description of the bacteria now known as Bibersteinia trehalosi was first published in 1921 by F.S. Jones with an original name of Bacillus bovisepticus group I [11]. In 1932 Newsom and Cross proposed a name change for the bacteria to Pasteurella haemolytica [12].

G.R. Smith proposed in 1959 that the Pasteurella haemolytica complex be divided into two biotypes, A and T, based on the ability of the different strains to ferment arabinose and trehalose respectively. He described the differences between the two strains
based on cultural and epidemiologic differentiation in sheep. He observed that the colonial morphology of type A strains appeared to be even and greyish with a central thickening. Type A was associated with enzootic pneumonia. Compared to type A strains, colonies of type T strains were larger when cultured and were associated with septicemia in lambs [13].

Ernst Biberstein was responsible for much of the early work done that characterized the organism, created the serotyping scheme, and indicated the unique nature of the taxon based on DNA-DNA relatedness studies [1]. Biberstein et al. subdivided the A and T biotypes into serotypes in 1960 using an indirect hemagglutination assay (IHA). This assay relied on differences in capsular polysaccharides between strains. Biberstein identified 16 serotypes with 12 belonging to the biotype A group and 4 belonging to the biotype T group [14]. The four serotypes belonging to the biotype T group were T3, T4, T10, and T15. These four serotypes show 98.7% similarity in a phylogenetic analysis based on 16S rRNA gene sequences [1].

Due to the inability of several isolates to be serotyped due to cross-agglutination reactions or nonreactivity to existing typing sera, a system of classification into biogroups and biotypes was developed by Bisgaard and Mutters in 1986 using 21 phenotypic characteristics [15]. The T biotype of Pasteurella haemolytica was officially recognized as a separate species from the A biotype and renamed Pasteurella trehalosi [16]. Jaworski et al. used the phenotypic classification system in 1998 to reclassify isolates of Pasteurella haemolytica biotypes A and T, Pasteurella trehalosi, and Pasteurella multocida in order to identify lineages with the highest pathogenicity. The previously identified serotypes 3, 4, 10, and 15 of Pasteurella trehalosi were all regrouped as biotype 2. This biogroup
predominated in wildlife species and also accounted for 21% of the Pasteurella isolates from domestic sheep, but were not present in the isolates from cattle. Fifty-six percent (259/464) of the Pasteurella isolates were classified as Pasteurella trehalosi in wildlife species. The Pasteurella trehalosi isolates all belonged to either biogroup 2 or a new group, biogroup 4. The biogroups were differentiated by isolates from biogroup 2 being positive for β-glucosidase activity while isolates from biogroup 4 were negative for β-glucosidase activity. Twenty-one percent (27/126) of the Pasteurella isolates from domestic ruminants were classified as Pasteurella trehalosi with 74% (20/27) belonging to biogroup 2. The remainder were classified as biogroup 4 or variants of either biogroup [17].

Blackall et al. demonstrated in 2007 that there was 62% or less DNA-DNA relatedness between Pasteurella trehalosi and the other members of the Pasteurellaceae family. Based on this difference, he proposed the bacteria name be changed to Bibersteinia trehalosi after Ernst L. Biberstein [1].

Miller et al. used 16S rRNA sequencing and leukotoxin A sequencing via PCR assay to clarify phylogenetic and epidemiologic relationships among Pasteurellaceae isolated from bighorn sheep in 2013 [9]. When this method was compared to the metabolism based method described by Jaworski et al. [17], only 16 of 25 trehalose-fermenting Bibersteinia trehalosi isolates were determined to be Bibersteinia trehalosi. The other 9 isolates formed a Bibersteinia trehalosi-like clade that was divergent from the reference strains of Bibersteinia trehalosi. Based on these findings, it has been determined that traditional metabolism based methods of identifying members of the Pasteurellaceae family lack sufficient accuracy and resolution for reliably discerning bacterial causes of
respiratory disease in bighorn sheep. Thus, if using metabolism based identification methods, strong consideration should be given to augmentation with molecular techniques for phylogenetic analysis [9].

**Treatment and Prevention**

According to the Iowa State University Diagnostic Lab susceptibility profile for bovine pathogens from 2010-2012, *Bibersteinia trehalosi* is most susceptible to treatment with drugs from the ceftiofur (88%), florfenicol (75%), and potentiated sulfa (75%) classes of antibiotics [18]. The presence of antimicrobial resistance genes in *Bibersteinia trehalosi* was first reported in 2006 in an isolate obtained from the lung of a calf with clinical signs of respiratory disease in France through the RESAPATH network. FloR-mediated resistance to chloramphenicol and florfenicol were associated with a plasmid which also carried functionally active genes for resistance to sulfonamides. The plasmid was composed almost exclusively of segments previously associated with other plasmids, transposons, or insertion sequences found in other members of the Pasteurellaceae family. This finding provides evidence of gene flow between Pasteurellaceae [2].

No vaccine is commercially available in the USA against *Bibersteinia trehalosi*. Several companies offer customized autogenous bacterins. However, evidence that substantiates vaccine effectiveness is lacking. Confer et al. demonstrated that serum antibody levels to LPS in vaccinated calves did not appear to correlate with protection in 1986 and 1987 studies [19, 20].
Bibersteinia trehalosi in Domestic and Wild Sheep

When Bibersteinia trehalosi was classified in the Pasteurella haemolytica complex it was reported that the organism was commonly carried in the nasopharynx and tonsils of apparently healthy domestic sheep. By 9 weeks of age 90% of tonsilar isolates from lambs were one of the T biotypes. The rate of nasal carriers of the T biotype of Pasteurella haemolytica was observed to peak in late autumn, coinciding with increased incidence of disease. No evidence was established indicating that increased carrier rate was the cause of disease. Pasteurella haemolytica type T was primarily associated with septicemia in older lambs and less commonly with sheep atypical pneumonia [21].

Septicemia

Septicemia typically occurred in spring born lambs during the months of September through December with a mortality rate between 2.5 and 3.2%. In general, reported clinical signs included sudden death, dullness, pyrexia, recumbency, frothy discharge of the mouth, dyspnea, and prostration leading to death within 6-8 hours. A higher mortality rate was seen on day one of outbreaks before decreasing over the next few days. Typical gross post mortem features of the disease included lambs in good body condition with frank blood exiting through the nostrils. Hemorrhages were noted on the pleura, epicardium, endocardium, heart valves, visceral peritoneum, mesenteric lymph nodes, and spleen. The trachea and bronchi were congested with blood stained froth. The lungs were congested, heavy, and bluish in color, with widespread focal hemorrhages. Clear, blood tinged exudate was observed in both pleural and peritoneal cavities. Fibrinous pericarditis, focal hemorrhagic hepatic necrosis, edematous lymph nodes, and blotchy kidneys were
also observed. Histologically alveolar edema, capillary congestion, and varying amounts of hemorrhage could be seen in lung tissue with colonies of bacteria forming thrombi in capillaries [21]. *Bibersteinia trehalosi* is still reported to cause systemic septicemia. A case was reported by Szeredi et al. in 2008 of systemic *Bibersteinia trehalosi* being diagnosed with an immunohistochemical method using rabbit hyperimmune serum raised against *Bibersteinia trehalosi* serotype 3 from a weaned lamb that died suddenly. Large numbers of *Bibersteinia trehalosi* were identified extracellularly in necrotic foci of the liver, tonsil, and nasal mucosa. Large numbers of the organism were also identified in the cytoplasm of inflammatory cells in alveoli and bronchioli. Low numbers of the organism were observed in the spleen, mediastinal lymph nodes, kidney and ileum. No organisms were observed in the heart muscle or brain [22].

**Respiratory Disease**

*Pasteurella haemolytica* biotype T was associated with about 10% of atypical pneumonia affecting sheep from 2-12 months of age. Atypical pneumonia was described as a proliferative, exudative pneumonia of the anterior and dependent parts of the lungs by Gilmour and Brotherston in 1963 [23]. It was defined pathologically by Stamp and Nisbet in 1963, who also proposed the name atypical pneumonia [24]. Other names for the disease have been apical, lobar, enzootic, chronic enzootic, and chronic non-progressive pneumonia. Clinical signs included coughing, dyspnea, hyperpnea exacerbated by exercise, dullness, and mucopurulent nasal discharge. Grossly the lungs appeared with either raised, firmly consolidated grey/pink areas or with sunken, dull red areas of atelectasis. Enlarged bronchial lymph nodes could also be observed post mortem.
*Pasteurella haemolytica* could reportedly be cultured from most early cases of atypical pneumonia, but was gradually eliminated from the lesions over a period of 8-11 weeks [21].

*Bibersteinia trehalosi* has also been associated with respiratory disease in adult domestic sheep with clinical signs of sudden, severe illness, inappetence, pyrexia, toxemia, and tachypnea consistent with endotoxemia. This presentation is similar to other gram negative infections. Differentiation is often not attempted due to positive response to IV treatment with oxytetracycline and NSAID therapy [25].

In a case study by Gonzalez et al. in 2013 describing *Mannheimia haemolytica* and *Bibersteinia trehalosi* serotypes isolated from lambs with ovine respiratory complex in Spain it was determined that the T4 serotype was most associated with a lack of consolidated lesions in lungs. Serotypes of a T3-T15 group were most often associated with lung consolidation. The researchers concluded that the T4 serotype had a greater degree of pathogenicity and was more likely to be associated with the septicemic form of disease [26].

Prevalence rate of *Bibersteinia trehalosi* in domestic sheep in the United States has been reported as 18% [27] and 6.7% in the highlands of Ethiopia [28]. The Gonzalez case study provided evidence of regional differences in the frequency of serotype isolation. The T4 serotype was the most common serotype isolated in the study in Spain, Donachie et al. [29] most frequently isolated the T15 serotype in Scotland, and Villard et al. [30] most frequently isolated the T3 serotype in France.
Bighorn Sheep

Large scale die offs of bighorn sheep populations in the western United States from pneumonia have recently been reported. Much attention has been given to investigate the etiologic agent(s) that may be responsible for the epidemics and have been focused on *Bibersteinia trehalosi*, *Mannhaemia haemolytica*, or viral causes [31].

*Pasteurella trehalosi* was shown to be isolated more frequently from healthy bighorn sheep than healthy domestic sheep. It was isolated at 63% (81/129) and 60% (27/45) in two geographically separate populations of bighorn sheep in the western United States [27]. It was only isolated in 18% of domestic sheep in areas geographically similar to the bighorn herds. *Bibersteinia trehalosi* has also been isolated from pneumonic bighorn sheep [31] at a higher rate than the isolation of *Mannhaemia haemolytica* [10, 32]. The majority of the studies show that the isolation of *Bibersteinia trehalosi* is not correlated to health status, in that it is isolated at a similar rate from the nasopharynx and lungs in both apparently healthy and diseased sheep [8]. Most isolates of *Bibersteinia trehalosi* in healthy bighorn sheep are non-hemolytic and leukotoxin negative [10, 27, 32].

While *Bibersteinia trehalosi* was frequently isolated in studies of pneumonia in bighorn sheep, prior to 2012 it was assumed that it was a secondary pathogen and required other contributing factors to cause respiratory disease. These included: sheep exposure to parainfluenza-3 virus, bovine respiratory syncytial virus, *Mycoplasma ovipneumoniae*, transport stress, harsh weather conditions, or other Pasteurellaceae from domestic ruminants [8, 31-33]. In one study, single doses of two different antibiotics were administered to bighorn sheep prior to translocation in an attempt to decrease the incidence of pneumonia due to transportation stress. The administration of antibiotics was
unsuccessful in eliminating Mannhaemia haemolytica and Bibersteinia trehalosi from the oropharyngeal cavity of the bighorn sheep and the effect on the incidence of pneumonia from transportation stress was undetermined [33].

In 2013 Dassanayake et al. demonstrated that leukotoxin positive Bibersteinia trehalosi caused severe and fatal pneumonia in bighorn sheep. Leukotoxin negative Bibersteinia trehalosi did not cause clinical pneumonia, death, or lung lesions in bighorn sheep. Further studies by the same group demonstrated that wild type and leukotoxin negative mutants of both Mannhaemia haemolytica and Bibersteinia trehalosi do not cause fatal pneumonia in bighorn sheep, suggesting that leukotoxin is the most important virulence factor of these organisms [10]. Tomassini et al. demonstrated that neutrophils derived from bighorn sheep appear to be more susceptible to leukotoxin that those from domestic sheep [27].

**Bibersteinia trehalosi in Cattle**

*Bibersteinia trehalosi* has been isolated from both healthy and diseased cattle. The organism is carried on nasal, conjunctival, and nasopharyngeal mucosa in healthy cattle [34, 35]. Conflicting reports exist about the prevalence of *Bibersteinia trehalosi* in the nasopharynx of healthy cattle. Some reports describe the organism as a ubiquitous commensal organism [36, 37]. Others report that the organism is not readily isolated from healthy, unstressed cattle [38]. Most published reports on *Bibersteinia trehalosi* in cattle originate from Europe. Very little is published about *Bibersteinia trehalosi* in cattle in the United States. All of the cattle field isolates that Blackall et al. [1] used for the reclassification of Pasteurella trehalosi as *Bibersteinia trehalosi* were from Europe. Three
of the isolates were from granulomas, one isolate was from a joint, and nine isolates were from lung tissue. No indication of disease status of the animals from which the isolates were obtained was provided in the study. None of the bovine isolates in the study demonstrated hemolytic ability. These finding are consistent with other studies that report when *Bibersteinia trehalosi* is isolated from cattle in the United Kingdom the isolates are often non-hemolytic [39].

**Respiratory Disease**

*Bibersteinia trehalosi* is infrequently identified as a respiratory pathogen in cattle. Isolates that are found in diseased animals are usually associated with bronchopneumonia [39]. Early reports of pathogenic *Bibersteinia trehalosi* in cattle are from Europe. Wray and Thompson [40] isolated *Pasteurella haemolytica* T3 in one instance from a calf with pneumatic lungs on post mortem. Allan et al. [34] cultured *Pasteurella haemolytica* T10 on 4 occasions from cattle involved in a respiratory disease outbreak on two separate farms. One isolate was from a yearling bull at the time of slaughter. Another isolate was from a 6 month old calf that died of pneumonia. The other two isolates were from sick 4 month old calves. Isolation of the organism was reportedly associated with proliferative bronchiolitis, alveolitis, and focal exudative fibrinous pneumonia, no site of isolation was given. Ball et al. [41] isolated *Pasteurella trehalosi* from various tissues and fluids in 27 cattle including lung, brain, nasal swab, milk, vaginal swab, peritoneal swab, mesenteric lymph node, feces and small intestine. Seven isolates were from the serotype T4, 12 isolates from the serotype T10, and 8 isolates from the T15 serotype. Only three of the cattle that the isolates originated from were exhibiting signs of pneumonia. Initial isolates
of *Bibersteinia trehalosi* associated with pneumonia in cattle originated in the northwest part of the United Kingdom in 2003, but have since spread through the rest of the country [35]. In 2009 Confer reported that *Bibersteinia trehalosi* associated pneumonia in young dairy calves had been described, but not documented in refereed literature [36]. In 2012 Cortese et al. published a series of case reports of peracute to fatal pneumonia in cattle caused by *Bibersteinia trehalosi* in the United States. The cases occurred in a group of mid lactation dairy cows, a group of feedlot bulls and steers, and a group of dairy calves. In all cases approximately 10% of the group was affected by respiratory disease that often resulted in death. On necropsy 50-75% of the lungs of each animal were affected, often with fibrinous bronchopneumonia. Affected animals were treated with a variety of antimicrobials labeled for use in bovine respiratory disease, but very little positive response to treatment was observed. *Bibersteinia trehalosi* was isolated either alone or with other bacterial respiratory pathogens such as *Pasteurella multocida* and *Mycoplasma bovis*. No viruses were usually detected in the reported cases. BVD was detected with PCR in only one calf. The researchers concluded that cross protection was observed within the groups when cattle were vaccinated with a *Mannhaemia haemolytica* vaccine containing leukotoxoid and cell wall antigen [42].

Clinical signs associated with *Bibersteinia trehalosi* in cattle include coughing, tachypnea, nasal discharge, pyrexia, malaise, lethargy, recumbency, wasting, poor body condition, and sudden death. Gross lesions that can be observed on post mortem examination include: purulent consolidating emphysematous pneumonia with fibrinous pleurisy, bronchial lymph node enlargement, pericarditis, necrotic laryngitis, and petechiation of lungs and subcutaneous tissues. Histologic examination typically
demonstrates fibrinopurulent bronchopneumonia and necrotizing fibrinous bronchointerstitial pneumonia. *Bibersteinia trehalosi* can be isolated most commonly from the center of consolidated lung tissue. All ages of cattle appear to be susceptible with an increased prevalence of disease in cattle under 12 months old. The number of cases peaks from October through the winter until about March. Disease is seen more often in housed cattle. *Bibersteinia trehalosi* is usually not found with a viral co-infection, but bacterial co-infection with other respiratory pathogens is not uncommon [35].

**Other Disease Presentations**

Although the majority of *Bibersteinia trehalosi* isolates associated with disease originate from pneumonic lung tissue, the organism can also be found in liver, spleen, and other tissues [35]. One report from the Scottish Agricultural Collage described a systemic infection of a 7 month old calf with *Bibersteinia trehalosi* that died rapidly [39]. Another case report from the UK describes a gravid 9 year old Limousin crossbred cow with a nursing calf that was found dead on pasture with no signs of illness on the day prior to death. The cow was in fair body condition. On post mortem examination fibrinous peritonitis and severe focal irregular hemorrhagic lesions on the liver with fibrinous exudate on the surface were observed. A hematoma and hemorrhagic lesions were seen on the gallbladder with thickening and calcification of the bile ducts. The spleen was swollen. Fibrinous serositis was observed in the cranial lung lobes and pericardial sac. A few small areas of consolidation were noted on the lungs below the area of pleurisy. *Bibersteinia trehalosi* was cultured from the liver, pleura, and spleen. A fluorescent antibody test showed positive staining for *Clostridium novyi* and *Clostridium chauveoi*. On histologic
evaluation acute necrotizing and hemorrhagic hepatitis was associated with sparse
degenerate leukocytes, venous thrombosis, and large numbers of gram negative
coccobacilli in thrombi and sinusoids. Clostridial organisms were not associated with
lesions indicating that they were likely post mortem invaders. Coccobacilli were also
found on the capsular surface of the liver and within gallbladder and lung lesions [39].

Spagnoli et al. [43] reported a case of a 3 year old Texas longhorn steer with a long
history of weight loss and progressive swelling of soft tissues of the jaw and neck. The
steer was euthanized due to a poor prognosis. Necropsy findings included multifocal to
coalescing dermal and subcutaneous pyogranulomas surrounded by fibrous tissue.
Submandibular and retropharyngeal lymph nodes were enlarged and 5-10% of the
cranioventral aspect of both lungs was consolidated and dark red. The pyogranulomas
contained aggregates of gram negative coccobacilli that were recovered in pure culture and
determined by molecular methods to be *Bibersteinia trehalosi*. This was the first reported
case of subcutaneous botryomycosis due to *Bibersteinia trehalosi*.

**Comparison of Bibersteinia trehalosi to Mannheimia haemolytica**

Historically *Bibersteinia trehalosi* and *Mannheimia haemolytica* were both
classified in the *Pasteurella haemolytica* complex. The organisms were first differentiated
from each other based on the ability of *Bibersteinia trehalosi* to ferment trehalose within 2
days, but not arabinose until at least day 10; whereas *Mannhaemia haemolytica* ferments
arabinose within 7 days, but does not ferment trehalose within 10 days. Subsequently
*Bibersteinia trehalosi* was designated as *Pasteurella haemolytica* type T and *Mannhaemia
haemolytica* was designated *Pasteurella haemolytica* type A [13].
The two types of *Pasteurella haemolytica* were further differentiated by clinical presentation in diseased animals. *Pasteurella haemolytica* type A was most commonly associated with pneumonia in cattle [38]. It was also associated with enzootic pneumonia in sheep. *Pasteurella haemolytica* type T was most commonly associated with septicemic disease in 6-12 month old lambs [13].

Differences between the two organisms in colonial morphology were reported when the colonies were examined by transmitted light after 18-24 hours. *Pasteurella haemolytica* type A colonies were described as even, grayish in color, and sometimes having a small clearly demarcated central thickening. *Pasteurella haemolytica* type T colonies were described as larger, up to 2 mm in diameter, with dark brownish centers and color fading toward the periphery [13].

Differences were also described at a cellular level. *Pasteurella haemolytica* type A was noted to have rough lipopolysaccharides while type T had smooth lipopolysaccharides. Neuraminidase was produced by most type A strains but not by type T strains. Neuraminidase removes terminal sialic acid residue from salivary glycoproteins resulting in a loss of viscosity and adhesiveness. This can potentially cause a reduction in the protective function of the epithelial mucous in that it becomes less able to entrap and remove invading microorganisms [6]. It was noted that type A strains were more sensitive to tetracycline and penicillin as penicillin failed completely to inhibit growth of the type T strains [6, 13]. Most recently, it has been reported that *Bibersteinia trehalosi* strains are more stable when compared to *Mannhaemia haemolytica* strains that have a higher degree of plasticity when analyzed with pulse field gel electrophoresis [44].
Organism Interaction

Due to differences observed between the *Bibersteinia trehalosi* and *Mannhaemia haemolytica* strains in clinical disease presentation and post mortem recovery rates in various ruminant species, Dassanayake et al. studied the interaction between the two organisms in vitro and in vivo. *Mannhaemia haemolytica* had been shown to consistently cause pneumonia in bighorn sheep in experimental studies, but *Bibersteinia trehalosi*, which had not been consistently proven to cause pneumonia in bighorn sheep, was isolated more frequently from pneumonic lung tissue. When grown separately *Bibersteinia trehalosi* had a doubling time of about 14 minutes. *Mannhaemia haemolytica* had a doubling time of about 27 minutes. In addition to a faster growth rate, *Bibersteinia trehalosi* also consistently achieved a 3-log higher cell density compared to *Mannhaemia haemolytica*. During co-culture *Mannhaemia haemolytica* growth was inhibited when *Bibersteinia trehalosi* entered the stationary phase (6 hours), resulting in a final cell density for *Mannhaemia haemolytica* that was 6-9 logs lower than expected with growth in the absence of *Bibersteinia trehalosi*. When grown in co-culture, but separated by a membrane *Bibersteinia trehalosi* still was able to inhibit *Mannhaemia haemolytica* growth by a proximity dependent mechanism. It was hypothesized that *Bibersteinia trehalosi* organisms overgrow *Mannhaemia haemolytica* organisms in pneumonic lung tissue of bighorn sheep due to their faster growth rate and higher final cell density in vitro. This hypothesis was supported by unpublished data from a study by the same group demonstrating that *Mannhaemia haemolytica* can be detected by PCR from pneumonic lung tissue from bighorn sheep that were negative by traditional culture methods [45].
Leukotoxin Analysis

Leukotoxin production has been associated with strains of both *Bibersteinia trehalosi* and *Mannhaemia haemolytica* and determined to be the most important virulence factor of the organisms [10]. The leukotoxin A gene encodes the protoxin which, after modification and secretion, targets and destroys ruminant lymphoid cells by binding to \( \beta(2) \) integrens. A high concentration of leukotoxin A causes pore formation in lymphoid membranes while a low concentration induces apoptosis. Sequencing based studies of the leukotoxin operon uncovered substantial levels of recombination and horizontal gene transfer among *Mannhaemia haemolytica* and *Bibersteinia trehalosi* isolated from domestic sheep and cattle [46]. Genetic sequencing of *Mannhaemia haemolytica* leukotoxin alleles demonstrates a higher diversity in sheep than in cattle suggesting that strain transmission from cattle to sheep is more common than from sheep to cattle [47]. *Bibersteinia trehalosi* strains tend to be more host specific than strains of *Mannhaemia haemolytica*. These findings may help explain why *Bibersteinia trehalosi* associated pneumonia is seen more in sheep than in cattle. The results of sequencing studies have demonstrated that contrary to the hypothesis of “ubiquitous dispersal,” the relationship between bacterial phylogeny and geography suggest that there may be some phylogeographic patterning to Pasteurellaceae evolution [46].

References


45. Dassanayake RP, Call DR, Sawant AA, Casavant NC, Weiser GC, Knowles DP, Srikumaran S: **Bibersteinia trehalosi inhibits the growth of Mannheimia haemolytica by a proximity-dependent mechanism.** *Applied and Environmental Microbiology* 2010, **76**(4):1008-1013.


CHAPTER 3

PATHOGENICITY OF BIBERSTEINIA TRELHOSI IN CALVES: RANDOMIZED CLINICAL TRIAL TO EVALUATE THE PATHOGENICITY OF BIBERSTEINIA TRELHOSI IN RESPIRATORY DISEASE AMONG CALVES

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Grant Dewell and Paul Plummer conceived and coordinated the experiment. Christy Hanthorn and Reneé Dewell performed the experiment. Vickie Cooper evaluated lungs. Chong Wang conducted statistical analysis. Timothy Frana helped with bacteriology guidance and draft of the manuscript. All authors read and approved the final manuscript.

Abstract

Introduction - Bibersteinia trehalosi causes respiratory disease in ruminants particularly in wild and domestic sheep. Recently, there has been an increased number of B. trehalosi isolates obtained from diagnostic samples from bovine respiratory disease cases. This study evaluated the role of B. trehalosi in bovine respiratory disease using an intratracheal inoculation model in calves.
Objective – Determine if *Bibersteinia trehalosi* causes respiratory disease in cattle alone or in combination with *Mannheimia haemolytica* and if it can be recovered and cultured from lung tissue or lung wash.

Animals – 35 cross bred dairy steers

Procedures - Calves were inoculated intra-tracheally with either leukotoxin negative *B. trehalosi*, leukotoxin positive *B. trehalosi*, *Mannheimia haemolytica*, a combination of leukotoxin negative *B. trehalosi* and *M. haemolytica*, or negative control. Calves were euthanized and necropsy performed on day 10 of study.

Results - *B. trehalosi* inoculated calves did not have increased lung involvement compared to control calves. Additionally, *B. trehalosi* was only cultured once from the lungs of inoculated calves at necropsy.

Conclusions - Based on these findings *B. trehalosi* may not be a primary pathogen of respiratory disease in cattle. Culture of *B. trehalosi* from diagnostic submissions should not be immediately identified as a primary cause of respiratory disease.

**Background**

*Bibersteinia trehalosi* is a known pathogen of ruminants and has been identified worldwide [1]. *B. trehalosi* was formerly included in a single species of *Pasteurella haemolytica* as biotype T [2]. This pathogen was recently reclassified as *B. trehalosi* on the basis of phylogenetic studies [3]. *B. trehalosi* has been associated with systemic pasteurellosis or septicemia in lambs [4] and pneumonia in bighorn sheep (*Ovis canadensis*) [5, 6].

Although *B. trehalosi* infections are considered rare in cattle, the agent is occasionally isolated from bovine lungs. Diagnostic reports of severe non-responsive Bovine Respiratory Disease (BRD) outbreaks associated with multi-drug resistant *B. trehalosi* have been
documented [7]. Clinical manifestations of these strains are often associated with multi-drug resistance and severe lung pathology. The reasons for development of these potentially highly virulent strains remain unclear. It has been hypothesized that *B. trehalosi* may have acquired increased pathogenicity from other bacteria. Some reports in the literature suggest that *B. trehalosi* and *Mannheimia haemolytica* can share genetic material [8, 9]. Earlier work has shown that nucleotide diversity of lktA from *B. trehalosi* is minimal (0.7 %) compared to *M. haemolytica* (22.0 %) and were genetically different from *M. haemolytica* [10]. This report included only *B. trehalosi* isolates from ovine samples. Bovine adapted isolates of *B. trehalosi* may have acquired some genetic material from *M. haemolytica* increasing its ability to infect cattle.

Understanding the role different pathogens play in BRD is critical to understanding and effectively treating clinical cases. The primary objective of this study was to evaluate the pathogenicity of *B. trehalosi* in respiratory disease among calves using field strains of *B. trehalosi*.

**Materials and Methods**

Two *B. trehalosi* isolates were identified from diagnostic submissions of bovine cases for the challenge study. Isolate identification was confirmed by 16S ribosomal RNA analysis. One isolate was PCR positive for the leukotoxin (lktA) gene [11] and the other isolate was negative. The lktA gene was amplified with a 5’ primer lktA9 (5’-TCAAGAAGAGCTGGCAAC-3’) and the 3’ primer lktA7 (5’-AGTGAGGGCAACTAAACC-3’). Amplification parameters were: denaturation at 94°C for 45 s, annealing at 62°C for 45 s, and extension at 72°C for 2 min. The leukotoxin-PCR-positive *B. trehalosi* isolate was cultured from the lungs, along with a *Pasteurella*
multocida, from a feedlot calf that had been treated with antibiotics multiple times for BRD that had fibrinosuppurative bronchopneumonia from a group of 273 kg Southeastern feedlot calves with 16% respiratory morbidity and 10% mortality. The leukotoxin-PCR-negative B. trehalosi isolate was cultured from the brain of a bull with multifocal, suppurative meningoencephalitis.

Thirty six 8-12 week old individually housed Holstein cross steer calves were obtained from a private calf raiser. Calves were tested for persistent infection by BVDV by immunohistochemistry prior to purchase and confirmed PI negative. Deep nasal swab samples were collected from each calf and submitted for bacterial culture to the diagnostic lab prior (Day -1) to inoculation. During the experimental challenge, calves were housed in a Biosecurity Level 2 facility at the Iowa State University Livestock Infectious Disease Isolation Facility. Each calf was confined separately in raised 0.9 x 1.8 meter pens that provided no opportunity for calf-to-calf contact in a room that held 12 calves at a time. Three groups of calves were utilized to accommodate room layout. Calves were provided free choice water and were fed mixed grass hay and a pre-mixed calf starter. Biosecurity procedures such as changing protective gloves and clothing between calves were employed by all research personnel working with calves. Calves were randomly assigned by a random number generator (www.Random.org) to one of five treatments. Treatments were randomly assigned to 1 of 3 replicates (Table 1). Eight calves were assigned to each bacterial challenge treatment and 4 calves to a negative control treatment. The 5 treatment groups were:

1) Negative control (Becton Dickinson BBL™ Brain Heart Infusion Broth 211059),
2) Leukotoxin negative B. trehalosi,
3) Leukotoxin positive *B. trehalosi*,

4) *M. haemolytica*

5) Combination of leukotoxin negative *B. trehalosi* and *M. haemolytica*

Prior to inoculation, each isolate was grown overnight on two blood agar plates and then transferred to Brain Heart Infusion broth immediately prior to inoculation to obtain an estimated $2.5 \times 10^9$ CFU of bacteria per mL. Isolates for group 5 had an estimated $2.5 \times 10^9$ CFU of bacteria per mL for both leukotoxin negative *B. trehalosi* and *M. haemolytica* for a total of $5 \times 10^9$ CFU of bacteria per mL. A small sample of the inoculum was enumerated on blood agar plates to determine exact concentration of the inoculum. Inoculums ranged on average from $1.7 \times 10^9$ to $3.3 \times 10^9$ CFU of bacteria per mL of broth.

A sterile 90 cm number 8 French Foley catheter (MILA International) was used to inoculate each calf. The researcher administering the inoculum was masked to the treatment being administered. For each inoculation, a sterile catheter was passed intranasal to approximately the bifurcation of the trachea and 20 mL of the inoculum was infused. Following inoculation, the calf’s head was briefly elevated.

Following inoculation, calves were monitored twice daily by trained personnel masked to treatment on days 1-10. Measurements recorded included rectal temperature, pulse rate, and respiration rate. Each calf was clinically assessed and assigned a respiratory and depression score during twice daily monitoring. The following scoring systems were used for the study.

*Respiratory Scores:*

0 – Normal, eyes are clear, nose is clean with no discharge, normal breathing

1 – Mild Respiratory, serous discharge from eyes and/or nose, slight cough
2 – Moderate Respiratory, muco-purulent discharge, cough, increased respiratory rate
3 – Severe Respiratory, excessive muco-purulent discharge, harsh cough, open mouth breathing

**Depression Scores:**

0 – Normal, calf is bright and alert, hold their head up and readily moves away from the observer

1 – Mild depression, calf’s attitude is slightly depressed but responds quickly to observer and appears normal

2 – Moderate depression, calf stands with head down, ears droop, abdomen lack of fill and may appear floppy, calf moves away slowly from observer

3 – Severe depression, calf stands with head down and very reluctant to move, very noticeable gauntness of abdomen

Any calf that was assigned a respiratory and/or depression score of three was considered a candidate for euthanasia. If calf was determined to be moribund from physical exam, it was humanely euthanized. All surviving calves were euthanized on day 10 of the study by use of captive bolt followed by a pithing rod. All euthanasias followed American Veterinary Medical Association guidelines [12].

Immediately following euthanasia, a necropsy was performed on each calf. The respiratory system was evaluated by a diagnostic pathologist. The percent abnormal lung was visually estimated for each lobe (Figure 1) and total lung involvement calculated as a percentage of total lung as described by Jericho and Langford [13]. Lungs and heart were removed from the calves and photographed (Figures 2-7). Samples of representative lung as determined by the pathologist were submitted ISU Veterinary Diagnostic Laboratory for
routine culture and histology. For culture, samples were plated on bovine blood agar and incubated aerobically at 35°C, sheep blood agar and incubated anaerobically at 35°C, and tergitol-7 agar and incubated aerobically at 35°C. All samples were incubated overnight and suspect hemolytic and non-hemolytic colonies were selected for identification by matrix-assisted laser desorption/ionization--time of flight (MALDI-TOF) (Bruker).

This protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#5-12-7352-B) and the Institutional Biosafety Committee (#12-I-0017-A). Personnel conducting daily monitoring, the pathologist and the statistician were blinded to calves treatment group.

**Statistical Analysis**

Our goal was to describe the magnitude and variation of our collected measurements and assess the association of these variables with BRD and *B. trehalosi*. The primary outcomes of interest included percent lung involvement, temperature, depression score, and respiratory score. The percent abnormal lung response was analyzed using mixed effect Analysis of Variance (ANOVA) model, with Treatment as fixed effect and Group as a random effect. The remaining responses (Temperature, Depression score, and Respiratory score) were analyzed using a repeated measures ANOVA model, with Treatment, time and their interaction as fixed effects and group as a random effect. Comparisons among treatment groups were performed using Tukey's t-tests. SAS® Version 9.2 (SAS® Institute Inc., Cary, NC, USA) was used in analyses. A p-value <0.05 was considered significant.
Results

Thirty-five of the thirty-six candidates were enrolled in the study. One calf assigned to the *M. haemolytica* group died prior to enrollment in study. *Pasteurella multocida* was isolated from deep nasal swab samples from 34 of 35 calves. *M. haemolytica* was isolated from 8 of 35 calves. *M. haemolytica* positive nasal swab calves were fairly evenly spread across all five treatment groups (1/4 in Control group, 1/8 in Leukotoxin negative *B. trehalosi* group, 2/8 in Leukotoxin positive *B. trehalosi* group, 1/7 in *M. haemolytica* group, 3/8 in Leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group). Six of the 35 enrolled calves were euthanized prior to day 10 of the study. Three of the euthanized calves (3/7) in the *M. haemolytica* group (days 2, 3 and 3) and three calves (3/8) in the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group (days 3, 5, and 8) were euthanized according to protocol because of a clinical assessment of moribund. The mean estimated percent lung involvement was highest for the *M. haemolytica* group (49%) followed by the mixed infection of *M. haemolytica* and leukotoxin negative *B. trehalosi* (26%). The leukotoxin positive *B. trehalosi* group had a mean 18% estimated lung involvement while the leukotoxin negative *B. trehalosi* mean lung involvement was estimated to be 13%. The control group had a mean lung involvement estimate of 13% (Table 2). Calves with lung involvement had histologic lesions that were consistent with bronchointerstitial pneumonia (26 of 35 calves) and 20 of these calves also had evidence of pyogranulomatous pneumonia.

*M. haemolytica* was isolated from 5 of 7 lungs of the *M. haemolytica* inoculated group and 4 of 8 lungs from the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group. *B. trehalosi* was isolated from one of 24 lungs that were inoculated
with *B. trehalosi*. Calf number 6 in the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination inoculation treatment group was euthanized on day 3 of the study as reported above. The calf had 71.6% lung involvement and both *M. haemolytica* and a *B. trehalosi* were isolated from the lung tissue. *B. trehalosi* was not isolated from the lung tissue of any other calves that were inoculated with a *B. trehalosi* isolate.

Rectal temperature (Figure 8), depression scores (Figure 9) and respiratory scores (Figure 10) followed an expected pattern after inoculation. Rectal temperatures ranged from 35.3-41.3° C. Depression scores ranged from 0-3 and respiratory scores ranged from 0-3. There were occasional time periods when differences in rectal temperatures, depression score or respiratory score between the *M. haemolytica* and the *B. trehalosi* or control groups were statistically significant (Figure 8, 9, and 10).

The calves from both groups inoculated with *B. trehalosi* demonstrated increased rectal temperature (>39.5° C) at 7 hours after inoculation compared to calves that had been inoculated with *M. haemolytica*. Temperatures for the two *B. trehalosi* only treatment groups returned to normal levels (37.5 – 39.5° C) within 12 hours. Calves in the *M. haemolytica* only inoculation group did not demonstrate increased rectal temperatures (>39.5° C) on average until 24 hours following inoculation and temperatures of these calves remained elevated except for morning of day 2. Calves in the combined inoculation group fluctuated above and below 39.5° C throughout the study (Figure 8).

Depression scores (Figure 9) for the *M. haemolytica* inoculation group began to increase 4 hours post inoculation and remained elevated at 1 or greater throughout the study. The combined inoculation group did not have increased depression scores (≥ 1) until day 4 of the study and never reached the severity of the *M. haemolytica* only inoculation...
group. Neither of the *B. trehalosi* inoculation groups had elevated depression scores. The average depression score for the control group was 0.2. Both *B. trehalosi* only inoculation groups had an average depression score of 0.3 while the *M. haemolytica* inoculation group had an average score of 1.3. The combined inoculation group had an average depression score of 0.6. Respiratory scores for the *M. haemolytica* inoculation group ranged from 0-3 and were not consistently abnormal (≥ 1) until day 4 of the study. None of the other groups had elevated average respiratory scores (Table 3).

**Discussion**

Results of this study indicate that the *B. trehalosi* isolates used in this study are not associated with significant disease in a research setting. Calves inoculated with either a leukotoxin positive (PCR positive for the leukotoxin (lktA) gene) or leukotoxin negative isolate of *B. trehalosi* did not demonstrate increased lung involvement compared to control calves. This finding contradicts some perceptions that *B. trehalosi* is an emerging primary respiratory pathogen of cattle [7]. In contrast, this study’s results are more supportive of *B. trehalosi*’s role in BRD as secondary and perhaps opportunistic bacteria. Reports of *B. trehalosi* associated pneumonia are from diagnostic submissions, often without access to history of inciting causes of respiratory disease, nutritional health of calves, additional stressors or information on antimicrobial therapies. In this challenge model healthy calves were challenged with field isolates of *B. trehalosi* and extensive lung involvement was not identified post inoculation.

The limitations of this study include the small size of the study, isolate credibility, the inoculation method and the health status of the calves prior to inoculation. The study
was designed as a pilot study to evaluate the pathogenicity of \textit{B. trehalosi} in cattle. The number of calves in each group was limited as much as possible to decrease the number of calves sacrificed while still providing important information. The small size of the study decreases the statistical power to identify statically significant differences even though numerically the difference between the negative control group (inoculated with broth only) and the positive control group (\textit{M. haemolytica}) were substantial.

The authors are unaware of any \textit{B. trehalosi} isolates that have demonstrated pathogenicity in a challenge model in cattle. The \textit{B. trehalosi} isolates selected for this study originated from field submissions to a diagnostic laboratory. Our approach was to use leukotoxin positive and negative \textit{B. trehalosi} isolates that had been associated with respiratory disease and could potentially be associated with lung pathology in the study calves.

Though unlikely, it is possible that, during the inoculation procedure, the dose was inadvertently misplaced into the esophagus instead of the lungs. There are multiple bovine respiratory disease challenge models cited in the literature including trans-tracheal injection, trans-thoracic injection, bronchoscope assisted, and using a nasal-tracheal approach that this study utilized. Confirmation that the catheter was correctly placed in the trachea as opposed to the esophagus can be difficult. However, the \textit{M. haemolytica} positive control group, had substantial lung involvement and a p-value of 0.07 compared to the negative control group suggesting that the inoculation technique and the challenge model was valid.

Calves in the study were conventionally reared dairy crossbred calves. Calves were purchased from a calf raiser and were considered clinically normal at the time of purchase
and did not exhibit signs of respiratory disease at enrollment. However, two calves in the control group had lung lesions (20-26 % lung involvement). Thus, this pathology was presumed to have resulted from a previous infection not associated with inoculation. Evidence of lung pathology from prior respiratory disease in some control calves may confound results between treatment groups. Histologic lesions characteristic of a previous BRSV infection were seen in 17 of the calves. Since two control calves demonstrated some lung pathology, the potential difference between treatment groups and the control group was affected.

Pre-study pharyngeal swabs did not identify any calves carrying *B. trehalosi* and the calf farm did not have a history of *B. trehalosi* disease. Despite this, it is still possible that calves could have had exposure to *B. trehalosi* prior to enrollment in the project. Given the age of the calves, it is also possible that passive immunity played a role in immune response. There is currently not a validated serologic test for cattle to determine previous exposure to *B. trehalosi*. It is possible that calves may have had previous unknown exposure to *B. trehalosi* that enhanced their immunological response.

It was surprising that calves inoculated with a combination of *M. haemolytica* and *B. trehalosi* tended to have numerically less lung involvement than that of *M. haemolytica* inoculation alone (difference was not significant). One would expect that mixed infections would potentiate increased lung involvement and subsequent pathology. Other research has suggested that *B. trehalosi* can inhibit the growth of *M. haemolytica*. Dassanayake et al. [14] reported that *B. trehalosi* inhibited the growth of *M. haemolytica* in co-culture when *B. trehalosi* enters the stationary phase. One possible explanation for this finding is that; when both bacteria are inoculated simultaneously, *B. trehalosi* may inhibit *in vivo* growth
of *M. haemolytica* until the immune system removes *B. trehalosi*. This postulated in vivo inhibition may delay the onset of clinical signs of BRD (such as a temperature spike and decrease the subsequent severity of lung involvement).

Our goal was to determine if *B. trehalosi* is really an emerging primary BRD pathogen as has been reported or if infection occurs secondary to some other viral/bacterial insult. Cortese et al. 2012 reported peracute fatal pneumonia in healthy adult cattle attributed to *B. trehalosi*. Other anecdotal reports concerning *B. trehalosi* and BRD suggest that *B. trehalosi* is associated with severe fibrinous pneumonia with consolidation (especially caudal lobes) and pleuritis that is unresponsive to antimicrobial therapy. The more minor role that *B. trehalosi* may play in BRD is supported by the isolation results described in this study. *B. trehalosi* was isolated from only one set of lungs post inoculation and in that case the sample was obtained only 3 days following inoculation from a calf that had been inoculated with both *B. trehalosi* and *M. haemolytica*. This finding suggests that *B. trehalosi* may not persist long term in the bovine lung. The temperature response of calves inoculated with *B. trehalosi* or *M. haemolytica* was different (p<0.05) over time. The early spike and rapid return to normal in rectal temperature from the *B. trehalosi* inoculated calves may indicate that the immune system in the lungs may more rapidly identifies and responds to a *B. trehalosi* bacterial insult compared to the *M. haemolytica* inoculated calves. The authors acknowledge that *B. trehalosi* isolates have been obtained from samples submitted from field cases. These “positives” suggest the occurrence of an opportunistic infection and proliferation of *B. trehalosi* after normal lung defenses have been compromised by other primary respiratory pathogens. The plausibility of *B. trehalosi* as a secondary, even an opportunistic pathogen
is a stronger argument than \textit{B. trehalosi} as an emerging primary BRD pathogen of economic importance.

\textbf{Conclusion}

This pilot study suggests that \textit{B. trehalosi} may not be an important primary pathogen of respiratory disease in cattle. The study’s results suggest that \textit{B. trehalosi} is a secondary, or perhaps opportunistic, BRD pathogen. Culture of this bacterium from diagnostic submissions should not necessarily be interpreted or cited as a primary cause of respiratory disease but may still have a role as a secondary pathogen or an opportunistic invader. Clinicians and diagnosticians should temper a \textit{B. trehalosi} culture in context of the clinical setting. Practically, identification of \textit{B. trehalosi} as the primary isolate from a submitted lung sample may suggest a failure in identification or treatment of a major BRD pathogen such as \textit{M. haemolytica}. Further challenge studies designed to assess \textit{B. trehalosi}’s interaction with other BRD pathogens (IBR, BVDV, \textit{Mycoplasma bovis}, \textit{Histophilus somni}) should be conducted. Additionally, a challenge model designed to evaluate if \textit{B. trehalosi}, inoculated after \textit{M. haemolytica} has already initiated lung damage, can lead to more severe BRD would be beneficial.

\textbf{References}


5. Wolfe LL, Diamond B, Spraker TR, Sirochman MA, Walsh DP, Machin CM, Bade DJ, Miller MW. **A bighorn sheep die-off in southern Colorado involving a Pasteurellaceae strain that may have originated from syntopic cattle.** *Journal of Wildlife Diseases* 2010, 46:1262-1268.


**Tables and Figures**

**Table 1.** Replicate and Treatment Group Assignments

Assignment of calves to treatment groups by replicate of study presented in table format.

<table>
<thead>
<tr>
<th>Replicate and Treatment Group Assignments</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Calves</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Leukotoxin negative <em>B. trehalosi</em></td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Leukotoxin positive <em>B. trehalosi</em></td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>M. haemolytica</em></td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><em>M. haemolytica</em> and leukotoxin negative <em>B. trehalosi</em></td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2. Percent Lung Involvement by Treatment Group

Individual and mean lung involved in disease process as a percentage of total lung volume.

Treatment group means that were statistically different from other treatment groups are indicated by different letters (a or b) in table. Adjusted p-values of treatment group from control group is reported.

Percent Lung Involvement by Treatment Group

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leukotoxin negative B. trehalosi</th>
<th>Leukotoxin positive B. trehalosi</th>
<th>M. haemolytica</th>
<th>M. haemolytica and leukotoxin negative B. trehalosi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calves</td>
<td>20.4</td>
<td>0</td>
<td>36.55</td>
<td>57.25</td>
<td>71.6</td>
</tr>
<tr>
<td></td>
<td>26.2</td>
<td>24.25</td>
<td>27.1</td>
<td>53.95</td>
<td>61.3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>21.7</td>
<td>28.1</td>
<td>26</td>
<td>62.85</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>32.1</td>
<td>27.1</td>
<td>33.15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18.05</td>
<td>14.2</td>
<td>28.6</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.15</td>
<td>1.5</td>
<td>63.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>11.35</td>
<td>75.1</td>
<td>4.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.45</td>
<td>6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean *</td>
<td>12.75 a</td>
<td>13.2 a</td>
<td>18.4 a</td>
<td>48.2 b</td>
<td>26.1 a, b</td>
</tr>
<tr>
<td>Adj P value **</td>
<td>0.9999</td>
<td>0.9981</td>
<td>0.0715</td>
<td>0.8049</td>
<td></td>
</tr>
</tbody>
</table>

*means with different letters are statistically different (p<0.05)

**Adjusted p value of treatment from the control group
### Table 3. Average Depression and Respiratory Score by Treatment Group

Average depression and respiratory scores over the course of the study are reported for each treatment group in table format.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Average Peak Depression Score (Range)</th>
<th>Average Peak Respiratory Score (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.25 (0-1)</td>
<td>1 (1-1)</td>
</tr>
<tr>
<td>Leukotoxin negative <em>B. trehalosi</em></td>
<td>0.75 (0-2)</td>
<td>1 (0-1)</td>
</tr>
<tr>
<td>Leukotoxin positive <em>B. trehalosi</em></td>
<td>1 (0-2)</td>
<td>1.25 (1-2)</td>
</tr>
<tr>
<td><em>M. haemolytica</em></td>
<td>2 (0-3)</td>
<td>1.7 (1-3)</td>
</tr>
<tr>
<td><em>M. haemolytica</em> and leukotoxin negative <em>B. trehalosi</em></td>
<td>1.25 (0-3)</td>
<td>1.25 (0-3)</td>
</tr>
</tbody>
</table>
Figure 1. Necropsy form

Form used to score percent lung involvement by lobe

Pathogenicity of Bibersteinia trehalosi

Necropsy Form

<table>
<thead>
<tr>
<th>Date/Time:</th>
<th>Calf ID:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathologist:</td>
<td>Recorder:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lungs</th>
<th>Percent Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Right Cranial Lobe cranial part</td>
</tr>
<tr>
<td>2</td>
<td>Right Cranial Lobe caudal part</td>
</tr>
<tr>
<td>3</td>
<td>Middle Lobe</td>
</tr>
<tr>
<td>4</td>
<td>Right Caudal Lobe</td>
</tr>
<tr>
<td>5</td>
<td>Left Cranial Lobe cranial part</td>
</tr>
<tr>
<td>6</td>
<td>Left Cranial Lobe caudal part</td>
</tr>
<tr>
<td>7</td>
<td>Left Caudal Lobe</td>
</tr>
<tr>
<td>8</td>
<td>Accessory Lobe</td>
</tr>
<tr>
<td>9</td>
<td>Trachea</td>
</tr>
<tr>
<td>10</td>
<td>Pleural surface</td>
</tr>
</tbody>
</table>
Figures 2 and 3. *Mannheimia haemolytica* inoculated lung demonstrating cranioventral consolidation

![Figure 2 and 3](image1)

Figure 4 and 5. *Bibersteinia trehalosi* leukotoxin positive inoculated lung demonstrating a smaller percentage of cranioventral consolidation

![Figure 4 and 5](image2)

Figures 6 and 7. BHI broth inoculated lung demonstrating normal lung tissue

![Figure 6 and 7](image3)
Figure 8. Temperature Over Time by Treatment Group

Line graph of rectal temperature by treatment group for each time point when temperature was recorded. Time points that were statistically different are indicated by different letters (a or b).

Temperature Over Time by Treatment Group

Points with different letters are statistically different (p<0.05)
**Figure 9. Depression Score Over Time by Treatment Group**

Line graph of depression score by treatment group for each time point when depression score was recorded. Time points that were statistically different are indicated by different letters (a or b).

**Depression Score Over Time by Treatment Group**

![Graph showing depression score over time by treatment group with different letters indicating statistical differences.]

Points with different letters are statistically different (p<0.05)
**Figure 10. Respiratory Score Over Time by Treatment Group**

Line graph of respiratory score by treatment group for each time point when respiratory score was recorded. Time points that were statistically different are indicated by different letters (a or b).

Respiratory Score Over Time by Treatment Group

Points with different letters are statistically different (p<0.05)
CHAPTER 4

SERUM CONCENTRATIONS OF HAPTOGLOBIN AND HAPTOGLOBIN-MATRIX METALLOPROTEINASE 9 (Hp-MMP 9) COMPLEXES OF BOVINE CALVES IN A BACTERIAL RESPIRATORY CHALLENGE MODEL

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Grant Dewell and Paul Plummer conceived and coordinated the experiment. Christy Hanthorn and Reneé Dewell performed the experiment. Vickie Cooper evaluated lungs. Chong Wang conducted statistical analysis. Jeffrey Lakritz performed the ELISA analyses. All authors read and approved the final manuscript.

Abstract

Objective—Evaluate potential diagnostic applications of serum Hp and Hp-MMP 9 concentrations in calves with BRD and establish a timeline for their detection in calves experimentally challenged with \textit{Bibersteinia trehalosi} and \textit{Mannheimia haemolytica}.

Animals—35 cross bred dairy calves
Procedures—Calves were inoculated via tracheal catheterization with either a PCR confirmed leukotoxin negative *B. trehalosi* isolate, a PCR confirmed leukotoxin positive *B. trehalosi* isolate, a *Mannheimia haemolytica* isolate, a combination of leukotoxin negative *B. trehalosi* and *M. haemolytica*, or a negative control. Serum samples were collected throughout the study. Calves were euthanized and necropsy performed on day 10 post inoculation.

Results—*M. haemolytica* inoculated calves had increased lung involvement. Serum Hp and Hp-MMP 9 concentrations were elevated compared to the other treatment groups. Increases in serum Hp and Hp-MMP 9 concentrations for the *M. haemolytica* group were significantly different from other study groups on day 7 of the study. *B. trehalosi* inoculated calves did not have increased lung involvement compared to control calves, but the leukotoxin positive *B. trehalosi* group demonstrated increased serum Hp-MMP 9 concentrations from day 3 to the end of the study compared to the pre-inoculation concentrations.

Conclusions and Clinical Relevance—Serum Hp-MMP 9 concentration is a useful diagnostic tool for detecting early pulmonary inflammation in calves challenged with *B. trehalosi* and *M. haemolytica*. Serum Hp-MMP 9 may also be a useful tool in detecting subclinical pulmonary inflammation in challenged calves.

**Introduction**

Bovine respiratory disease (BRD) has been shown to affect various biomarkers in cattle [1-7]. Two biomarkers that have been identified for diagnostic potential are serum
haptoglobin (Hp), matrix metalloproteinase 9 (MMP 9), and the complexes that they are able to form (Hp-MMP 9) [1, 6-9].

Free Hp is an alpha-2 globulin that is primarily synthesized in the liver [3]. Free Hp’s primary function is to bind to free hemoglobin in the blood. By scavenging the free hemoglobin, Hp helps to prevent oxidative tissue damage and conserve iron by returning the heme residue to the host’s metabolic process. This scavenging mechanism also serves to decrease the risk of free hemoglobin being utilized for bacterial pathogen growth [7, 8].

MMP 9 is a collagenase belonging to the gelatinase B group [5]. The gelatinase B group is a family of MMP’s that are zinc dependent proteinases capable of degrading at least one component of the extracellular matrix or basement membrane. This capability assists in the migration of white blood cells, mostly neutrophils, from the blood to the site of inflammation [4]. MMP 9 is stored in tertiary granules within the bovine neutrophils and is released when neutrophil degranulation is induced by either chemical or microbial stimuli [1, 5, 10, 11]. After release, MMP 9 is able to cleave interleukin 8, if present in the microenvironment into active interleukin 8. This creates a positive feedback loop for neutrophil migration [10]. While only neutrophils have been demonstrated to store MMP 9 for immediate release [8], other cells such as alveolar macrophages can be induced to produce MMP 9 [5, 12].

Free Hp and MMP 9 are secreted by multiple cellular sources in response to a variety of challenges; Hp-MMP 9 complexes have been shown to form exclusively in neutrophils [1]. These complexes are stored until neutrophil degranulation at which time they are released [1, 6, 8]. Hp has been demonstrated to have a high diagnostic sensitivity for detecting infectious or inflammatory conditions. Due to its poor specificity, it cannot
be used as the primary diagnostic test for any one condition. Free Hp is also poor at differentiating between acute and chronic inflammation [1, 7]. Free MMP 9 is more specific to acute inflammation, but it is not specific enough to differentiate between healthy and diseased cattle [1, 4]. Hp-MMP 9 complexes are capable of differentiating between acute and chronic inflammation as their release from neutrophils is associated with acute inflammatory responses [1, 8].

Various biomarkers potentially associated with BRD have been studied. In previous studies Hp was demonstrated to increase after calves were exposed to *Mannheimia haemolytica* via intra-tracheal inoculation with the earliest detection occurring at 24 hours, and peak concentrations occurring at 3 days post inoculation [6, 7]. In another challenge study, increases in serum Hp concentrations in calves infected with BVDV were not detected until 7-9 days post infection [7]. The purpose of this study was two-fold: to evaluate the diagnostic applications of serum Hp-MMP 9 concentrations in calves with BRD and to establish a timeline for their detection in calves undergoing experimental challenge with *Bibersteinia trehalosi* and *Mannheimia haemolytica*.

**Materials and methods**

Prior to initiating the study, the protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC 8-11-7187-B) and the Institutional Biosafety Committee (IBC#11-D-0017-A). This study is a hypothesis generating study representing a secondary use of calves enrolled in a challenge study designed to evaluate the pathogenicity of *Bibersteinia trehalosi* in respiratory disease among bovine calves [13]. The rationale for the secondary use of these calves to meet additional objectives is
consistent with using the 3R principles to maximize information obtained from animal research [14].

Calves were inoculated via tracheal catheterization with either a PCR confirmed leukotoxin negative \textit{B. trehalosi} isolate, a PCR confirmed leukotoxin positive \textit{B. trehalosi} isolate, a \textit{Mannheimia haemolytica} isolate, a combination of leukotoxin negative \textit{B. trehalosi} and \textit{M. haemolytica}, or a negative control as previously described [13].

Blood samples were collected from each calf via jugular venipuncture on Days 1 (pre-inoculation), 3, 5, 7, 9, and 10 (immediately prior to euthanasia). Blood samples were centrifuged at 4000 rpm for 15 minutes. Following centrifugation, samples were placed on ice before being transferred, using a transfer pipette, to an appropriately labeled cryovial. Boxes containing cryovials were placed immediately in an ultra-low freezer and stored at -70°C until being shipped to The Ohio State University on dry ice for analysis.

All surviving calves were euthanized on day 10 of the study, necropsied, and evaluated for percent abnormal lung involvement as previously described [13].

**Serum Haptoglobin-Matrix metalloproteinase 9 (Hp-MMP 9) ELISA assay**

The ELISA for heteromeric serum complexes of bovine Hp-MMP 9 was performed as described previously with minor alterations [1, 6, 15]. The ELISA is designed to capture bovine MMP 9 and detect haptoglobin bound to immobilized MMP 9. Un-conjugated antibodies prepared against purified bovine MMP 9 (MAb 10.1; in 100 µL, 2 µg antibody per well in 0.1 M sodium carbonate (pH 9.6), was allowed to bind to the wells of 96 well ELISA plates overnight at 4°C. After capture antibody binding, the plates were washed with 50 mM Tris buffered saline (TBS; pH 7.5) containing 0.05% tween 20, 5
times. After washing, the wells were tapped on dry paper towels and the wells were individually coated with 300 μL of SuperBlock T20 (TBS) (ThermoScientific, Cat #37536) at 4°C for 120 minutes to prevent non-specific protein binding. After blocking, the wells were again washed with 50 mM Tris buffered saline (TBS) containing 0.05% tween 20 and 100 μL of serum standards and unknown serum samples from experimental calves were added and incubated on a plate shaker (120 min at 21°C). The serum samples were each diluted 1:100 with sample diluent (TBS + 1% Bovine serum albumin + 0.05% Tween 20). Standards consisted of serial dilutions of serum from a cow possessing a concentration of 912.6 ng heteromeric complexes of Hp-MMP 9 complex/mL and were created by diluting serum in sample diluent (TBS + 1% Bovine serum albumin + 0.05% Tween 20). Standard concentrations ranged from 3.56 - 228 ng/mL. These standard concentrations represent the upper and lower limits of quantitation respectively. After analyte binding, standard and sample wells were vigorously washed 5 times with TBS+0.05% Tween 20. After washing, a rabbit anti-bovine Hp-horseradish peroxidase (HRP) conjugate was added as a detection antibody (1:5,000; secondary antibody 100 μL, containing 0.02 μg per well in TBS + 0.1% BSA; Immunology Consultants laboratory RHPT-10P; HRP conjugate, 1 mg/mL) [1]. The unbound HRP conjugate was removed by repeated washing (as above), after which 100 μL of TMB (SureBlue reserve TMB microwell peroxidase substrate; Kirkegaard-Perry Labs, Cat# 53-00-01) was added to each well to detect bound Hp-MMP 9. The wells were incubated for 20 minutes at 22°C and the reaction stopped by addition of 100 uL of 0.1N hydrochloric acid. The concentrations of Hp-MMP 9 were determined using the linear portion of the equation of the line described by absorbance of the calibrators at 450 nm and the known concentration of these calibrators. Any sample remaining outside of the range
of the standard curve (> 228 ng/mL) were diluted further and re-analyzed. Linear regression analysis of the serum standard concentration versus absorbance curves on each plate was created to determine the serum concentrations of heteromeric Hp-MMP 9 complexes in the experimental calves. Concentrations determined from the slope and intercept of the standard curve were corrected for dilution (x 100) and reported in ng/mL. The between plate variability in calibrators was 3% (range 0.98-2.7%) and the average coefficient of correlation of the calibration curves for this assay is 0.91 (range 0.85-0.91)

Serum total haptoglobin ELISA assay

Serum Hp concentrations were determined as described (Bovine haptoglobin 96-well ELISA, Life Diagnostics, West Chester, PA 19380) using commercial Bovine haptoglobin ELISA test kits, according to manufacturer’s instructions. Standard curves were prepared using purified bovine haptoglobin standard (2.5 μg/mL) included with the kit at a concentration range from 7.8 – 250 ng/mL. Serum samples were diluted according to the kit instructions (1:2,000 dilution) and were run in duplicate. Controls included were normal bovine serum, 5% BSA in TBS and blank wells. Linear regression of the Hp calibrator concentration versus absorbance was used to determine the equation for the line. The slope and intercept of this line was used to calculate the concentration of serum total Hp in the unknown animal samples. These concentrations were corrected for the dilution factor (x 2,000) and the concentrations reported in μg/mL.
**Statistical analysis**

Hp- MMP 9 and Hp were analyzed by repeated measures analysis of variance (ANOVA), with treatment, time, and their interaction as fixed effects and animal as subject of repeated measures. Lung involvement was analyzed by analysis of variance model, with treatment as explanatory variable. Correlations were calculated among the values of lung involvement, Hp, and Hp-MMP 9 for both Hp and Hp-MMP 9 serum concentrations as both ranked and absolute values prior to the challenge as well as averaged over the time of the study. SAS® Version 9.2 (SAS® Institute Inc., Cary, NC, USA) was used in analyses. A p-value <0.05 was considered significant.

**Results and Discussion**

**Lung involvement**

Prior to inoculation all calves appeared clinically normal. *Pasteurella multocida* was isolated from pharyngeal swab samples from 34 of 35 calves. *M. haemolytica* was isolated from 8 of 35 calves. *M. haemolytica* positive pharyngeal swab calves were spread across all five treatment groups (1/4 in control group, 1/8 in leukotoxin negative *B. trehalosi* group, 2/8 in leukotoxin positive *B. trehalosi* group, 1/7 in *M. haemolytica* group, 3/8 in leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group).

Six of the 35 enrolled calves were euthanized prior to day 10 of the study. Three calves (3/7) from the *M. haemolytica* group (days 2, 3 and 3) and three calves (3/8) from the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination treatment (days 3, 5 and 8) were euthanized according to protocol because of a clinical assessment of moribund.
The mean estimated percent lung involvement was highest for the *M. haemolytica* group (49%). The mixed infection group of leukotoxin negative *B. trehalosi* and *M. haemolytica* had a mean lung involvement of 26%. The leukotoxin positive *B. trehalosi* had a mean lung involvement of 18% while the leukotoxin negative *B. trehalosi* mean lung involvement was estimated to be 13%. The control group had a mean lung involvement estimate of 13%. There was evidence of a statistically significant (p=0.018) difference for mean percent total lung involvement between the *M. haemolytica* group and the leukotoxin negative *B. trehalosi* group. There were no significant differences between the other treatment groups. Even though the control group had a lower mean percent lung involvement than the leukotoxin negative *B. trehalosi* treatment group, the low number of study subjects in the group did not lend sufficient power to the group mean to make it significantly different from the *M. haemolytica* treatment group. The mean and median values were similar in all treatment groups except the mixed infection group. This group had a high amount of variability within it as evidenced by a mean percent lung involvement of 26% and a median percent lung involvement of 5%. The high variability made drawing conclusions difficult in this group (Figure 1).

**Free Hp**

Other studies have found that serum Hp is nearly undetectable in healthy cattle [8]. Concentrations up to about 200 μg/mL are considered acceptable for healthy animals [2, 7, 9, 16]. Concentrations between about 200 and 400 μg/mL are associated with mild inflammation and concentrations greater than about 400 μg/mL suggest severe
inflammation [7]. Serum Hp concentration does not differ with age or sex in cattle [2, 7, 9].

In this study, 11 calves (2/4 in the control group, 2/8 in the leukotoxin negative *B. trehalosi* group, 4/8 in the leukotoxin positive *B. trehalosi* group, 1/7 in the *M. haemolytica* group, and 2/8 in the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group) had serum Hp concentrations greater than 200 μg/mL prior to inoculation. Two of the calves had low serum concentrations of Hp-MMP 9 indicating the presence of chronic inflammation. *M. haemolytica* was cultured from the pharyngeal of both calves. Nine of the calves with high serum concentrations of Hp also had high serum concentrations of Hp-MMP 9 indicating the presence of chronic inflammation which was associated with an acute inflammatory event. Three of these calves were culture positive for *M. haemolytica* from pharyngeal swabs. Nineteen calves had no detectible serum Hp prior to inoculation.

The interaction between treatment group and bleeding date was a statistically significant effect (p=0.01). The mean serum Hp concentration for the leukotoxin positive *B. trehalosi* treatment group was significantly (p=0.008) different from the other treatment groups for the pre-inoculation bleeding date, indicating that more calves with evidence of chronic inflammation prior to commencement of the study were randomly assigned to this treatment group. On the fourth and sixth bleeding dates (days 7 and 10) there was a significant (p<0.05) difference in the mean Hp concentration between the *M. haemolytica* treatment group and the leukotoxin negative *B. trehalosi* treatment group and the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination group. The *M. haemolytica* treatment group demonstrated significant elevations in serum Hp
concentrations from the first two bleeding dates to the third bleeding date (day 5) (p=0.02). The elevation in serum Hp concentration remained significant (p<0.02) throughout the study. This finding is in agreement with other studies that have demonstrated an increase in serum Hp after *M. haemolytica* infection [4, 6]. Concentrations in this study peaked on day 7 rather than day 3 as previously reported. A decrease in the serum Hp concentration for the mixed infection group can be observed between the second and third bleeding dates (days 3 and 5). This observation supports findings by Dassanayake et al. suggesting that *B. trehalosi* is capable of inhibiting the growth of *M. haemolytica* in vivo [17]. In this study the difference in serum Hp concentration is not significantly different from the other treatment groups. Two calves from this treatment group were euthanized before the third bleeding time point. The smaller treatment group likely did not have enough power for a statistical difference to be observed (Figure 2).

**Hp-MMP 9**

The mean serum Hp-MMP 9 concentration for all treatment groups prior to inoculation was 104 ng/mL, with a minimum value of 0 ng/mL, a maximum value of 752.2 ng/mL, and a median value of 4.15 ng/mL. Little to no Hp-MMP 9 should be detectible in the serum of healthy calves [6]. Sixteen calves had no detectible serum Hp-MMP 9 concentration prior to inoculation. For this study a serum concentration greater than 20ng/mL was considered high. Thirteen calves had high serum Hp-MMP 9 concentrations prior to inoculation. Four of the thirteen calves had low serum concentrations of Hp, suggesting the presence of a mild acute inflammatory process. None of these 4 calves
were culture positive for *M. haemolytica* from the pharyngeal swabs. Nine of the thirteen calves had high serum concentrations of Hp and were discussed previously.

Treatment group, bleeding date, and their interaction were all significant (*p*<0.01) effects. On the fourth bleeding date (day 7) the mean serum Hp-MMP 9 concentration for the *M. haemolytica* group became significantly different from the negative control, the leukotoxin negative *B. trehalosi*, and the leukotoxin negative *B. trehalosi* and *M. haemolytica* combination groups (*p*=0.01, *p*=0.009, and *p*=0.004 respectively). This difference continued throughout the study. On the fifth bleeding date (day 9) the mean serum Hp-MMP 9 concentration for the *M. haemolytica* group became significantly different from the leukotoxin positive *B. trehalosi* group (*p*=0.006) and remained different throughout the rest of the study. The *M. haemolytica* group demonstrated significant elevations in serum Hp-MMP 9 concentrations from the first three bleeding dates to the last three bleeding dates (*p*<0.02). The leukotoxin positive *B. trehalosi* treatment group demonstrated a small but significant (*p*<0.004) elevation in serum Hp-MMP 9 concentrations from the pre-inoculation bleeding date to the second bleeding date (day 3) that continued throughout the study (Figure 3). This finding is consistent with other studies that have demonstrated the rapid recruitment and accumulation of neutrophils at the onset of BRD [6] as well as the action of leukotoxins to stimulate the active degranulation of bovine neutrophils [5].

**Correlations**

There was statistically significant (*p*<0.0001) evidence of a strong (*r*=0.7) correlation between Hp and Hp-MMP 9 serum concentrations as both ranked and absolute
values prior to inoculation. Evidence of a relationship remained statistically significant over time (p<0.0001), but the strength of the relationship varied depending on if a Pearson (absolute values) or Spearman (ranked data) correlation coefficient was calculated. The Pearson correlation coefficient was r=0.63; whereas, the Spearman correlation coefficient was r=0.79. This strong relationship was expected since both substances have been established as biomarkers for inflammation and the test for free Hp measures Hp-MMP 9 contribution also.

There was statistically significant evidence (p<0.05) of a moderate correlation (r=0.44 and r=0.35) that became stronger over time (r=0.67 and r=0.48) between Hp-MMP 9 and percent lung involvement for both ranked and absolute values respectively. There was statistically significant evidence (p=0.04) of a moderate (r=0.35) correlation between free Hp and percent lung involvement for ranked, but not absolute values prior to inoculation. This correlation became stronger (r=0.54, p=0.0008) when values were averaged over the time of the study. The correlation between free Hp and percent lung involvement for absolute data was also statistically significant when averaged over time (r=0.41, p=0.015).

This correlation data indicates that Hp-MMP 9 serum concentrations may be a good ante-mortem diagnostic indicator of lung damage that is found at the time of the calf’s death. This data also supports the conclusion from other studies that Hp-MMP 9 serum concentration is a better diagnostic test for lung damage than free Hp serum concentration [1]. Future studies should be conducted to determine reference ranges of serum Hp-MMP 9 for different levels of disease severity.
Conclusion

All study calves appeared to be clinically normal prior to inoculation in this study. Culture results of pharyngeal swabs taken from calves prior to inoculation showed that all but one calf was positive for *P. multocida*. The presence of this bacterium did not appear to have an impact on pre-inoculation values of serum Hp or Hp-MMP 9 since many of the calves had serum concentrations of these two biomarkers that were within limits considered acceptable for healthy animals [2, 7, 9, 16]. The pharyngeal swabs for 8 calves were culture positive for *M. haemolytica* prior to inoculation. This finding could have potentially affected the development of lung lesions as well as increases in serum Hp and Hp-MMP 9 concentrations in calves that were members of treatment groups other than the *M. haemolytica* group. The effect was not large enough to preclude seeing statistically significant results from the challenge in the *M. haemolytica* group. The development of a rapid, inexpensive test for serum Hp-MMP 9 concentrations could have clinical applications as a screening test for subject enrollment in BRD studies. Subjects with elevated serum concentrations could be excluded from participation.

Significant differences were observed between the *M. haemolytica* group and the other treatment groups in percent total lung involvement, serum Hp, and serum Hp-MMP 9 concentrations. The difference in percent total lung involvement demonstrates that the inoculation technique was appropriate for this challenge model. The increase in serum Hp and Hp-MMP 9 concentrations at day 7 are consistent with other reports of these two biomarkers having diagnostic potential with respect to BRD. Results suggest a different, slightly later, timeframe for using them as diagnostic tools.

No significant difference was observed between the leukotoxin positive *B. trehalosi*
treatment group and the negative control, leukotoxin negative *B. trehalosi*, and combination of leukotoxin negative *B. trehalosi* and *M. haemolytica* groups with respect to percentage of total lung involvement or serum Hp concentrations. A small but significant increase was noted in the serum Hp-MMP 9 concentrations of the leukotoxin positive *B. trehalosi* treatment group from day 1 to day 3, indicating that serum Hp-MMP 9 concentrations may be a more sensitive test for pulmonary inflammation than visible lung damage or serum Hp concentrations. This finding also provides evidence that the leukotoxin positive strain of *B. trehalosi* induced more neutrophil degranulation leading to a higher release of Hp-MMP 9 complexes than the leukotoxin negative strain of *B. trehalosi*. In this study the earlier rise in the serum Hp-MMP 9 concentration for the leukotoxin positive *B. trehalosi* treatment group than for the *M. haemolytica* treatment group may indicate that bovine neutrophils respond faster to a *B. trehalosi* infection than to a *M. haemolytica* infection.

The stronger significant correlation between serum Hp-MMP 9 concentration and percent lung involvement than between serum Hp concentration and percent lung involvement support the conclusions of other studies that serum Hp-MMP 9 concentrations can be an effective tool for early diagnosis of BRD [6] and that it is a better diagnostic tool for acute inflammation than serum Hp [1]. Future studies to determine a reference range for serum Hp-MMP 9 concentration and to develop a rapid, inexpensive test would help to make serum Hp-MMP 9 concentration a clinically useful diagnostic tool.

References


Figures

Figure 1. Distribution of percent lung involvement by treatment group

Treatment group 0: Negative control group, Treatment group 1: leukotoxin negative *B. trehalosi* group, Treatment group 2: leukotoxin positive *B. trehalosi* group, Treatment group 3: *M. haemolytica* group, Treatment group 4: Combination of leukotoxin negative *B. trehalosi* and *M. haemolytica* group

Means with different letters are significantly different (p<0.05)

Diamonds represent the group mean. Boxes represent the middle two quartiles of individual values, divided by the center line which represents the median. Bars adjacent to the boxes represent the outer two quartiles of individual values.
Figure 2. Serum Hp concentration in micrograms/mL over time by treatment group

Treatment group 0: Negative control group, Treatment group 1: leukotoxin negative *B. trehalosi* group, Treatment group 2: leukotoxin positive *B. trehalosi* group, Treatment group 3: *M. haemolytica* group, Treatment group 4: Combination of leukotoxin negative *B. trehalosi* and *M. haemolytica* group

Circles above trend lines indicate significant differences from other treatment groups.

Triangles below trend lines indicate significant differences from prior bleeding dates.

**Serum Hp concentration over time by treatment group**
**Figure 3.** Serum Hp-MMP 9 concentration in nanograms/mL over time by treatment group

Treatment group 0: Negative control group, Treatment group 1: leukotoxin negative *B. trehalosi* group, Treatment group 2: leukotoxin positive *B. trehalosi* group, Treatment group 3: *M. haemolytica* group, Treatment group 4: Combination of leukotoxin negative *B. trehalosi* and *M. haemolytica* group

Circles above trend lines indicate significant differences from other treatment groups.

Triangles below trend lines indicate significant differences from prior bleeding dates.

**Serum Hp-MMP 9 concentration over time by treatment group**
Conclusions

During identification and selection of *Bibersteinia trehalosi* isolates to be used as challenge strains, it was determined that bacterial identification using 16S ribosomal RNA gene sequencing and matrix-assisted laser desorption/ionization--time of flight (MALDI-TOF) was faster and more accurate than identification using traditional bench top metabolism based methods. This finding for cattle strains of *B. trehalosi* is in agreement with the finding of Miller et al. According to Miller et al., metabolism based methods of identifying members of the Pasteurellacea family lack sufficient accuracy and resolution for reliably discerning bacterial causes of respiratory disease in bighorn sheep. He suggested that metabolism based methods should be augmented by molecular techniques for phylogenetic analysis [1]. Based on experience in this study, the researchers would suggest replacing metabolism based identification methods with molecular identification methods for faster and more accurate bacterial identification.

Conflicting reports exist describing the prevalence of *B. trehalosi* in the nasopharynx of healthy cattle. Some reports describe the organism as an ubiquitous commensal organism [2, 3]. Others report that the organism is not readily isolated from healthy, unstressed cattle [4]. *B. trehalosi* was not isolated from the nasopharynx of any of the study subjects prior to inoculation. This finding supports the position that the organism is not readily isolated from healthy, unstressed cattle. However, the study calves were obtained from a single source, thus limiting the scope of inference.
No published reports exist describing the prevalence of *Pasteurella multocida* in the nasopharynx of cattle in the United States. One report from the United Kingdom reported a prevalence of 17% of *P. multocida* in the nasopharynx of suckling age beef and dairy calves in Scotland [5]. *P. multocida* was isolated from 34/35 calves prior to inoculation in this study. The prevalence rate for this group of calves of 97% was much higher than previously reported. The high prevalence rate observed in this group of calves may be a true reflection of the prevalence rate of conventionally reared dairy calves in the United States compared to Scotland. Alternatively, the prevalence rate may be artificially inflated in this group of calves due to previous viral challenges on the farm of origin. The cause of the observed high prevalence rate was outside the scope of this study. A study to determine an accurate prevalence rate of various bacterial organisms in the nasopharynx of young bovine calves would be justified.

No significant differences were found between the groups of calves challenged with *B. trehalosi* alone or in conjunction with *M. haemolytica* and the calves inoculated with BHI broth with respect to percent lung involvement, rectal temperature, respiratory or depression score, or serum Hp or Hp-MMP 9 concentration in this study. During the challenge study, measured parameters from the groups of calves inoculated with *B. trehalosi* were more similar to the measured parameters from the negative control group which was inoculated with brain heart infusion (BHI) broth than to the positive control group which was inoculated with a virulent strain of *Mannheimia haemolytica*.

Significant differences in percent lung involvement, rectal temperatures, depression and respiratory scores, and Hp and Hp-MMP 9 serum concentrations did exist between the groups of calves challenged with *B. trehalosi* alone or in conjunction with *M. haemolytica*.
and the calves inoculated with *M. haemolytica* alone. Percent lung involvement for the *M. haemolytica* challenged group of calves was significantly higher when compared to the other study groups. Rectal temperatures for calves in the *B. trehalosi* groups peaked at 7 hours post inoculation then decreased to within normal range. Rectal temperatures for calves in the *M. haemolytica* group did not peak until 24 hours post inoculation and remained elevated throughout the rest of the study. Depression and respiratory scores were significantly higher for the calves in the *M. haemolytica* group than for the calves in the *B. trehalosi* groups from days 5 and 7 to the end of the study respectively. Serum concentrations of Hp and Hp-MMP 9 were significantly higher for the calves in the *M. haemolytica* group than for the calves in the other groups from day 7 to the end of the study. The faster peak in rectal temperature and rise in serum concentrations of Hp and Hp-MMP 9 may indicate that the immune system in the lungs may more rapidly identify and respond to a *B. trehalosi* bacterial insult compared to a *M. haemolytica* insult in inoculated calves.

Dassanayake et al. has reported studies suggesting that leukotoxin is the most important virulence factor of *B. trehalosi* and *M. haemolytica* [6]. In the challenge study calves in the group inoculated with leukotoxin positive *B. trehalosi* and in the group inoculated with *M. haemolytica* demonstrated significant increases in serum concentrations of Hp-MMP 9 when compared to pre-inoculation concentrations. The increase occurred at day 3 for the calves in the leukotoxin positive *B. trehalosi* group and at day 7 for the calves in the *M. haemolytica* group. Several conclusions can be drawn from this observation. Our findings support those of Dassanayake et al. [6] that the ability of an organism to produce leukotoxin is an important virulence factor. Serum concentrations of Hp-MMP 9
appear to be a more sensitive diagnostic test for acute inflammation in calves with bacterial respiratory disease than serum concentrations of Hp. The faster rise in serum concentrations of Hp-MMP 9 for calves challenged with leukotoxin positive *B. trehalosi* than for calves challenged with *M. haemolytica* also supports our finding that the immune system in the calves’ lungs may more rapidly identify and respond to the leukotoxin insult from *B. trehalosi* compared to *M. haemolytica*.

Dassanayake et al. has also demonstrated the ability of *B. trehalosi* to inhibit the growth of *M. haemolytica* in vitro and has suggested that the same inhibition may occur in vivo in bighorn sheep [7]. The lack of a significant difference between the group of calves inoculated with a combination of *B. trehalosi* and *M. haemolytica* and the group of calves inoculated with BHI broth with respect to percent lung involvement, rectal temperature, respiratory or depression score, and serum Hp or Hp-MMP 9 concentration supports the hypothesis that the inhibition occurs in vivo in cattle as well.

In our study *B. trehalosi* was not isolated from the lung of inoculated calves after day 3 post inoculation. This suggests that by itself *B. trehalosi* does not persist long term in the bovine lung. In field cases where it is cultured from pneumonic bovine lung, it is biologically plausible that *B. trehalosi* is a secondary invader that has overgrown *M. haemolytica* which was responsible for the primary lung damage.

In conclusion, this study’s results suggest that *B. trehalosi* may not be an important primary pathogen of respiratory disease in cattle. It is likely a secondary, or opportunistic, BRD pathogen. Culture of this organism from diagnostic submissions should not necessarily be interpreted or cited as a primary cause of respiratory disease but may still have a role as a secondary pathogen or an opportunistic invader. Based on the results of
this study, *B. trehalosi* may be a component of BRDC, but it should not be considered a sufficient cause of BRDC in cattle.

**Suggestions for Future Research**

It may be prudent to repeat this challenge model using different strains of *B. trehalosi* as more research is reported and understanding increased about the importance of an organism’s ability to produce leukotoxin and other potential virulence factors. Endoscope assisted inoculation may also remove potential differences in diagnostic results from misplaced inoculum. Future studies should also consider the interaction of *B. trehalosi* with other bovine respiratory pathogens.

If serum concentrations of Hp-MMP 9 are to be used commercially as an ante-mortem diagnostic test for BRD a rapid, economical test will need to be developed and a reference range for healthy cattle established.

**References**


multocida and other respiratory pathogens in the nasal tract of Scottish calves. *Veterinary Record* 2010, **167**(15):555-560.
