

ACUTE PHASE PROTEINS IN NATURALLY OCCURRING RESPIRATORY
DISEASE OF FEEDLOT CATTLE: A NOVEL APPROACH TO DIAGNOSIS

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ABSTRACT

ACUTE PHASE PROTEINS IN NATURALLY OCCURRING RESPIRATORY DISEASE OF FEEDLOT CATTLE: A NOVEL APPROACH TO DIAGNOSIS

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Bovine respiratory disease (BRD) is the most costly disease of feedlot cattle in the United States. Costs associated with BRD have been estimated from \$13.90 to \$15.57 per head with annual losses to the cattle industry exceeding \$750 million. A presumptive diagnosis of BRD is usually based on clinical signs including elevated rectal temperatures. Physical exam alone lacks high sensitivity and specificity, leading to misclassification and unnecessary treatment or failure to treat true cases. More sophisticated diagnostic tests exist but are not practical in feedlot settings. The aim of this study was to evaluate three acute phase proteins [Haptoglobin (Hpt), Lipopolysaccharide binding protein (LBP) and Transferrin (Tf)] in feedlot cattle with naturally occurring respiratory disease diagnosed by a calf health scoring chart (CHSC). Seventy-seven beef calves was observed for signs of Bovine Respiratory Disease (BRD) during the first 28 days after arrival at the feedlot. Fourteen cases and pen matched controls were selected based on the CHSC. BRD cases were defined as a score of ≥ 5 , while controls were defined as a score ≤ 4 . The mean CHSC score in cases was 6.9 which was significantly greater than the controls 2.8 ($P < 0.01$). Mean plasma LBP and Hpt concentrations were significantly greater in cases than controls ($P < 0.01$). Our study results show that measurement of Hpt and LBP could be useful in detecting respiratory disease in feedlot conditions. Transferrin concentrations between the two groups were not statistically different.

Chapter 1

Bovine respiratory disease and acute phase proteins in cattle

Literature review

Introduction

Bovine respiratory disease (BRD) is the most common disease among feedlot cattle in the United States, accounting for approximately 75% of feedlot morbidity and 50%-70% of all feedlot deaths (Edwards, 2006). BRD causes between \$800 million and \$900 million annually in economic losses in the United States alone (Edwards, 2010; Wittum et al., 1996; USDA, 2007). Costs associated with its prevention, treatment, morbidity, and mortality have been estimated from \$13.90 to \$16.26 per head (Snowder et al., 2006; Faber et al., 1999; USDA, 2000a). Economic losses stem from cost of prevention, treatment, and production costs, as well as from decreased performance of beef calves. Annual losses to the US cattle industry are estimated to approach \$900 million and preventive and treatment costs are over \$3 billion annually (Griffin, 1997). The development of BRD is multifactorial and is influenced by a combination of host, environment and pathogen factors. Susceptibility to viral and bacterial pathogens is influenced by anatomy, physiology and management of beef cattle.

Susceptibility to BRD

A series of events is typically associated with BRD, starting with management related stressors, including prolonged transport, commingling, overcrowding, adverse weather,

dust, weaning, processing, high humidity, poor ventilation, and sudden ration changes (Loneragan et al., 2001; Sanderson et al., 2008). This stress compromises the defense mechanisms of the immune system in the host. Viruses invade the nose and lungs due to weakened immune barriers. Viruses damage the epithelium lining of the upper airways and compromise the mucociliary apparatus (Ames, 2002; Caswell et al., 2007). The thin fluid film of mucus in the apparatus traps foreign particles (dust, mold and pollen) and microorganisms (Ackermann et al., 2010), and along with coordinated movements of the cilia towards the pharynx, will aid in clearing the respiratory tract, as the contents are either swallowed or expelled via coughing. Compromise of the mucocilliary apparatus, allows overgrowth of normal respiratory inhabitants as well as harmful bacterial pathogens, and allows the pathogens to gain access to deeper airways.

Cattle are very susceptible to BRD because of anatomical conformation like small nostrils limiting airflow, resulting in increased breathing effort and a propensity to drying and irritation of the narrow throat passage, allowing viruses and bacteria to invade a small lung field.

The pathogens most commonly implicated in BRD are bovine herpesvirus 1 (Infectious Bovine rhinotracheitis virus, IBR), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), bovine parainfluenza virus 3 (PI-3), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* (Schneider et al., 2009, Traven et al., 2001). *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*, are ubiquitous and normal bacterial flora of the bovine

nasopharynx (Apley 2003; Cooper et al., 2010; Edwards 2010; Nikunen et al., 2007; Panciera et al., 2010).

Bovine respiratory disease diagnosis

Diagnosis of BRD is often difficult. A presumptive diagnosis of BRD is usually based on clinical signs including depression, inappetence and elevated rectal temperatures.

Physical exam alone lacks high sensitivity (61.8 %) and specificity (62.8 %), leading to misclassification and unnecessary treatment or failure to treat true cases (White et al., 2009). Clinical signs and gross pathology in respiratory disease are rarely pathognomonic, which makes it difficult to identify the causative agent(s). Necropsy and diagnostic testing for BRD pathogens is still considered the gold standard test to diagnose BRD (Fulton et al., 2012). More intricate modalities such as thoracic ultrasound and radiography, are also available to help in the diagnosis of BRD.

Abutarbush et al., (2012) assessed thoracic ultrasonography in commercial feedlot cattle related to the first diagnosis of presumptive BRD. Based on thoracic ultrasonography at enrollment, animals diagnosed with severe clinical BRD (post-arrival fever cases) were more likely to have lung lesions than animals diagnosed with less severe clinical disease (post-arrival no fever cases or post-arrival controls). However, the identification of lung lesions at enrollment was not associated with subsequent animal health outcomes (treatment, wastage, or mortality) in any of the explorations, and ultrasonography, therefore, would not be considered useful in general commercial production settings. These results suggest that clinical assessment combined with measurement of rectal temperature may be used successfully to classify animals into groups with higher and

lower probabilities of lung lesions based on thoracic ultrasonography (Aburtabush et al., 2012). Rabeling et al., (1998) assessed the value of ultrasonography as an imaging technique for the assessment of respiratory diseases in 18 calves, using postmortem findings as the gold standard. The investigators calculated the sensitivity and specificity of the technique for the detection of bronchopneumonia to be 0.85 and 0.98, respectively. Reinhold et al., (2002) inoculated 17 calves intratracheally with *Pasteurella multocida*, found statistically significant positive correlations between the ultrasound scores and the pathological findings. The results of radiography and ultrasonography were compared on 56 horses and cows with lower respiratory tract disease (Reef et al., 1991). They concluded that ultrasonography was more sensitive than radiography for the detection of small pleural effusions and consolidations in large animals and that radiography is the best technique to characterize lesions deep within the lung when the periphery of the lung is normal. A different study by Braun et al., (1996) found that ultrasonography of the lungs, pleura, and mediastinum in healthy cows provides information that can be used as a reference when examining cattle with suspected disease of the thorax. The sensitivity of thoracic auscultation to detect lung consolidation was 5.9% in a study that looked at 106 calves (Buczinski et al., 2013). When adding calf respiratory scoring chart and previous BRD treatment by the producer, sensitivity of detection increased to 71.4% (40/56). This study showed that thoracic auscultation is of limited value in diagnosing lung consolidation in calves and that ultrasonographic assessment of the thorax could be a useful tool to assess BRD. A retrospective study looking at medical records of 42 cattle that underwent both thoracic radiographic and postmortem examinations, determined the sensitivity and specificity of radiographs for identifying cows with thoracic lesions to be

94% and 50%, respectively. In this study, with a prevalence of thoracic lesions of 86%, the positive- and negative-predictive values were 92% and 57%, respectively (Masseau et al., 2008). The conclusion was that bovine thoracic radiographs are useful in detecting thoracic lesions in cows (Masseau et al., 2008). Tegtmeier et al., (2000) found a good agreement between the radiological and post mortem findings, when looking at thoracic radiology as a tool to diagnose pulmonic lesions in young calves.

The diagnostic approach of bovine respiratory disease (BRD) in cattle is becoming more sophisticated. The laboratory diagnostics of BRD is directly connected with the isolation and identification of pathogens linked to BRD, including viruses and bacteria. Currently diagnostic laboratories use two diagnostic approaches. The first approach mainly consist of serological methods, including tests like virus neutralization tests and virus isolation tests. These tests were historically thought to be the reference assays for the diagnosis of BRD. The second approach to BRD diagnosis are polymerase chain reaction assays (PCR and real time-PCR) (adapted from Cooper et al., 2010; Timsit et al., 2010; Larsen et al., 1999; Nicholas et al., 2003). Detection of virus-specific DNA or RNA with the polymerase chain reaction (PCR) method provides a sensitive and specific diagnostic approach and combined reverse transcription–PCR (RT-PCR) assays have been developed for diagnosis of several RNA viruses (Belak et al., 1993; Larsen et al., 1999). Klima et al., (2014) observed high concordance between nasopharyngeal swabs and lung tissue for PCR detection of *Mannheimia* spp. and BVDV, indicating that nasopharyngeal swabs may provide a representative profile of the involvement of these agents in acute

BRD mortalities. Table 1. In appendix is a nice summary comparing uses of diagnostic tests along with their strengths and weaknesses.

These assays are not routinely used to diagnose field based BRD because of expensive cost, specialized training, inability to provide instant results chute side and impracticality of their use. There have been six Clinical scoring systems for BRD described in the literature. Thomas et al., (1977) developed the first system to classify the severity of BRD in calves experimentally inoculated with BRSV or BVDV. This system is far too complex and impractical to be used in field settings as it monitors 17 predictors, including hematologic data. A more user friendly system based on identification of five clinical signs was developed by McGuirk (1998) at the University of Wisconsin at Madison. The calf health scoring chart (CHSC) assigns each calf the sum of the nasal discharge, rectal temperature, cough scores and the greater one of the two scores from the ocular discharge and head/ear carriage (McGuirk, 2008). Calves whose total score is ≥ 5 are categorized “BRD cases”, while healthy animals are defined as a score ≤ 4 . A third system known as DART (Depression, Appetite, Respiration and Temperature) was developed to identify beef cattle for BRD treatment in feedlots, but it is difficult to standardize because the clinical sign weights and decision points are not defined (Panciera et al., 2010). Love et al., (2014), developed three additional scoring systems where the individual assesses the presence or absence of ocular discharge, nasal discharge, ear droop or head tilt, respiratory quality and spontaneous coughing. Calves with abnormal ear or head carriage, or calves with nasal discharge and one other clinical sign, or calves that have any three clinical signs are BRD cases based on the BRD scoring

system. Only calves with nasal discharge or calves with two other clinical signs (spontaneous coughing, ocular discharge or abnormal respiratory) would require handling the calf to measure its rectal temperature and confirm BRD status if the temperature is >102.5°F. These aforementioned tests are based on subjective evaluations from the observers for the diagnosing of BRD.

Acute phase proteins (APP's) are a new frontier in the effective diagnosis of BRD in veterinary clinical practice. These might prove to be very helpful, and as objective data, could complement the use of subjective diagnostic aids such as the calf health scoring charts. Acute phase proteins are a large heterogeneous group of proteins mainly secreted by hepatocytes (Ruminy et al, 2001) whose plasma concentrations increase or decrease in response to tissue injury, infection, inflammation, parasite infestation, stress or trauma. Acute phase proteins have been widely studied in human medicine, as biomarkers of diseases, inflammatory processes and various infections (Jain et al., 2011). They have been integrated into the process of diagnosing and monitoring disease response and treatment. The use of different acute phase proteins as biomarkers of disease in veterinary medicine is less well documented, especially in monitoring health and detection of diseases in cattle. In recent years, increased focus has been placed in the research and Acute phase proteins in veterinary species.

The Acute Phase Response

The acute phase response (APR) in animals is a complex process by which the host system responds to alterations in homeostasis, caused by tissue injury, infection, inflammation, parasite infestation, stress or trauma, leading to a range of metabolic and

biochemical cascades. These processes cause the synthesis of reactants (APP's) during the APR, with the ultimate objective of restoring homeostasis in the host. The APR is considered a part of the innate immune system, a nonspecific host immune response, and a first line of defense against invading pathogens. This initial process is a major pathophysiologic phenomenon, allowing the body time to mount the highly specific adaptive immune response. The innate immune system's objective is to contain the infection regardless of the invading offender, and to prevent further injury of organs and to limit growth of infective pathogens. During this initial phase, the efforts of the host are directed towards removal or neutralization of the inflammatory stimulus, initiation of repair and restoration of homeostasis. Key metabolic changes observed during this initial response are the increased or decreased liver production of acute phase proteins. Various stimuli from tissue injury, bacterial and viral infections, parasite infestation, neoplasia, foment the complex cascade of events in the initial APR. The inflammatory process is initiated at the site of the insult or injury through a collaborative effort of plasma, tissue macrophages, platelets and endothelial cells. Bacterial infections usually lead to a strong systemic acute phase response (Alsemgeest et al., 1994), due to the strong reaction of monocytes and macrophages. These cells release primary inflammatory mediators, such as histamine, leukotriene's, prostaglandins, and the pro-inflammatory cytokines IL-1 β , tumor necrosis factor- α (TNF- α) and interferon-gamma (INF- γ) (Martin et al., 1999). TNF- α and IL-1 β are activated in response to endotoxin (Schindler et al., 1990). The Acute phase response is normally milder in response to viral infections (Alsemgeest, 1994). These pro-inflammatory cytokines are responsible for induction of a number of physical changes clinically characterized by fever, anorexia, catabolism of muscle cells

and activation of white blood cell precursors in the bone marrow, and macrophages (Dinarello, 1983; 1989; van Miert, 1995). TNF- α , IL-1 β and IFN γ also play an important role in induction of IL-6, IL-8 and other metabolic components such as prostaglandins, leukotrienes and nitric oxide (van Miert, 1995, Eckersall et al., 2010, Petersen et al., 2004). During the APR, TNF- α , IL-1 and IL-6 play a pivotal role (IL-6 being the main player) in activating hepatocyte receptors in order to initiate the synthesis of varying acute phase proteins (Heinrich et al., 1990). Ligand-cytokine receptor interaction results in tyrosine phosphorylation and the activation of STAT proteins 3 and 1 (Gregory et al., 1998). Homodimers and heterodimers composed of activated STAT3 and STAT1 subsequently translocate from the cytoplasm to the nucleus where they bind characteristic enhancer elements and initiate gene transcription (Gregory et al., 1998). After activation, the Kupffer cells form IL-6 and present it to the hepatocytes. IL-6 depresses mononuclear phagocytic production of IL-1 and TNF- α (Schindler et al., 1990) thus mitigating the whole cascade reaction. Down-regulation of the hepatic APR is achieved by rapid hepatic removal of circulating cytokines (Heinrich et al., 1990) and release of IL-10 by the Kupffer cells which results in suppression of the local IL-6 production (Knolle et al., 1995).

The initial APR is accomplished once the infective organisms are isolated and destroyed, followed by a repair phase mediated mainly by macrophages (Serhan et al., 2005). In order to effectively transition from inflammation to healing phase, it is crucial to switch from pro-inflammatory mediators (i.e. prostaglandins) to anti-inflammatory mediators (i.e. lipoxin) (Serhan et al., 2005). Lipoxins inhibit recruitment of neutrophils and,

instead, promote the recruitment of circulating monocytes, which remove dead cells and initiate tissue remodeling (Serhan et al., 2005). If the inflammatory response is effective at palliating the effects of the causative phenomena, the level of acute phase proteins returns to normal within days or weeks, and the host returns to normal function. The strength of the stimulus for inflammation determines the degree of change in the concentration and duration of acute phase proteins in plasma. Levels of acute phase protein will continue at high levels as long as the stimulus remains. When the host does not adequately mitigate an insult, this local response may escalate into a systemic reaction characterized by the induction of changes such as, pain, fever, and increased release of systemically acting mediators like vasopressin, insulin-like growth factor (IGF), corticotropin-releasing factor (CRF), corticotropin, and others. In more severe instances, the host will undergo further systemic changes, including; hematopoietic (i.e. leukocytosis and thrombocytosis), metabolic (cachexia) and lipid metabolism changes (Berczi et al., 1996). Deregulations in the delicate balance between pro-inflammation and repair, could lead to chronic inflammation and disease.

Acute Phase Proteins

The study of serum proteins in animals was commonly conducted in research investigations in the mid-1900s but a more precise emphasis in the study of acute phase proteins in veterinary medicine was not reported until the early 1990s. Application of acute phase proteins in the diagnosis and prognosis of disease in animal species are an area that has received much attention in the veterinary research world in the past twenty years. Acute phase proteins are phylogenetically old and found in mammals, birds,

marsupials and fish (Murata et al., 2004). The acute phase proteins can be produced by both hepatocytes and peripheral tissues. The plasma concentrations of the acute phase proteins are related to the severity of the disorder and the extent of tissue damage (Hagbard et al., 2004). Positive acute phase proteins are those whose concentrations increase with disease or inflammation. These are further classified as major, moderate, or minor, depending on the magnitude of changes from base line in blood. Major acute phase proteins will increase 10-100 fold, moderate acute phase proteins will increase 2-10-fold, and minor acute phase proteins will only slightly increase 1-5 fold. Negative acute phase proteins are those whose concentrations decrease from their normal baseline during inflammatory responses (Ceron et al., 2005; Eckersall & Bell, 2010). Major acute phase proteins are normally low in serum concentrations in the serum of healthy animals, but will increase 10-100 fold on stimulation, reaching a peak 24-48 hours after the insult and fall rapidly during recovery (Niewold et al., 2003). Moderate acute phase proteins will increase their concentrations 5-10 fold, reaching a peak concentration 2–3 days after stimulation and decrease more slowly than major acute phase proteins (Eckersall, 2006). Minor acute phase proteins show a gradual increase of 50-100 % over normal values. Acute phase proteins have a wide range of functions in the host. Studies in domestic animals have led to the identification of several acute phase proteins originally described in humans and laboratory animals (Eckersall et al., 1988). Of the numerous acute phase proteins which have been identified in humans, a number have been examined in cattle. Despite the uniform nature of the APR, there are numerous differences in the acute phase characteristics between different animal species (Pyörälä, 2000). C-reactive protein is a good example of this phenomenon: in healthy humans it is practically negligible, but has

a high relative increase during infections, whereas in healthy cattle it is present, but does not increase markedly during the acute phase response (Steel et al., 1994). Biochemical, physiological and clinical investigations into haptoglobin (Hp), lipopolysaccharide binding protein (LBP), Serum amyloid A (SAA), fibrinogen, α 1-acid glycoprotein (AGP), ceruloplasmin, albumin, Tf and C-reactive protein of cattle amongst others have been reviewed with the emphasis on their role in response to tissue damage. Although most acute phase proteins are regarded as biomarkers of inflammation, in this paper, we focus on Hp, LBP and Tf.

Haptoglobin

Hp is the most studied acute phase proteins in cattle. The main function or activity of Hp is centered on the formation of an Hp–Hemoglobin (Hb) complex (Murata et al., 2004). Hp is the principal scavenger of free hemoglobin in blood. Hp has bacteriostatic effects by binding free Hb, thus making iron unavailable for bacteria growth and proliferation. Binding Hb serves an additional anti-oxidant role of iron stabilization, resulting in a diminished oxidative damage to albumin, lipids and tissues. Hp also plays a key role in the recruitment of neutrophils in the early phase of inflammation. Hp can dampen the inflammatory reactions by binding of the complex Hp–Hb to CD163 of monocytes/macrophages resulting in the up regulation of anti-inflammatory mediators (i.e. IL10) (Nielsen et al., 2006). Angiogenesis enhancement and chaperone activity are also well documented activities of Hp (Park et al., 2009). Many studies have indicated the significance of Hp as a clinically useful parameter for measuring the occurrence and

severity of inflammatory responses in cattle. Skinner et al., (1991) evaluated serum Hp in inflammations in cattle and introduced Hp as an inflammatory indicator.

Haptoglobin and bovine respiratory disease

Using an experimental model of BRD induced by a challenge of calves with bovine herpesvirus type-1 and *Mannheimia haemolytica*, Godson et al., (1996) observed a temporal relationship between the increase in Hp concentration in serum and the onset of bacterial infection. They concluded that quantification of Hp could be a valuable diagnostic and prognostic aid in animals with BRD. Heegaard et al., (2000) found strong and reproducible acute phase responses for Hp and SAA, peaking at around 7–8 days after inoculation of BRSV. The magnitude and the duration of the Hp response was found to correlate well with the severity of clinical signs (fever) and with the extent of lung consolidation while SAA responded most rapidly to infection. Serum Hp concentration upon arrival to the feedlot was not an effective predictor of BRD incidence and had no significant effect on net returns or the number of BRD treatments in a backgrounding phase. Serum Hp concentration increased as the number of antimicrobial treatments increased and could potentially be used to predict the number of treatments a calf would require (Berry et al., 2004). Retrospective analysis of acute phase protein concentrations in growing calves suffering from bronchopneumonia, showed that, both Hp and fibrinogen (Fb) could be used for identification of calves requiring an anti-inflammatory treatment. Hp and Fb were useful predictors of inflammation severity; most of the animals (80%) that received anti-inflammatory treatment after clinical examination

presented pathological Hp and/or Fb values upon onset of disease. Hp alone was able to confirm > 75% of case decisions whether diseased calves were treated or not (Humblet et al., 2004). In a study by Grell et al. (2005), calves experimentally infected with BRSV displayed the highest Hp concentrations at approximately 7-9 days post-inoculation. Calves presenting with the most severe clinical symptoms (respiratory rate, rectal temperature) had the highest Hp levels, while calves presenting with the mildest clinical symptoms displayed the lowest levels of acute phase reaction. Ulutas et al., (2011) demonstrated that serum concentrations of Hp and SAA were increased in persistently infected cattle animals with BVDV and that the measurement of SAA and Hp could be of value indicating suspected persistently infected animals with BVDV in screening herds for general health status. Gånheim et al., 2003 reported similar Hp results between calves infected with Bovine Viral Diarrhea (BVD) virus alone and a coinfection between BVD virus and *Mannheimia haemolytica*. Experimental induction of pneumonia by inoculation of *P. multocida* A3 irrespective of the number of bacteria has shown Hp plasma concentrations to increase, thus making Hp a valuable protein biomarker for the evaluation of disease (Dowling et al., 2002). In feedlot cattle with clinical respiratory tract disease a high but variable Hp response was observed by Wittum et al., (1996). Carter et al. (2002) found that serum Hp concentrations in transported feedlot cattle were greater in calves treated more than once, compared to calves not treated or treated only once. On days 0 and 7, Hp concentrations were higher in calves requiring more than one treatment compared to calves with one treatment determination and evaluation of serum Hp showed that this protein could be a valuable factor in the diagnosis of inflammatory diseases (Alsemgeest et al., 1994). Estimation of acute phase proteins has also been

looked at as a tool for controlling the effectiveness of vaccination program. In a study by Stefaniak et al. (1997), nine calves were immunized with Somnuvac® vaccine at the fourth and eighth weeks of life. The calves were on a farm with a high incidence of respiratory tract infection. No elevated (> 0.2 g/l) Hp levels were observed and no clinical signs of disease occurred in the experimental group. Among nine control, non-immunized calves at 8, 9 and 12 weeks of life, every time one (but not the same) animal had increased Hp levels, then two others were found coughing and with an elevated body temperature (>39.5 °C). The determination of Hp levels confirmed the protective influence of vaccination against respiratory tract inflammation in the endangered herds.

Haptoglobin and bovine mastitis

Hirvonen et al., (1996) reported an increase in Hp as an inflammatory factor in heifers with mastitis. Hp concentrations increase in milk from cows with clinical mastitis and subclinical mastitis (Hiss et al., 2007) and in milk from cows with experimentally induced chronic sub-clinical *Staphylococcus aureus* mastitis (Grönlund et al., 2005). In this study they found that healthy udders had undetectable levels of Hp and Hp levels were significantly increased in challenged cows. In a different study, the quantity of bacterial DNA in milk samples was associated with concentrations of Hp activity in the milk (Kalmus et al., 2013). There was a linear correlation between the severity of mastitis and Hp concentrations. Hp in these two studies provided a good indicator of the mammary gland inflammation in milk, and provided useful support for the bacteriological diagnosis of mastitis. Other studies also found serum concentrations of Hp and SAA to be increased in cases of cattle mastitis (Gerardi et al., 2009; Petersen et al.,

2004; Safi et al., 2009). Wenz et al., (2010) found Hp levels in milk to be higher in cows affected by clinical mastitis versus cows with mild systemic disease. Another research project (Roozbahani et al., 2011), studied the relationship between somatic cell count (SCC) and blood concentrations of haptoglobin (Hp) and serum amyloid A (SAA), to determine the ability of these proteins as reliable indicators for monitoring udder health and subclinical mastitis with SCC in dairy cows. The results showed a positive significant correlation ($P < 0.01$) between SAA and SCC but this correlation for Hp and SCC were not significant. In a different study by a different group (Gultiken et al., 2012), they found a good correlation between SCC and Hp. Hirvonen et al., (1996) found serum Hp as the most effective factor in the diagnosis of severity and prognosis of mastitis. Salonen et al., (1996) observed an increase in serum Hp in cows suffering from *E. coli* mastitis. Eckersall et al., (2001) reported elevation of Hp in serum and milk in mild mastitis.

Haptoglobin and bovine gastro intestinal tract disease

Studies by Okamoto et al. (1998) and Jawor (2007) demonstrated increased serum Hp concentrations in calves with diarrhea, and subsequent decrease during treatment of the animals. In the study by Okamoto et al. (1998), 35 of 73 calves with diarrhea (47.9%) had detectable serum Hp levels (ranging from 0.05 to 0.7 g/l). In Jawor (2007) study, 40% of calves had an Hp concentration > 0.1 g/l. Deignan et al., (2000) found median Hp levels significantly increased within 3 days of experimentally infected challenge with a mixture of three *Salmonella* serotypes (*S. Dublin*, *S. Enteritidis* and *S. Heidelberg*) (0.21 g/l) and decreased by day 5. Serum Hp concentrations were found to significantly correlate with

clinical measures of disease severity: fecal and morbidity scores. Serum levels of Hp and SAA have been measured in cows with left displaced abomasum, right displaced abomasum or abomasal volvulus and the values were most strongly associated with liver fat percentage than with the alteration in abomasum's so an increase in SAA or Hp may indicate the presence of hepatic lipidosis in cattle with abomasal displacement (Guzelbektes et al., 2010).

Haptoglobin and bovine reproductive tract disease

Parturition increases the Hp concentration but not the concentration of Alpha-1-acid glycoprotein in cows (Uchida et al. 1993) and Hp was detectable in the serum of all animals within 3 days after calving (Alsemgeest et al., 1993). In a study by Chan et al. (2010), cows with acute puerperal metritis had significantly higher Hp concentrations than those in the healthy group throughout the 6 months after delivery, but the higher concentrations of SAA in the metritis group were significantly different in a shorter period (between 4 days and 2 months postpartum). The highest Hp concentration was found in the period of 0-3 day's postpartum (1.1 ± 0.43 g/l), whereas for SAA it was 4-7 days postpartum (85 ± 23 mg/l). The Hp concentration may be also used to evaluate the efficacy of therapy (Mordak, 2008). Cows with Retained placentas were divided into two groups with or without manual removal of the membrane. Ten days after calving the highest Hp concentration was found in the group where the placenta had been expelled after 4 days (2.22 ± 0.36 g/l), and the lowest was found in cows where the placenta had been easily removed manually (0.9 ± 0.3 g/l). For cows where placental removal was not

complete or where they had been expelled within 4 days, the Hp concentrations were not statistically different (1.83 ± 0.2 vs. 1.53 ± 0.27 g/l, respectively).

Haptoglobin, animal welfare and carcass quality

In slaughtered cattle without evident disease activity Hp-values were unmeasurable, whereas in most cases with disease activity Hp values were elevated. Serum amyloid A values were found to be elevated in animals with overt lesions, but Hp appears to be a useful variable to discern cattle without disease activity from those with lesions (Gruys et al., 1993). Saini et al. (1998) proposed that a potential application of acute phase proteins might be in screening and separating healthy from diseased animals. In emergency slaughtered cattle it was found that Hp and AGP levels were raised, indicating that examination of the acute phase proteins could help in improving food safety (Hirvonen et al., 1997). The determination of acute phase protein levels (Hp, Fb and albumin levels) seems to be one of better methods allowing the health and welfare of animals in large herds, based on the examination of blood or milk samples obtained from representative groups of animals (Nikołajczuk et al., 2000).

Haptoglobin and bovine neonates

Increased Hp concentrations (>0.1 g/l) were found in the serum of 32% of calves at the 48th hour of life. By the 25th day of life, 31% of the calves showed elevated Hp levels. Because Hp is not commonly detected in healthy cattle (Eckersall et al., 1988), it was surprising that a high rate of calves showed elevated Hp levels at the 48th hour and the 25th day of life. It was concluded that the calves had been at risk of an inflammatory

process from first few hours of life, since the increase in Hp concentrations after infection with the bacterial agent occurs within 24 hours (Ganheim et al., 2003). The highest Hp levels, as well as the highest individual differences, occurred in the calves of the group showing failure of passive transfer (Ig levels below 5 g/l at the 48th hour of life).

Haptoglobin and other bovine diseases

Hp has also been widely investigated in other inflammatory diseases in cattle.

Concentration of Hp in serum increases following abscess formation, endotoxin administration and post-operation (Alsemgeest 1994). Increased serum or plasma Hp concentration in cattle was found after trauma (Earley et al., 2002; Fisher et al., 2001), experimental local aseptic inflammation (Eckersall et al., 1988), various acute infections under field conditions (Alsemgeest et al., 1994; Skinner et al., 1991), castration (Earley et al., 2002; Fisher et al., 2001), metritis (Smith et al., 1998), severe uterine bacterial post-partum infection (Sheldon et al., 2001), off feeding for 3 days (Kato et al., 2002; Lipperheide et al., 1997), transportation for 2 days (Murata et al., 1993) and after major injuries at slaughter/culling (Hirvonen et al., 1997).

Lipopolysaccharide binding protein

Lipopolysaccharide binding protein (LBP) is a soluble 50 kDa polypeptide that binds to bacterial lipopolysaccharide (LPS) to elicit innate immune responses. LBP presents the LPS to important cell surface (membrane bound) pattern recognition receptors CD14 and TLR4, found in monocytes, macrophages and granulocytes. LBP binds to the amphipathic Lipid A functional group of LPS, facilitating the process of LPS degradation

into a monomer and further impelling the transfer to membrane bound CD14. This presentation via LBP enhances their pro-inflammatory activity by 100 to 1000 fold. LBP can also bind Lipoteichoic acid (LTA), a molecule exposed on the cell wall of Gram-positive bacteria. The interaction between LBP and LTA triggers a pro-inflammatory cascade via TLR-2 activation and its subsequent activation of innate immunity. Like other acute phase proteins, LBP is also an opsonin, and as such will bind to bacteria and their byproducts to promote their phagocytosis. Although the liver is probably responsible for the production of most of circulating LBP, Rahmana et al., (2010) provided information, previously unavailable, of a widespread extra hepatic expression of LBP in several normal bovine tissues. This study showed that many of the tissues which are likely to be involved in mucosal innate defense may locally produce LBP that is expressed even in non-pathological conditions. This study also demonstrated LBP expression in glial cells in brain and cerebellum, paralleling increased LBP concentrations found in cerebrospinal fluid of patients with neurological disorders (Heumann et al., 1995). Consistent with previous reports in humans (Vreugdenhil et al., 1999) and mice (Vreugdenhil et al., 2000), LBP was found throughout the bovine intestinal epithelium (rumen, reticulum and omasum). The finding of LBP in bovine salivary glands (parotid and submandibular) parallels that of human gingival tissue (Ren et al., 2004 and Ren et al., 2005).

Lipopolysaccharide binding protein and bovine respiratory disease

Bovine LBP along with SAA, Hp and alpha1-acid glycoprotein (AGP) was evaluated as inflammatory markers during an outbreak of bovine respiratory disease (BRD) caused by

bovine respiratory syncytial virus (BRSV). A group of 10 Holstein Friesian calves (7 males, 3 females) was followed weekly over a 6-week period starting 1 week (week 0) before the manifestation of the first clinical signs of BRD. Concentrations of SAA and LBP increased at week 1, peaked in values at week 3 and decreased at week 4 during this outbreak. The researchers concluded that both LBP and SAA were sensitive markers of respiratory infection (Orro et al., 2011). Horadagoda et al., (1995), demonstrated a four-fold increase in the serum concentration of LBP 36 h after a single intratracheal inoculation of *Manheimia haemolytica* A1. The findings of this study also indicated that cattle possess a LPS detection mechanism comparable to that described in man and experimental animals in which LBP forms complexes in serum with circulating LPS enhancing the signal to the immune system to mount a host response. Nikunen et al., (2007) studied calves with respiratory disease and determined that *Pasteurella multocida* was associated with significant increases in LBP, fibrinogen, Hp, SAA and AGP serum concentrations. Schroedl et al., (2001) measured plasma levels of LBP in a group of 20 calves experimentally infected with Gram-negative *Mannheimia haemolytica* in comparison to Hp. In infected calves, LBP levels rose significantly 6 h after infection, reaching a maximum at 24 h. Hp concentrations significantly rose after 12 h, and peak responses were measured 48 h after infection. The production of bovine LBP by respiratory type II epithelial cells corresponds to what has been reported in humans and mice lung (Dentener et al., 2000). The local presence of LBP may contribute to host defense against bacterial endotoxins and bacteria, and confirms the respiratory epithelial cells as an additional micro anatomical compartment where a local acute phase reaction

can develop, since another acute phase protein, namely AGP, can be produced by respiratory epithelial cells in bovine tissues (Lecchi et al., 2009).

Lipopolysaccharide binding protein and bovine mastitis

LBP has been previously identified in milk acting as an acute phase protein during innate immunity responses to intramammary infection (Bannerman et al., 2004) or in naturally occurring mastitis (Zeng et al., 2009). Blood and milk concentrations of the acute phase protein lipopolysaccharide-binding protein (LBP) were evaluated in cows with naturally occurring mastitis. Blood and milk samples were collected from 101 clinically healthy dairy cows and 17 dairy cows diagnosed with clinical mastitis. Microbiological testing identified coagulase-negative staphylococci (CNS) as the most prevalent pathogen among the subclinically infected quarters. The most commonly isolated CNS species in the study herds were *S. epidermidis*, *S. simulans*, *S. chromogenes*, *S. hemolyticus*, and *S. xylosus*. The second most prevalent pathogen isolated was *Staphylococcus aureus*. Together, these 2 pathogens accounted for 84% of the subclinical intramammary infections in this study. Concentrations of LBP (measured by ELISA) were greater in the blood and milk of cows with clinical mastitis than in those with healthy quarters. Concentrations of LBP also differed between uninfected and subclinically infected quarters with low somatic cell count. Blood concentrations of LBP in cows with subclinical intramammary infections could not be differentiated from those of cows with all healthy quarters.

Lipopolysaccharide binding protein and bovine neonates

Orro et al., (2008) demonstrated that LBP, Hp, SAA and AGP serum concentrations were high after birth, and concentrations showed a gradual decrease during the first 3 weeks of life. The lowest concentrations were at 21 days of age, after which concentrations stabilized. The results indicated that post-partum elevation of acute phase proteins is associated with the birth process and/or factors in colostrum and not necessarily with disease-related processes. This stresses the importance of considering a calf's age when interpreting acute phase proteins concentrations in serum. High concentrations of LBP in the host prove to reduce the toxicity of endotoxins, thus contributing to the neutralization of LPS effects, an event that ultimately drives to their detoxification and protection from septic shock (Wurfel et al., 1994 and Lamping et al., 1998).

Transferrin

Transferrin (Tf) is an iron-binding blood plasma glycoprotein synthesized by the liver, with a molecular weight of around 80 KD and contains two specific high-affinity Fe (III) binding sites. Tf is the major circulating iron binding protein and correlates with total iron binding capacity of plasma. Tf is responsible for 50% to 70% of the iron binding capacity of serum. The physiological role of decreased synthesis of such proteins is generally to save amino acids for producing "positive" acute-phase proteins more efficiently. Each Tf molecule can carry two iron ions in the ferric form (Fe^{3+}), with each ion coupled with a carbonate ion. The protein contains an array of amino acids that are perfectly arranged to form four bonds to the iron ion, which locks it in place. Once it finds iron atoms, Tf flows through the blood until it finds a Tf receptor on the surface of a

cell (Ogunnariwo et al., 1990). Tf binds tightly to the Tf receptor on the surface of a cell (importantly, to erythroid precursors in the bone marrow), and is consequently transported into the cell in a vesicle. Acidification of the vesicle in the cell, causes Tf to be released and thus allowing transport of the receptor through the endocytic cycle back to the cell surface (recycled back), ready for another round of iron uptake. Triggered by the neutral pH of the blood, the receptor releases the empty Tf, and it continues its job of gathering iron. Tf is also associated with the innate immune system. Tf is found in the mucosa and binds iron, thus creating an environment low in free iron, where few bacteria are able to survive. The levels of Tf decrease in inflammation, appearing contradictory to its function. A decrease in the amount of Tf would result in hemosiderin in the liver. Tf has a bactericidal effect on bacteria, in that it makes Fe³⁺ unavailable to the bacteria. Tf imbalance can have serious health effects for those with low or high serum Tf levels. An increase in Tf is seen in iron deficiency anemia, characterized by microcytosis and hypochromasia. Conversely, it is decreased in conditions associated with increased protein loss, such as nephrotic syndrome, chronic renal failure, severe burns, and protein-deficiency states and in severe liver disease. The negative acute-phase proteins are less dramatic in their response to acute inflammation compared to the positive acute-phase proteins

Transferrin and ruminant respiratory disease

A study by McNair et al. (1998) demonstrated that, the negative acute phase protein Tf could also be useful in calves. Although serum Tf levels remained within the reference range, in calves that were experimentally infected with *Histophilus somni*, its

concentration was associated with lung lesions. In fact, the lowest Tf concentrations were measured in calves with extensive lesions, and there were significant decreased differences between mean values (on days 1-6) between the group with no lesions and the group with extensive lesions.

Transferrin and bovine mastitis

Secretions from quarters infected by a major pathogen contained on average a higher concentration of Tf versus non infected quarters. The correlation coefficient between transferrin and somatic cell-count (0.65) in the study shows that a relationship exists between transferrin milk content and inflammation (Rainnard et al., 1982). Sanchez et al., (1988) found the ratio transferrin/albumin to be four times greater in colostrum than in mature or mastitic milk. Colostrum contains lactoferrin and transferrin, important glycoproteins which are key parts of the innate immune system. Transferrin binds iron, creating an environment low in free iron – which in turn, makes it difficult for any invasive bacteria to survive.

Transferrin and other bovine diseases

Rioux et al., (2008) performed a study looking at *Fasciola hepatica* infection of Corriedale sheep and analyzed different biomarkers, including Tf, during the first 12 weeks of infection. The results showed an increase in serum transferrin levels in response to induced anemia in sheep during fasciolosis. Tf levels increased in intensity during the biliary phase of infection at week 9 post infection.

Moser et al., (1994) determined Tf (Tf) concentrations in cattle in various physiological states, in energy-deficient (ketotic) cows, in situations of several acute and chronic infections, after endotoxin administration and in animals with bovine leucocyte adhesion deficiency (BLAD). Tf concentrations varied between 1.5 and 8.5 g/l and in healthy animals were in the range of 2.0 and 6.6 g/l. Tf concentrations in adult animals were smaller than in young animals and increased in veal calves with iron deficiency above 8 g/l, resulting in a negative correlation between Hb and Tf. Chronic infectious diseases (such as Johne's) were characterized by relatively low Tf levels (below 2 g/l), while during acute infections, after endotoxin-administration and during ketosis Tf concentrations were not changed. In spite of the severe liver congestion and renal loss of Tf, cattle with cardiomyopathy had considerably higher concentrations of serum Tf than healthy cattle. This increase may help to compensate for the low serum concentration of iron in the affected animals.

Conclusion

Acute phase proteins offer a new frontier into a more dynamic and effective diagnostic process of BRD in veterinary clinical practice. There is an increasing body of evidence in the literature to support the utilization of acute phase proteins as biomarkers of inflammation in cattle. Acute phase proteins are valuable indicators of the manifestation and severity of pathological conditions in clinical and other field studies of calves. As previously discussed acute phase proteins are very sensitive, but also non-specific, and potential confounding factors, such as age, stress factors and presence of other diseases and infections should be carefully kept in mind while interpreting results. There is a

broad spectrum of possible applications of acute phase proteins-based diagnostics for use with cattle. LBP may prove to be a more sensitive diagnostic marker in cattle infection and is faster than Hp in detecting sepsis (Schroedl et al., 2001). In terms of response time, LBP Acute phase proteinsears to be superior to Hp as an early marker of infection, as its concentrations in blood increased by 4 hours versus 8 hours with Hp. Although SAA (Horadagoda et al., 1999) and LBP have been shown to be more sensitive acute phase protein than Hp, some studies have concluded that SAA is not useful marker of respiratory disease in field conditions (Berry et al., 2004, Carter et al., 2002). Serum SAA concentrations have been reported to be influenced by physical stress, which may partially explain its unpredictability. There is a renowned need for the development and optimization of rapid field tests that allow rapid determination of acute phase proteins after collection of blood from animals. Increased availability of assays to measure the circulating concentrations of the acute phase proteins should give a further stimulation to the use of acute phase proteins in clinical diagnosis, monitoring of health in production animals and research studies. The presence of strongly elevated acute phase protein concentrations before institution of treatment, combined with prognosis, may be helpful in making more informed decisions about whether or not to establish therapy. Acute phase proteins concentrations could also aid in monitoring the course of the disease and the efficacy of the chosen therapy. The nondiscriminatory determination of animal health is important due to the increasing focus of consumers and farmers on the welfare of animals, and antibiotic resistance, called by some experts as one of the world's most pressing public health problems. This literature review supports the usefulness of acute phase proteins measurements to determine the magnitude of inflammatory changes in the

animal, to abet and facilitate electing proper therapy, and to monitor its efficacy. This in turn should facilitate application of acute phase proteins for the more proficient determination and diagnosis of respiratory diseases in feedlot cattle.

HYPOTHESIS AND SPECIFIC AIMS

The objective of this study was to determine the concentration of three acute phase (Hpt, LBP and Tf) proteins in field cases of naturally occurring bovine respiratory disease as diagnosed by a calf health scoring chart (University of Wisconsin School of Veterinary Medicine). Acute phase proteins offer a new frontier into a more dynamic and effective diagnostic process of BRD in veterinary clinical practice. There is an increasing body of evidence in the literature to support the utilization of acute phase proteins as biomarkers of inflammation in cattle.

We hypothesized that LBP and Hpt levels would be higher in BRD cases vs controls in our study as diagnosed by the WI calf health scoring chart. We also hypothesized that Transferrin, being a negative acute phase protein would be lower in BRD cases versus healthy controls.

Chapter 2

Manuscript

TITLE PAGE

Short communication: Acute phase proteins in naturally occurring respiratory disease of feedlot cattle.

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Short communication: Acute phase proteins in naturally occurring respiratory disease of feedlot cattle. I. Idoate, B. Vander Ley, L. Schultz, Meera Heller

Abstract

The aim of this study was to evaluate three acute phase proteins [Haptoglobin (Hpt), Lipopolysaccharide binding protein (LBP) and Transferrin (Tf)] in feedlot cattle with naturally occurring respiratory disease diagnosed by a calf health scoring chart (CHSC). Seventy-seven beef calves was observed for signs of Bovine Respiratory Disease (BRD) during the first 28 days after arrival at the feedlot. Fourteen cases and pen matched controls were selected based on the CHSC. BRD cases were defined as a score of ≥ 5 , while controls were defined as a score ≤ 4 . The mean CHSC score in cases was 6.9 which was significantly greater than the controls 2.8 ($P < 0.01$). Mean plasma LBP and Hpt concentrations were significantly greater in cases than controls ($P < 0.01$). Our study results show that measurement of Hpt and LBP could be useful in detecting respiratory disease in feedlot conditions. Transferrin concentrations between the two groups were not statistically different.

Keywords: bovine respiratory disease, acute phase proteins, haptoglobin, lipopolysaccharide binding protein, transferrin

Introduction

Bovine respiratory disease (BRD) is the most common disease among feedlot cattle in the United States, accounting for approximately 75% of feedlot morbidity and 50%-70% of all feedlot deaths (Edwards, 2010). BRD causes between \$800 million and \$900 million annually in economic losses (Edwards, 2010; Wittum et al., 1996; USDA, 2007).

Economic losses stem from prevention costs, treatment, death, associated costs and from diminished average daily gain, and decreased feed efficiency.

The development of BRD is multifactorial and is influenced by a combination of host, environment and pathogen factors. Susceptibility to viral and bacterial pathogens is influenced by anatomy, physiology and management of beef cattle (Taylor et al., 2010).

Respiratory disease diagnosis can be confirmed using an assortment of methods.

Necropsy and detection of BRD pathogens remain the gold standard tests to diagnose BRD, however use of clinical scoring systems is widespread and useful for lay people to systematically evaluate and classify sick cattle. There have been six Clinical scoring systems for BRD described in the literature which rely on evaluation of a variety of clinical signs and assignment of scores based on the evaluator's impression (Thomas et al., 1977; McGuirk, 1998; Panciera et al., 2010; Love et al., 2014).

Bacterial infections usually lead to a strong systemic acute phase response (Alsemgeest et al., 1994), due to the marked activation of monocytes and macrophages and release of inflammatory mediators, such as histamine, leukotriene's, prostaglandins, and the pro-inflammatory cytokines IL-1 β and tumor necrosis factor- α (TNF- α) (Martin et al., 1999). TNF- α , IL-1 and IL-6 play a pivotal role in activating hepatocyte receptors (HepG2 or Hep3B cells) to initiate the synthesis of various APPs (Heinrich et al., 1990, 1998; Gruys et al., 2005). Binding of IL-6 to cell surface receptors, results in tyrosine phosphorylation and the activation of STAT pathway to upregulate gene transcription (Gregory et al., 1998). Down-regulation of the hepatic APR is achieved by rapid hepatic removal of circulating cytokines (Heinrich et al., 1990, 1998) and release of IL-10 by the Kupffer cells which results in suppression of the local IL-6 production (Knolle et al., 1995;

Baumann et al., 1994). The initial APR is down regulated once the infective organisms are isolated, cellular debris is removed, and macrophages initiate tissue repair (Serhan et al., 2005).

Haptoglobin (Hpt) is a positive APP, and is the principal scavenger of free hemoglobin in blood (Murata et al., 2004). Haptoglobin elicits bacteriostatic effects by binding free Hb, thus making iron unavailable to proliferating bacteria. Iron utilization is a conserved process that has been identified in multiple bacterial pathogens, including *Vibrio sp.*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella flexneri*, and *Bacillus subtilis*. (Skaar, 2010). Hpt also plays a key role in the recruitment of neutrophils in the early phase of inflammation (Riollet et al., 2000).

LBP is a soluble polypeptide that binds to bacterial lipopolysaccharide (LPS) and presents the LPS to pattern recognition receptors CD14 and TLR4, found on monocytes, macrophages and granulocytes. The presentation of LPS via LBP enhances the pro-inflammatory activity of these innate immune cells by 100 to 1000 fold (Fierer et al., 2002). LBP can also bind Lipoteichoic acid (LTA), a pathogen recognition molecule exposed on the cell wall of Gram-positive bacteria. The interaction between LBP and LTA triggers a pro-inflammatory cascade via TLR-2 activation (Mogensen, 2009).

Transferrin (Tf) is an iron-binding blood plasma glycoprotein found in mucosa. Tf has a single polypeptide chain of about 700 amino acids and contains two specific high-affinity Fe³⁺ binding sites, for the transport of iron in the circulation (Ceron et al., 2005; Oliveira et al., 2014). Tf is a negative APP whose concentration falls during the acute phase response (Nguyen, 1999). Acute phase response to infection or inflammation can lead to marked anemia (Feldman et al., 1981). Tf elicits indirect bacteriocidal effects by binding

free iron, thus making iron unavailable to proliferating bacteria, in a response that may be mediated by lipocalin (Flo et al., 2004).

Previous studies using challenge models have shown that all three of these APPs may be useful biomarkers for bacterial pneumonia in calves, however no studies have been done to assess APP levels in naturally occurring pneumonia in a feedlot setting (Ganheim et al., 2003; Conner et al., 1989; Schroedl et al., 2001; Dowling et al., 2002; Heegard et al., 2000; McNair et al., 1998).

The purpose of this study was to evaluate three acute phase proteins [Haptoglobin (Hpt), Lipopolysaccharide binding protein (LBP) and Transferrin (Tf)] in naturally occurring respiratory disease of feedlot cattle diagnosed by calf health scoring chart (CHSC). The hypothesis was that there would be a significant difference between the acute phase protein levels evaluated, between the clinical cases and their matched healthy controls, as diagnosed by a CHSC.

Materials and Methods

Animals

For this study, 77 mixed breed beef steer calves were purchased at three area livestock auction barns for enrollment in a nutritional study. Animals were transported 30-230 miles to and housed at the University of Missouri Beef Research and Teaching Farm (BRTF). At arrival the animals were weighed, ear tagged [both Allflex visual identification (VID) & Radio-frequency identification (RFID)], vaccinated with Bovi-Shield Gold® 5 and One Shot Ultra® 8 (Zoetis, Florham Park, NJ, USA) and treated with Cydectin® Pour-On (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA).

All procedures were approved by the University of Missouri Institutional Animal Care and Use Committee. The calves were part of a post-weaning feedlot performance and feed efficiency tests using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). All calves were housed six per pen. Pens were of open construction, measuring 4.9×8.84 m. Frost-free waterers were shared between two pens, and feed bunks and slabs were under sloped roof shades (4.9×6.7 m).

Clinical examination

Bovine respiratory disease was diagnosed by an experienced animal technician based on a calf health scoring chart developed at the University of Wisconsin. The CHSC assigns each calf the sum of the nasal discharge, rectal temperature, cough scores and the greater one of the two scores from the ocular discharge and head/ear carriage (Table 1. McGuirk, 2008). Calves whose total score was ≥ 5 were categorized “BRD positive”, while controls were defined as a score ≤ 4 .

Fourteen steers were selected as cases based on their score and were not diagnosed with any other diseases during the study. Control animals ($n=14$) were selected from contemporary pen mates and sampled at the same time point.

Animals in study were identified with electronic identification and correspondent visual identification tags in parenthesis.

1. Case group: Electronic I.D 225999 (7502), 226020 (7503), 51258 (7506), 51238 (7508), 226081 (7512), 51285 (7516), 51304 (7518), 51216 (7520), 51229 (7522), 51365 (7524), 51279 (7526), 51267 (7528), 51247 (7532) and 51156 (7534).

2. Control group: Electronic I.D 226051 (7505), 226051 (7505), 51216 (7507), 51152 (7509), 51222 (7513), 51315 (7517), 51151 (7519), 51164 (7521), 51295 (7523), 225990 (7525), 226002 (7527), 51239 (7529), 51236 (7533) and 51239 (7535).

Samples

Blood samples were collected from BRD clinical cases and their matched healthy controls selected from the same pens, using the coccygeal veins. The blood was allowed to clot at 4°C. After clotting, the samples were centrifuged at 2800 xg for 20 min at 4°C. Serum was harvested and frozen at -20°C for later analyses.

Acute phase proteins analysis

Concentration of LBP was assayed using a commercially available Human LBP ELISA kit (Cell Sciences Inc., Canton, MA) that recognizes both human and bovine LBP, as described previously (Bannerman et al., 2003). Plasma samples were diluted 1:100 and assayed according to the manufacturer's instructions. The mean optical density (OD) of standard duplicates, reference serum, and the samples were calculated. A standard curve was designed by plotting the mean OD of the standards (b-f, y axis) and the LBP concentration (x axis).

LBP ELISA assay procedure:

Prior to initiation of the assay, all reagents and samples were allowed to reach room temperature.

Samples

1. 100 µl of standards (50, 25, 12.5, 6.25, 3.12, 1.5 ng/ml = vial a-f) reference serum, and diluted samples were pipetted in duplicate into the corresponding wells of the pre-coated modules.

The 1/200 dilution of sample was accomplished in two steps.

A. 50 µl of serum sample was placed in a tube and 950 µl of Dilution buffer was added (this gave us a 1/20 dilution).

Dilution buffer was prepared by adding contents of Vial 6 with 50 ml PBS. 50 µl of Tween 20 were then added.

B. 100 µl of above mixture (1/20 dilution) was placed into 900 µl of the prepared dilution buffer (this provided us with the desired 1/200 dilution).

2. The plate was incubated for 1 hour at room temperature using a plate shaker.

3. The wells of the plate were manually washed three times using prepared wash buffer.

The PBS/Tween 0.05% wash buffer was prepared by dissolving 1 tablet phosphate buffered saline (PBS, Vial 5) in 200 ml distilled water, and by adding 100 µl Tween 20.

4. Detection antibody (ready to use as supplied in vial). 100 µl detection antibody was added to each well and incubated the plate at room temperature for 1 hour using a plate shaker.

5. The plate was manually washed three times with prepared PBS/Tween 0.05% wash buffer.

6. Substrate (ready to use as supplied in vial). 100 μ l substrate solution was pipetted to each well. The plate was then incubated for 11 + 1 min in the dark at room temperature without shaking.
7. Stopping reaction. 100 μ l of stop solution (ready to use as supplied in vial) was pipetted to each well. The plate was then tapped gently to mix.
8. The absorbance of the wells were read at 450 nm using a VersaMax ELISA Microplate Reader.
9. LBP concentration were calculated using the mean optical density (OD) of standard duplicates, reference serum, and the samples.
10. A standard curve was then designed by plotting the mean OD of the standards (b-f, y axis) and the LBP concentration (x axis).
11. We calculated the LBP concentration from the mean OD of samples from the standard curve and multiply by the dilution factor (in this case 200).

Haptoglobin concentrations were determined with a commercially available Bovine Haptoglobin ELISA kit (Immunology Consultants Laboratory, Inc., Portland, OR) according to the manufacturer's instructions. Samples were diluted at 1:50, 1:200, 1:1,000 and 1:2,000 ratios and assayed according to manufacturer's instructions. Samples were diluted until observed values fell within the standard curve.

Haptoglobin ELISA assay procedure:

Prior to initiation of the assay, all reagents and samples were allowed to reach room temperature.

Samples

1. 100 μL of standards, standard 0 (0.0ng/ml), standard 1 (15.6ng/ml), standard 2 (31.25ng/ml), standard 3 (62.5ng/ml), standard 4 (125ng/ml), standard 5 (250ng/ml), standard 6 (500ng/ml) and standard 7 (1000ng/ml) were pipetted in duplicate.

2. 100 μL of 1/50 dilution sample were pipetted in duplicate into pre designated wells

To prepare a 1/50 dilution of sample, 5 μL of serum sample was transferred to 245 μL of 1X diluent. The Diluent Solution supplied was a 5X Concentrate and was diluted 1/5 with distilled or deionized water as instructed (1 part buffer concentrate, 4 parts dH₂O).

3. The micro titer plate was incubated at room temperature for fifteen (15 ± 2) minutes. The plate was kept covered and level during incubation.

4. The wells of the plate were manually washed four times using prepared wash buffer.

The Wash Solution supplied was a 20X Concentrate and was diluted 1/20 with distilled or deionized water as instructed (1 part buffer concentrate, 19 parts dH₂O). The wells were completely filled with wash buffer, and then inverted the plate then pour/shake out the contents in a waste container. This step was followed by sharply striking the wells on absorbent paper to remove residual buffer.

5. 100 μL of appropriately diluted Enzyme-Antibody Conjugate was pipetted to each well. The plate was incubated at room temperature for fifteen (15 ± 2) minutes. The plate was kept covered in the dark and level during incubation.

The Enzyme-Antibody conjugate was prepared by calculating the required amount of working conjugate solution for each microtitre plate test strip by adding 10 μL Enzyme-Antibody Conjugate to 990 μL of 1X Diluent for each test strip to be used for testing.

6. The plate was washed and blotted as described in Step 4.
7. 100 μL of TMB Substrate Solution (ready to use as supplied) was then pipetted into each well.
8. The plate was incubated in the dark at room temperature for precisely ten (10) minutes.
9. After ten minutes, add 100 μL of Stop Solution (ready to use as supplied) was pipetted to each well.
10. The absorbance of the wells were read at 450 nm using a VersaMax ELISA Microplate Reader.

Steps 1-10 were repeated for dilutions of 1/100, 1/1000 and 1/2000 in order to accommodate for results outside of reading range.

- 1/100 dilution was prepared by transferring 10 μL of serum sample to 990 μL of 1X diluent.
- 1/1000 dilution was prepared by transferring 100 μL of 1/100 serum sample to 900 μL of 1X diluent.
- 1/2000 dilution was prepared in three steps.

Step 1. 50 μL of sample into 950 μL of 1X diluent (1/20 dilution).

Step 2. 100 μL of 1/20 dilution sample into 900 μL of 1X diluent (1/200 dilution)

Step 3. 100 μL of 1/200 dilution sample into 900 μL of 1X diluent (1/2000 dilution)

Transferrin concentrations were determined with a commercially available Bovine Transferrin ELISA kit (Immunology Consultants Laboratory, Inc., Portland, OR) according to the manufacturer's instructions. Samples were diluted at 1:40,000 and assayed according to manufacturer's instructions.

Transferrin ELISA assay procedure:

Prior to initiation of the assay, all reagents and samples were allowed to reach room temperature.

Samples

1. 100 μL of standards, standard 0 (0.0ng/ml), standard 1 (18.75 ng/ml), standard 2 (37.5ng/ml), standard 3 (75ng/ml), standard 4 (150ng/ml), standard 5 (300ng/ml) and standard 6 (600ng/ml) were pipetted in duplicate.

2. 100 μL of 1/40,000 dilution sample was pipetted in duplicate into pre designated wells

To prepare a 1/40,000 dilution of sample, 5 μL of serum sample was transferred to 495 μL of 1X diluent (resulting in 1/100 dilution). Then, 2 μL of the 1/100 dilution sample was added to 798 μL of 1X diluent solution (resulting in 1/40,000 dilution). The Diluent Solution supplied was a 20X Concentrate and was diluted 1/20 with distilled or deionized water as instructed (1 part buffer concentrate, 19 parts dH₂O).

3. The micro titer plate was incubated at room temperature for (30 \pm 2) minutes. The plate was kept covered and level during incubation.

4. The wells of the plate were manually washed four times using prepared wash buffer.

The Wash Solution supplied was a 20X Concentrate and was diluted 1/20 with distilled or deionized water as instructed (1 part buffer concentrate, 19 parts dH₂O). The wells were completely filled with wash buffer, and then inverted the plate then pour/shake out the contents in a waste container. This step was followed by sharply striking the wells on absorbent paper to remove residual buffer.

5. 100 µL of appropriately diluted Enzyme-Antibody Conjugate was pipetted to each well. The plate was incubated at room temperature for fifteen (20 ± 2) minutes. The plate was kept covered in the dark and level during incubation.

The Enzyme-Antibody conjugate was prepared by calculating the required amount of working conjugate solution for each microtitre plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing.

6. The plate was washed and blotted as described in Step 4.

7. 100 µL of TMB Substrate Solution (ready to use as supplied) was then pipetted into each well.

8. The plate was incubated in the dark at room temperature for precisely ten (10) minutes.

9. After ten minutes, add 100 µL of Stop Solution (ready to use as supplied) was pipetted to each well.

10. The absorbance of the wells were read at 450 nm using a VersaMax ELISA Microplate Reader.

All samples for the three APP studied were analyzed with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at a wavelength of 450 nm.

Statistical analysis

Differences in BRD scores, LBP, Hpt and Tf between cases and contemporary control animals were analyzed and carried out using Stata® 13 software analysis system (StataCorp LP, College Station, Texas, USA). The Shapiro test was used to appraise normal distribution. Since all values were not normally distributed or in the case of LBP and BRD scores were normally distributed but had unequal variances, a non-parametric test, Kruskal-Wallis equality-of-populations rank test was performed. A P-value 0.001 was used as the level of significance for all tests.

Results and Discussion

BRD scores, serum LBP and Hpt concentrations were considerably higher for BRD clinical cases than for their matched healthy controls (see Fig 1) as diagnosed by the Wisconsin calf health score chart. The differences were statistically significant between cases and controls ($p=0.001$). The negative APP Tf evaluated in our study, proved to be statistically not different between cases and controls ($p=0.5656$). Our study result shows that measurement of HPT and LBP is associated with clinically diagnosed respiratory disease under field conditions, and the levels correlate with those previously described in challenged experimental studies (Ganheim et al., 2003; Conner et al., 1989; Schroedl et al., 2001; Dowling et al., 2002; Heegard et al., 2000; McNair et al., 1998).

Schroedl et al. (2001) showed an early significant increase in LBP concentration in a group of 20 calves experimentally infected with Gram-negative *Mannheimia haemolytica*

6 hours after bacterial inoculation. In this same study, haptoglobin levels did not significantly rise before 12 hours. Ganheim et al., 2003 studied Hpt levels in calves experimentally challenged with bovine viral diarrhea virus (BVDV) and/or *M haemolytica*, demonstrating Hpt levels similar to the ones found in our study. Conner et al., 1989 and Dowling et al., 2002, challenged calves experimentally with *M haemolytica* and *Pasteurella multocida* respectively. In both studies, the concentration levels were significantly higher in the challenged animals versus the study controls.

Receiver operating characteristic (ROC) curves were calculated in our study to determine an ideal cutoff value for LBP and Hpt concentrations. The ROC curve is created by plotting the fraction of true positives out of the total actual positives (TPR = true positive rate) vs. the fraction of false positives out of the total actual negatives (FPR = false positive rate), at various threshold settings. Sensitivities, specificities and percent correct classifications are listed as a function of cutoff value in Table 2. Another important measure of the accuracy of the clinical test is the area under the ROC curve. A perfect classification is an area that equals to 1.0. This test is 100% accurate because both the sensitivity and specificity are 1.0 so there are no false positives and no false negatives. The area under ROC curve for Hpt in our study was 0.9235 and for LBP was 0.9668 (Fig 2). This data suggests a very accurate classification for both LBP and Hpt ideal concentration cutoffs for our study. On the other hand a test that cannot discriminate between normal and abnormal corresponds to an ROC curve that is 0.5. The calculated area under ROC curve for Tf was 0.4674, suggesting that Tf concentrations were not accurate in differentiating BRD cases from healthy controls.

In our study, the WI calf health scoring chart used to diagnose BRD ended up being a very user friendly and efficient scoring system at differentiating BRD cases from controls, even in a feedlot setting, where calves were screened for naturally occurring BRD. Although originally instituted for neonatal dairy calves, the system proved efficient discriminating cases and controls in our study, as it did in the study by Love et al., (2014) where they looked at developing a novel clinical scoring system for on-farm diagnosis of bovine respiratory disease in pre-weaned dairy calves. Other clinical scoring systems are also available for diagnosing BRD. DART (Depression, Appetite, Respiration and Temperature) is a clinical scoring system developed to identify beef cattle for BRD treatment in feedlots, but it is difficult to standardize because the clinical sign weights and decision points are not defined (Panciera et al., 2010). Love et al., (2014), developed three additional scoring systems where the individual assesses the presence or absence of ocular discharge, nasal discharge, ear droop or head tilt, respiratory quality and spontaneous coughing. These scoring system determines an animal to be BRD positive, when it has an abnormal ear or head carriage, or calves with nasal discharge and one other clinical sign, or calves that have any three clinical signs. These clinical scoring tools are of practical interest in the cattle industry because they are based on clinical signs that can be easily identified by producers. Buczinski et al., (2014) showed that adding the clinical score assessment to thoracic auscultation and thoracic ultrasonography, increased sensitivity of BRD diagnosis in pre-weaned Dairy Calves, although decreases specificity when compared with lung consolidation findings. The recommendation of the WI CHSC is to treat calves because of high BRD presumption if

the score is ≥ 5 , and to monitor calves with scores of 4. Calves with scores of ≤ 3 are considered healthy.

Although APPs appear to be very sensitive, we must also recognize that they are also non-specific, and potential confounding factors, such stress factors, age of the animal and presence of concurrent diseases should be cautiously conserved in mind while deciphering results. When bovine respiratory disease (BRD) outbreaks occur, veterinarians might steer away from expensive, complex ancillary ante mortem tests requiring long turnaround times. Instead, the initial actions are often directed toward electing the appropriate therapy. More specific diagnostics are needed in gaining knowledge to help guide future vaccination, metaphylaxis, or treatment programs. There is a prominent demand for the development and optimization of rapid diagnostic field tests.

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Table 1. Comparison of uses of diagnostic tests along with their strengths and weaknesses

Test	Use	Positives	Negatives
Serology	Ab detection	Detect vaccine responses and past infections. High sensitivity. Effective even relatively late in course of disease.	Titers do not infer resistance and are not able to differentiate vaccine induced vs infection acquired Ab's. Requires convalescent serum, results not available >3weeks.
Culture-nasal, nasopharynx, trachea, BAL	Detect bacteria and viruses	Demonstrate colonization and active infection. Can sample cases and controls.	Positive culture does not necessarily mean lung infection. Extended time to get results. Viral infections are transient. Bacterial isolates from nasal samples are of dubious significance.
Culture-lung lesions	Detect bacteria and viruses	Need active replication of pathogen at time of death. Can determine antibiotic resistance	Sensitivity not best. Extended time to get results.
IHC	Detects antigen in lung lesion	Localize infectious agent within lesion.	Sensitivity and Specificity depend on available monospecific immune serum or monoclonal Ab's to specific infectious agent.
Single PCR-nasal, nasopharynx, trachea, BAL	Detects genetic material of pathogen in sample	Provides evidence of presence of pathogen	Cannot differentiate incidental from concurrent infection. Cannot determine antimicrobial resistance

Single PCR-lung	Detects region of pathogen genome	Potential evidence that pathogen associated with disease	May not differentiate causative pathogen from MLV vaccine
Multiplex PCR- nasal, nasopharynx, trachea, BAL, lung	Detects regions of several pathogen genomes	Single tests detects several pathogens. More info than single PCR.	May not represent causative infection pathogen versus MLV vaccine

Adapted from Fulton et al., 2012; Cooper 2012; Fulton et al., 2012; Montgomery 2009.

Table 2. Adapted from McGuirk SM. 2008

Calf Health Scoring Chart			
0	1	2	3
Rectal Temperature			
100-100.9 °F	101-101.9 °F	102-102.9 °F	≥ 103 °F
Cough			
None	Induce single cough	Induced repeated coughs or occasional spontaneous cough	Repeated spontaneous cough
Nasal discharge			
Normal serous discharge	Small amount of unilateral cloudy discharge	Bilateral, cloudy or excessive mucus discharge	Copious bilateral mucopurulent discharge

Eye scores			
Normal	Small amounts of discharge	Moderate amount of bilateral discharge	Heavy ocular discharge
Ear scores			
Normal	Ear flick or head shake	Slight unilateral droop	Head tilt or bilateral droop

Table 3. The table represents the ultimate cut point concentrations for Hpt and LBP.

APP	Cutpoint	Sensitivity %	Specificity %	Correctly classified
Hpt	≥ 0.81 (mg/ml)	92.86	85.71	89.29
LBP	≥ 0.33 (µg/ml)	92.86	92.86	92.86

Fig 1. The figures represent higher BRD scores and LBP and HPT concentrations for cases versus controls in the study

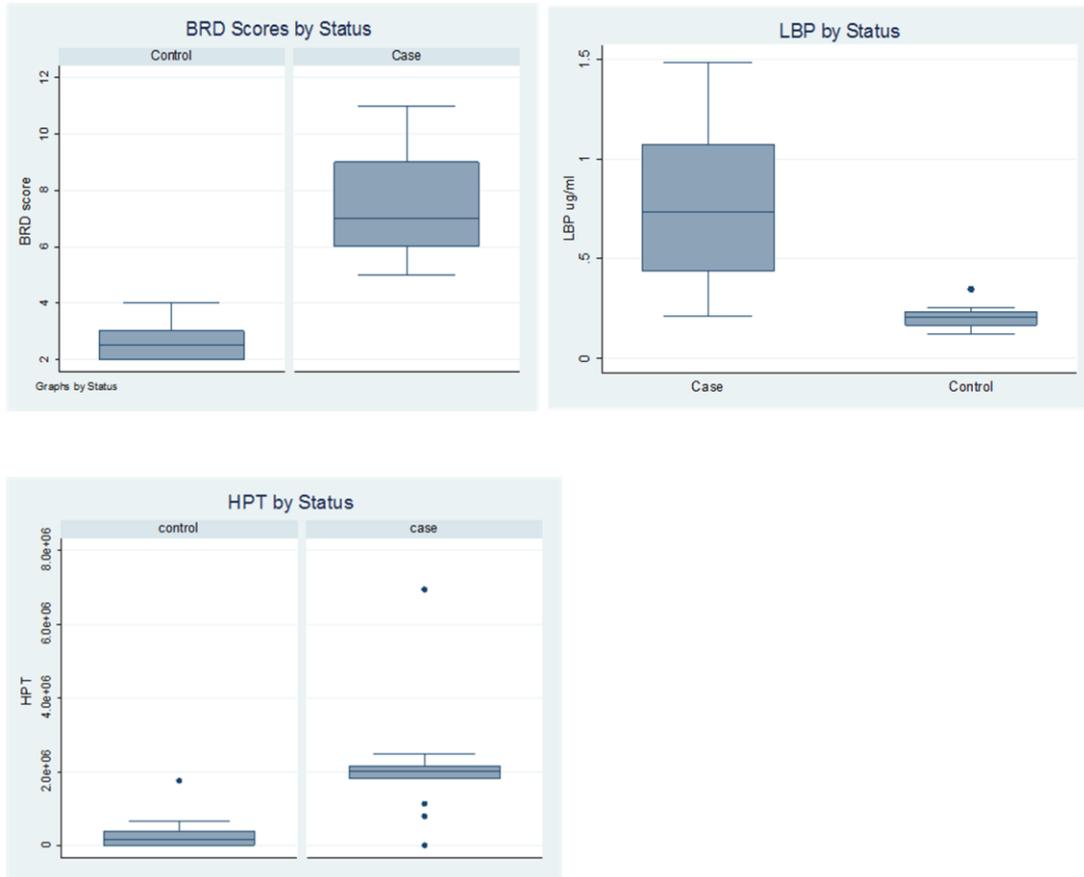


Fig 2. The two graphs show ROC curves for LBP and Hpt shows ROC curves in comparison to a calf heath scoring chart. The tests were able to accurately separate BRD cases from controls. An area of 1 represents a perfect test; an area of .5 represents a worthless test.

