Selection of animals from affected/at risk groupings

Selection of animals for sampling should consider relevance to the problem at hand. The animals selected should have the following characteristics: 1) Relationship to contemporary problems, 2) Be in the initial phases of disease; and 3) Should not have received treatment for this disease. Sampling of animals should focus on those showing the same signs as affected animals, albeit earlier on in the process.

Sampling animals after treatment or after death will have less value due to impact of antimicrobial or other therapeutics on the disease process. Animals in the “at risk” or affected groups which have chronic problems will either be of limited value or lead to findings that complicate interpretation of the problem.

Animal information should include 1) Age of animals in the group and age of those selected for sampling; 2) number of days in the group (or days on feed); 3) environmental concerns (housing, bedding, cleaning, animal movement), 4) Group morbidity and mortality and how these correlate to prior groups, 5) Duration of problem in group and time since diagnosis (duration of disease) in sampled animals; 6) Clinical signs, treatment(s) and the animal responses, vaccination(s), ancillary treatments, and clinical diagnoses (working diagnoses) for the group.

Nasopharyngeal or nasal swabs.

These are probably of value for viral testing, although sampling upper respiratory bacterial flora is of lower value in some cases. Deep nasal or nasopharyngeal swabs demonstrate good correlations with lung isolates in the diagnosis of *M. hemolytica* and *M. bovis* in some studies.

Transtracheal aspiration.

Trans-tracheal wash, or trans-tracheal aspiration allows one to obtain sterile culture specimens via puncture of the inter-tracheal membrane, allowing access of a small bore catheter. Producing a washing of the airway and collecting a variable amount of fluid containing cells, mucous and other debris as well as the fluid introduced in a manner which bypasses the upper airway is considered the gold standard for antemortem testing. Samples obtained, represent contributions from both the left and right lungs. In older calves and adults, this procedure is a challenge to complete and obtain sterile fluid without injuring the patient and/or the operator. Appropriate restraint of the animal is critical. Fluid collected can be used for cytologic examination, biochemical analyses and viral and bacterial identification, culture and antimicrobial susceptibility. **YOU MUST KNOW THE STATUS OF RECENT ANTIMICROBIAL THERAPY FOR THESE PROCEDURES TO BE USEFUL. Obtain specimens from untreated animals.**

Bronchoalveolar lavage.

Bronchoalveolar lavage (BAL) can be conducted in standing and restrained animals (2 halters with each tied above and to the left or right of the animal). Passing the single lumen BAL catheter via the nares into the trachea, lung and lodging in the airway is followed by instilling 20
– 120 mL saline for lavage. Bronchoalveolar lavage catheters are made with a cuff requiring inflation prior to this procedure. In this authors experience, lodging the catheter in the airway is a suitable alternative to obtaining samples from the patient. However, in this procedure, only one lung or lung lobe is sampled, and the process of blindly passing the catheter into one lung/lobe may result in sampling non-predisposed or un-affected lung. It is nearly impossible to lodge the catheter into the cranial and ventral lung lobes using either a BAL catheter or bronchoscope in an awake animal. The procedure performed on awake, un-sedated animals results in struggling and un-expected movements, resulting in lower, albeit sufficient recovery for cytology and culture. One should also consider that passage of the BAL tube via the nares with respect to results in collection of fluids, secretions and flora of the upper airway. It should be expected that bacterial pathogens associated with BRDC will be recovered in healthy animals when using the BAL procedure. Others suggest this is of less concern and culture is a viable option when using the BAL procedure. **YOU MUST KNOW THE STATUS OF RECENT ANTIMICROBIAL THERAPY FOR THESE PROCEDURES TO BE USEFUL.** Obtain specimens from untreated animals.

**Lung biopsy.**

Lung biopsy is possible, albeit rarely utilized in the diagnosis of lung injury such as that associated with BRDC. Lung biopsy can be performed with or without US guidance and provide sufficient tissue for cytologic examination (impression smears), histopathology (cores) or culture. Adversities associated with lung biopsy include hemorrhage which can vary from a mild trickle from the nares to acute death. Rapid respiratory rates are potentially likely to result in hemorrhage. Another potential problem could be pneumothorax. I have not observed this to be a problem in cattle. It is usually unilateral when present. **YOU MUST KNOW THE STATUS OF RECENT ANTIMICROBIAL THERAPY FOR THESE PROCEDURES TO BE USEFUL.** Obtain specimens from untreated animals.

**Necropsy**

Depending upon the nature of the operation, euthanasia and necropsy may be more cost effective than treatment. In larger operations, necropsy of dead or moribund animals is critical to health and welfare of the other animals in the group. Submission of bacterial cultures from non-treated animals, with compatible clinical signs and are in the early stages of the disease will provide information about organisms cycling through animals, their antibiograms and resistance (if any). **YOU MUST KNOW THE STATUS OF RECENT ANTIMICROBIAL THERAPY FOR THESE PROCEDURES TO BE USEFUL.** Obtain specimens from untreated animals.

**Inflammation and the Acute Phase Response**

Most laboratory (blood) work will reflect the summation of acute or chronic inflammation present at the time of sampling. A number of investigators have evaluated the utility of the measurement of acute phase proteins as markers of inflammation and whether or not their use is predictive of inflammation. Acute phase proteins are in general produced by the liver in response to infection, or inflammation whether by infectious, toxic or neoplastic process.

When sentinel cells, (macrophages and dendritic cells), are activated by invading microbes or microbial products, these cells release a variety of signals including cytokines (TNF-α, IL-1, IL-6), chemokines, oxidants (O$_2^-$, H$_2$O$_2$, ‘OH, and NO$^-$), and lipids (leukotrienes and prostaglandins). These molecules are responsible for “turning on” the inflammatory responses,
recruiting other cells such as neutrophils, activating other immune cells, vasodilation, increasing vascular permeability, and stimulating production of acute phase proteins.

The body’s overall response to inflammation is a complicated process involving gene expression, protein expression, and changes in physiologic responses which together form what is known as the acute phase response. Many of these changes also linger into the chronic stages of disease. The acute phase response can be defined as the change in cytokines levels, leading to changes in many plasma proteins (acute-phase proteins) as well as a large number of behavioral, physiologic, biochemical, and nutritional changes such as fever, somnolence, anorexia, leukocytosis, and decreased gluconeogenesis. A drawback of the use of acute phase proteins (APP) in clinical medicine is that it requires some degree of time for the expression of genes and transcription of genes encoding the APP.

**Acute Phase Proteins (APP)**

In human medicine an acute phase protein (APP) has been defined as one whose plasma concentration changes (either positive or negative) by at least 25 percent during inflammatory disorders. During inflammation, serum concentrations of APPs increase largely due to changes in hepatocyte production in response to up-regulation of APP genes. This gene up-regulation is stimulated by cytokines, IL-6 being the chief stimulator of the production of most acute-phase proteins.

In cattle, several APP have been evaluated to determine their usefulness as biomarkers of inflammation. These include haptoglobin (Hp), α-1 proteinase inhibitor (α-1 antitrypsin), ceruloplasmin, α-1 acid glycoprotein (AGP), serum amyloid-A (SAA), fibrinogen, and lipopolysaccharide (LPS) binding protein (LBP).

In a study examining acute mastitis, increases were shown in 3 acute phase proteins: ceruloplasmin, α1 antitrypsin, and haptoglobin. Haptoglobin (Hp) was the only biomarker absent in healthy cattle, having sharp increases associated with inflammation. Another study examined APPs during an outbreak of natural respiratory disease caused by Bovine Respiratory Syncytial virus (BRSV). This study demonstrated increases in serum SAA and LBP in weeks 1 and 3; however, the highest concentrations of Hp were observed at week 3. The investigators assumed that the second peak of SAA and LBP as well as the high concentration of Hp on week 3 was a response to secondary bacterial infection. In contrast Heegaard et al. demonstrated that, experimental infection with BRSV induced Hp and SAA concentrations at similar times with peak levels at 6-7 days post infection. The animals in this study the fastest Hp response were diagnosed at necropsy to be infected with *Pasteurella multocida*. A third study attempted to determine chronicity of disease based on APPs. Cattle were categorized into 4 groups: healthy, acute inflammation, subacute inflammation, and chronic inflammation. Significant differences were found in serum Hp concentrations between the acute and chronic stages; however, there were no significant changes in SAA. The serum Hp concentrations in cattle with chronic inflammation were significantly greater than the acute or subacute disease categories. **These studies and others indicate that Hp is the most reliable APP used as an indicator of inflammation in cattle.**

**Haptoglobin**

The main function of haptoglobin is to protect tissue from the negative effects of the inflammatory response. Hp binds free hemoglobin (Hb) through the formation of very high-affinity complexes. These complexes (but not Hp or Hb alone) bind to a macrophage receptor (CD163) that protects the host through endocytosis and subsequent intracellular degradation of Hb. This serves several functions. One is the reduction in glomerular filtration loss of free Hb
therefore supporting the recycling of iron. Another is the immediate removal of Hp-Hb complexes as source of iron that could be used for microbial growth. Finally, this clearance is important because heme and iron released from free Hb may participate in generation of reactive oxygen species (ROS) and promote tissue injury.

Other than the previously mentioned hepatocyte production of APPs, Hp is produced and stored in leukocytes, specifically neutrophils. Hp is synthesized in MYs (myelocytes/metamyelocytes) and remains stored in cytosolic granules throughout granulocyte differentiation. Also, Hp is found to be co-localized with lactoferrin, a designated marker protein of specific granules, in neutrophils and is shown to be released in response to PMA and various inflammatory stimuli.

In cattle, increased concentrations serum Hp can be found in a variety of inflammatory diseases such as bovine respiratory disease and mastitis as well as with endotoxin administration and inflammation caused by intramuscular injection of oil and turpentine. On the other hand, it is also found in a variety of non-inflammatory (metabolic) conditions such as abomasal displacement and fatty liver, as well as stress. Hp is a good indicator of response to therapy, although it appears to be unrelated to case severity or need for treatment.

Recent studies in this investigator’s laboratory demonstrated that a protein-protein complex produced by neutrophils and stored in their granules is comprised of haptoglobin and matrix-metalloproteinase 9 (MMP 9). These two proteins are stored in gelatinase granules of neutrophils covalently linked and are released upon stimulation of neutrophils after chemical induction of degranulation in vitro. The presence of protein-protein complexes are of interest to the protein biochemist in our laboratory, since their source from terminally differentiated cells: suggests a multi-step pathway with multiple functions: 1) Be targeted to bovine macrophage receptors with important functionality; 2) implication in the pathogenesis of inflammation, and 3) developmental processes and neoplasia.

We are interested in determining whether this orphan discovery would have relevance in clinical disease. Both Hp and MMP 9 are considered acute phase proteins, one has a known macrophage receptor and the other is incriminated in a variety of disorders. We selected serum from clinical cases to evaluate whether the presence of Hp-MMP 9 complexes is predictive of the type of inflammation (acute, chronic) and whether. Selection of serum was based upon as accurate a clinical diagnosis as possible, follow-up necropsy confirmation of the clinical diagnosis in comparison to the serum concentrations of this protein. Case definition was defined through the use of records review by 3 Food Animal clinicians into acute and chronic disease. Ten serum samples were obtained from healthy lactating cows at a local dairy. Once the clinical diagnosis was arrived at, these serum samples were analyzed by a 4th individual (a protein biochemist) who was blinded to the clinical diagnoses. Serum concentrations of haptoglobin, Hp-MMP 9 complexes and MMP 9 were assay using ELISA. The serum concentrations of each analyte were assigned to each case (acute or chronic) and the results (disease classification vs serum Hp, Hp-MMP 9 and MMP 9) were examined for statistical relationships by a 5th investigator (a statistician).

Although our preliminary study (a clinical study, retrospective in nature) has some obvious draw backs in terms of experimental design. It was clear that serum concentrations of Hp-MMP 9 were useful in detecting cattle with acute septic diseases. Whereas they were not present in all but 2 cows with chronic disease. One of which was a case of disseminated neoplasia (lymphosarcoma). In contrast, serum Hp was elevated in all cases of acute and chronic inflammation. Normal healthy cows had negligible concentrations of serum Hp, and no detectable serum Hp-MMP 9. Finally, serum concentrations of MMP 9 were not correlated with
either acute or chronic inflammation and were often elevated to levels present in acute or chronic inflammation in healthy, lactating animals.

We have also analyzed serum from calves given a bolus of LPS IV and demonstrated a small, but detectable peak of Hp-MMP 9 at approximately 1.5 hours post LPS infusion, which decreased to undetectable by 2 hours. In all of the time points measured (0 hours to 8 hours at 0.5 hour increments) serum concentrations of Hp remained in the normal range. These data support our hypothesis that serum concentrations of Hp-MMP 9 reflect PMN degranulation and appear to be short lived after a single LPS bolus. However, in those cows with acute onset, septic peritonitis, they were elevated due to the continuing stimulus associated with bacteremia and sepsis syndrome. Analysis of serum samples from feedlot animals at admission and periodic samples obtained during their feeding period is underway. Although the analysis is not complete, the data suggest that serum Hp is sometimes elevated when cattle have reduced rates of gain and serum Hp-MMP 9 appears to precede these elevations in serum Hp.

REFERENCES