DIAGNOSIS OF SYSTEMIC INFLAMMATORY DISEASE IN MANATEES

(Trichechus manatus latirostris)

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2004
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by

Kendal Elizabeth Harr
This thesis is dedicated to my daughters, Lillian and Maeve, whose laughter and commentary always remind me what is important and give me perspective on life.
ACKNOWLEDGMENTS

This work was supported by a grant from the Florida Fish and Wildlife Conservation Commission (s018) and could not have been completed without the exceptional mentoring provided by my committee, Dr. Nancy Denslow, Dr. William Winter, Dr. Ruthie Francis-Floyd, and especially my primary mentor, Dr. John Harvey. I would like to acknowledge the technical support of Marjorie Chow, Scott McClung, Melanie Pate, Tina Conrad, Kati Allison, Lavonne Williams, Patricia Kindland, and Maxine Sacher at the University of Florida College of Veterinary Medicine and Angie Jones at Lowry Park Zoo. I would like to thank Dr. Dave Murphy, Bob Bonde, Dr. Mark Sweat, Dr. Lucy Keith, and Dr. Cathy Walsh for help in the field and all of the biologists working with the state and federal government whose tracking and capture expertise enabled the sampling required for this study. I am also indebted to Dr. Hollis Erb for assistance with biostatistical analysis and Dr. Iske Larkin for advice and moral support. Finally, I would like to acknowledge my husband, Dr. Darryl Heard, for editing, continual commentary, and the loving support that I know that he tries to give both me and our children.
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Manatees (*Trichechus manatus latirostris*) are commonly afflicted with inflammatory and infectious disease secondary to human interaction, such as boat strike and entanglement, as well as “cold stress syndrome.” White blood cell count and fever, primary indicators of systemic inflammation in most species, are insensitive in diagnosing inflammatory disease in manatees. Acute phase response (APR) proteins, such as haptoglobin and serum amyloid A (SAA), have proven to be sensitive measures of internal inflammation/infection in domestic large animal species. This study assessed diagnosis of generalized inflammatory disease by different methods including complete blood count (CBC), albumin:globulin (A:G) ratio, gel electrophoresis analysis, C reactive protein (CRP), alpha1 acid glycoprotein, haptoglobin, fibrinogen, and SAA. Samples were collected from 71 normal and 27 abnormal animals during diagnostic medical examination. Serum amyloid A, measured by enzyme linked immunosorbent assay (ELISA), followed by A:G ratio, measured by plasma gel electrophoresis, were most
sensitive in diagnosing inflammatory disease with diagnostic sensitivity and specificity of approximately 90%. The reference interval for SAA is <10-50 µg/ml with an equivocal interval of 50-70 µg/ml. The reference interval for A:G ratio by plasma gel electrophoresis is 0.7-1.1. Albumin:Globulin ratio, calculated using biochemical techniques, was not accurate due to overestimation of albumin by bromcresol green dye-binding methodology. A:G ratio, measured by serum gel electrophoresis, has a low sensitivity of 15% due to the lack of fibrinogen in the sample. Haptoglobin, measured by hemoglobin titration, had a reference interval of 0.4-2.4 mg/ml, a diagnostic sensitivity of 60%, and a diagnostic specificity of 93%. Fibrinogen, measured by heat precipitation, has a reference interval of 100-400 mg/dl, a diagnostic sensitivity of 40%, and a diagnostic specificity of 95%.

Measurement of SAA has not been done previously in manatees and the antibody-based ELISA used was validated in this novel species. Validation of this serum amyloid A ELISA was performed according to the Clinical Laboratory Improvement Amendments. Accuracy, precision, analytical specificity, analytical sensitivity, linear range of the assay, and reference interval were determined and evaluated as adequate. This assay was assessed as a good diagnostic after it satisfied these validation criteria.

Further investigation of other potential diagnostically useful proteins that currently can not be assessed with commercially available assays was performed. Two dimensional gel electrophoresis was performed on two healthy manatees and one manatee with subacute injury. The gels were overlayed and four different proteins unique to the sick animal were identified for identitification by Edmann sequencing.
CHAPTER 1
INTRODUCTION

Leukocyte count and fever are sensitive indicators of internal inflammation and infection in many mammalian species. In a few species, e.g., cattle, leukocytosis and left shifting do not occur unless disease is severe and then may rapidly progress to leukopenia as leukocyte utilization exceeds bone marrow production. For this reason, leukocyte count, though occasionally useful in bovine species, is not a sensitive indicator of inflammation. Clinical reports from veterinarians indicate manatee leukocyte responses are similar to cattle. Additionally, adequately assessing the core temperature of a 500 kg aquatic animal is inaccurate at best. Acute phase response (APR) proteins, such as haptoglobin, are used as a primary indicator in horses and cattle because APR proteins have proven to be sensitive measures of internal inflammation/infection.\textsuperscript{1,2} In Europe, appropriate APR proteins are used as screening tests for inflammatory disease in livestock.\textsuperscript{3} There is a distinct possibility that APR proteins could be a valuable screening diagnostic for inflammation in manatees. Currently, there is no published literature on acute phase proteins and their use for identifying inflammation in manatee species.

Several practical factors must be considered when identifying and validating an APR protein for diagnosis of inflammation in manatees. These include physiologic activity of the protein within manatees, stability of the protein over time during transport with potential temperature change, accuracy of the methodology employed in identifying the protein in this species, and clinical use in different disease states specific to manatees. All of these factors must be evaluated prior to clinical use as a diagnostic test.
The purpose of this study was to identify and validate APR protein methodologies that can be applied to manatees. Several specific aims were identified within this objective and appropriate hypothesis driven experimentation was carried out according to the following outline.

Specific Aim #1: Identified methodologies that detected APR protein in manatee serum and/or plasma.

Hypothesis: Commercially available antibodies crossreact with manatee APR protein.

Specific Aim #2: Determined intra-assay variation and assessed precision

Hypothesis: Intra-assay variation of commercially available assays were within 15%

Specific Aim #3: Determined degradation and loss of the protein over time at room temperature, 4 degrees C, -20 degrees C, and –70 degrees C.

Hypothesis: The APR protein was stable at the above temperatures over time.

Specific Aim #4: Established protein reference intervals for healthy manatees and assessed sensitivity and specificity of the protein assay in predicting underlying disease.

Hypothesis: At least one of the proteins assayed was highly sensitive in detecting nonspecific inflammatory and traumatic disease.

Specific Aim #5: Identified the acute phase protein, detected by ELISA or turbidometric method, at the molecular level to assess analytical specificity.

Hypothesis: The assay was specific in detecting the protein in question.

Specific Aim #6: Identify plasma proteins produced by ill or injured manatees that may be investigated for diagnostic potential.

Hypothesis: Additional proteins to those detected by the commercially available methodologies are made during inflammation.
The following APR proteins were identified as likely diagnostic candidates based on their usage in domestic animals. Rejection of one or more hypotheses would result in rejection of the APR protein assay. It was expected that some of the antibodies tested would not crossreact with manatee protein. Therefore, at least some of the methodologies would be eliminated early in the project. Tests that are not antibody based exist for both fibrinogen and haptoglobin. Therefore, it was assumed that we would obtain results for at least these two proteins.

Acute Phase Proteins

The APR is a nonspecific response to inflammation (infection, autoimmune disease, etc.) or tissue damage (trauma, surgery, or tumors). Positive APR proteins (haptoglobin, C-reactive protein, fibrinogen, serum amyloid A, alpha_{1} acid glycoprotein, and others) are produced by the liver during APR in response to cytokines released at the site of injury to either protect the body or to combat a potential pathogen. In humans, APR proteins are nonspecific indicators of disease similar to fever or leukocyte counts. Plasma concentrations of the individual proteins change at different rates after the initial insult thus providing useful information not only about the inflammation, but also about duration of disease. Sequential measurement can also aid in assessment of treatment response.

Serum Amyloid-A

Serum amyloid-A (SAA) is produced by the liver in inflammatory states and circulates complexed to a lipoprotein. It is also elevated in many autoimmune diseases, polyarthritis, granulomatous disease, and neoplasia. Deposits of amyloid-A (AA) protein are most often found in the kidneys, liver, and spleen in chronic disease states but may be found in any organ. In horses, SAA has been reported to be a very sensitive indicator of
inflammation. In equine species, SAA is found in trace amounts in healthy animals and increases dramatically in nonspecific inflammatory states, especially bacterial and viral infections.

**Haptoglobin**

Haptoglobin (Hp) is an alpha2-glycoprotein that irreversibly binds hemoglobin (Hb). Hp-Hb complexes are large enough to prevent or greatly reduce renal filtration of free Hb in plasma and its iron. The complexes are removed rapidly by hepatocytes and Kupffer cells which degrade the proteins, and iron and amino acids are reutilized. The Hp-Hb complex is also a peroxidase, that hydrolyzes peroxides released by neutrophils at sites of inflammation. Hp also functions as a natural bacteriostatic agent for iron-requiring bacteria by preventing the utilization of hemoglobin iron by these organisms.

Haptoglobin is quickly consumed in hemolytic syndromes. Severe hepatocellular disease also results in decreased synthesis of haptoglobin. It is increased during the acute phase response. In humans, it is also increased in response to exogenous glucocorticoid and non-steroidal anti-inflammatory drug (NSAID) administration, during some protein losing syndromes such as nephrotic syndrome, and during severe biliary obstruction. Haptoglobin is undetectable in the blood of healthy cattle. In cattle with inflammation or infection, e.g., mastitis, metritis, pyometra, traumatic reticulitis, abomasal displacement, bacterial nephritis, and hepatic lipidosis, haptoglobin levels increase markedly. It has proven to be a sensitive indicator of inflammatory disease in cattle.

**Fibrinogen**

Fibrinogen was the first APR protein recognized. Increased production by the liver results in increased levels in inflammatory states, as well as pregnancy. It is integral in platelet aggregation. In the coagulation cascade, it is cleaved by thrombin to form fibrin,
the backbone of the thrombus. Though, only mildly increased concentrations are present in many species during inflammatory disease, it has proven very useful in detecting inflammation in ruminant species, specifically cattle. Clinical reports from Sea World veterinarians indicate that hyperfibrinogenemia is also the diagnostic of choice in cetacea. Hypofibrinogenemia can occur in disseminated intravascular coagulation, liver failure, and cachexia.

A heat precipitation method is used as a quick estimate of fibrinogen concentration. More accurate methods include modifications of the Ratnoff-Menzie assay, measurement of clot weight, and quantification of immunoprecipitate formed with specific antifibrinogen antisera.

**C-Reactive Protein**

C-reactive protein (CRP) has been useful as a marker for numerous inflammatory disease states in humans. In humans, dogs, and rabbits, CRP is one of the first APR proteins to increase in inflammatory disease and exhibits a dramatic increase in concentration. It is clinically useful for screening for organic disease, assessment of the activity of inflammatory diseases, detection of concurrent infections in systemic lupus erythematosus, in leukemia or after surgery, and management of neonatal septicemia and meningitis.4,10,11

In the presence of calcium, CRP binds not only polysaccharides present in many bacteria, fungi, and protozoal parasites but also phosphorylcholine, phosphatidylcholine and polyanions (such as nucleic acids). In the absence of calcium, CRP binds polycations, such as histones.12 When bound, CRP activates the classic complement pathway starting at C1q. Like antibodies, CRP initiates opsonization, phagocytosis, and lysis of invading organisms including bacteria and viruses. CRP also binds toxic
autogenous substances released from damaged tissue and aids in their clearance from blood.

**Alpha₁ Acid Glycoprotein**

Alpha₁ acid glycoprotein (AAG), also known as orosomucoid, contains a high percentage of carbohydrate with a large number of sialic acid residues, is highly water soluble, and is the major constituent of the seromucoid fraction of plasma. AAG’s true physiological role is still unknown; however it has been shown to bind and inactivate basic and lipophilic hormones, including progesterone and several drugs, including the benzodiazepines. AAG increases during the APR especially with gastrointestinal inflammation and neoplasia in humans. Iatrogenic increases in AAG concentration are caused by administration of steroids and non-steroidal anti-inflammatory drugs. Levels are low in protein losing syndromes due to loss and in women with increased estrogen concentrations through decreased production.

**Summary**

Commercially available diagnostic assays that measured the positive acute phase proteins haptoglobin, C-reactive protein, fibrinogen, serum amyloid A, and alpha₁ acid glycoprotein were evaluated for utility in diagnosing inflammatory disease in manatees. Additionally, identification of novel proteins was attempted using proteomic analysis.
CHAPTER 2
COMPARISON OF METHODS USED TO DIAGNOSE GENERALIZED INFLAMMATORY DISEASE IN MANATEES (Trichechus manatus latirostris)

Introduction

The West Indian or Florida manatee (Trichechus manatus latirostris) is a federally endangered aquatic mammal living in Florida coastal waterways. Common causes of morbidity and mortality include boat strike, entanglement, brevetoxicosis, and “cold stress syndrome” which can lead to secondary bacterial or fungal infection.14 Response to therapy in resolving wounds can be difficult to assess in chronic infectious conditions such as subdermal abscesses, osteomyelitis, and pyothorax.

In many mammalian species, leukocytosis and fever are moderately sensitive indicators of internal inflammation and infection. However, in a few species, such as cattle, leukocytosis and left shifting do not occur unless disease is severe and then may decrease precipitously as leukocyte utilization exceeds production by the bone marrow. For this reason, leukocyte count, though sometimes useful in bovine species, is not a sensitive indicator of inflammation. Clinical reports from veterinarians indicate that manatees’ leukocyte response is similar to that of cattle. Additionally, determining the core temperature of a 500 kg aquatic mammal with internal anatomic countercurrent heat exchange mechanisms is not accurate.

The APR is a nonspecific response to tissue damage (trauma, surgery, or tumors) or inflammation (infection, autoimmune disease, etc.). The goals of the APR is to neutralize pathogens, minimize tissue damage, promote repair, and return the body to
homeostasis. Positive APR proteins (haptoglobin, CRP, fibrinogen, SAA, alpha\textsubscript{1} acid glycoprotein, and others) are produced by the liver during the APR in response to cytokines released at the site of injury to either protect the body or to combat a potential pathogen. In humans, APR proteins are nonspecific indicators of disease similar to fever or leukocyte counts. Plasma concentrations of the individual proteins change at different rates after the initial insult, thus providing useful information not only about the presence of inflammation but also about the duration of disease. Sequential measurements can also aid in assessment of response to treatment.

APR proteins, such as CRP, haptoglobin, and SAA, are sensitive measures of internal inflammation and infection in domestic species. In Europe, appropriate APRs are used as screening tests for inflammatory disease in livestock. It is likely that APRs would be a valuable tool in the diagnosis of inflammation in manatees as they are in cattle and other domestic animals. Currently, there is no published literature on APR proteins in sirenian species for identification of inflammatory diseases.

This study compared the effectiveness of complete blood count (CBC), albumin:globulin ratio (A:G) ratio, and concentrations of CRP, alpha\textsubscript{1} acid glycoprotein, haptoglobin, fibrinogen, and SAA. Methods were compared for diagnostic utility using diagnostic specificity and sensitivity as well as likelihood ratios.

**Materials and Methods**

**Animals and Sample Collection**

This project was preapproved by the University of Florida Institutional Animal Care and Use Committee approval #A726 and under United States Fish and Wildlife Service Permit #MA791721-3. Blood was collected during routine diagnostic health assessment from the brachial vascular bundle (also known as the pectoral arteriovenous
plexus) from 71 apparently healthy and 27 ill manatees during free-living animal captures performed by government agencies (n=44) and from rehabilitating animals housed at Lowry Park Zoo (n=25), Miami Seaquarium (n=17), and other institutions (n=12). Fresh blood samples from 45 apparently healthy and 18 diseased manatees had concurrent CBC, biochemical analysis, and gel electrophoresis performed at the University of Florida College of Veterinary Medicine Clinical Pathology Laboratory. Haptoglobin and SAA were measured in frozen serum samples from 26 healthy and nine diseased animals with known disease course and response to therapy. These samples did not have concurrent CBC and biochemical assays performed.

An attending veterinarian either at the rehabilitation facility or in the field performed a physical examination, including behavioral and external assessment, heart rate, and respiratory rate. History and reevaluation were available for every animal housed in a facility and seven recaptured in the field. Animals were assessed as healthy based on case history, physical examination, CBC, and biochemical data by clinicians blinded to the data collected in this study.

Crossreactivity of antibody-based assays with the homologous serum or plasma protein in manatees was assessed using samples from four to five obviously sick, acutely injured animals. An assay was determined to lack crossreactivity if the results in these acutely injured animals were comparable to the negative serum control.

**Laboratory Analysis**

Total white blood cell (WBC) count and red blood cell indices were determined using a Cell Dyn 3500 (Abbott Laboratories, Abbott Park, IL, USA) previously calibrated for manatee blood cells by Harr. Differential leukocyte counts were determined manually by counting 200 cells on Wright Giemsa stained blood preparations. Manual
packed cell volume (PCV) was determined using microhematocrit centrifugation for three minutes at 13,500 g and compared to the automated hematocrit for quality control. White blood cell count was estimated from each slide by multiplying the square of the objective by the average cell number per magnified field. This number was compared to the automated white cell count for quality control purposes. Nucleated red blood cells (NRBC) were counted concurrently with 100 WBC and as a percentage of RBCs. They were reported as per 100 WBC or the relative percentage was multiplied by the total RBC number to generate an absolute NRBC count per microliter.

Biochemical analysis of heparinized plasma was performed on a Hitachi 911 (Boehringer Mannheim/Roche Applied Science, Indianapolis, IN, USA) using Sigma reagents (Sigma-Aldrich Corp., St. Louis, MO, USA). Plasma albumin:globulin (A:G) ratio was determined using two different methods. In the first method, total protein and albumin concentrations were determined using the biuret method and bromcresol green methodology respectively.\textsuperscript{4,23} Globulin values were calculated by subtracting albumin from total plasma protein concentration. During assay development A:G ratio determined using biochemical methods using the Hitachi 911 using sigma reagent was found to be highly inaccurate. Albumin concentration in manatee plasma is overestimated by bromcresol green methodology by approximately 30% when compared to protein gel electrophoresis measurement causing marked error in the calculation of both globulin and A:G ratio. For this reason, further determination of plasma protein concentrations using the Hitachi was discontinued.
Table 2-1. A comparison of three methods used to quantitate albumin (g/dl) in captive and free-living manatees.

<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>Albumin</th>
<th>Globulin</th>
<th>A:G ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (+/- SD)</td>
<td>Range</td>
<td>Mean (+/- SD)</td>
</tr>
<tr>
<td>Serum Gel electrophoresis</td>
<td>56</td>
<td>7.4(+/- 0.57)</td>
<td>5.8 - 8.7</td>
<td>3.5(+/- 0.41)</td>
</tr>
<tr>
<td>Plasma Gel electrophoresis</td>
<td>45</td>
<td>7.7(+/- 0.63)</td>
<td>6.4 - 9.2</td>
<td>3.2(+/- 0.46)</td>
</tr>
<tr>
<td>Plasma Hitachi 911</td>
<td>60</td>
<td>7.9(+/- 0.58)</td>
<td>6.3 - 9.2</td>
<td>4.9(+/- 0.46)</td>
</tr>
</tbody>
</table>

Note that samples assayed by both serum and plasma gel electrophoresis have similar albumin concentrations. The Hitachi analyzer, using a nonspecific bromcresol green methodology, yields results that are approximately 30% higher. Since globulin and A:G ratio calculations are based on albumin concentrations, inaccurate albumin concentrations cause inaccurate globulin concentration and A:G ratio.

In the second method, albumin and globulin concentrations were determined by densitometry after protein gel electrophoresis. (Beckman Appraise Densitometer, Beckman Coulter, Inc., Fullerton, CA, USA)

Five different APR proteins were investigated using commercially available tests. Manufacturer recommended methodologies were followed. An alpha 1 acid glycoprotein methodology was investigated: an anti-bovine radial immuno-diffusion assay (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland). Two methodologies were investigated for CRP: an anti-porcine CRP ELISA (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) performed manually and an anti-human CRP turbidometric assay (Tina-quant CRP – high sensitivity, Roche Diagnostics Corporation, IN, USA) performed on the Hitachi 911 (Boehringer Mannheim/Roche Applied Science, Indianapolis, IN, USA). Two methodologies were investigated for haptoglobin: a turbidometric test involving precipitation of hemoglobin-haptoglobin complexes (Tri-
Delta Development Limited, Maynooth, County Kildare, Ireland) performed on the Hitachi 911 (Boehringer Mannheim/Roche Applied Science, Indianapolis, IN, USA) and an anti-human haptoglobin based ELISA (Tina-quant Haptoglobin, Roche Diagnostics Corporation, 9115 Hague Road Indianapolis, IN, 46250, USA) performed manually.

Fibrinogen was assessed by the heat precipitation method. When performing the heat precipitation method, plasma protein concentration was measured in an anticoagulated sample using a refractometer. A second plasma protein concentration was measured after the microhematocrit tube was heated to 57°C for three minutes. The difference between the precipitated and unprecipitated proteins was assumed to represent fibrinogen.24 One SAA assay was investigated: an anti-bovine SAA ELISA (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) performed manually.

**Statistical Analysis**

Descriptive statistics, T tests, sensitivity, specificity, and likelihood ratios were performed manually or using a commercial statistics software program (Minitab Inc., State College, PA, USA). Sensitivity was calculated by dividing true positives by the sum of true positives and false negatives. Specificity was calculated by dividing true negatives by the sum of true negatives and false positives. Positive likelihood ratios were calculated by dividing sensitivity by 1 minus specificity.25 Graphs were created using a commercially available graphing software program (Sigma Plot, SPSS, Inc, Chicago, IL, USA) Reference intervals of normally distributed values were generated by mean +/- 1.96 standard deviations. Reference intervals for analytes that failed normality testing were generated based on the use of percentiles.26 Outliers greater than three standard
deviations from the mean were deleted from the sample set used to generate reference intervals.

**Results**

Monoclonal antibodies against human CRP and human haptoglobin did not crossreact with those proteins in manatees. Similarly, the monoclonal antibodies against porcine CRP and bovine alpha1 acid glycoprotein (AAG) did not crossreact with those of manatees.

Mean, median, 95% reference interval, diagnostic specificity, and diagnostic sensitivity for all assays that exhibited antibody crossreactivity or are not antibody based including CBC, serum and plasma A:G ratio, haptoglobin, fibrinogen, and SAA are presented in Table 2-1 and depicted in Figure 2-1.

In this study, SAA is a very sensitive (85%) and specific (90%) diagnostic indicator of disease using the reference interval of <10 to 50 µg/ml, with an equivocal range of 50-70µg/ml. (See Table 1) The positive likelihood ratios calculated indicate that a result in the equivocal range would be approximately eight times as likely to be seen in a manatee with inflammatory disease as a manatee without inflammatory disease. A:G ratio determined by plasma gel electrophoresis with an established reference interval of 0.7-1.1 had the highest diagnostic sensitivity of any assay. Diagnostic sensitivity and specificity at this level was 88% and 94% respectively. A:G ratio determined using serum protein electrophoresis had a much lower sensitivity of 15%.

Haptoglobin, measured by hemoglobin titration, was detectable in the blood of manatees and had a reference interval of 0.4-2.4 mg/ml. This high reference interval results in a lower diagnostic sensitivity of approximately 60% with a diagnostic specificity of 93%. Fibrinogen, measured by heat precipitation, has a reference interval
Table 2-2. Diagnostic sensitivity and specificity for seven tests of inflammation in captive and free-living manatees. Values are listed from highest to lowest diagnostic sensitivity. Plasma A:G ratio and SAA have higher diagnostic sensitivities than other tests. Haptoglobin and fibrinogen may also be useful if positive but may more frequently yield a false negative result. A:G ratios were determined by protein gel electrophoresis. Fibrinogen was determined by the heat precipitation method. A:G = albumin:globulin, SAA = Serum amyloid-A, NRBC = Nucleated red blood cell, WBC = White blood cell.

<table>
<thead>
<tr>
<th>Test</th>
<th>Units</th>
<th>HEALTHY</th>
<th></th>
<th>DISEASED</th>
<th></th>
<th>Diagnostic Sensitivity</th>
<th>Diagnostic Specificity</th>
</tr>
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<tr>
<td>Plasm A:G ratio</td>
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<td>20</td>
<td>0.8 (+/-0.11)</td>
<td>0.8</td>
<td>0.7-1.1</td>
<td>11</td>
<td>0.6 (+/-0.1)</td>
</tr>
<tr>
<td>SAA</td>
<td>µg/ml</td>
<td>71</td>
<td>22 (+/-25)</td>
<td>&lt;10</td>
<td>&lt;10-50</td>
<td>38</td>
<td>266 (+/-398)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>mg/ml</td>
<td>71</td>
<td>1.4 (+/-0.7)</td>
<td>1.3</td>
<td>0.4-2.5</td>
<td>38</td>
<td>2.5 (+/-1.0)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>mg/dl</td>
<td>27</td>
<td>200 (+/-115)</td>
<td>200</td>
<td>100-400</td>
<td>16</td>
<td>400 (+/-240)</td>
</tr>
<tr>
<td>NRBC</td>
<td>/100 WBC</td>
<td>27</td>
<td>0.1 (+/-0.4)</td>
<td>0</td>
<td>0-2</td>
<td>16</td>
<td>6.6 (+/-15.6)</td>
</tr>
<tr>
<td>WBC</td>
<td>X10^3/µL</td>
<td>27</td>
<td>6.8 (+/-2.63)</td>
<td>6.4</td>
<td>3.7-10.6</td>
<td>16</td>
<td>6.7 (+/-1.72)</td>
</tr>
<tr>
<td>Serum A:G ratio</td>
<td>none</td>
<td>24</td>
<td>0.9 (+/-0.14)</td>
<td>0.9</td>
<td>0.7-1.2</td>
<td>11</td>
<td>0.7 (+/-0.13)</td>
</tr>
</tbody>
</table>
Figure 2-1. Horizontal whisker box plots of five values in healthy and diseased populations. Outliers=black dots. 50% of the population is contained within gray rectangle. Median is represented by a solid line. Note that there is a marked population difference between healthy and diseased manatees in values with a high diagnostic sensitivity.
of 100-400 mg/dl, a diagnostic specificity of 95%, and a diagnostic sensitivity of approximately 40% in the manatee population studied.

The median SAA concentration of the normal population was below detectable limits (<10 µg/ml). Greater than 80% of the healthy population had concentrations less than or equal to 20µg/ml. Positive likelihood ratios calculated for SAA at 20, 40, 50, 60, 70, 80 µg/ml were 3.63, 6.0, 7.7, 8.2, 8.6, and 9.5 respectively.\(^{25}\)

**Discussion**

SAA and A:G ratio, measured by plasma gel electrophoresis, were much more reliable inflammatory disease diagnostic markers in manatees than either WBC or NRBC. This study confirmed previous clinical impressions of poor diagnostic utility of WBC count and NRBC numbers for the diagnosis of inflammatory disease. In boat struck manatees, increased NRBC may be regenerative due to hemorrhage and decreased red cell mass or in response to severe inflammation. Haptoglobin and fibrinogen may also be diagnostically useful but have a lower diagnostic sensitivity.

**SAA**

SAA is highly evolutionarily conserved across vertebrate species from fish to humans, indicating that its roles in wound healing and inflammation are likely integral to returning the body to homeostasis during the APR.\(^{27}\) It is produced by hepatocytes in response to cytokines, primarily IL-1, IL-6, and tumor necrosis factor that are secreted at local sites of inflammation. SAA circulates complexed to lipoprotein, predominantly high density lipoprotein (HDL) in humans.\(^{28}\) It plays a role in lipid metabolism although its exact role is still speculative. SAA is reported to increase cholesterol transport through HDL to tissues from the liver to facilitate tissue regeneration.\(^{29}\) Other studies
indicate that SAA facilitates removal of large quantities of cholesterol liberated at sites of tissue damage.\textsuperscript{30}

SAA reportedly has an immunomodulatory function that promotes wound healing. A-SAA (an induced form) in humans can induce extracellular matrix (ECM) degrading enzymes, such as collagenase, stromelysin, and matrix metalloproteinases 2 and 3, which are important for repair and reorganization after tissue damage.\textsuperscript{31,32} In vitro studies have demonstrated that SAA can act as a chemoattractant for neutrophils, monocytes, mast cells, and T lymphocytes.\textsuperscript{33-35}

In horses, SAA has been reported to be a very sensitive indicator of inflammation.\textsuperscript{36} Manatees appear to be similar to horses, their evolutionary cousins, in that SAA is found in only trace amounts in healthy animals and increases dramatically in nonspecific inflammatory states, especially bacterial infection.

There are physiologic interactions with hormones in other species that may be important when using SAA as a diagnostic test in manatees. Exogenous or endogenous glucocorticoids may affect (most commonly cause a decrease in) the concentration of SAA.\textsuperscript{37} Additionally, both estrogens and androgens can affect cytokine production and therefore SAA concentration. Pregnancy, a state of decreased estrogen and increased progesterone concentrations, generally causes mildly increased SAA concentrations in humans.\textsuperscript{38} Though administration of glucocorticoids or pregnancy may, therefore, mildly change the diagnostic specificity and sensitivity of SAA concentration in manatees, the analysis of this healthy and diseased manatee population showed that SAA is a very useful inflammatory indicator in manatees.
SAA is the serum precursor of amyloid A (AA) protein, the principal component of reactive amyloid deposits found in a heterogeneous group of chronic inflammatory disorders including rheumatoid arthritis and tuberculosis in humans.\textsuperscript{39} Deposits of AA protein are most often found in the kidneys, liver, and spleen in chronic disease states but it may be found in any organ. The morbidity due to amyloidosis in manatees has yet to be determined.

**A:G Ratio**

A:G ratio is often a sensitive indicator of inflammation as it incorporates both the negative APR protein, albumin, in the numerator and positive APR proteins in the globulin fraction, in the denominator. Three methodologies for measuring A:G ratio were evaluated, two using heparinized plasma and one using serum. Of the three methodologies evaluated, plasma gel electrophoresis was by far the most diagnostically useful. Serum samples had significantly decreased diagnostic sensitivity. Fibrinogen, an important positive APR protein, is removed in the clotting process and therefore accurate positive detection of inflammation is markedly decreased in serum samples. A:G ratio determined using bromcresol green methodology using the Hitachi 911 was inaccurate and should not be used. Albumin concentration in manatee plasma is overestimated by by approximately 30% with large variation by this biochemical method when compared to protein gel electrophoresis measurement. This inaccurate measurement, likely due to nonspecific binding of bromcresol green dye with other proteins such as fibrinogen, results in a higher A:G ratio and increased numbers of false negative results.\textsuperscript{40}

**Haptoglobin**

Haptoglobin (Hp) is a glycoprotein that migrates in the alpha-2 globulin fraction and binds hemoglobin (Hb) irreversibly. The large Hp-Hb complexes greatly reduce
renal loss of free Hb and its iron. The complexes are removed rapidly by hepatocytes and Kupffer cells in the liver where they are degraded and iron and amino acids are recycled. The Hp-Hb complex is also a peroxidase, capable of hydrolyzing peroxides released by neutrophils at sites of inflammation. Hp also functions as a natural bacteriostatic agent for iron-requiring bacteria by preventing the utilization of hemoglobin iron by these organisms.\textsuperscript{41}

Haptoglobin is the characteristic APR protein in cattle and was therefore investigated in this study of manatees.\textsuperscript{42} Haptoglobin is undetectable in the blood of healthy cattle. Haptoglobin levels may increase 50 fold or more in cattle with inflammation or infection, such as mastitis, metritis, pyometra, traumatic reticulitis, abomasal displacement, and bacterial nephritis.\textsuperscript{43-46} It has therefore proven to be a sensitive indicator of inflammatory disease in cattle.

Haptoglobin had a reference interval of 0.4-2.4 mg/ml which is more comparable to the reference interval in dogs, which ranges up to approximately 2.6 mg/ml, than the undetectable values (0.2 mg/ml) in cattle. This high reference interval results in a lower diagnostic sensitivity of approximately 60% with a diagnostic specificity of 93%. Haptoglobin concentration, measured by hemoglobin titration, is decreased by hemolysis in the sample. Consequently, moderate hemolysis in samples may decrease the sensitivity of this assay. Haptoglobin is quickly utilized during intravascular hemolysis, and severe hepatocellular disease also results in decreased synthesis of haptoglobin. In humans, it is also increased in response to exogenous progesterone, glucocorticoids, and NSAID administration, during some protein losing syndromes (such as nephrotic syndrome), and during severe biliary obstruction.\textsuperscript{41}
**Fibrinogen**

Fibrinogen was the first APR protein recognized. In the coagulation cascade, it is cleaved by thrombin to form fibrin, the backbone of the thrombus. Fibrinogen is also integral in the aggregation of platelets. Although, only mildly increased fibrinogen concentrations are observed in many species, it has proven very useful in detecting inflammation in ruminant species, specifically cattle and cetaceans. Increased production by the liver, stimulated by locally produced cytokines, results in increased fibrinogen concentrations in inflammatory states as well as pregnancy in humans. Low concentrations of fibrinogen can occur in disseminated intravascular coagulation, liver failure, and cachexia.

Fibrinogen measured by heat precipitation, though useful in assessing inflammatory lesions, is not as sensitive as either SAA, haptoglobin, or plasma A:G ratio determined by gel electrophoresis. Increased fibrinogen concentration, measured by heat precipitation, only accurately diagnosed inflammatory disease in approximately half of the diseased population. The heat precipitation method is a quick estimate of fibrinogen concentration and should not be considered quantitative. More accurate methods include modifications of the Ratnoff-Menzie assay, measurement of clot weight, and quantification of immunoprecipitate formed with specific antifibrinogen antisera. These quantitative methods should be assessed.

**Wild-Caught Versus Captive Animals**

Reference intervals generated included samples for both captive (n=54) and wild caught (n=44) animals. All nine false positive animals were wild-caught animals. False positive status was based on veterinary physical exam as the gold standard. Of the nine false positive animals based on SAA analysis, three wild-caught animals were
categorized as false positive on both haptoglobin and SAA assays. This indicates that these animals likely had a subclinical bacterial or viral disease that was not detected during the one to two hour assessment performed on the beach. Due to the constraints of the original study design and our inability to prove disease in these free living animals, their classification as false positive was maintained for statistical analysis. If data were generated for captive animals only, i.e. animals at facilities with regular keeper and veterinary observation, diagnostic specificity and positive predictive value of the SAA ELISA and haptoglobin titration would approach 100%. Therefore, a positive test result provides excellent evidence of inflammatory disease.

Inflammatory disease has not been assessed in the wild population due to lack of available reliable diagnostics. It has been reported that manatees are naturally resistant to infectious disease.\textsuperscript{48} The wild-caught animals that were assessed in this study were caught in winter during months of cold stress. During this time period, large numbers of hypothermic animals are clustering at natural and man-made warm water outflows. This is the time period when manatees would be most likely to contract viral or bacterial disease and so our assessment of subclinical inflammatory disease is not surprising.

Of the nine diseased animals assessed as false negatives at facilities, three were diagnosed with uncomplicated brevetoxicosis. The paralysis and drowning associated with this intoxication would not be expected to cause an inflammatory lesion. If these animals were eliminated from our diseased population, sensitivity of the SAA and haptoglobin assay would be approximately 90% and 70% respectively. Four of the other false negative animals had pectoral wounds caused by crab trap entanglement. These peripheral, infected wounds may have been walled off with a fibrotic reaction and
therefore did not induce the systemic inflammatory response necessary to cause increased concentrations of APR proteins.

**Prognostic Value**

Four rehabilitating animals in the study population, housed at two facilities, died during the study period. Three of the four animals had SAA concentration exceeding 1200µg/ml, the maximum measurable concentration with 10 fold dilution. The deceased animals maintained this >100 fold increase over normal SAA concentration over one to two months. Two other live animals also had >1200µg/ml SAA on presentation. The SAA concentration decreased dramatically in the first two weeks of treatment to approximately 100µg/ml and these animals recovered from their injuries. The low number of manatees with this markedly increased SAA concentration precludes statistical analysis. However, these preliminary findings indicate that markedly increased SAA concentration especially when assessed over time may be prognostically useful.

**Conclusion**

SAA, measured by ELISA, and A:G ratio, measured by plasma gel electrophoresis, were most sensitive in diagnosing inflammatory disease in manatees with diagnostic sensitivity and specificity approaching 90%. A:G ratio measured using other techniques was not diagnostically sensitive and, therefore, not useful in the diagnosis of inflammatory disease. Haptoglobin, measured by hemoglobin titration, had a lower diagnostic sensitivity of 60%, likely caused by the relatively high concentration of haptoglobin in normal animals. This assay may give important information about response to therapy. The fibrinogen concentration estimate assessed in this study was more sensitive than white blood cell count but only diagnosed half of the true positive inflammatory lesions that were diagnosed by SAA and A:G ratio.
CHAPTER 3
VALIDATION OF A COMMERCIALLY AVAILABLE SERUM AMYLOID A (SAA) ELISA FOR MEASUREMENT OF SAA CONCENTRATION IN MANATEES (Trichechus manatus latirostris)

Introduction

The West Indian or Florida manatee (Trichechus manatus latirostris) is a federally endangered aquatic, herbivorous mammal living in Florida coastal waterways. Standard markers of inflammatory disease are not useful diagnostic tools in manatees. For example, the diagnostic sensitivity of increased leukocyte count in manatees was 17% in a population studied. Additionally, the measurement of temperature in a 1,000 pound aquatic mammal with numerous internal thermal countercurrent exchange mechanisms is inaccurate. Serum amyloid A (SAA) has been found to be a useful, major acute phase reaction (APR) protein in manatees. The concentration of SAA in healthy manatees is low (<10-50µg/ml) but shows a rapid, several fold increase in concentration at the onset of acute inflammatory disease.

The purpose of this investigation was to assess the reliability of a commercially available ELISA for measuring sirenian SAA concentration in plasma and serum. The Tridelta Phase range SAA kit (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland), a solid phase sandwich Enzyme Linked Immunosorbent Assay, is designed for use in multiple domestic animals including bovine, equine, canine, feline, porcine, and human species. The monoclonal antibody specific for SAA distributed in this kit does crossreact with SAA in manatees. According to the Clinical Laboratory Improvement Amendments (CLIA) guidelines (http://cms.hhs.gov/clia/) for assays
developed in house, this study assessed precision, detection limit, analytical specificity and sensitivity, and established a reference interval in healthy animals.

**Materials and Methods**

**Blood Samples**

Blood samples from a total of 98 manatees were included in the trial in accordance with the University of Florida Institutional Animal Care and Use Committee approval #A726 and under United States Fish and Wildlife Service Permit #MA791721-3. Extra blood was taken during routine diagnostic health assessment from the brachial vascular bundle (also known as the pectoral arteriovenous plexus) from 71 apparently healthy and 27 ill manatees during wild animal captures performed by the Florida Fish and Wildlife Commission and from rehabilitating animals housed at Lowry Park Zoo, Homosassa Springs State Wildlife Park, and Miami Seaquarium. Manatees were assessed as healthy based on history, physical examination, CBC, and biochemical analysis by a veterinarian. Serum and plasma samples from healthy and ill animals were used in statistical assessment of accuracy and precision across the linear range of the assay.

**SAA Analysis**

The Tridelta Phase range ELISA (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) was performed manually, with a standard curve generated using a known quantity of bovine serum amyloid A calibrator, according to the manufacturer’s instructions. Serum from a healthy horse and one with septic peritonitis were used as negative and positive controls respectively during each analysis. All samples were assayed in duplicate and the average of the two wells was reported. Samples were rerun if concentrations in the duplicate assays differed by greater than 30%. 
Assay Characteristics of SAA ELISA

A 95% reference interval was constructed using percentiles from data from a population of 66 healthy animals. Interassay precision was assessed using ten serum samples with SAA concentrations evenly distributed between <10 to >120 µg/ml. Each serum sample was analyzed on two plates with six replicates each to total 12 replicates. A dilutional experiment was performed as a test of analytical sensitivity. Three samples that contained 49, 106, and 120 µg/ml SAA were diluted 1:1, 1:2, 1:4, 1:8, and 1:16 with 1x dilution buffer. To assess analytical specificity and interaction with other compounds, each of four samples, containing <10, 50, 95, and >120 µg/ml SAA, were spiked with purified human high density lipoprotein (Fitzgerald Industries International, Inc., Concord, MA, USA) to sample concentration of 0, 50, 100, and 300 mg/dl. Additionally, ten paired manatee serum and plasma samples were analyzed to determine if a heparin interaction could be detected.

Protein Purification and Sequence

The monoclonal biotinylated anti-SAA antibody (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) used in the serum amyloid A sandwich ELISA was immobilized on a Seize X Protein G immunoprecipitation column (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer’s directions. The antibody was crosslinked to the column using disuccinimidyl suberate (DSS). Manatee serum containing >120 µg/ml SAA was passed over one column while diluent was passed over a second column, serving as a negative control. Bound antigen was eluted from the column using buffer of pH 2.5 provided with the Seize X Protein G immunoprecipitation column. All eluted fluid was saved. Elutions were mechanically concentrated using a Microcon YM-3 centrifugal filter unit (Millipore Corporation, Bedford, MA, USA) that
retains proteins with mass over 3,000 daltons. The concentrated antigen was separated on precast, stacked 16.5% tris-tricine, SDS-PAGE Criterion polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) with tris-tricine running and sample buffers designed for peptide separation (Bio-Rad Laboratories, Hercules, CA, USA) using Criterion gel electrophoresis apparatus. SeeBlue Plu2 Pre-Stained Protein Standard (Invitrogen, Carlsbad, CA, USA) was used as a molecular weight marker. Gels were stained with Biosafe-Coomassie Blue (Bio-Rad Laboratories, Hercules, CA, USA) and destained with water.

**Western Blot and Edmann Sequencing**

Gels were transferred to a PVDF membrane for Western blotting analysis using a Bio-Rad Criterion Blotter (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer’s directions. The membrane was blocked with 5% Blotto (5% Carnation instant non-fat dry milk in 20mM tris pH 7.6, 0.15 NaCl, 0.05% Tween 20, 0.02% sodium azide). Approximately 0.03 ml stock monoclonal biotinylated anti-SAA (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) was diluted in SAA ELISA dilution buffer (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) and the membrane was incubated at room temperature for approximately three hours. The membrane was rinsed three times with SAA ELISA dilution buffer (Tri-Delta Development Limited, Maynooth, County Kildare, Ireland) and incubated with streptavidin conjugated alkaline phosphatase (Pierce Biotechnology, Rockford, IL, USA) at a 1:1000 ratio with TBSTZ (20 mM tris pH 7.6, 0.15 M NaCl, 0.05% Tween, 0.02% sodium azide) for two hours. The substrate (0.3% NBT and 0.15% BCIP in 0.1 M tris pH 9.5, 0.1 M NaCl, 5 mM MgCl₂) was added and the blot was allowed to develop for 2-4 minutes. Reaction was quenched using 20mM EDTA when bands were visible.
PVDF membrane was digested using endoproteinase – Lys C (Roche Diagnostics, Indianapolis, IN). Subsequent fractions were separated on an HPLC and N-terminally sequenced on an Procise 494HT Protein Sequencer. (Applied Biosystems,Foster City, CA, USA)

Statistical Analysis

Statistical analysis including descriptive statistics, coefficient of variation for assessment of precision, a paired T test to determine if heparinized samples were significantly different than serum samples, and simple linear regression to evaluate accuracy of diluted sample measurement were performed using a commercial statistical program. (Minitab Inc., State College, PA, USA) and Microsoft Excel 2000 (Microsoft Corporation, Seattle, WA, USA). A p value of <0.05 was accepted as significant.

Statistical Analysis. Sensitivity was calculated by dividing true positives by the sum of true positives and false negatives. Specificity was calculated by dividing true negatives by the sum of true negatives and false positives. Positive likelihood ratios were calculated by dividing sensitivity by 1 minus specificity. Reference intervals of normally distributed values were generated by mean +/- 1.96 standard deviations. Reference intervals for analytes that failed normality testing were generated based on the use of percentiles. Outliers greater than three standard deviations from the mean were deleted from the sample set used to generate reference intervals.

Results

Assay Characteristics of SAA ELISA

The linear range of the standard curve generated using the manufacturer’s bovine SAA calibrator was <10 \( \mu g/ml \) to >120 \( \mu g/ml \) (Fig. 3-1). The 95% reference interval for apparently healthy manatees was <10 to 50 \( \mu g/ml \). The interassay coefficient of variation


ranged from 3 to 14%. Samples near the high end of the reference interval (50 µg/ml) produced a coefficient of variation (CV) of 12%. This coefficient of variation was high but acceptable according to CLIA. Therefore, an equivocal reference interval of 50 to 70 µg/ml was established by adding three standard deviations of replicates at 50 µg/ml to 50 µg/ml. Values >70 µg/ml indicate active inflammatory disease.

Investigation of inaccuracy by dilution of samples from three animals produced a linear regression equation in which x represented the predicted concentration according to sample dilution and y represented the measured level. (Fig 3-1) The slope was equal to 0.9865 and the intercept was 4.17. The equation produced an $R^2$ value of 96.1% and $p<0.001$.

**Analytical Sensitivity and Specificity**

Three separate attempts at accuracy evaluation, by spiking manatee serum with bovine SAA calibrator in the commercial ELISA (Tri-Delta Development Limited, Unit 7, Block F, Maynooth Business Campus, Maynooth, County Kildare, Ireland), were performed. Stock solution, containing 3,000 ng/ml and 1,000 ng/ml bovine SAA calibrator, was diluted with manatee serum containing undetectable concentrations of SAA for a final sample concentration between 30 to 600 µg/ml. All experiments were unsuccessful and, regardless of expected final concentration, all measured concentrations were undetectable (<10 µg/ml). It is hypothesized that a compound present in manatee serum binds SAA and covers the epitope of the monoclonal antibody used in the ELISA kit. This results in unmeasurable quantities of bound SAA regardless of concentration added. Once all of the binding sites of this unknown compound are filled by SAA, any extra, free SAA is then measured by the assay. This may account for the differences observed between the sick and apparently healthy populations. Approximately 80% of
the healthy animals had SAA concentration <20 µg/ml while the median concentration of
diseased animals was 100 µg/ml, a 5 fold increase.

Figure 3-1. Graph of the linear range of the standard curve for the Tridelta Phase range
SAA ELISA using a bovine SAA calibrator. Note that final concentration is
multiplied by the dilution factor of 500. The top graph is representative of the
standard curve used in an ELISA assay in this project. The bottom graph
includes the final point recommended by the manufacturer but out of the
linear region of the curve. This type of graph was not used in this project.
Figure 3-2. Linear regression analysis of manatee serum SAA dilutions as an assessment of accuracy. Note the high $R^2$ value and $p<0.001$ indicating that manatee serum SAA concentration was linear and proportional upon dilution by the Tridelta Phase range SAA ELISA.

Since human SAA binds HDL, interaction between manatee SAA and HDL was assessed. Testing with HDL spiked samples revealed a decrease of approximately 5% of original concentration with each addition of 50 mg/dl HDL. Addition of 300 mg/dl HDL, resulted in a decrease of approximately 25%. (Table 3-1 and Fig 3-3)

Table 3-1. Effects of the addition of HDL on the measured concentration of SAA in three manatee serum samples. Note that high concentrations of HDL lowers measured SAA.

<table>
<thead>
<tr>
<th>Added HDL (mg/dl)</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41</td>
<td>89</td>
<td>122</td>
</tr>
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<td>50</td>
<td>38</td>
<td>84</td>
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</tr>
<tr>
<td>300</td>
<td>30</td>
<td>75</td>
<td>87</td>
</tr>
</tbody>
</table>
Figure 3-3. Three manatee serum samples showed significant decrease in SAA concentration with proportional increase of HDL concentration. This may result in false negative results in obese animals or animals with increased HDL concentration.

Heparinized plasma and serum samples were not significantly different and the raw data revealed no clinically significant difference between plasma and serum samples.

**Western Blot and Edmann Sequencing**

Antibody contamination from the immunoprecipitation column was present in the elutions from the negative control column as well as columns treated with manatee serum. Western blot revealed a protein of approximately 12,000 daltons in size in elution one and two from the column treated with serum. Lesser quantities of a protein approximately 3,000 daltons in size was also identified. Edmann sequencing of the 12,000 dalton protein revealed that the protein is N terminally blocked. Digestion produced a protein fragment with the sequence ASP, PRO, ASN, HIS, PHE, ARG, PRO, GLU, GLY, LEU, PRO, ASP, LYS. When this fragment was blast searched in the National Center for Biotechnology Information (NCBI) data base, serum amyloid A from
the brown rat, Rattus norvegicus, the house mouse, Mus musculus, and the domestic cat, Felis catus, yielded an E value of $3 \times 10^{-4}$ and serum amyloid A from the domestic dog, Canis familiaris, and European cattle, Bos Taurus, yielded an E value of $9 \times 10^{-4}$.

**Discussion**

The Clinical Laboratory Improvement Amendments (CLIA) were finalized as guidelines for human laboratory medicine in 2003. The guidelines provide an excellent, well-reviewed basic standard for assay validation and quality control practices within laboratory medicine. Although veterinary diagnostic laboratories are not legally required to abide these guidelines for human laboratories, validation of this serum amyloid A ELISA was performed according to CLIA Section 493.1253(b)(2), Non-Waived Test Modified or Developed In-House.

This section requires assessment of accuracy in comparison to a gold standard, precision analysis, analytical sensitivity and specificity, linear range of the assay, and reference interval for healthy individuals. We were unable to assess accuracy of this ELISA in comparison to a gold standard as there is no other way to measure serum amyloid A in manatees at this time. Manatee serum SAA concentration was measured in a linear and proportional manner upon dilution by the Tridelta Phase range SAA ELISA.

Interassay precision analysis across the linear range of the assay showed that the coefficient of variation remained below 15% which, though higher than the 10% reported by the manufacturer, is still acceptable according to good laboratory practice standards. The relatively high coefficient of variation at the high normal reference interval (50 µg/ml) was concerning as the imprecision of the assay may have resulted in incorrect diagnosis of inflammatory disease. The equivocal reference interval of 50-70 µg/ml
prevents incorrect diagnosis which may result in costly treatment. However, the data is not of Gaussian distribution. When the population used to generate the reference interval is analyzed, the median SAA concentration is trace (<10 µg/ml). Additionally, greater than 80% of the animals had <20 µg/ml SAA. Positive likelihood ratios revealed that a test result in the equivocal range would be eight times as likely to be found in an animal with inflammatory disease as a healthy animal.

SAA circulates in blood complexed to lipoprotein, predominantly HDL in humans.28 HDL binds at the N terminal region of SAA which is also the most conserved region of the protein and a likely epitope for the monoclonal antibody used in the sandwich ELISA. Investigation of HDL interaction revealed that measurement of SAA concentration is proportionally decreased with increasing concentrations of purified human HDL. This indicates that increased HDL concentration in manatees will likely result in inaccurate, decreased concentrations of SAA in animals with inflammatory disease. This warrants concern for false negative results in obese animals and animals with high HDL concentration.

The predominant protein isolated by Western blot had a mass of approximately 12 kD which is comparable to the mass of SAA of known sequence in other species (11.5 to 12.5 kD). Sequencing of the whole protein was impossible due to N-terminal blocking. SAA in other species has also been shown to be blocked when sequence attempts by Edmann degradation were performed.51 There was excellent homology for SAA in numerous species when partial sequence of the immunopurified protein was analyzed. (Fig. 3-4)
Sequencing of the complete protein is currently underway. A second, smaller protein, approximately 3 kD in size, was also isolated during immunopurification and observed on the SDS PAGE gel. Partial sequence analysis of this protein by Edmann degradation resulted in an E value greater than 300 and identification was therefore not possible. We hypothesize that this protein is noncovalently bound to SAA and may have allosteric interaction that alters binding of SAA to lipoproteins. Further study is warranted.

In conclusion, the Tridelta Phase range SAA ELISA accurately and precisely measures SAA concentration in manatee serum and plasma. However, HDL interacts with SAA such that a clinically significant decrease in SAA concentration is possible in obese animals.
Figure 3-4. Multiple sequence alignment for serum amyloid A from the NCBI bioinformatics database for multiple species showing a highly conserved protein across species. The SAA fragment isolated from manatee serum has high homology for the carboxyl terminal end of the protein.
FURTHER INVESTIGATION: TWO DIMENSIONAL GEL ELECTROPHORESIS

Manatee plasma proteins were separated, using two dimensional gel electrophoresis, to identify potential inflammatory proteins for investigation for which no commercial diagnostic assay is currently available. Manatee A was an adult female with subacute open wounds due to recent trauma from a boat propeller. Manatees B and C were healthy adult females. Fifty µg of each labeled protein, mixed with 500 µg of each unlabeled manatee plasma protein, was focused in a pH 3 to 10 non linear IPG strip. The proteins contained in this strip were then analyzed with 8 to 16% Tris Glycine SDS PAGE. Two overlays of the two dimensional gel electrophoresis manatees B and C plasma and manatee A plasma revealed the same four distinct red, Cy-5 labeled proteins. One isoform separated at approximately 35 kD and three other proteins separated at approximately 15, 16, and 18 kD. These proteins will be sequenced using Edmann chemistry.
Figure 4-1. Two dimensional gel electrophoresis of apparently healthy versus diseased manatee. a) Coomassie brilliant blue stained blot after proteins were transblotted from scanned gel to PVDF membrane. b) Control manatee (B), labeled with Cy-3, is green in color. c) Experimental manatee (A) is labeled with Cy-5 and is red. d) Overlay of the healthy and diseased animal reveals proteins that are unique to the disease animals are red. Proteins that are present in both healthy and diseased animals are yellow.
Figure 4-2. Two dimensional gel electrophoresis of apparently healthy versus diseased manatee. a) Coomassie brilliant blue stained blot after proteins were transblotted from scanned gel to PVDF membrane. b) Control manatee (C), labeled with Cy-3, is green in color. c) Experimental manatee (A) is labeled with Cy-5 and is red. d) Overlay of the healthy and diseased animal reveals proteins that are unique to the disease animals are red. Proteins that are present in both healthy and diseased animals are yellow.
CHAPTER 5
SUMMARY

This study examined the innate, inflammatory, acute phase response in manatees to
determine a practical approach for inflammatory disease diagnosis. An immunoassay to
measure SAA, as well as assays to measure haptoglobin, fibrinogen, and A:G ratio, were
found to be potentially useful in manatees. These assays were assessed in a large
population of healthy and diseased manatees for diagnostic sensitivity and specificity.
SAA and plasma A:G ratio were the most sensitive and specific indicators of
inflammatory disease. SAA appears to be a major acute phase protein in manatees that
increases acutely by 50 to 100 fold from trace concentrations and decreases quickly after
resolution of inflammation. Haptoglobin and fibrinogen appear to be moderate acute
phase proteins with a less dramatic and lingering increase in concentration over time.

SAA sandwich ELISA validation revealed a precise and accurate method within the
linear range of <10->120µg/ml. Analytical specificity assessment showed an interaction
with human HDL results in decreased SAA concentration. This indicates that the
physiology of SAA in manatees is likely similar to humans. SAA circulates in blood
complexed to lipoprotein, predominantly HDL in humans.28 HDL binds at the N terminal
region which is also the most conserved region of the protein and a likely epitope for the
monoclonal antibody used in the sandwich ELISA. Investigation of HDL interaction
revealed that measurement of SAA concentration is proportionally decreased with
increasing concentrations of purified human HDL. This indicates that increased HDL
concentration in manatees will likely result in inaccurate, decreased concentrations of
SAA in animals with inflammatory disease. This may result in false negative results in obese animals and animals with high HDL concentration. Comparison of paired serum to plasma samples revealed no interaction. The ability to use plasma is practical in that the same volume of manatee blood will yield approximately twice as much plasma as serum.

Stability over time at varying temperatures was not assessed in this study. Manufacturer data shows this is a well conserved protein that is highly stable when frozen for at least three years and is minimally affected by freeze thaw cycles.

Analysis of the protein isolated by the ELISA revealed the predominant protein, isolated by Western blot, had a mass of approximately 12 kD. This is comparable to the mass of SAA of known sequence in other species (11.5 to 12.5 kD). Sequence of the whole protein was impossible by Edman degradation due to N-terminal blocking. SAA in other species has also been shown to be blocked when sequence attempts by Edmann degradation were performed. When partial sequence of the immunopurified protein was analyzed, there was excellent homology for SAA in numerous species. Complete sequence of the protein is planned.

Manatee plasma proteins were separated by two dimensional gel electrophoresis to identify potential inflammatory proteins for which no commercial diagnostic assay is currently available. Two overlays of the two dimensional gel electrophoresis of each control animal's plasma and the boat struck manatee's plasma revealed the same four distinct, red, Cy-5 labelled proteins. One isoform separated at approximately 35 kD and three other proteins were separated at approximately 15, 16, and 18 kD. These proteins will be sequenced using Edmann chemistry.
In conclusion, this study successfully accomplished all specific aims set out in the original master's proposal by identifying and validating an inflammatory disease marker that can be analyzed by available commercial diagnostics as well as identifying other potential proteins for inflammatory disease diagnosis.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Dr. Kendal Harr is the daughter of Alma and Kenneth Harr. She was born in 1969 and raised in Glen Cove, Long Island, New York. Dr. Harr obtained her B.S. from Cornell University, Ithaca, New York, in 1990, majoring in animal science and minoring in marine biology. She graduated from the College of Veterinary Medicine, Cornell University, in 1995 with a D.V.M. Following three years in small animal practice, Dr. Harr subsequently completed a residency training program in clinical pathology at the University of Florida, Gainesville, Florida. She is a Diplomate of the American College of Veterinary Pathology. She has two beautiful daughters, Maeve and Lillian, and a wonderful husband, Darryl.