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AN ABSTRACT OF THE THESIS OF

Trina L. Westerman for the degree of Master of Science in Veterinary Science presented on August 8, 2014.

Title: Evaluation of Serum Amyloid A and Haptoglobin as Prognostic Indicators for Horses in a Referral Population

Abstract approved:

Keith P. Poulsen

Abstract:

Acute phase protein (APP) measurement can be used to characterize and rapidly detect inflammation. Serum amyloid A (SAA) and haptoglobin (HP) may be useful for diagnostic and prognostic purposes in horses presenting for colic and other inflammatory diseases. The objective of this thesis was to evaluate the use of SAA and HP in horses presenting for colic and inflammatory diseases for prognostic application of case outcome. We examined case outcomes such as development of complications, survival outcome, duration and cost of hospitalization, and requirement for surgical intervention in horses presenting for colic. Specific laboratory values measured included total white blood cell count, neutrophil count, fibrinogen, SAA, HP, which were compared in control horses and horses admitted for colic and inflammatory diseases. Clinicopathologic values were compared in medical and surgical colic cases to test the ability of APPs to predict indication for surgical intervention. Survival outcome, development of complications, and hospitalization

cost and duration were analyzed in both in horses presenting for colic and inflammatory diseases.

In horses presenting for colic, SAA was significantly higher in the surgical group compared to both the control and medical colic groups. Haptoglobin concentration was not significantly different between all groups. Horses with elevated SAA were more likely to require surgical intervention than those medically managed. Euthanasia due to poor prognosis or the development of serious complications were more likely to occur in horses presenting for colic with an elevated SAA. In horses presenting for inflammatory diseases, admission SAA and HP concentrations were not significantly associated with survival or the development of complications. Increased HP concentration on admission was associated with longer duration of hospitalization in hoses with inflammatory diseases. Horses with an increasing SAA between 24-72 hours compared to admission SAA were more likely to be euthanized or develop complications. The findings of this study show that SAA and HP measurement could be a potential useful diagnostic and prognostic tool in horses presenting for colic and inflammatory diseases.

Evaluation of Serum Amyloid A and Haptoglobin as Prognostic Indicators for Horses in a Referral Population

by
Trina L. Westerman

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APPROVED:

Major Professor, representing Veterinary Science

Dean of the College of Veterinary Medicine

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Trina L. Westerman, Author

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CONTRIBUTION OF AUTHORS

Dr. Keith Poulsen was involved with study design and manuscript preparation of chapters two and three. Dr. Susan Tornquist was involved with method development and manuscript preparation of chapters two and three. Crystal Foster assisted with method development and final approval of the manuscript of chapters two and three.

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Chapter 1

Introduction

The Acute Phase Response

The immune system is comprised of diverse cellular mechanisms and pathways to protect the host against disease. The two main arms of the host immune response are made up from innate and acquired immunity mechanisms. The innate immune response plays the role of early host defense against infection and is responsible for activating the acquired immune response [1]. The innate system includes physical barriers, immune cells, the complement system, pattern recognition receptors and inflammatory mediators and is activated by inflammatory stimuli such as infection, trauma and neoplasia [1]. When an inflammatory stimulus is present, diverse local cells including macrophages, neutrophils, fibroblasts and endothelial cells are activated, which produce proinflammatory cytokines that are primarily responsible for cell signaling [2]. Interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are the major proinflammatory cytokines primarily responsible for activating the acute phase response (APR) [3]. These cytokines are released into systemic circulation which results in induction of the APR by the liver which results in modulation of protein synthesis [4]. Proteins with a change in plasma concentration by more than 25% are considered acute phase proteins (APPs) and are classified as either positive APPs (upregulated) or negative APPs (downregulated) [3]. Positive APPs are categorized according to their response to inflammation. Major APP plasma concentrations increase greater than 10 fold in

response to inflammation, whereas moderate APPs increase 2 to 10 fold and minor APPs increase less than 2 fold [5]. The APR is present in all species however differences exist between the magnitude of response of positive APPs between species [4]. For example, serum amyloid A (SAA) is a major APP with fibrinogen and haptoglobin being moderate APPs in horses, whereas in cattle both SAA and haptoglobin are major APPs [4]. Albumin is the main negative APP in nearly all species [4]. Acute phase proteins are of clinical interest as they can be identified in serum and used to detect and characterize inflammation. This is of particular interest as early recognition of systemic inflammation with current standard of care diagnostic tests is not ideal. Therefore APP measurement has a high potential improve clinical diagnostic and prognostic abilities of veterinary practitioners.

Acute Phase Proteins

Equine SAA is an apolipoprotein produced primarily by hepatocytes during the acute phase response. The physiologic roles of SAA include induction of leukocyte chemotaxis, inhibition of lymphocyte and endothelial cell proliferation, activation of inflammatory mediator synthesis and inhibition of platelet aggregation [6-8]. Serum amyloid A, a major APP in horses, has very low or undetectable levels in healthy horses with normal concentrations reported from less than 5 to 20µg/ml. During inflammation, SAA concentration can increase greater than 100 fold within 6-12 hours following inflammatory stimuli. These characteristics allow measurement of SAA concentrations to aid in rapid identification of inflammation. Serum amyloid A has a relatively short half-life which also makes it an ideal marker for serial

analysis to monitor therapeutic response [9]. Increased serum concentration of SAA in horses has been reported in numerous disease processes including both infectious and non-infectious respiratory disease, neonatal septicemia and colic [10-12]. In a study by Vanderplas et. al, SAA was evaluated in horses presenting for colic of both non-inflammatory (such as intestinal obstructions and bloat) and inflammatory (such as peritonitis and colitis) etiologies. SAA was significantly higher in horses with inflammatory causes of colic and in non-survivors [12].

Equine haptoglobin is an α2-globulin with the primary function of binding free hemoglobin within systemic circulation to prevent loss of iron and prevent hemolysis caused by excess free hemoglobin [13]. Haptoglobin also has the ability to bind free myoglobin [14]. Haptoglobin is a moderate acute phase protein in horses and has a slower response time compared to major APPs [15]. Haptoglobin concentrations typically begin to increase 12-24 hours following an inflammatory insult with peak concentrations at 72-120 hours [6]. Haptoglobin is always present in the plasma of healthy horses however it can be quickly consumed to low or undetectable levels in cases of intravascular hemolysis and rhabdomyolysis [16,17]. Similar to moderate APP measurement in small animals, recent studies have shown haptoglobin's usefulness in equine medicine is most likely as a marker of chronic inflammation [15,18]. For example, in a model of acute exacerbation of equine recurrent airway obstructive disease, haptoglobin has been shown to remain elevated weeks following normalization of SAA [10]. Divergences between major and moderate APPs have been shown to help differentiate between acute and chronic inflammation and when monitoring chronic disease in other species [18,19].

Fibrinogen is a soluble plasma glycoprotein and is the only positive acute phase protein regularly measured in equine medicine. Fibrinogen is a moderate acute phase protein with the primary function of stabilizing blood clots. Fibrinogen begins to increase 24-72 hours after an inflammatory stimulus with peak concentrations not being reached until 72-144 hours [9]. Fibrinogen's long response time and relatively wide reference interval makes it a relatively insensitive marker of inflammation in acute disease [6]. Similar to haptoglobin, fibrinogen is always present in plasma of healthy horses and it can be consumed in coagulation abnormalities such as disseminated intravascular coagulation. Fibrinogen is typically measured by a heat precipitation method because it is simple and inexpensive test however it is not sensitive enough to detect decreased fibrinogen. Fibrinogen has also been shown to not be as sensitive as haptoglobin as an indicator of chronic inflammation in experimentally induced placentitis and arthritis [20,21].

Measurement of Acute Phase Proteins

The development of commercially available equine specific SAA and haptoglobin ELISAs have increased the ability to study APPs in horses however these assays are not suitable for practical applications and real-time monitoring of patients [22]. Recent validation of assays utilizing automated serum biochemistry machines for SAA and haptoglobin measurement has allowed for evaluation of APP concentrations in horses during case management [7,23]. Lack of test availability and the paucity of literature regarding the diagnostic and prognostic use of SAA and

haptoglobin have likely contributed to the continued underutilization APPs in equine practice.

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Chapter 2

Evaluation of serum amyloid A and haptoglobin as prognostic indicators for horses presented for colic in a referral population

Abstract:

Reasons for performing study: Acute phase protein (APP) measurement can be used to characterize and rapidly detect inflammation. Serum amyloid A (SAA) and haptoglobin (HP) may be useful for diagnostic and prognostic purposes in horses presenting for colic.

Objective: Evaluate the use of SAA and HP for prognostic application of case outcome in regards to requirement for surgical intervention, development of complications, and duration and cost of hospitalization.

Methods: Total white blood cell count, neutrophil count, fibrinogen, SAA, HP were compared in 20 control horses and 42 horses admitted for colic. Clinicopathologic values were compared in medical and surgical colic cases to test the ability of APPs to predict indication for surgical intervention, development of complications, and duration and cost of hospitalization.

Results: Serum Amyloid A was significantly higher in the surgical group compared to both the control and medical colic groups ($p<0.01$). Haptoglobin concentration was not significantly different between all groups. Horses with SAA >20µg/mL (upper limit reference interval) were more likely to require surgical intervention than those medically managed with an odds ratio (OR) 6.6, (95% confidence interval [CI] 1.4-29.4), $p<0.05$). Euthanasia due to poor prognosis or the development of serious complications (thrombophlebitis, laminitis) were more likely to occur in horses with a

SAA >20µg/mL (OR 8.8, CI1.2-62.2, $p<0.05$). A weak positive correlation was observed between cost and SAA (r=0.30; $p<0.05$). No significant correlation between duration of hospitalization and clinicopathologic variables was present. Horses with complications (euthanasia due to poor prognosis n=4, thrombophlebitis n=3) were more likely to have an elevated SAA (>20µg/mL) (OR 8.8, CI1.2-62.2, $p<0.05$).

Conclusions: Horses presenting for colic with an elevated SAA were more likely to require surgical intervention, develop complications and be euthanized due to poor prognosis despite treatment.

Introduction:

The acute phase response (APR) serves as part of the innate immune response to inflammatory stimuli such as infection, trauma and neoplasia [1]. Human physicians utilize acute phase protein (APP) measurement for characterization and rapid detection of inflammation. Acute phase proteins are used for diagnostic and prognostic purposes for diverse disease processes including cardiovascular disease, cancer and sepsis [2-4]. Lack of data in the veterinary literature and limited test availability due to species specificity has resulted in underutilization of APP measurement, beyond measurement of fibrinogen. Recent development and validation of commercially available equine specific APP ELISAs has facilitated study of APPs in horses. However, these assays are not suitable for practical applications and real-time patient monitoring [5]. Recent validation of serum amyloid A (SAA) and haptoglobin (HP) assays performed on automated serum biochemistry machines has allowed for evaluation of APP concentrations in horses during case management however test availability remains relatively limited [6,7].

Increased serum concentrations of APPs in horses have been reported in colic, infectious diseases, and following elective and non-elective surgery [8-10]. APPs can either increase (positive APPs) or decrease (negative APPs) in response to inflammation. Positive APPs are categorized according to the degree of their response to inflammation. Major APP plasma concentrations increase greater than 10 fold in response to inflammation, whereas moderate APPs increase 2 to 10 fold and minor APPs increase less than 2 fold [11]. Fibrinogen, a moderate APP, is the most commonly utilized APP in equine medicine. However, it is a relatively insensitive

marker of inflammation due to its wide reference interval, response time (24-72 hours following an inflammatory event), minimal fold increase in inflammatory conditions, and consumption in certain disease states [12]. Serum amyloid A, a major APP in horses, is present in very low or undetectable levels in normal horses, can increase greater than 100 fold within 6-12 hours following inflammatory stimuli, and has a relatively short half-life making it an ideal marker for early stages of inflammation and monitoring therapeutic response [12]. Serum amyloid A has multiple functions including induction of leukocyte chemotaxis and activation of inflammatory mediatory synthesis [13]. Haptoglobin, a moderate APP in the horse, begins to increase 12-24 hours following an inflammatory event and may be useful as an indicator of chronic inflammatory disease [14]. Haptoglobin's main function is to bind free hemoglobin to prevent iron loss [15].

Previous studies have evaluated the use of SAA in equine acute abdominal pain [8,16]. The objectives of this study were to (1) compare serum SAA and HP concentrations in horses referred to a tertiary care facility for colic in relation to medical or surgical treatment requirement outcome, (2) evaluate the association of serum SAA and HP concentration with duration of hospitalization and the development of complications or death in horses treated for colic, and (3) associate hospitalization cost with serum SAA or HP concentrations. The null hypothesis was that serum SAA and HP would not be different in horses treated for medical compared to surgical colic.

Materials and Methods:

Animals

All procedures and sample collection were done with approval of the Oregon State University Institutional Animal Care and Use Committee. Blood samples were obtained from 20 clinically normal horses from the Oregon State University Teaching Herd and privately owned animals to serve as controls. Experimental horses (42) were those admitted to the Veterinary Teaching Hospital for evaluation of colic. Horses were grouped as either medical or surgical colics. The medical group included horses with non-strangulating disorders of the gastrointestinal tract (such as displacements, impactions, bloat), which resolved medically. The surgical group included horses presented for colic where surgical intervention was recommended to correct the underlying etiology. Horses with peritonitis, colitis or other systemic diseases not related to the primary gastrointestinal disease were excluded from the study. Horses were also grouped according to survival outcome. Only survivors (horses discharged from hospital) were included in analysis of duration of hospitalization; non-survivors (horses that died during hospitalization for any reason) were excluded. For analysis of cost and complications, horses were included if treatment was elected on admission following minimum database case work-up. Horses euthanized due to financial constraints were not included in analysis of cost, length of hospitalization, and complications.

Experimental design and sample collection

This was a prospective convenience study of horses referred to the tertiary

care facility at Oregon State University. Complete blood counts and automated serum biochemistry assays for SAA and HP were performed on all control and experimental samples. Additional data collected from hospitalized patients included diagnoses, requirement for surgical intervention, development of complications, survival and cost and duration of hospitalization. Complications included in analysis were the development of thrombophlebitis (n=3) and laminitis (n=0) or the recommendation for euthanasia during treatment or surgery due to poor prognosis (n=4).

When horses presented to the veterinary teaching hospital, blood was collected from the jugular vein into EDTA, sodium heparin, and tubes without anticoagulant. Additional samples in 10 horses presenting with colic signs were obtained at the time of normal blood sampling throughout hospitalization to monitor changes in SAA and HP concentrations. Plasma from heparinized blood was collected by centrifugation at 2200 x *g* for 10 minutes. Complete blood cell count and serum biochemistry analysis were performed on EDTA and heparinized plasma, respectively by automatic analyzers either at the Oregon State University Diagnostic Laboratory[a,b] during business hours or Oregon State University Teaching Hospital[c,d] during emergency hours. Plasma fibrinogen was analyzed by the heat precipitation method. Blood collected in the serum tube was allowed to clot and centrifuged at 3000 x *g* for 10 minutes followed by removal and storage of serum -80^{0}C for later batch analysis of SAA and HP.

Serum amyloid A and haptoglobin analysis

An automated chemistry analyzer[b] was used to measure SAA and HP using commercially available assays[e,f] previously validated for equine use [6,7]. The assays were validated as per manufacturer instructions and according to standard operating procedure for new tests in the Oregon State University Veterinary Diagnostic Laboratory.

Data analysis

Total white blood cell count (WBC), neutrophil count, fibrinogen, SAA and HP were tested for normality using the Shapiro-Wilk test. All data from horses presented for colic had non-normal distributions ($p<0.005$), therefore median and interquartile ranges (IQR) were reported. Kruskall-Wallis test and Dunn's multiple comparison post test were used to identify significant differences of clinicopathological variables between the control, medical and surgical groups. Nonparametric Spearman correlation was used to assess the association between the cost and duration of hospitalization in relation to WBC count, neutrophil count, fibrinogen, SAA and HP concentration. Mann-Whitney U test was used to identify significant differences of clinicopathologic variables between horses with and without complications. Fisher's exact test was performed on categorical data. Additional samples obtained at later time points during hospitalization in a subset of horses did not undergo statistical analysis and were presented solely in a descriptive manner. Statistical analysis was performed using commercially available statistical software[g]. $P<0.05$ was considered significant.

Results

Forty-two horses met the inclusion criteria and were included in the study. The medical and surgical groups were both comprised of 21 horses. Twenty eight horses (66.6%) survived, 10 horses (23.8%) were euthanized due to financial constraints and 4 horses (9.5%) were euthanized due to poor prognosis.

All control horses had SAA concentrations below the quantitation limit of the assay (<5µg/mL). Serum amyloid A concentrations >20µg/mL for SAA concentration were considered elevated. Haptoglobin concentrations from control horses were normally distributed. The mean +/- two standard deviations was used to determine the reference interval (10-70mg/dL). Haptoglobin concentrations >70mg/dL were considered elevated.

The distribution of colic horses with abnormal clinicopathologic findings are reported in Table 2-1. Median WBC, neutrophil count, fibrinogen, SAA and haptoglobin of control, medical and surgical colic groups were also compared (Table 2-2). The medical and surgical colic groups had significantly higher neutrophil counts compared to the control group ($p<0.01$). Abnormal total white blood cell count (<6000 or >12000 cells/µL), neutrophil count (<3000 or >6000 cells/µL) and haptoglobin were not significantly associated with medical or surgical outcome. Serum amyloid A was significantly higher in the surgical colic group compared to both healthy control and medical colic groups ($p<0.01$). Horses with SAA >20µg/mL were more likely to have had surgical intervention than horses with colic treated medically (OR 6.6, CI 1.4-29.4, $p<0.05$). Haptoglobin concentration was not significantly different between all groups.

Cost analysis of treated animals (n=32) using a Spearman correlation revealed poor correlation between financial cost compared to WBC, neutrophil count and haptoglobin. A weak negative correlation between cost and fibrinogen (n=11) was present (r=-0.56; $p<0.05$). A weak positive correlation was observed between cost and SAA (r=0.30; $p<0.05$).

No significant correlation between duration of hospitalization and clinicopathologic variables was present. Horses with complications (euthanasia due to poor prognosis n=4, thrombophlebitis n=3) were more likely to have an elevated SAA (>20µg/mL) (OR 8.8, CI1.2-62.2, $p<0.05$). Abnormal total white blood cell count, neutrophil count and haptoglobin were not associated with the development of complications.

Ten horses had SAA and HP run on additional days of hospitalization (Table 2-3). Horses 1-4 presented with large intestinal disease (i.e. displacement, impaction) and SAA and HP remained normal throughout hospitalization of these horses. Horse 5 with a large intestinal displacement had normal SAA and HP on Day 1 and 2 prior to surgical intervention on Day 2. Horse 6 was diagnosed with colic of unknown etiology with an elevated SAA on Day 1 and 2, which resolved with medical treatment. Horses 7 and 8 were treated for gastric impaction. Horse 7 presented with elevated SAA and HP which remained elevated on Day 2. Horse 7 was a poor surgical candidate due to deteriorating condition and severe leukopenia and subsequently was euthanized on day 2. In contrast to the previous cases, horse 8 had normal SAA and HP on presentation but these elevated following surgical intervention. Horses 9 and 10 were diagnosed with eosinophilic enteritis during

exploratory laparotomy. Horse 9 presented with a normal SAA and HP whereas horse 10 presented with elevated SAA and HP. Both horses had elevated SAA and HP following surgical intervention.

Discussion

The clinical application of acute phase protein measurement in equine practice for characterization and rapid detection of inflammation has been limited compared to its use in human medicine. APPs are widely used in human medicine for diagnostic and prognostic purposes and for monitoring response to anti-inflammatory treatment and chemotherapeutics. In this study, serum amyloid A and haptoglobin concentrations were evaluated in horses presented for colic in relation to requirement for surgical intervention, development of complications and duration and cost of hospitalization. Serum amyloid A was the most sensitive clinicopathologic parameter analyzed associated with requirement for surgical intervention or the development of complications in horses presented for acute abdominal pain. Serum amyloid A was significantly higher on presentation in horses with colic requiring surgical intervention in comparison to those with medical colic. More than half (57%) of horses requiring surgical intervention had a SAA value greater than the reference interval compared to 14% of patients with medical colic. Although elevated SAA was associated with higher odds of surgical intervention, overlap between groups demonstrates an increase in SAA at admission alone does not differentiate between medical and surgical colic. This difference should also be emphasized regarding the study population and exclusion of horses with peritonitis and colitis, although these horses can present with signs of abdominal pain. Peritonitis and colitis, as inflammatory disorders, have previously been shown to be associated with an increased acute phase response in horses [16]. These disorders were excluded from the study population since clinical signs, physical examination and diagnostics (ie.

diarrhea, fever, neutropenia) often differentiate these from horses presenting with colic that may require surgical intervention.

The utility of haptoglobin as a diagnostic or prognostic tool in acute colic was not shown in this study. Elevated haptoglobin did not predict need for surgical intervention or the development of complications. Haptoglobin may not be useful as a predictive tool for surgery in acute colic due to its slower increase following an inflammatory stimulus compared to SAA but HP likely still has clinical value in colic cases. An elevated haptoglobin at admission may suggest the presence of a more chronic inflammatory disorder. For optimization of acute phase protein use in clinical cases, inclusion of at least one positive major and one positive moderate acute phase protein has been recommended [17]. Evaluation of both allows more complete interpretation due to the more rapid, marked elevation and relatively fast decline of major acute phase proteins, in contrast to the slower elevation and decline seen in moderate acute phase proteins. Analysis of both allows a more detailed assessment of the severity and duration of the inflammatory process in a patient.

All control horses had SAA below the quantitation limit of the assay which is similar to previously reported values in clinically normal horses [8,9]. This low constitutive expression in combination with the rapid and marked increase (>10 fold) during the acute phase response makes SAA an ideal marker for inflammation. Multiple studies have utilized the same kit (LZ Test ‘Eiken’) for evaluation of SAA in horses and results have been consistent across studies [8,9,12]. In contrast, most recent publications on equine haptoglobin use different test methods than those used in this study. The haptoglobin reference interval determined in this study by an

immunoturbidimetric assay was 10-70mg/dL. The upper limit of the reference interval is lower compared to recent publications evaluating equine haptoglobin where the upper limit reported is approximately 200mg/dL utilizing colorimetric and hemoglobin binding capacity assays [14,18]. This difference emphasizes the importance of using laboratory specific intervals particularly when different methodologies are used.

Serum amyloid A and haptoglobin were performed on additional days of hospitalization in a small portion of the study population (Table 3). Horses treated medically (#1-4) and surgically (#5) for large intestinal disorders such as displacements and impactions did not have elevated SAA or HP at any time point. Horse #5 underwent surgery on Day 2 for a right dorsal displacement of the colon illustrating continued absence of SAA elevation in a non-inflammatory disease process despite more pronounced and longer duration of colic signs. Horse #6 had colic of unknown etiology with elevated SAA, which had decreased on day 2. Horse #6 exemplifies the utility of acute phase protein testing, as no other diagnostics had revealed the underlying etiology of the colic episode but SAA was increased on admission and decreasing by day 2 revealing the colic was likely due to a resolving inflammatory cause. Horses #8 and 10 both had normal SAA on admission followed by an elevation after surgical intervention. Postoperative elevations in acute phase proteins have previously been reported for horses undergoing other types of surgeries [10,19]. Further studies to investigate the normal post-surgical acute phase response in horses with colic are warranted to improve monitoring for the occurrence of

postoperative complications such as thrombophlebitis, laminitis and incisional infections.

The major limitations of this study include small sample size. In particular the high number of animals euthanized due to financial constraints in the surgical group led to decreased sample size for analysis of development of complications, cost analysis and duration of hospitalization. Due to the low number of non-survivors (n=4) when excluding euthanasia due to financial restraints, an effective survival analysis could not be performed. In addition, daily serial testing in all patients could have provided a great amount of additional information regarding the normal acute phase response following medical and surgical colic episodes. Additional information serial SAA levels may have provided includes prediction of hospitalization duration and development of complications. Another study limitation was the absence of fibrinogen testing in all patients. Previous studies have shown the lack of elevation in plasma fibrinogen in acute colic in adult horses [20,21], therefore increasing the sample number would likely not have led to statistical significance.

In conclusion, this study showed horses requiring surgical intervention for colic typically have an elevated SAA whereas horses treated medically frequently had normal SAA concentrations. In addition, horses with an elevated SAA on admission were more likely to be euthanized due to poor prognosis despite treatment or develop significant complications such as thrombophlebitis. The results of this study indicate SAA measurement could be a potential useful diagnostic and prognostic tool in horses presenting for colic.

Endnotes

a)Siemens Advia 120, Munich Germany
b)Beckman Coulter AU480, Brea, CA
c)Heska Hematrue Analyzer, Loveland, CO
d)Heska Element DC, Loveland, CO
e)LZ Test ‘Eiken’ SAA, Tokyo, Japan
f)Haptoglobin assay OSR6165, Beckman Coulter, Inc, Brea, CA
g)Graphpad Prism, La Jolla, CA

	Control (n=20)	Medical (n=21)	Surgical (n=21)
Leukopenia	25%	19%	19%
Leukocytosis	0%	5%	14%
Neutropenia	10%	14%	14%
Neutrophilia	0%	33%	52%
Hyperfibinogenemia	0%	0%	0%
Elevated SAA	0%	14%	57%
Elevated haptoglobin	0%	5%	19%

Table 2-1: Percentage of horses with abnormal clinicopathologic findings in control, medical and surgical groups. Leukopenia <6000 cells/µL, leukocytosis >12000 cells/µL, neutropenia <3000 cells/µL, neutrophilia >6000 cells/µL, hyperfibrinogenemia >400mg/dL, elevated SAA >20µg/mL, elevated haptoglobin >70mg/dL.

	Control (n=20)			Medical Colic				Surgical Colic			
Variable	Median (IQR)	Minimum	Maximum	Sample number	Median (IQR)	Minimum	Maximum	Sample number	Median (IQR)	Minimum	Maximum
WBC (cells/µl)	6690 (5933-7178)	4820	9800	21	7800 (6605-9100)	4500	14140	21	7490 (6500-9405)	2900	20100
Neutrophils (cells/µL)	3365 (3120-3830)	2653	4711	21	5500[a] (3120-3830)	2600	12443	21	6142[a] (3950-7600)	388	17100
Fibrinogen (mg/dL)	200 (125-300)	100	400	6	300 (175-300)	100	300	7	200 (100-300)	100	300
SAA (µg/mL)	<5 N/A	<5	<5	21	<5 N/A	<5	429.6	21	31.3[a,b] (<5-172.2)	<5	2113
Haptoglobin (mg/dL)	42.8 (27.6-49.8)	0.6	60.5	21	30.7 (19.4-44.2)	0.5	72.9	21	28.1 (15.5-50.4)	1	124.3

Table 2-2: Comparison of clinicopathologic values in control, medical colic and surgical colics. [a]Significant difference between colic group and control at $p<0.01$. [b]Significant difference between medical and surgical group at $p<0.01$. Interquartile range for control and medical colic groups reported as N/A due to all values within IQR <5µg/ml.

Horse	Group	Day of hospitalization	HP (mg/dL)	SAA (µg/mL)
1	Medical-Large intestine	1	4	0
		2	22	6.2
		5	34.4	0
2	Medical-Large intestine	1	0.5	1.3
		2	3.2	0
3	Medical-Large intestine	1	20.9	0.4
		2	28.7	0.8
		5	34.3	0.4
4	Medical-Large intestine	1	20.5	1
		3	41	0.5
5	Surgical-Large intestine	1	28.1	1.6
		2	19.4	1.4
6	Medical-Unknown etiology	1	47.8	346.9
		2	58.2	268.3
7	Medical-Gastric impaction	1	124.3	455
		2	128.1	428.3
8	Surgical-Gastric impaction	1	54	4.3
		2	65.2	2153.7
		4	62	2500
9	Surgical-Eosinophilic enteritis	1	80.9	2112.5
		2	116.7	2500
10	Surgical-Eosinophilic enteritis	1	31.9	0
		2	46.2	1170.3
		5	104.6	398.4

Table 2-3: Serial analysis of SAA and HP during hospitalization. SAA >20µg/mL, HP >70mg/dL considered elevated.

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Chapter Three

Evaluation of serum amyloid A and haptoglobin as prognostic indicators for horses presented for inflammatory disease in a referral population

Abstract:

Reasons for performing study: Acute phase proteins (APPs) can be used to rapidly detect and characterize inflammation. Serum amyloid A (SAA) and haptoglobin (HP) may be useful for diagnostic and prognostic purposes in horses with inflammatory diseases.

Objective: Evaluate the use of SAA and HP for prognostic application of case outcome in inflammatory diseases in regards to mortality, development of complications, and duration and cost of hospitalization.

Methods: Total white blood cell count, neutrophil count, albumin, fibrinogen, SAA and HP concentrations were compared in 20 control horses and 53 horses admitted with suspected inflammatory and infectious diseases. Clinicopathologic values on admission were compared in surviving and euthanized horses to test the ability of APPs to predict indication for mortality, development of complications, and duration and cost of hospitalization. Additional horses (n=22) had SAA and HP monitored during hospitalization to test the ability of serial analysis to predict the development of complications or euthanasia due to poor prognosis.

Results: Neutrophil count, SAA and HP were significantly different in both surviving and euthanized horses on admission compared to the control group. Albumin was the only clinicopathologic variable tested with a significant difference ($p<0.05$) between survivors and nonsurvivors. Analysis of hospitalization duration of treated animals

revealed a moderate positive correlation between duration and haptoglobin (r=0.355; p<0.05). No significant correlation was found between the clinicopathologic variables tested on admission and the development of complications. Horses with an increased SAA between 24-72 hours compared to admission SAA were more likely to be euthanized or develop complications (OR 7.0, CI 1.1-45.9, p<0.05).

Conclusions: Admission SAA and HP concentrations were not significantly correlated with survival outcome in horses presenting for inflammatory conditions. Acute phase proteins likely have more utility in serial analysis for horses presenting with inflammatory conditions rather than testing at a single time point.

Introduction:

The acute phase response (APR) is part of the innate immune system triggered by inflammatory stimuli such as trauma, infection and neoplasia [1]. Activation of the APR results in increased or decreased synthesis of acute phase proteins (APPs) by the liver. Positive APPs are categorized by their response to inflammation as major (>10 fold response), moderate (2-10 fold increase) and minor (<2 fold increase) [2]. When the liver responds to inflammation by producing positive APPs, the production of negative APPs is downregulated. Albumin is the most widely measured negative APP [3]. The most widely measured positive APP in equine practice is fibrinogen, a moderate APP in the horse. Fibrinogen's wide reference interval, moderate increase and slow response time of 24-72 hours following an inflammatory insult make it a relatively insensitive APP, particularly as a predictive marker of inflammation [3]. In contrast, serum amyloid A (SAA) is a major APP in horses characterized by low or undetectable levels in normal horses. Following inflammatory stimuli SAA has a rapid and robust response with increases of 10 to >100 fold within 6-12 hours and a short biologic plasma half-life of approximately 20-35 hours in other species [4-6]. This response makes SAA a sensitive marker during the early inflammatory response and useful for monitoring treatment efficacy [4]. Haptoglobin (HP), another moderate APP in the horse, is normally present in plasma, increases 12-24 hours following an inflammatory stimulus, and has a longer half-life of approximately 3.5 days [4, 5, 7].

Acute phase protein (APP) measurement is becoming routine in human health care for both diagnostic and prognostic purposes in numerous disease processes.

Measurement of APPs has not gained widespread use in equine practice due to limited test availability and turnaround time limiting its clinical usefulness. Veterinary practitioners who measure APPs often consider them to be of high clinical value as one of the most important biomarkers of inflammation [8]. Validation of equine assays utilizing automated serum biochemistry analyzers has facilitated APP evaluation during case management however test availability remains limited [9, 10]. Along with limited availability, the demand for these assays in equine practice currently remains low due to the small number of studies showing the ability of APP measurement to improve diagnostic and prognostic abilities of clinicians. This is likely to change with recent studies showing increased APP serum concentrations in horses with inflammatory diseases such as enterocolitis, pneumonia, placentitis and septicemia in foals [11-13].

The objectives of this study were to (1) compare serum SAA and HP concentrations in horses referred to a tertiary care facility for evaluation of suspected inflammatory or infectious disease in relation to survival outcome, (2) evaluate the association of serum SAA and HP concentration with duration and cost of hospitalization and the development of complications, and (3) evaluate the utility of serial analysis of SAA and HP concentration in predicting survival outcome and complication development. The null hypothesis was that serum SAA and HP would not be different in surviving versus euthanized horses.

Materials and Methods:

Animals

Procedures and sample collection were performed under approval of the Oregon State University Institutional Animal Care and Use Committee. Blood samples were collected from 20 clinically normal horses from the Oregon State University Teaching Herd and privately owned animals to serve as controls. Experimental samples were obtained from horses (n=53) admitted to the Veterinary Teaching Hospital for evaluation of suspected inflammatory diseases. Horses euthanized for financial reasons with no treatment attempted were excluded from analysis. Horses presenting for acute colic were included only if an underlying inflammatory disease was diagnosed such as peritonitis and colitis. Horses presenting for treatment of surgical and medical colic such as intestinal obstruction, strangulation, bloat and unknown etiologies were excluded. Horses included in the study were grouped according to survival outcome. Horses euthanized due to poor prognosis either following initial examination and diagnostics or during treatment comprised the non-survivor group. For analysis of cost and complications, horses (n=51) were included if treatment was elected on admission following initial examination and diagnostic tests. Analysis of hospitalization duration was performed only on surviving horses (horses discharged from hospital).

Experimental design and sample collection

This was a prospective convenience study of horses evaluated at the tertiary care facility at Oregon State University. Complete blood counts and automated

serum biochemistry assays for SAA and HP were performed on all control and experimental samples. Albumin was evaluated in 48 experimental horses and not assayed in control horses. Additional data collected from hospitalized patients included diagnosis, development of complications, cost and duration of hospitalization, and survival outcome. Complications were defined as new problems that developed during hospitalization that were not present on admission.

On hospital admission, blood was collected from the jugular vein into EDTA, sodium heparin, and tubes without anticoagulant. Additional samples for SAA and HP measurement were obtained in 22 horses up to once daily at the time of normal blood sampling during hospitalization. Plasma from heparinized blood was collected by centrifugation at 2200 x *g* for 10 minutes. Complete blood cell count and serum biochemistry analysis were performed on EDTA and heparinized plasma, respectively, using automatic analyzers and standard methods either at the Oregon State University Diagnostic Laboratory[a,b] during business hours or Oregon State University Teaching Hospital[c,d] during emergency hours. Plasma fibrinogen was analyzed by the heat precipitation method. Blood collected in the serum tube was allowed to clot and centrifuged at 3000 x *g* for 10 minutes followed by removal and storage of serum at -80^{0}C for later batch analysis of SAA and HP.

Serum amyloid A and haptoglobin analysis

Serum amyloid A and HP were measured by an automated chemistry analyzer [b] using commercially available assays[e,f] previously validated for equine use [9, 10]. The assays were validated according to manufacturer instructions and standard

operating procedure for new tests in the Oregon State University Diagnostic Laboratory.

Data analysis

Total white blood cell count (WBC), neutrophil count, fibrinogen, albumin, SAA and HP were tested for normality using the Shapiro-Wilk test. White blood cell count, neutrophil count, albumin concentration and HP concentration had normal distributions. Fibrinogen and SAA had non-normal distributions ($p<0.05$). Mean and 95% CI were determined for normal data however median and interquartile range were reported for all variables for continuity of data presentation. One-way ANOVA was performed to identify significant differences between the control, survivor and non-survivor groups in normally distributed data (WBC, neutrophil count, HP). Albumin was not evaluated in control horses therefore a *t* test was performed to identify significant differences between survivors and non-survivors. Kruskall-Wallis test and Dunn's multiple comparison post-test were performed on data with non-normal distributions (fibrinogen and SAA) to identify significant differences between the control, survivor and non-survivor groups. Pearson correlation was used to assess the association between cost and hospitalization duration in relation to WBC count, neutrophil count, albumin and HP concentration. Nonparametric Spearman correlation was used to assess the association between cost and hospitalization duration in relation to SAA and fibrinogen. Mann-Whitney U test was used to identify significant differences of clinicopathologic variables between horses with and without complications. Peritonitis and colitis subgroup survival data were analyzed by a Mann-Whitney U test. Categorical data were compared using a

Fisher's exact test with odds ratio and 95% confidence interval reported when significant. Significance was set at $p<0.05$ for all statistical analysis. Statistical analysis was performed using commercially available statistical software [g].

Results

Fifty-three horses met the inclusion criteria and were included in the study. Diagnosis included peritonitis (n=16), colitis (n=15), trauma (n=4), renal insufficiency (n=3; two with concomitant cystitis), pneumonia (n=2), cellulitis (n=2), fever of unknown origin (n=2), lymphangitis (n=1), chronic laminitis (n=1), septic osteomyelitis (n=1), hyperammonemic encephalopathy (n=1), urethral obstruction with bladder rupture (n=1), tooth root fracture and abscess (n=1), middle uterine artery rupture (n=1), pyometra (n=1) and placentitis (n=1). Thirty-six horses (68%) survived and 17 horses (32%) were euthanized due to poor prognosis.

The distribution of abnormal clinicopathologic findings are reported in Table 3-1. Forty-four of 53 horses had increased SAA concentrations (>20µg/ml) at the time of admission. Of the nine horses with normal SAA concentrations on admission, four horses had elevated HP concentrations with all of these horses having clinical signs for ≥5 days including laminitis, renal insufficiency, pyometra and placentitis. Five horses had both normal SAA and HP concentrations with all presenting for clinical signs of ≤2 days duration. Five horses in this study had decreased haptoglobin concentrations with the two survivors undergoing rhabdomyolysis on admission, two non-survivors with disseminated intravascular coagulation and one non-survivor with hemorrhagic colitis.

White blood cell count, neutrophil count, albumin, fibrinogen, SAA and HP concentrations of control and experimental samples are summarized in Table 3-2. Neutrophil count, SAA, and HP of the control group were significantly different compared to both the survivor and non-survivor groups ($p<0.05$). Non-survivors had

a significantly lower albumin compared to the survivors ($p<0.05$). No significant difference in survival outcome was associated with WBC count, neutrophil count, fibrinogen, SAA and HP. Survival outcome in horses diagnosed with peritonitis (n=16) and colitis (n=15) were analyzed as separate subgroups with no clinicopathologic variables significantly associated with survival outcome in either subgroup.

Cost analysis of treated animals (n=51) revealed a moderate positive correlation between cost and fibrinogen ($r=0.342$; $p<0.05$) and weak negative correlation between cost and albumin ($r=-0.279$; $p<0.05$). No significant correlation between cost and SAA and HP concentrations was found. Analysis of hospitalization duration of treated animals revealed a moderate positive correlation between duration and haptoglobin ($r=0.355$; $p<0.05$).

Complications that were included in statistical analysis were the development of phlebitis/thrombophlebitis (n=3), laminitis (n=2), right dorsal colitis (n=1), polysynovitis (n=1), retained placenta following dystocia (n=1), abortion (n=1), large colon impaction (n=1), septic peritonitis (n=1), and bladder rupture (n=1) in a total of twelve patients. No significant correlation was found between the clinicopathologic variables tested on admission and the development of complications compared to survivors with no complications.

Horses with serial analysis of SAA and HP concentrations performed between 24-72 hours of hospitalization (n=22) were categorized as increased or decreased concentration compared to presentation to assess correlation with the outcome of euthanasia or development of significant complication. Horses with a >2500µg/ml

SAA concentration at admission and at successive samples were placed in the increased category which included one survivor with a complication and one survivor with no complications. Horses with an increasing SAA between 24-72 hours compared to admission SAA were more likely to be euthanized or develop complications during hospitalization (OR 7.0, CI 1.1-45.9, $p<0.05$). No significant correlation was present between serial HP concentrations and survival outcome or the development of complications.

Serial data for a subset of samples is presented (Tables 3-3 and 3-4) to demonstrate the pattern of SAA, HP and fibrinogen concentrations during treatment in both surviving and non-surviving horses. Data for surviving horses shows SAA concentration generally decreased during hospitalization and treatment, but SAA concentrations did rise despite institution of treatment in some horses which did not always correspond with the development of complications. In particular, SAA of non-survivors tended to increase. Haptoglobin generally reflected inflammation more quickly than fibrinogen. In addition, serial analysis was performed on two horses with decreased haptoglobin concentrations (horse #1 with rhabdomyolysis and horse #13 with disseminated intravascular coagulation).

Discussion

Acute phase proteins are routinely used by human physicians for diagnostic purposes and to monitor response to treatment in hospitalized patients. The use of APPs is becoming more widely recognized in veterinary medicine with increasing numbers of published studies and availability of automated assays. The purpose of this study was to evaluate the clinical significance of SAA and HP measurement in horses admitted to a tertiary care facility for inflammatory and infectious diseases. In this study, SAA and HP concentrations were evaluated in horses in regards to survival outcome, development of complications, and duration and cost of hospitalization.

Serum amyloid A was consistently increased in the horses presented in this study with more than 80% of horses having increased SAA concentrations at the time of admission. Previous studies have shown SAA to be a reliable marker of inflammation in diverse processes including diseases caused by both bacterial and viral etiologies and aseptic processes such as surgical trauma [13-15]. Serum amyloid A was more consistent in identification and characterization of the severity of the inflammatory response at the time of initial evaluation compared to measurement of fibrinogen alone. Fibrinogen allows assessment of the magnitude of the inflammatory response days after the initial insult due to the slow response of time of 24-72 hours with peak concentrations not being reached until 72-144 hours. Although typically increased in horses admitted to this study, SAA may not have been increased in some horses presented early in the APR prior to up-regulated synthesis of SAA, which occurs at 6-12 hours with peak response up to 48 hours after the initial insult [4, 15]. Another potential reason for normal SAA concentration on

admission would be resolving inflammation and the short plasma half-life of SAA, which would have caused our initial measurement to be too late to find increased SAA in that particular horse. Although the half-life of equine SAA has not been determined, prior studies have shown SAA serum concentrations can return to normal within days following an inflammatory insult [14, 16]. Previous studies evaluating clearance of serum amyloid A in mice report half-lives of 30-75 minutes [17, 18]. Species difference and ongoing inflammation likely affects biologic half-life in disease states as reported in patients with community acquired pneumonia with a half-life of 35 hours [6]. Similarly, SAA half-life was approximately 20 hours in sheep being treated for *Psoroptes ovis* infestations [5].

Moderate APPs such as fibrinogen and haptoglobin with a longer APR remain valuable in APP measurement due to the information they provide regarding chronicity. Divergences between major and moderate APPs have been shown to provide useful information in dogs and cattle in regards to differentiating between acute and chronic inflammation and when monitoring chronic disease [6, 19]. In this study, four horses had normal SAA concentrations with increased HP concentrations. Each of these horses had chronic disease and although it was a small subset of the tested population, haptoglobin may be a valuable diagnostic tool for monitoring inflammation and treatment efficacy in diseases such as chronic laminitis or placentitis. Haptoglobin, although not significantly correlated with survival, had a moderate positive correlation with hospitalization duration. This correlation likely reflects that horses with chronic ongoing inflammation may have required longer hospitalization for appropriate treatment. Haptoglobin may also be useful in

identifying hemolytic processes. Haptoglobin's main function is to bind free hemoglobin however it can also bind free myoglobin [20]. Haptoglobin concentration can be overwhelmed by large amounts of free hemoglobin or myoglobin and concentrations will become abnormally low in cases of intravascular hemolysis and rhabdomyolysis [21, 22]. Only a few horses (n=5) in this study had abnormally low haptoglobin concentrations including two survivors with rhabdomyolysis due to trauma, three euthanized horses with disseminated intravascular coagulation (n=2), and hemorrhagic colitis (n=1). If SAA and HP measurement are used to monitor horses with inflammatory diseases, HP may have the additional benefit as an indicator of hemostatic diseases such as disseminated intravascular coagulation in patients already at increased risk. Future studies are necessary to determine if haptoglobin will be a reliable biomarker in horses with hemostatic disease.

No significant association with admission SAA concentration and survival outcome was identified in this study. A prior study had found a significant association with survival outcome and increased SAA concentrations found in horses presenting for all types of colic however this may reflect the typical survival outcome associated with inflammatory disorders such as colitis or peritonitis compared to non-inflammatory disorders such as intestinal obstructions or bloat [23]. Recently we have shown a significant association of horses presenting for non-inflammatory causes of colic with SAA and the likelihood of complications developing or euthanasia due to poor prognosis (Westerman et al. (2014) unpublished data). Based on the differences identified in these studies, it is important when assessing APPs to

analyze them in context of the primary disease process. In this study, SAA on admission was typically increased, which would be expected in inflammatory diseases, however it was not associated with survival outcome. These findings are reflected in human APP literature where admission APP does not correlate well with survival while serial analysis is a much better predictor of mortality [24]. For example, rising procalcitonin and C reactive protein, both APPs utilized in human medicine, are significantly associated with increased mortality [19, 25]. Although only a small number of horses (n=22) had sequential analysis performed, rising SAA was significantly associated with the development of a significant complication during hospitalization or recommendation for euthanasia. Serial analysis of SAA likely will have the most benefit in individual case management of cases with a known inflammatory component rather than single point analysis.

In conclusion, this study showed SAA and HP concentrations evaluated on admission in horses admitted for inflammatory conditions were not significantly associated with survival outcome. Haptoglobin will likely have the most utility in monitoring cases of chronic inflammation with further investigations regarding specific disease processes needed. Single point analysis of SAA is likely to identify the presence of inflammation however serial analysis during treatment is likely to be a more effective diagnostic and prognostic tool in horses presenting for an inflammatory condition.

Endnotes

a)Siemens Advia 120, Munich Germany
b)Beckman Coulter AU480, Brea, CA
c)Heska Hematrue Analyzer, Loveland, CO
d)Heska Element DC, Loveland, CO
e)LZ Test ‘Eiken’ SAA, Tokyo, Japan
f)Haptoglobin assay OSR6165, Beckman Coulter, Inc, Brea, CA
g)Graphpad Prism, La Jolla, CA

	Control (n=20)	Survivors (n=36)	Non-survivors (n=27)
Leukopenia	25%	6%	18%
Leukocytosis	0%	22%	12%
Neutropenia	10%	19%	24%
Neutrophilia	0%	50%	47%
Hypoalbuminemia	N/A	22%	44%
Hyperalbuminemia	N/A	4%	6%
Hyperfibinogenemia	0%	37%	40%
Elevated SAA	0%	81%	88%
Decreased haptoglobin	5%	6%	18%
Elevated haptoglobin	0%	64%	47%

Table 3-1: Values correspond to percentage of horses with abnormal clinicopathologic findings. Leukopenia <6000 cells/µL, leukocytosis >12000 cells/µL, neutropenia <3000 cells/µL, neutrophilia >6000 cells/µL, hypoalbuminemia <2.8mg/dL, hyperalbuminemia >3.9mg/dL, hyperfibrinogenemia >400mg/dL, elevated SAA >20µg/mL, elevated haptoglobin >70ml\g/dL, decreased haptoglobin <10mg/dL,

	Controls (n=20)			Survivors				Non-survivors			
Variable	Median (IQR)	Minimum	Maximum	Sample number	Median (IQR)	Minimum	Maximum	Sample number	Median (IQR)	Minimum	Maximum
WBC (cells/µL)	6690 (5933-7178)	4820	9800	36	8525 (5598-11450)	1300	19500	17	7400 (6600-10665)	2000	18750
Neutrophils (cells/µL)	3365 (3120-3830)	2653	4711	36	6228[a] (3294-8575)	932	16380	17	5600[a] (3941-8744)	857	15188
Albumin (mg/dL)	N/A	N/A	N/A	32	3.1[b] (2.9-3.5)	2.4	4.1	16	2.8[b] (2.2-3.4)	1.9	4.1
Fibrinogen (mg/dL)	200 (125-300)	100	400	19	300 (200-500)	100	900	10	300 (100-525)	100	700
SAA (µg/mL)	<5	<5	<5	36	401[a] (120-1437)	<5	2500	17	392[a] (273-959)	<5	2500
HP (mg/dL)	42.8 (27.6-49.8)	0.6	60.5	36	87[a] (58-112)	0.9	213	17	56[a] (20-127)	0	242

Table 3-2: Comparison of clinicopathologic values in control, survivors and non-survivors. [a]Significant difference between experimental group and control at $p<0.005$. [b]Significant difference between survivor and non-survivor group at $p<0.05$.

Horse	Diagnosis	Complication	Day of hospitalization	Fibrinogen (mg/dL)	Haptoglobin (mg/dL)	SAA (µg/mL)
1	Peritonitis	Admission - rhabdomyolysis	1		0.9	2500
			2	400	4.3	2500
			3		20.9	413.8
			16	300	32.9	5
2	Peritonitis		1	500	53.7	459.3
			2	500	61.4	694.4
			7	400	81.5	62.3
3	Peritonitis		1		51.1	331.5
			2	400	55	1101
			4	500	65.9	614
4	Colitis	Day 2 - thrombophlebitis	1		125.9	2500
			2	300	95.3	2500
			7	500	89.5	300.5
5	Colitis	Day 6 - polysynovitis	1	400	131.1	310.5
			4	900	124.7	369.9
			7	800	116.6	2500
			11	700	137.2	1334.2
6	Colitis	Day 2 - thrombophlebitis	1		83.9	1709
			2	400	70.4	1606
			3	200	78.6	1504
7	Colitis		1	300	31.5	901.9
			4	400	49.9	407.9
			6	100	44.1	58.4
8	Colitis		1	200	111.8	2500
			3	400	66.5	371.1
			5	200	68.2	367.9
9	Pneumonia		1	500	125.5	963
			3	900	160.3	359.7
10	Pneumonia		1	900	213.1	379.8
			3	900	208.8	374.4
			6	800	209.5	368.6
11	Uterine artery rupture	Day 1 - dystocia and retained placenta	1		60.3	5
			3	500	92.7	2347.2
			5	500	111.6	481.3
12	Head trauma		1	300	57.8	236.8
			7	500	69.8	153.1

Table 3-3: Serial APP measurement on a subset of surviving horses. SAA >20µg/mL, haptoglobin <10 or >70mg/dL, fibrinogen >400mg/dL considered abnormal

Horse	Diagnosis	Complications	Day euthanized	Day of hospitalization	Fibrinogen (mg/dL)	Haptoglobin (mg/dL)	SAA (µg/mL)
13	Fibrinonecrotizing colitis	Admission - DIC	3	1		9	2500
		Day 3- septic peritonitis		2	100	1.6	365
14	Right dorsal colitis	Admission - laminitis	9	1	100	94.7	936.5
		Day 7 - thrombophlebitis		2		83	1352.1
				3		82.1	1440.2
				4	300	88.5	1132.5
				7	500	128.9	2110.5
15	Necrohemorrhagic ulcerative colitis	Day 2 - laminitis	2	1		27.2	331
				2		31.8	619.7
16	Urethral obstruction	Day 2 - bladder rupture	2	1	100	21.7	425.6
				2		55.1	1501

Table 3-4: Serial APP measurement on a subset of euthanized horses. Disseminated intravascular coagulation abbreviated DIC. SAA >20µg/mL, haptoglobin <10 or >70mg/dL, fibrinogen >200mg/dL considered abnormal

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Chapter 4

Conclusions

The objective of this thesis was to evaluate the use of serum amyloid A and haptoglobin in horses presenting for colic and inflammatory diseases to a tertiary care facility. Serum amyloid A and haptoglobin concentrations were evaluated for their potential as prognostic indicators compared to clinicopathologic variables typically evaluated in diseased horses including total white blood cell count, neutrophil count and fibrinogen. The hypothesis was that increased serum amyloid A and haptoglobin concentrations in hospitalized horses presented for colic and inflammatory disease would be better negative prognostic indicators of case outcome compared to total white cell count, neutrophil count and fibrinogen in regards to mortality, development of complications, duration and cost of hospitalization and requirement for surgical intervention.

Serum amyloid A concentration on admission was significantly associated with failure to survive and the development of complications in horses evaluated for colic but not for horses presenting for primary inflammatory diseases. Serum amyloid A was the only clinicopathologic variable assessed on admission associated with poor outcome in horses with colic. Horses were only included in the colic group if presenting for primary non-inflammatory causes such as intestinal obstruction and bloat and had no evidence of a primary inflammatory etiology (i.e. colitis and peritonitis). Although the underlying cause of a colic episode may not be inflammatory, it does not preclude the development of inflammation as a result of the primary disease process. For example, horses presenting with strangulating

gastrointestinal lesions will develop an inflammatory response due to compromised or dead bowel [1]. Horses with colic requiring surgical intervention (57%) were more likely to have an elevated SAA concentration compared to those medically managed (14%). Serum amyloid A may have use as an ancillary diagnostic test in horses presenting for colic to help determine the need for surgical intervention.

Admission SAA concentration was associated with the development of the significant complications of laminitis and thrombophlebitis in horses with colic. In contrast, no association with admission SAA concentration with survival or the development of complications was observed in horses presenting with inflammatory diseases including separate analysis of the peritonitis (n=16) and colitis (n=15) subgroups. This difference emphasizes the importance of considering the primary etiology of the disease when utilizing admission SAA concentration as a prognostic indicator. Further larger-scale investigations into specific inflammatory disease processes will help elucidate the prognostic abilities of single point analysis SAA in regards to morbidity and mortality.

Serum amyloid A may also have utility in assessing colic patients by ambulatory practitioners. Colic is the most common cause for a horse to require emergency evaluation [2]. Need for hospitalization and potential surgery is not always evident when a horse is evaluated for colic. Due to the association of SAA with the requirement for surgical intervention and complications, a point-of-care SAA could provide additional evidence for the need for referral. As with any diagnostic test, the result must be interpreted in context of all physical examination findings and diagnostics as an elevated SAA can also indicate a primary inflammatory disease

process. In the case of an elevated SAA performed by an ambulatory practitioner during a colic examination, even if the underlying cause of colic is an inflammatory cause such as peritonitis or colitis and not necessarily a surgical lesion, these patients often require hospitalization. Evidence of significant inflammation may lead to earlier referral, hospitalization and treatment which could improve patient outcomes.

Admission serum amyloid A and haptoglobin were not strong predictors of cost or duration of hospitalization in horses presenting for colic and inflammatory diseases. A weak positive correlation was observed between cost and SAA ($r=0.30$; $P<0.05$) in horses presenting for colic which is most likely due to the association between elevated SAA and the requirement for surgical intervention. A moderate positive correlation between hospitalization duration and haptoglobin was present ($r=0.355$; $P<0.05$) in horses presenting for inflammatory disease which is likely associated with the need for longer term treatment with more chronic disease processes.

Serum amyloid A had much better prognostic abilities in horses with inflammatory disease when measured in serial analysis compared to single point analysis. Resolution of inflammation following initiation of treatment has previously been difficult to evaluate. Fibrinogen is most frequently utilized however it has a lengthy response time following an inflammatory insult [3]. Horses with an acute inflammatory process will often present with a normal fibrinogen which will subsequently increase days following the initial insult even with a positive clinical response to therapy. Serial analysis of SAA provides a more accurate real time assessment of the patient's inflammatory status. Elevation of SAA following

institution of treatment was associated with failure to survive and development of complications. This finding is similar to a previous study where horses with an elevated SAA following elective castration were more likely to develop post-operative complications [4]. Serial SAA analysis may prove useful in identifying horses at high risk of complications promoting early therapeutic intervention to improve clinical outcomes. Acute phase proteins, such as C reactive protein and procalcitonin, are frequently used as serial tests in human medicine to monitor disease progression to improve early therapeutic intervention and to determine the effectiveness of treatments [5, 6]. Although not performed in the colic study, evaluation of serial analysis in horses recovering from colic surgery may aid in early identification of common post-operative complications such as laminitis and thrombophlebitis to allow earlier institution of appropriate therapy. As APP measurement becomes easier to measure due to automated analyses on standard biochemistry machines and cost decreases, serial analysis may become more commonly performed to improve diagnostic and prognostic abilities in horses presenting with a variety of disease processes.

Major APP measurement, such as SAA, provides information regarding acute inflammation which is complemented by the ability of moderate acute phase protein measurement to identify chronic inflammation. Previous reviews of the clinical use of acute phase protein measurement in veterinary medicine have stressed the importance of measuring both major and moderate APPs [7]. Although haptoglobin was not significantly associated with survival outcome, clinically it provides relevant information. Identification of chronic inflammation, particularly when not evident by

the history or clinical course, can provide valuable information regarding the disease process or evidence of an underlying chronic disease. Haptoglobin will likely be most useful in equine medicine in disease processes where SAA may not be persistently elevated in chronic disease such as recurrent airway obstruction [8]. The ability of haptoglobin to aid in monitoring chronic disease, such as chronic laminitis, requires further investigation. Serial analysis of haptoglobin may be useful as an indicator of deterioration of chronic disease prior to clinical decline and to evaluate the efficacy of long-term treatments.

Another application of haptoglobin in acute disease may include identification of hemostatic diseases such as disseminated intravascular coagulation (DIC). In this study, haptoglobin concentration was decreased with evidence of hemostatic abnormalities including DIC (n=2) and hemorrhagic colitis (n=1). Currently, testing for DIC in horses at tertiary care facilities may be limited during the hospital diagnostic laboratory business hours. In addition, testing of hemostatic parameters such as prothrombin and partial thromboplastin times may require control animals depending on method utilized which impacts reference intervals. If haptoglobin proves to be a reliable marker of DIC, its potential use as an automated assay available during emergency hours may help identify horses at risk of DIC. Rapid identification and institution of therapy for these horses is critical due to the worse clinical outcomes associated with this disease [9]. A larger scale study evaluating the ability of haptoglobin to identify horses with hemostatic abnormalities is needed. Based on the horses identified in this study, a coagulation/DIC panel is recommended for horses with systemic illness and a decreased haptoglobin.

Analysis of serum amyloid A and haptoglobin in horses presenting for both colic and inflammatory disease in this study show they have excellent promise as potential diagnostic and prognostic tools. Further investigations evaluating specific disease processes and larger scale studies will improve the ability to use APPs in equine medicine. Serum amyloid A and haptoglobin have potential to become routinely utilized diagnostics in equine medicine as more scientific evidence becomes available, cost decreases and availability of these assays increase.

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