Serum amyloid A in healthy, septic and diarrheic foals and its association with length of hospitalization.

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Sandra Martinez Saldivar

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Approved by:

Major professor:
Katherine M Delph Miller, DVM, MS,
DACVIM

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Abstract

Title: Serum Amyloid A in Healthy, Septic, and Diarrheic Foals and Association with Length of Hospitalization

Background: Serum amyloid A (SAA) can be a marker of systemic inflammation when elevated in neonatal foals, but investigation with enterocolitis has not been performed.

Hypothesis/Objectives: Measure and describe SAA in healthy, septic, and diarrheic foals. We hypothesized that 1) SAA will be higher on admission in diarrheic than in septic foals and 2) higher SAA concentration on admission will be associated with length of hospitalization (LOH).

Animals: 35 foals <1 month of age. Foals were categorized into 3 groups: healthy control (n=14), diarrheic (n=17), and septic (n=4). The healthy foals were from a single university-associated farm, and both diarrheic and septic foals were a hospitalized population.

Methods: Prospective observational cohort study. In healthy foals, SAA was measured daily on days 1-3. For both diarrheic and septic foals, SAA was measured on admission and then daily, until SAA was < 100 µg/ml or discharge/humane euthanasia. Serum amyloid A was compared between groups, and the association between SAA and LOH was determined.

Results: On admission, SAA (median in µg/mL; IQR) was higher in both diarrheic (919; 770-1104) and septic (1122.5; 814.2–1393.2) compared to healthy foals (2; 0-15) ($p < 0.001$ and $p < 0.01$, respectively). No difference was detected between diarrheic and septic foals ($p = 0.6958$).

Foals with higher SAA on admission were hospitalized (median days; IQR) longer (8; 6-10.5) than foals with lower SAA (6; 5-7) ($p < 0.01$).

Conclusions and Clinical Importance: Admission SAA in both diarrheic and septic foals can provide information regarding LOH to guide decision making since hospitalization requires a significant financial investment.

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Dedication

To my sister: you are my best friend. I am just so grateful for your existence; without your company and support, this life would be unbearable.

To my parents: thank you for giving me my sister and for always trying your best.

To my second parents: thank you for taking on such a huge responsibility, even when it was not yours. Without you, I wouldn't be here, and none of this would have been possible. I will be forever in your debt.

To my friends, the ones that are still here: thank you for staying, even when life makes it difficult.

To my dog Tripie: I was not looking for you when I found you, but you were a blessing and the perfect match. I would have loved to finish this adventure with you by my side, but you will always be in my heart.

To myself: you had to change and grow so much, and this journey was harder than expected, but you never gave up. I'm so proud of you.

Chapter 1 - Literature Review

Diarrhea in neonate foals.

Diarrhea is defined as an increase in the rate of defecation and/or changes in fecal firmness, which indicates increase in water content (1). Diarrhea is a common issue among foals (2) frequently necessitating the expertise of a veterinarian for appropriate management (3).

The etiology of diarrhea can vary widely, including both noninfectious and infectious causes. Noninfectious causes include foal heat diarrhea, dietary imbalances, necrotizing enterocolitis, luminal irritants, neonatal encephalopathy, and secondary lactose intolerance. Infectious organisms that may be involved include Rotavirus, *Clostridium perfringens* types A and C, *Salmonella* species, *Clostridioides difficile*, and Coronavirus. While less common, parasitic causes include *Strongyloides westeri* or *Cryptosporidium* (4). A description of differentiating features for each follows:

- Foal heat diarrhea: present between 5 to 15 days of age without clinical signs other than diarrhea; it affects 75 to 80% of foals for 3-4 days. Previously it was believed to be secondary to changes in the mare's hormones; however, this condition has been noted in orphaned foals. Now it is believed to be to a secretory-type diarrhea led by maturation changes in the GI tract microflora (5).
- Dietary imbalances: foals that are fed milk replacers, caprine milk, or bovine milk are prone to develop diarrhea due to a high amount of fat in these products compared to equine milk (1).
- Necrotizing enterocolitis: the mucosa and submucosa of the GI tract suffer a necrotizing injury, with the distal small intestine and proximal colon primarily involved. While the

cause is not completely understood, it has been speculated to be secondary to prematurity, previous mucosal injury, milk feeding, bacterial invasion, hypoxic insult, thrombosis, and decreased perfusion (4).

- Luminal irritants: secondary to pica, which leads to ingestion of significant amount of sand, gravel, or abrasive materials that can cause irritation to the GI tract (6).
- Neonatal encephalopathy/asphyxia-associated gastroenteropathies: due to hypoxic or ischemic insult secondary to neonatal encephalopathy (4).
- Rotavirus: the most common virus causing diarrhea in foals (5 to 35 days). Both group A and B rotaviruses are reported to cause outbreaks in equine neonates, and they are highly contagious with a morbidity of 100% in neonates. Transmission is direct or indirect (fecal-oral route or fomites), incubation period is from 1 to 4 days, and it is ubiquitous. It causes microvillous atrophy of the small intestine, leading to maldigestion, malabsorption, lactose intolerance, and increase in secretion (1,4).
- *Clostridium perfringens:* can be a primary cause of diarrhea in foals <10 days of age or may be present in healthy animals (type A). However, type A can also cause hemorrhagic diarrhea with a mortality of 28%, while type C has a mortality of 83%. A risk factor for acquiring these bacteria is parturition occurring in sand or dirt environments. In contrast to other causes of diarrhea or infection, it is believed that trypsin inhibitors present in colostrum protect toxins against gastric degradation, which make foals prone to develop this condition (1,4).
- *Clostridioides difficile:* can be a primary cause of diarrhea in foals but can also be present in healthy animals. It expresses toxin A, which is an enterotoxin, and toxin B, which has a cytotoxin; additionally, it has binary ADP ribosyltransferase toxins (1,7).

- *Salmonella:* typically, *Salmonella typhimurium* is involved, and it can affect foals from any age, causing enterocolitis, colic, and hemorrhagic diarrhea; additionally, other structures can be affected, causing uveitis, synovitis, osteomyelitis, and physitis. It causes inflammation and mucosal impairment of the ileum and colon by using the invasion-associated type III secretion system.
- Coronavirus: can be present in healthy animals. While uncommon, it has been documented to cause diarrhea in foals. In one case, it caused ischemic necrosis of the distal extremities, which led to hoof wall detachment (1,4).

Diagnosis of diarrhea can be challenging; however, a comprehensive assessment with a detailed review of history, age, physical examination, fecal testing, clinicopathologic abnormalities, and blood culture are essential for recognition of the underlying cause.

A valuable diagnostic tool in these cases is abdominal ultrasound, which facilitates the evaluation of the GI tract by measuring intestinal wall thickness, evaluating luminal contents, and assessing motility and for the presence of pneumatosis intestinalis, which is associated with necrotizing GI disease (3,8).

Fecal testing, encompassing culture, polymerase chain reaction (PCR), and fecal egg count, play an important role in definitive diagnosis to formulate specific treatment, prevention, and management strategies. Sepsis remains an important complication of diarrhea, with approximately 50% of diarrheic foals testing positive on blood culture, emphasizing its need in diagnostic protocols (9).

Animals with diarrhea can face numerous secondary complications, such as endotoxemia, metabolic issues like acidosis and hypovolemic shock, bacteremia, and septicemia, which increases in likelihood if it is associated with an underlying infectious enteritis/enterocolitis (1).

The most common clinical signs demonstrated by neonates with diarrhea are colic, variable fecal consistency, abdominal distention, anorexia, lethargy, fever or hypothermia, weakness, teeth grinding, and clinical signs associated with Systemic Inflammatory Response Syndrome (SIRS) such as tachycardia, tachypnea, congested/injected mucous membranes, weak pulses, and cool extremities (4).

Sepsis.

According to the consensus definition proposed by the 2018 Dorothy Russell Havemeyer Working Group Meeting on Foal Sepsis, sepsis was defined as the combination of SIRS and suspected or confirmed infection; while SIRS was defined as the presence of at least 2 of 4 of the following parameters out of range: rectal temperature, heart rate, respiratory rate, white blood cell count (blood culture), with one of them having to be either abnormal temperature or blood culture. The next two parameters were added in 2020: venous lactate and venous blood glucose (10).

That definition changed in 2024, where sepsis was defined as a life-threatening condition with confirmed or suspected infection and systemic manifestations of infection, recommending that SIRS criteria no longer be used for newborn foals (birth to 3 days old) (10). For proven infection, either positive blood culture or culture from two different sites is necessary; while for suspected infection, neutropenia and left shift are necessary (10). Knowing the definition of sepsis is key in the equine veterinary world, since it is the leading cause of morbidity and mortality in neonates (11,12).

The most common bacteria involved in sepsis in neonates are Gram negative, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella* species, *Enterobacter* species, and *Actinobacillus* species. Regarding Gram positive bacteria, commonly

involved organisms are *Streptococcus* species, *Enterococcus* species, or *Staphylococcus* species (13).

It is worth noting how the relative abundance of identified organisms Gram positive and Gram negative in the blood culture have been changing recently, with Gram positive slowly increasing in frequency. As reported by Wong *et al.* in 2018 41% of the total blood culture samples had growth with 53% being Gram negative and 47% being Gram positive bacteria (14). Compared to a previous study by Marsh and Palmer in 2001, 28.5% of the total blood culture samples had growth with 66.2% being Gram negative and 33.8% were Gram positive (15). Similarly in 2008, Russell *et al.* reported 25.8% growth on all blood culture samples, with 40.3% being Gram negative and 57.3% being Gram positive (16).

How can sepsis be diagnosed?

A notorious dilemma with blood culture interpretation is a false positive result, that is mostly considered secondary to contamination. Bacteria regarded as potential contaminants have included the skin organism coagulase-negative *Staphylococcus* species (17,18), *Corynebacterium* species and related genera, *Bacillus* species (except *Bacillus anthracis*), *Micrococcus* species, *Cutibacterium acnes* and related species, *Aerococcus* species and *Proprionibacterium* species. *Enterococci*, viridans group streptococci, and *Clostridium* species are of variable clinical significance, since they can either be contaminants or pathologic. In contrast, the following bacteria do not tend to be contaminants: *Staphylococcus aureus*, *Streptococcus pneumoniae*, beta-hemolytic streptococci, *Listeria monocytogenes*, *Escherichia coli* and other *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Haemophilus influenzae*, anaerobic Gram

negative rods (*Bacteroides* species, *Fusobacterium* species) and *Candida* species (19,20), and *Acinetobacter* species, which can be consider non-pathogenic or pathogenic, such as *Acinetobacter baumanni,* a nosocomial microorganism (14,21).

Some variables that help distinguishing between a pathogen or a contaminating organism includes culturing the same bacteria in different samples at different time points and from different locations, which increases the possibility that the organism is truly not a contaminant, and whether the patient does or does not have clinical signs suggestive of sepsis (17).

Another reason why blood culture could be positive in the absence of disease is bacteremia. It has been well documented that multiple species, including foals, can experience bacteremia without clinical disease, and it is suspected that this is secondary to colonization of the GI and respiratory tracts when they are neonates. A clear example is Hackett *et al.*, where blood culture samples were obtained multiple times after birth from 9 healthy foals shortly after foaling. Nine of the 70 samples were positive, mainly before foals were 12 hours old. The organisms isolated were *Bacillus* species, *Micrococcus* species, *Streptococcus* species, *Staphylococcus* species and *Propionibacterium* species (22).

While blood culture has been considered the gold standard for diagnosis of sepsis, it is well known it has low sensitivity (Se) (14,23,24), which could be due to low blood volume, low circulating bacteria, or previous antibiotic therapy administration. Additionally, given that results can take up to 48 to 72 hours its utility for determining timely judicious antimicrobial therapy is compromised (14,25).

Hence, other tools or diagnostic tests have been sought to increase Se in the timely diagnosis of sepsis in neonates. One of these tools is the sepsis score, with the first sepsis score

being developed by Brewer and Koterba in 1988. Multiple variables such as history, laboratory tests, and clinical assessment are considered, with points granted for each variable, and sepsis is diagnosed at a cutoff >11. In the initial study, a Se of 93% and a specificity (Sp) of 86% was reported (14,26).

Subsequent studies using modified sepsis scores obtained lower Se and Sp. Corley and Furr reported a Se of 67% and a Sp of 76% with a negative predictive value (NPV) of 84% and a positive predictive value (PPV) of 55% (27). Weber *et al*. used a modified sepsis score and reported a Se of 56.4% and Sp of 73.4% using the original cutoff >11 for diagnosis of sepsis; additionally, they obtained a receiver operating characteristic (ROC) with an optimal cutoff >7 with a Se of 84.4% and a Sp of 41.8% (28). Wong *et al.*, evaluated Se and Sp of 3 modified sepsis scores including SIRS to detect sepsis, with results showing that the original sepsis score had similar Se and Sp to the modified sepsis scores (14).

Overall, it has been concluded that sepsis scores have moderate Se and Sp; therefore, other diagnostic tools, biomarkers, or laboratory tests have been proposed, to increase the Se and Sp of this score and optimal diagnosis of sepsis in neonates. It is essential to highlight the importance of sepsis since this condition is life threatening and there are no specific tests for its diagnosis; that is why this is a major area of research.

Acute Phase Response and Acute Phase Proteins.

According to Whicher, cited by Ceron, the acute phase response (APR) is a reaction triggered by an injury, trauma, or infection (29) that alters the concentration of plasma proteins synthesized primarily by the liver, in order to protect the body from microorganisms and decrease tissue damage (30). These plasma proteins are called acute phase proteins (APP) and can be

categorized based on their change in concentration as minor (>0.5-2 fold), moderate (>5-10 fold) (31) and major (>100 fold); and positive or negative based on a rise or decline in the levels in blood (32) (33). The next table illustrates APP classification in horses (34).

Table 1. Classification of APP in horses with estimated time of increase and decrease, and peak. Adapted from Jacobsen and Andersen 2007 **(34)**.

APP	Normal plasma concentration (µg/mL)	Plasma concentration with inflammation (µg/mL)	Start increase or decrease (hours)	Peak (hours)
Major				
Serum amyloid A	0.5-20	50-800	6-12	48
Moderate and minor:				
-Haptoglobin	200-1000	400-2700	12-24	72-120
- α1-acid glycoprotein	70-90	100-250	24	72
-C-reactive protein	7.5	10-35	24	72-120
-Fibrinogen	2000-4000	3000-11000	24-72	72-144
-Ceruloplasmin	300-400	700-900	120	168-336
Negative (g/L)				
Albumin	30	27.5	144	192-240

What is serum amyloid A?

Two of the most important APP in horses are fibrinogen and serum amyloid A (SAA). SAA is the precursor of amyloid A protein (35). SAA is apolipoprotein, with three isoforms each of which are 12 kDa synthesized primarily in the liver, that are typically associated with high density lipoprotein (HDL), except in levels > 500 fold, when it becomes dissociated from HDL. They can be divided into homeostatic SAA (SAA4), which is present in a healthy animal, and acute phase SAA (SAA1 and SAA2), which increases during inflammation (36–38).

Fibrinogen is already present in healthy animals and is considered a positive, minor APP that tends to increase 24 – 72 hours after the insult and peaks around 72 – 144 hours (34). In contrast, SAA values are very low or even zero in healthy horses and increase significantly (10-

100 fold) during inflammation 6 to 12 hours after the insult. Because of this, it is considered the only major APP in horses (34,39). Another advantage of SAA is that when the insult disappears, its concentrations decreases within 12 hours because it has a short life (70-90 min.) in laboratory rodents (40–42).

How to measure serum amyloid A?

Pepys *et al*. measured SAA concentrations first in circulating blood in horses using an electro-immunoassay, and found only trace levels in healthy animals (43,44). Other methods for measuring SAA historically include single radial immunodiffusion (SRID) (43), sandwich enzyme linked immunosorbent assay (ELISA) (45), and chemiluminescence enzyme immunoassay (46). The chemiluminescence enzyme immunoassay was developed as the authors were seeking a more efficient diagnostic test since the electro-immunoassay and SRID assays took more than 24 hours to provide results (46). Subsequently, Wakimoto developed a semi latex agglutination test that took 60 minutes of incubation at room temperature. Although the results correlated with the sandwich ELISA, the test provided only semi-quantitative measurements. (47). A further advance came with Stoneham *et al.*, who adapted a latex agglutination immunoturbidimetric assay from Wada *et al*. to use in horses (48,49).

Point of care (POC) for SAA evaluation.

While the standard laboratory reference method for quantitative SAA measurement in horses is turbidimetric immunoassay (TIA), (50) other tests were developed seeking convenience with a stall side test. One of these is the Stablelab point-of-care lateral-flow membrane-based immunoassay (Epona Biotech, now marketed by Zoetis) (51,52). This assay was used multiple

times before it was validated by Schwartz *et al*. in 2018 (51). In their study, they assessed linearity, where they found that POC had acceptable performance with serum/plasma up to at least 1000 µg/mL. Precision displayed an intra-assay coefficient of variation (CV) from 13.3 to 17.8% and an inter-assay (inter-batch) CV from 13.6 to 45.5%. They also compared results between whole blood and serum/plasma, which showed a Passing-Bablok correlation of 0.908, indicating a good correlation. However, there was a significant difference between SAA results in whole blood vs serum/plasma ($p = 0.002$), and a negative bias was found in whole blood samples when SAA concentrations were higher than 500 µg/mL. Additionally, they compared POC results with the results of a previously validated TIA to see if they were interchangeable, and they found that the higher the SAA concentration, the worse the overestimation of SAA concentration with POC compared to TIA (51).

The Stablelab test is not the only point of care for SAA measurement in horses available on the market. Karam *et al*. validated a POC lateral flow immunoassay (LFIA) from VMRD, Pullman WA, USA using whole blood and plasma following the manufacturer's recommendations. The analytical performance of the device was evaluated by assessing linearity, with a $R^2 = 0.98$, which is high. The results between whole blood and plasma were compared. Both were positively correlated, and there was no significant difference between them ($p = 0.05$). However, whole blood tended to give higher SAA values. Both intra and inter – assay coefficient of variation (CV) ranged from 7.8 to 13.3% and 5.7 to 12%, respectively. Additionally, POC LFIA and TIA were positively correlated, and the overall agreement between both methods was 80% (53). Due to these results, both Schwartz and Karam recommended using the same kind of sample each time and ideally the same batch, to minimize variability (51,53).

Agreement between TIA and POC.

Since multiple fundamental studies on this topic have been published using TIA, it is important to consider the agreement between TIA and these newly available POC assays. Kiemle *et al*. in 2022 compared two TIA (LZ-SAA TIA-Hum and VET-SAA TIA-Vet) from Eiken Chemical with POC Stablelab from Zoetis (52). First, the inter-method comparison showed a significant difference in median SAA in all devices, with POC Stablelab yielding the highest and TIA-Vet the lowest values. The correlation between POC Stablelab and TIA-Hum was $r_s = 0.77$ and between POC Stablelab and TIA-Vet was $r_s = 0.69$, which is fair. However, all 3 had a growing absolute bias when SAA concentration increased.

POC Stablelab intra-assay and inter-assay CVs were higher than the quality requirement derived from human SAA biological variation (CV <12.5%), the only exception was intra-assay CV with the medium concentration range (301-1000 μg/mL). The total observed error between POC and both TIA was >37%, exceeding the total error accepted (TEa) indicating that POC Stablelab and TIA are not interchangeable; however, it is important to note that this TEa was established for humans, since there is no TEa specific for equids.

Subsequently, it was verified that with both TIA-Vet and POC bilirubin and hemoglobin had a negative bias (falsely low concentrations); while a positive bias was noted with the presence of intralipid (falsely high concetrations).

Additionally, they found a hook effect when SAA was > 3000 μg/mL, which saturates the test's detection system, causing a falsely low SAA levels.

So overall, while the Stablelab POC is a useful test, values obtained previously with TIA are not comparable with values obtained using POC. This does not rule out the Stablelab POC as a useful tool, but emphasizes the importance of consistency with regard to sample and device type

used in order to reduce variability (52). This is important to consider, since multiple studies have obtained SAA basal values in healthy horses using different analytical methods.

For example, Nunokawa *et al.* results showed a constant SAA concentration of 19.37 ± 9.41 μg/mL in normal foals <12 months of age and 21.53 ± 9.81 μg/mL in normal horses >18 months of age. Horses with experimentally-induced inflammation had the highest SAA value (15 times higher than basal value before insult) at day 2 post insult: horses <12 months old with inflammation had values from 95 to 178 μg/mL and horses >18 months old with inflammation had values ranging from 94 to 114 μg/mL (43).

Satoh *et al.,* calculated SAA reference intervals of 21.23 ± 12.20 μg/mL in healthy foals ≤12 months old and 14.93 ± 9.07 μg/mL in healthy horses ≥18 months old. Additionally, foals ≤12 months old with enteritis had a mean SAA concentration of 216.62 ± 178.56 μg/mL and foals with diarrhea had mean SAA concentration of 110.30 ± 114.67 μg/mL. Both sick groups showed a significant difference when compared with clinically normal horses. In horses ≥18 months old with induced inflammation (castrated horses and one with jejunojejunostomy), values peaked 2 days later, being around 2.5 to 50 times higher than previous insult. Finally, horses ≥18 months old with signs of inflammation had values of SAA ranging from 151 to 195 μg/mL, and a significant difference was found between these horses and clinically normal horses. These results were similar to those reported by Nunokawa *et al.* (43,45).

Stoneham *et al*. measured SAA in foals divided in cohorts, where the medians were 2.20 μg/mL in healthy foals, 279 μg/mL in septic foals, 195 μg/mL in foals with focal infection, 5.10 μg/mL in foals with failure of transfer of passive immunity (FTPI), 3.10 μg/mL in foals with noninfectious disease (NI), and 0 μg/mL in foals with a naval infection. There was a significant

difference between the control group and both the septic and focal infection groups; however, there was no statistically significant difference between control group and both FTPI and NI (48).

Clinical utility of SAA.

With the currently-available research, a SAA range for healthy adult horses has been established at 0 – 20 μg/mL, with a Se of 53%, a Sp of 94%, and a diagnostic accuracy of 75% for discriminating healthy horses from horses with inflammation (42,50,54). Interestingly, it has been acknowledged that SAA concentrations are different between horses with systemic or local inflammation, as noted by Hooijberg *et al*. In that study, horses with systemic inflammation had a mean SAA concentration of 1583 μg/mL (range: 688 to 4000), horses with local inflammation had a mean of 343 μg/mL (range: 37 to 1609), and horses with no inflammation had a mean SAA of 5.6 μg/mL (range: 1.8 to 14.5) (55,56). However, these reference intervals were obtained using testing methodology different from the POC assays, and according to Kiemle *et al.*, POC and TIA are not interchangeable (52). Recently, there has been more research using POC assays. Ludwig *et al*. assessed serum and synovial fluid SAA concentrations with POC Stablelab. In this study, it was found that, at concentrations $\leq$50 μg/mL, the agreement between both POC and TIA was good, but at SAA concentrations >100 μg/mL the concordance decreased. Additionally, 9 horses were used and the basal samples for SAA evaluation using serum with POC were 0 μg/mL (56). Since then, multiple studies have been conducted to evaluate SAA in foals using POC assays.

SAA in foals.

In the past, multiple authors have investigated SAA concentrations in different equine populations, including neonatal foals. As previously noted, Nunokawa *et al.* obtained a mean SAA

concentration of 19.37 μg/mL in healthy foals ≤12 months using SRID (43); Satoh *et al.* found a mean SAA concentration of 21.23 μg/mL in healthy foals ≤12 months of age with a sandwich ELISA (45); and Stoneham *et al*. found a median SAA concentration of 2.20 μg/mL in healthy foals using a latex agglutination immunoturbidimetric assay (48). Westerman went a step further and evaluated SAA serially and its association with outcome in adult horses; therefore, eventually, this serial measurements were done in foals also (39). For example, Hultén and Demmers obtained a median SAA concentration of <0.5 μg/mL in foals with diarrhea using non-competitive chemiluminescence enzyme immunoassay (46).

Recently, multiple authors have been using POC assays as a diagnostic tool for research. Giguère *et al*. use the POC Stablelab to evaluate serum SAA in foals between 3 weeks and 5 months old. The foals were divided into cohorts: control (n = 44), pneumonia due to *Rhodococcus equi* (n = 31) or pneumonia due to another etiologic agent (n = 23). Measurements were obtained at the beginning of treatment and 2 weeks later. Control foals had a median SAA concentration of 0 μg/mL, foals with *R. equi* pneumonia had a median SAA concentration of 212 μg/mL, and foals with other causes of pneumonia had a median SAA concentration of 27 μg/mL. SAA levels were significantly different in both pneumonia groups compared with the healthy group. There was no significant difference between survival and non-survival. In their population, some foals with pneumonia had a SAA of <5 μg/mL, and some healthy foals had a SAA >100 μg/mL; furthermore, there was no correlation between pulmonary lesions and SAA concentrations (57).

Barr and Nieman sampled foals <36 hours old and divided them into 3 different cohorts: healthy, sick non-septic, and septic, finding median serum SAA concentrations using the POC Stablelab to be 0, 1.5, and 114 μg/mL respectively. Kruskal Wallis showed a significant difference in at least one of the 3 groups, indicating that there is a relationship between SAA concentration

and health condition. The area under the curve was 0.80 which indicates a good SAA discrimination ability between septic and healthy foals. A particularly noteworthy element of that study was that four different cutoff values were evaluated to determine which one was the best to distinguish healthy foals from septic ones. Sensitivity (Se), Sp, NPV, PPV, and accuracy were calculated for each cutoff value evaluated, and the best cutoff value was 100 μg/mL with a Se of 52.9%, a Sp of 97.5%, a NPV of 93.7%, a PPV of 75%, and an accuracy of 92.1%. It was noted that some septic foals had a low SAA, that eventually increased, so it was recommended to perform serial measurements (24).

Additionally, Hoeberg *et al.* evaluated if SAA on admission could predict sepsis and outcome in 409 hospitalized foals up to 14 days old. They divided foals into four different groups: healthy, sick non-septic, septic, and uncertain sepsis status. It is worth noting that foals from 5 different hospitals were used, and SAA measurements were performed with different analytic methods, which could increase variability.

In this study, SAA on admission differed significantly between the sick non-septic and septic groups, with a value of 312 $\pm$ 685.4 μg/mL in the sick non-septic group and 1079.7 $\pm$ 1254.5 μg/mL in the septic group. Serum amyloid A concentrations were significantly different between groups, with median SAA concentrations in the sick non-septic group of 52 μg/mL, in the septic group of 718 μg/mL, and in the uncertain sepsis group of 123 μg/mL.

It was concluded was that while SAA measurement can be an useful tool to diagnose sepsis or predict the outcome, it should be used as a complement to other tests, such as CBC or blood culture, because it has low Se (23).

Van den Brom-Spierenburg *et al*. went further by obtaining SAA concentrations on admission, day 1, and day 2 and evaluating its association with SIRS, blood culture results, and

survival in foals <14 days old. Heparinized blood was used to obtain concentration using the POC Stablelab. Subjects were classified into four different cohorts: SIRS, nonSIRS, positive on blood culture, and negative on blood culture (58).

From admission to day 1, SAA concentration significantly increased in all subjects from 132 to 572 μg/mL. Foals with SIRS had a higher SAA concentration on admission compared to nonSIRS; however, by day 2, there was no significant difference. In contrast, there was a difference on day 2 between positive and negative blood culture groups.

Regarding survival, SAA on admission showed no clinically-significant difference between survivors and non-survivors, but median SAA was higher in non-survivors on day 1. It should be noted that euthanized foals were excluded when it was due to financial constraints, and that for day 2, just a subset of 40 foals out of 90 was sampled. In their sample, 46% of the ill foals and 59% of the nonSIRS foals had an SAA of <100 μg/mL on admission, which demonstrates the limitation of sampling SAA once and highlights the importance of sequential sampling (58).

De Bruijn *et al*. assessed SAA using whole blood with the POC Stablelab assay test in 127 foals <14 days old (59). The objective of this study was to evaluate the predictive value of one SAA measurement on admission or two SAA measurements (admission and day 2 or 48 hours later) for diagnosing sepsis and as a survival indicator. Additionally, previous SAA cutoffs were assessed for utility of diagnosing sepsis. They used multiple sepsis definitions: neutropenia, positive blood culture, and a combined definition of neutropenia, positive blood culture, or both.

The hazard ratio (HR) evaluates how quickly one event occurs in two different groups during a period of time, the higher it is, the risk is. Sepsis was associated with a higher risk of death under the previously explained definitions: positive blood culture had a HR of 2, neutropenia

had a HR of 2.5, or combined definition had a HR of 3.7. Additionally, SIRS had the highest HR of 6.8, implying that foals with SIRS had almost 7 times more risk of death.

SAA on admission, day 2, or the change between both days was not significantly associated with outcome. However, SAA on admission was significantly associated with blood culture positivity, neutropenia, and the combined group, but not with SIRS.

It is important to note that for the second sample, from the 127 foals, only 97 were sampled due to euthanasia/death or missing data.

It was concluded that an additional sample on day 2 did not improve the diagnostic ability of the test on admission, and that SAA is useful as a complementary tool, with other diagnostic tests. It was suggested that the lack of improved diagnostic ability with an additional sample on day 2 is due to the fact that SAA peaks physiologically in healthy foals at 48 hours of life (59).

SAA optimal cutoff for diagnosis of sepsis in foals.

Stoneham *et al*. suggested a cutoff of 100 µg/mL as highly suggestive of infection in foals. This cutoff was suggested based on the value they obtained from their cohorts, with the median of the control group at 2.2 µg/mL and 27 µg/mL at the 95% percentile; while the median of the septic group was 279.9 µg/mL. An important factor to consider is that an immunoturbidimetric method was used, not a POC (48).

Giguère *et al*. calculated 2 cut-points by ROC in their pneumonia foals. The first one to diagnose pneumonia was at 5 µg/mL with a sensitivity of 72.2% and a specificity of 77.3%; the second one to diagnose pneumonia due to *Rhodoccoccus equi* was 53 µg/mL with a Se of 64% and a Sp of 77% (57). As is evident, this cut-point was much lower than the one mentioned by Stoneham *et al*. in 2001 (48).

Hoeberg *et al.* calculated an optimal SAA cutoff of 1050 μg/mL to detect sepsis, with a Se of 30.2% and a Sp of 90.7% (23). Based on nonstatistical observation, it was noted that 69% of the survivors had SAA levels <300 μg/mL, while 31% of non-survivors had values typically >300 μg/mL. Additionally, there was a significant difference between survivors and non-survivors; the median SAA in non-survivors was 191 μg/mL, while the median of SAA in survivors was 93 μg/mL. The optimal SAA cutoff obtained to predict non-survival was 1250 μg/mL, with a Se of 22.1% and a Sp of 90.8% (23).

De Bruijn *et al.* ran a ROC analysis on SAA using foals divided into foals with neutropenia, positive blood culture, and the combined group of neutropenia, positive blood culture or both. This displayed an SAA on admission cutoff of 419 μg/mL for positivity in the blood culture (AUC: 0.68) and a better performance for the combined group (AUC: 0.82) with a Se of 80% and a Sp of 81%; while, with neutropenia, there was a lower cutoff at 379 μg/mL.

The SAA cutoffs on day 2 were 845 μg/mL with neutropenia and 933 μg/mL for the combined group.

Regarding external validation of previous cutoffs and blood culture positivity, 100 μg/mL had Se of 84.5% and Sp of 33%, while 1050 μg/mL had Se of 48.3% and Sp of 78.3% (59).

Based on this data, SAA has shown promising results as a complementary diagnostic tool for septic foals. While the research into SAA in septic foals is extensive, there is limited research contemplating SAA in neonatal foals with diarrhea, with only one study using foals with diarrhea and measured SAA using a noncompetitive chemiluminescence enzyme immunoassay where it was SAA was significantly higher in the bacterial group (positive to blood culture or transtracheal wash) with a median of 65 μg/mL vs 1.6 μg/mL in the non-bacterial group. An important caveat in this study is that a control group was not included (60).

Taken together, these findings show that data in neonatal foals with diarrhea is limited to older studies that used non-comparable assays or did not present validated cutoffs. There is still a significant gap regarding this topic. Because this is a common condition in foals, our objectives were to evaluate SAA in this population also.

Chapter 2 - Introduction.

Diarrhea, defined as an increase in frequency or water consistency of feces is a common problem in foals, which can lead to complications including endotoxemia and sepsis and be life threatening (1). Sepsis, defined as a life-threatening condition with confirmed or suspected infection and systemic manifestations of infection (10), is the leading cause of morbidity and mortality in neonatal foals (11,12).

The most common tool for diagnosis of neonatal sepsis in foals is blood culture and/or sepsis score; however, both have a low to moderate Se, which has prompted researchers to seek additional biomarkers to improve Se in order to start treatment as soon as possible to increase the survival rate and decrease financial impact (23,24).

Therefore, research into biomarkers that could help provide prognostic information and evaluate response to therapy are essential.

One biomarker that has previously been evaluated in young foals is SAA, an acute phase protein (APP) that increases significantly and rapidly in horses during inflammation or infection, being present at very low levels in healthy animals. While there have been multiple publications regarding neonatal sepsis (23,24,58,59), there are few publications investigating SAA in neonatal diarrhea (43,45,60). Additionally, publications evaluating serial measurements of SAA in foals with neonatal diarrhea are lacking.

Therefore, the objectives of this thesis are:

1. Measure and compare SAA for initial 3 days in foals presenting with diarrhea (Diarrhea), septic foals without gastrointestinal (GI) disease (Septic), and a control group of apparently healthy foals (Healthy).

2. To describe the change in SAA over initial 3 days in foals with Diarrhea, Septic and Healthy.
3. Evaluate if Septic and Diarrhea foals differ in the rate of SAA change from Day 1 to Day 2 and from Day 1 to Day 3.
4. Monitor pattern of SAA for Diarrhea and Septic foals throughout their hospitalization.
5. Determine if SAA on admission is associated with blood culture result (Gram positive vs Gram negative vs no growth).

We hypothesized that:

1. SAA would be higher on admission in Diarrhea than in Septic or Healthy foals.
2. Foals with a higher SAA on admission would stay hospitalized longer.
3. Foals with a higher peak SAA through hospitalization would stay hospitalized longer.

By doing this project, we expect to contribute to the research field and elucidate if serial SAA measurements during hospitalization can increase sensitivity in diagnosing sepsis and provide information regarding the length of hospitalization.

Chapter 3 - Material and Methods

Study design

A prospective observational cohort study was conducted from May 2024 to July 2025 by the Equine Internal Medicine service at the academic referral hospital Veterinary Health Center (VHC) at Kansas State University (KSU) in Manhattan, Kansas. The project was approved by the KSU Institutional Animal Care and Use Committee (IACUC protocol number 5004).

Study population

A total of 35 neonatal foals were recruited as subjects including client-owned foals from the VHC at KSU and a single university-associated farm to perform serial SAA measurements. Informed client consent was obtained for all foals.

Inclusion and exclusion criteria

Neonatal foals born on the university-associated farm were considered for inclusion in the healthy group while they were less than 1 month of age. Neonatal foals less than one month of age presenting to the VHC for illness were considered for inclusion in the septic foals with no signs of GI disease or foals with diarrhea groups. During consideration for inclusion, these neonatal foals < 1 month of age, of indistinct breed or sex were categorized into one of the following three cohorts:

1. Healthy: Foals with normal parameters on physical examination; normal age-appropriate bloodwork including complete blood count (CBC), serum biochemistry, and immunoglobulin G (IgG) measurement; and normal sepsis score (<11) with no signs of GI disease were included in this group. The foal must fulfill all three

requirements. Foals with diarrhea or development of illness or wounds were excluded from this group.

2. Septic foals with no signs of GI disease (Septic): Hospitalized foals with clinical findings of at least one of the following (fever, injected mucous membranes and/or sclera, joint or limb swelling, uveitis, respiratory distress); no clinical signs of GI disease (reflux, colic, abdominal distention, straining to defecate/meconium impaction) during the first 3 days of hospitalization; at least one of the following abnormal bloodwork parameters (abnormal total white blood cell count, abnormal neutrophil count, left shift, toxic changes to neutrophils, or elevated fibrinogen); and abnormal sepsis score (> 11) were included in this group. The foal must fulfill all four requirements.
3. Foals with diarrhea (Diarrhea): Hospitalized foals with a history of diarrhea within 24 hours of presentation, diarrhea on intake, evidence of diarrhea on their tail/hind end on intake or development of diarrhea within 12 hours of intake were included in this group. Thirty-nine foals were considered for inclusion and 35 met inclusion criteria to be included in the study and were categorized accordingly.

Of the four foals not included in the Healthy group, one had evidence of omphalitis (but did not meet criteria for the Septic group), one had a left shift (but did not meet criteria for the Septic group), one developed an infected laceration during the collection days, and one had diarrhea (but did not meet criteria for the Diarrhea group as it was not hospitalized).

Miniature horses, donkeys and mules were excluded from the study.

Sample collection

Complete physical examinations were performed on all foals on intake during consideration for inclusion in the study and each time of sample collection. Samples for CBC, serum biochemistry, IgG measurement, SAA, and blood culture were collected on presentation (Day 1) on all foals.

Subsequent serial SAA measurements were obtained every 24 hours ± 2 hours thereafter. Serial SAA measurements were discontinued on Septic and Diarrhea group foals at discharge, death or until the SAA value was < 20 ug/mL, whichever was last. Serial SAA measurements were only collected at 24 hours (Day 1) and 48 hours (Day 2) after the initial sample for Healthy group foals.

For initial sample collection, jugular venipuncture was performed using a 20 gauge, 1-1.5-inch needle following cleansing with 70% isopropyl alcohol. Blood was collected into a red-top tube and a purple-top, EDTA tube and was analyzed within 24 hours for CBC and biochemistry and within 3 hours for SAA and IgG measurement. CBC and serum biochemistry were performed by the Kansas Veterinary Diagnostic Lab (KVDL). Measurement of SAA was performed using the Zoetis StableLab test kit and HandHeld reader according to manufacturer's instructions. Measurement of IgG was performed via a semi-quantitative ELISA (IDEXX Foal IgG Snap Test) according to manufacturer's instructions.

For blood culture sample collection, 8-12 mL of blood was collected following clipping and aseptic preparation of the jugular vein using 2% chlorohexidine and 70% isopropyl alcohol. Using aseptic technique, blood was collected via either direct aseptic jugular venipuncture (Healthy) or immediately following routine and clinically indicated intravenous jugular catheter placement (Septic and Diarrhea) before any extension lines were connected or before any

medications were administered through the catheter. Blood was then aseptically injected into a blood culture vial (Thermo Scientific SIGNAL blood culture system), incubated at 37°C for 30-60 minutes, and vented before being submitted to the KVDL for routine aerobic and anaerobic culture and susceptibility testing.

Serial SAA samples were collected through either direct venipuncture (Healthy) or via the intravenous catheter (Septic and Diarrhea). In the latter case, 12 ml of blood was obtained and discarded from the intravenous catheter, then 3 ml were obtained for the SAA sample and placed in a purple-top, EDTA tube, and finally the catheter was flushed with 10 mL of 0.9% saline and then 5 mL of heparinized saline (2 unit/ml concentration).

A fecal sample was collected for submission of a Foal & Neonate GI/Diarrhea PCR panel on Day 1 to Day 2 for Diarrhea group foals. The fecal samples were collected via free catch during defecation, manual collection from the ground, or via rectal mucosal swab using a synthetic flocked swab (Copan ESwab). Free catch or manually-collected feces were contained in a clean specimen cup, and the flocked swabs were contained within the ESwab's plastic tube with liquid medium system (ESwab Liquid Based Collection and Transport). Fecal samples were kept at 4°C until submission to the University of California (UC), Davis Veterinary Medicine PCR Laboratory via overnight shipment.

Data collection

Data collected included signalment (age, breed, sex) and history including gestational age, any disease of the mare during gestation, any abnormality at birth, whether parturition was attended, timing of the foal standing and nursing, clinical signs displayed at home prior to presentation, treatments administered prior presentation, including plasma, antibiotics, anti-

inflammatories, colostrum replacer/supplement/milk replacer, or others. This historical data was collected using a client questionnaire Appendix A - .

Data collected during the course of hospitalization or inclusion in the study included initial physical exam findings; blood work including initial CBC, serum biochemistry, SAA, and IgG measurement and serial SAA concentrations; intake sepsis score calculation; diagnostics including ultrasonography, fecal diagnostics, and blood culture; treatments administered; progress; complications; length of hospitalization; and outcome (survival or non-survival). For surviving foals, a follow-up phone call was made to determine longer-term outcome (at 7 days following discharge).

Statistical analysis

Statistical analysis was performed using R studio. Normal distribution of the data was assessed using the Shapiro-Wilk test per group per day with significance at $p < 0.05$. The Healthy group rejected normality in all 3 days; therefore, non-parametric tests were applied. Descriptive statistics were calculated and reported. For inferential statistics, whisker boxplots and scatter plots were used to evaluate the behavior of the data.

Objective 1

To measure and compare SAA for the initial 3 days in Healthy, Septic and Diarrhea foals, a boxplot with raw scale was used to visualize the distribution of the SAA concentrations per day and group.

Since our data was non-parametric, for this objective a Kruskal Wallis test was performed on day 1, day 2, and day 3 to compare each group's SAA values. To acknowledge which group was different, a Dunn's post hoc with Holm correction was conducted.

To evaluate if groups differed in total inflammation over 72 hours, an area under the curve (AUC) was calculated for each subject using the trapezoidal method; however, one foal from the Diarrhea group was excluded from the analysis, since the value on day 3 was not obtained. A Kruskal Wallis test was performed to rule out any differences between groups, and then Dunn's post hoc was performed to know which group was different.

Objective 2

In order to describe the change of SAA over initial 3 days in Healthy, Septic and Diarrhea foals, a line graph with ribbons was used to visualize and explain the variability per day and group.

Additionally, the percentages of SAA change from day 1 to 2 and from day 1 to 3 were obtained, medians and interquartile ranges (IQRs) were described, and results were displayed in boxplots only on both sick groups, since the Healthy group had low basal levels and the interpretative value was considered insignificant.

Objective 3

To determine if Diarrhea and Septic foals differ in the rate of SAA change from Day 1 to Day 2 and from Day 1 to Day 3, a Wilcoxon rank sum test was performed to compare percentage change between both groups.

Objective 4

To monitor the pattern of SAA in foals from the Diarrhea and Septic groups throughout their hospitalization, a boxplot was generated to visually describe the pattern on both groups. Additionally, median and first to third quartiles (Q1-Q3) were presented in a table.

Objective 5

To determine if SAA on admission is associated with blood culture result (gram positive vs gram negative vs no growth) only the Diarrhea and Septic groups were considered, since the Healthy foals did not fulfill the inclusion criteria for "sick", SAA values and CBC/Chemistry results were within normal limits, and they did not display clinical signs associated with disease. Results were shown in a boxplot, and a Kruskal Wallis test was performed to determine if there was an association.

Hypothesis 1

To evaluate if SAA would be higher on admission in the Diarrhea group compared to the Septic or Healthy groups, medians, IQR, and range values were obtained. A Kruskal Wallis test was used to compare differences across all the groups. Subsequently, Dunn's post hoc with Holm correction was performed to assess which group was significantly different, and all this was displayed in a boxplot.

Hypothesis 2

A Spearman's rank correlation was performed to evaluate the relationship between SAA concentration on admission and length of hospitalization in sick (Diarrhea and Septic) foals < 1 month old. LOESS smoothing was applied for visualization, and results were displayed in a scatterplot.

To determine a clinically relevant threshold at which SAA at admission could predict a prolonged length of stay, a receiver operating characteristic (ROC) curve analysis was performed to establish an optimal cutoff in our sample, and this was visualized in a boxplot.

Hypothesis 3

A Spearman's rank correlation was run to evaluate the relationship between peak SAA concentration and length of hospitalization in foals < 1 month old. LOESS smoothing was applied for visualization, and results were displayed in a scatterplot.

Chapter 4 - Results

A total of 39 foals < 1 month old were considered but four did not meet inclusion criteria; therefore 35 foals were enrolled and categorized into 3 cohorts: Healthy (n = 14), Diarrhea (n = 17) and Septic (n = 4).

Demographics

Table 2. Demographics of the study population per group.

	Healthy (n = 14)	**Diarrhea (n = 17)**	**Septic (n = 4)**
Age (days)	Mean: 10.9 Median: 10 Range: 1 – 18	Mean: 4.2 Median: 2 Range: 1 – 21	Mean: 3.25 Median: 2.5 Range: 1 – 7
Sex	Filly: 7 Colt: 7	Filly: 6 Colt: 11	Filly: 3 Colt: 1
Breed	AQH: 14	AQH: 11 Arabian: 1 Appendix: 1 Gypsy Vanner: 1 Mustang: 1 Missouri Fox Trotter: 1 Paint: 1	AQH: 3 Gypsy Vanner: 1

AQH: American Quarter Horse.

Descriptive statistics

Table 3. SAA concentration (medians and IQR) by group and day.

		SAA, median (Q1-Q3)		
Group	**n**	**Day 1**	**Day 2**	**Day 3**
Healthy	14	2 (0–15)	1.5 (0–12.2)	1 (0–18)
Diarrhea	17	919 (770–1104)	1377 (959–1686)	619.5 (117.8–1177)
Septic	4	1122.5 (814.2–1393.2)	819.5 (389.5–1245)	973.5 (87.5–1891.8)

Kruskal-Wallis by day: Day 1, $p < 0.001$; Day 2, $p < 0.001$; Day 3, $p < 0.001$.

Table 4. Blood culture results in all groups.

Group	Blood culture positive Gram positive	Blood culture positive Gram negative	Blood culture negative
Healthy	n = 8/14 (57%) *Cutibacterium acnes* *Bacillus* species *Corynebacterium stationis* *Aerococcus* species *Streptococcus* species (α hemo) *Bacillus licheniformis*	n = 8/14 (57%) *Acinetobacter variabilis* *Acinetobacter lwoffii* *Acinetobacter pseudolwoffii* *Acinetobacter* species	n = 6/14 (43%)
Diarrhea	n = 3/17 (18%) *Bacillus cereus* *Streptococcus* species *Listeria monocytogenes*	n = 5/17 (29%) *Escherichia coli* *Salmonella* species *Salmonella anatum*	n = 9/17 (53%)
Septic	n = 0/4 (0%)	n = ¾ (75%) *Campylobacter jejuni* *Escherichia coli* *Actinobacillus lignieresi*	n = 1/4 (25%)

Table 5. PCR diarrhea panel results in Diarrhea group.

Diarrhea panel	Number of positive foals
Clostridioides difficile	1
Clostridium perfringens	8
C. difficile & C. perfringens	4
Salmonella species	2
Negative	2
Total	17

Table 6. Toxinotypes from foals positive to PCR Diarrhea panel.

Microorganism	n (%)
Clostridium perfringens **(n = 12)**	
Type A	10 (83.3%)
Type C	1 (8.3%)
Toxins genes not detected	1 (8.3%)
Beta 2 toxin	10 (83.3%)
Enterotoxin (cpe)	3 (25%)
netF	3 (25%)
Clostridioides difficile **(n = 5)**	
Toxins A and B	5 (100%)

Table 7. Sepsis score per group.

Group	Healthy	Diarrhea	Septic
Sepsis score	0 (n = 4)	3 (n = 1)	11 (n = 2)
	1 (n = 3)	5 (n = 1)	12 (n = 1)
	3 (n = 3)	6 (n = 1)	19 (n = 1)
	4 (n = 1)	8 (n = 2)	
	5 (n= 3)	9 (n = 1)	
		10 (n = 1)	
		11 (n = 3)	
		12 (n = 1)	
		16 (n= 2)	
		18 (n = 1)	
		19 (n = 1)	
		21 (n = 1)	
		24 (n = 1)	

Table 8. Length of hospitalization in sick groups.

Group	Diarrhea	Septic
Length of Hospitalization (days)	5 (n = 4) 6 (n = 4) 7 (n = 3) 8 (n = 2) 9 (n = 1) 10 (n = 1) 11 (n = 1) 17 (n = 1)	5 (n = 1) 6 (n = 1) 9 (n = 1) 12 (n = 1)
Median (days) **Q1** **Q3**	7 6 8	7.5 5.75 9.75

Table 9. Day of peak SAA concentration and time to peak, by group, presented as median (Q1-Q3).

Group	n	Day 1, n	Day 2, n	Day 3, n	Median (Q1-Q3)
Healthy	14	8	4	2	1 (1-3)
Diarrhea	17	5	11	1	2 (1-2)
Septic	4	2	1	1	1.5 (1-2.25)

In the Diarrhea group, the median day to SAA normalization (SAA <100 μg/mL) was day 5 (Q1-Q3: 4-6.25), and in the Septic group, the median day to SAA normalization was day 3 (Q1-Q3: 3-5). One foal was excluded from this analysis in each group because its SAA concentration did not reach an SAA concentration of <100 μg/mL during hospitalization.

Inferential statistics

Objective 1

Measure and compare SAA for initial 3 days in Healthy, Septic and Diarrhea foals.

The control group maintained low SAA concentrations across all three days with medians of 2, 1.5 and 1 μg/mL on days 1, 2 and 3, respectively. In contrast, both Diarrhea and Septic foals demonstrated substantially elevated SAA concentrations relative to controls, as illustrated in Table 3 and Figure 4.1.

SAA concentrations differed significantly among the three groups for day 1 with a $\chi 2 = 24.44$, df = 2 and $p < 0.001$; day 2 $\chi 2 = 25.2$, df = 2 and $p < 0.001$; and day 3 $\chi 2 = 21.45$, df = 2 and $p < 0.001$.

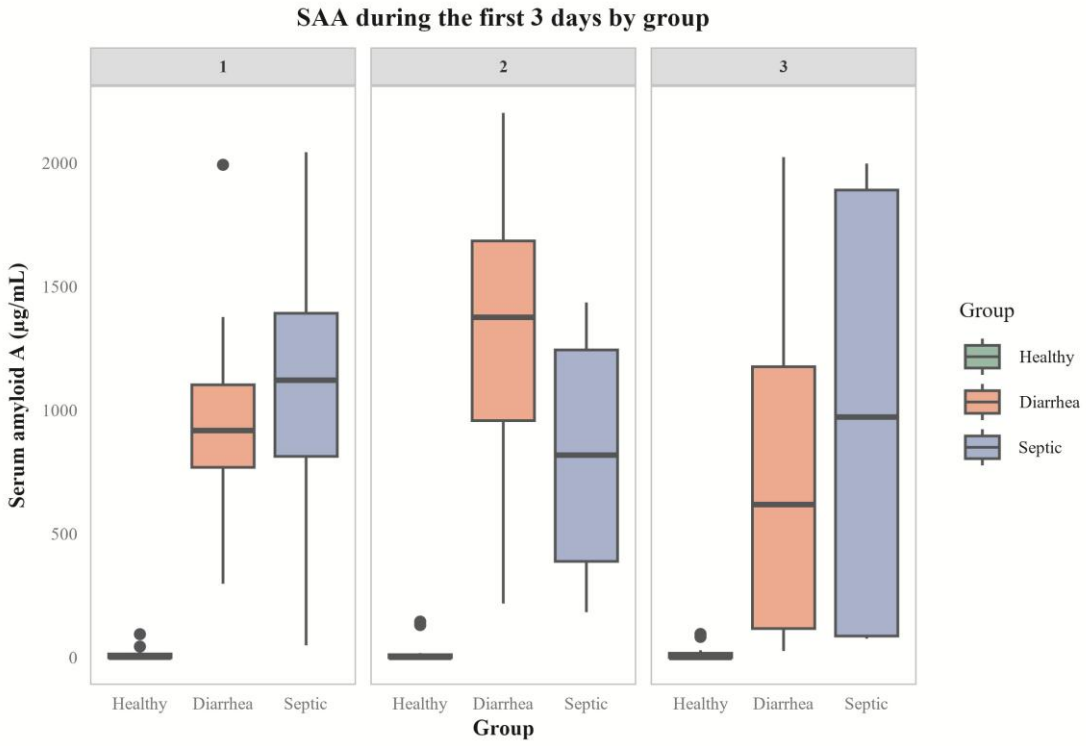


Figure 4.1. Serum Amyloid A (SAA) concentrations (μg/mL) in Healthy, Diarrhea and Septic foals at Day 1, Day 2 and Day 3. Box and whisker plots display the median, interquartile range and outliers for each group. The boxplot is presented in raw scale for better clinical interpretation.

There was a significant difference on day 1, 2 and 3 between the Diarrhea and Healthy groups, and between the Septic and the Healthy group, with both sick groups having higher SAA

concentrations compared to the Healthy group. Between both sick groups, there was no significant difference as noted in Table 3.

Table 10. Pairwise comparisons of SAA between groups by day (Dunn's post hoc test, Holm-adjusted p values), *p <0.05.

Comparison	Day 1	Day 2	Day 3
Healthy vs. Diarrhea	<0.001*	<0.001*	<0.001*
Healthy vs. Septic	0.002*	0.031*	0.008*
Diarrhea vs. Septic	0.696	0.447	0.973

Over the initial 72 hours, SAA concentrations per group showed a $\chi^2 = 24.07$, df = 2 and $p < 0.001$, which implies a significant difference between groups; as displayed on Table 11.

Table 11. SAA area under the curve (AUC) over 72 hours by group.

Group	n	AUC, median (Q1–Q3)
Healthy	14	4.5 (0.5–25.8)
Diarrhea	16	2198 (1265–2904)
Septic	4	1903 (840–2923)

Kruskal-Wallis: $\chi^2 = 24.07$, df = 2, $p < 0.001$.

There was a significant difference between Diarrhea and Healthy groups with a p value < 0.001, with the Diarrhea group having the highest AUC.

Between the Healthy and the Septic groups, the AUC was higher in the Septic group (p = 0.008), which implies a significant difference also.

Finally, when both the Diarrhea and Septic groups were compared a value of p = 0.866 was obtained, we did not find a significant difference.

Collectively, these findings show that both sick groups had a higher SAA value compared to the Healthy group over the first 72 hours of hospitalization.

Objective 2

To describe the change in SAA over initial 3 days in foals presenting with Diarrhea, Septic and Healthy foals.

Healthy foals consistently exhibited low SAA concentrations with minimal variability as noted in Figure 4.2. On the other hand, Diarrhea foals tended to increase their SAA concentration on Day 2, decreasing by Day 3 and had a significant variability, as we can see in the same figure. Finally, the Septic group displayed the widest variability of the three groups, with the SAA concentrations tending to increase the most at Day 1 and 3 Figure 4.2.

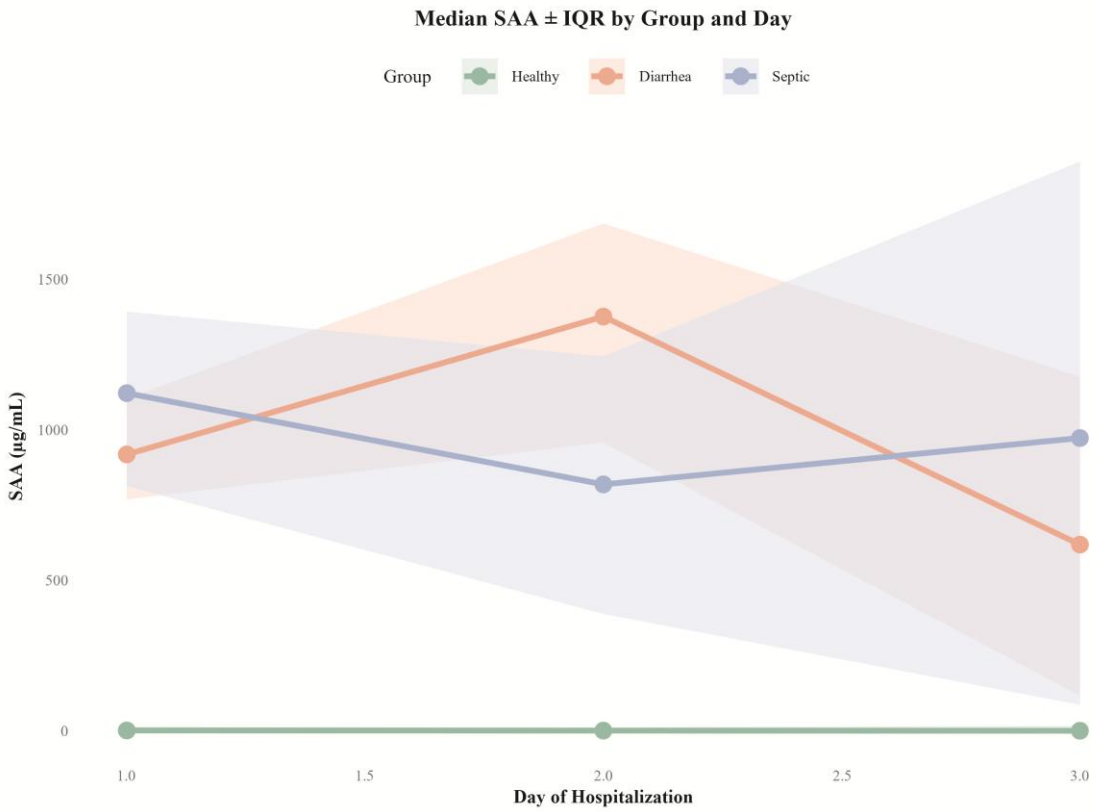


Figure 4.2. Median SAA concentrations (µg/mL) in Healthy, Diarrhea and Septic foals on Day 1, Day 2 and Day 3 of hospitalization. Lines are the median SAA, and the variability is illustrated with the shaded ribbons indicating IQR.

On both sick groups, the changes from Day 1 to Day 2 (Figure 4.3) and from Day 1 to Day 3 (Figure 4.4) were evaluated with percentages. The median percentage change in SAA from Day 1 to Day 2 was 24.73% (Q1: -25.24, Q3: 74.58) in Diarrhea foals and -14.65% (Q1: -36.59, Q3: 67.32) in Septic foals (W = 24, p = 0.41) (Figure 4.3).

The median percentage change in SAA from Day 1 to Day 3 was – 38.06% (Q1: -76.68, Q3: 27.78) in Diarrhea foals and 22.38% (Q1: -29.8, Q3: 58) in Septic foals (W = 41, p = 0.44) (Figure 4.4).

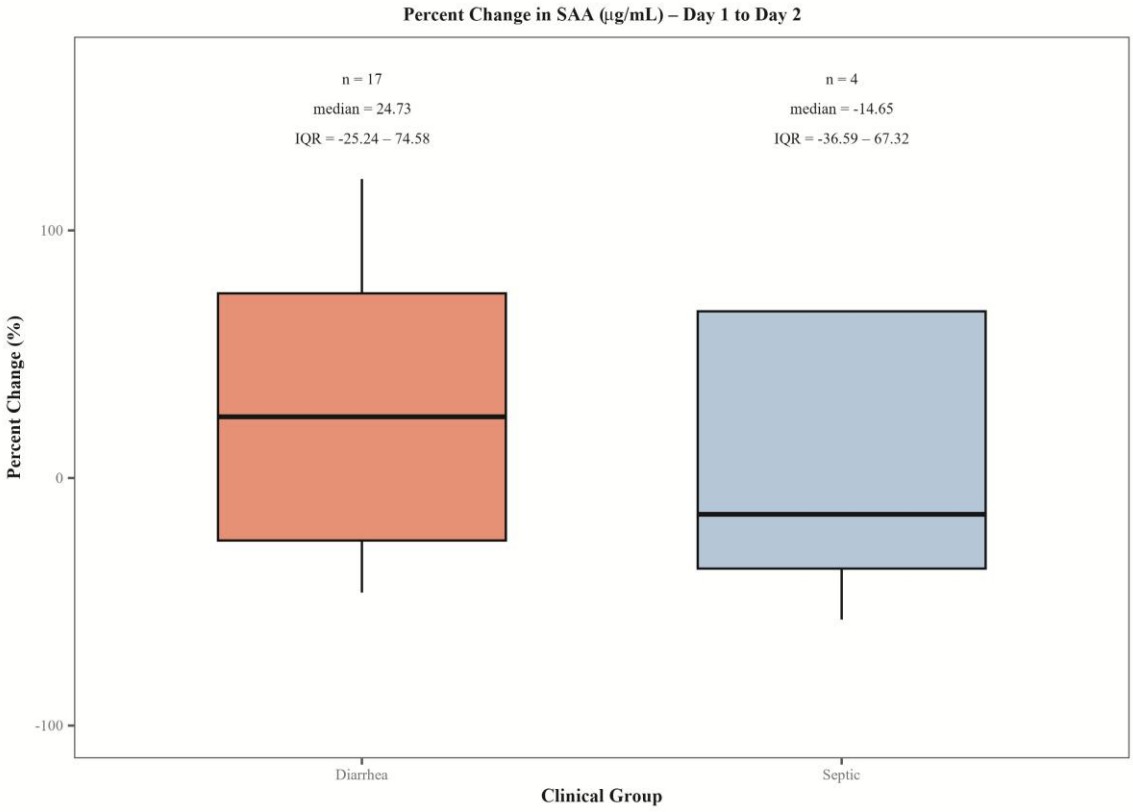


Figure 4.3. Percentage change on SAA from Day 1 to Day 2 on Diarrhea and Septic foals.

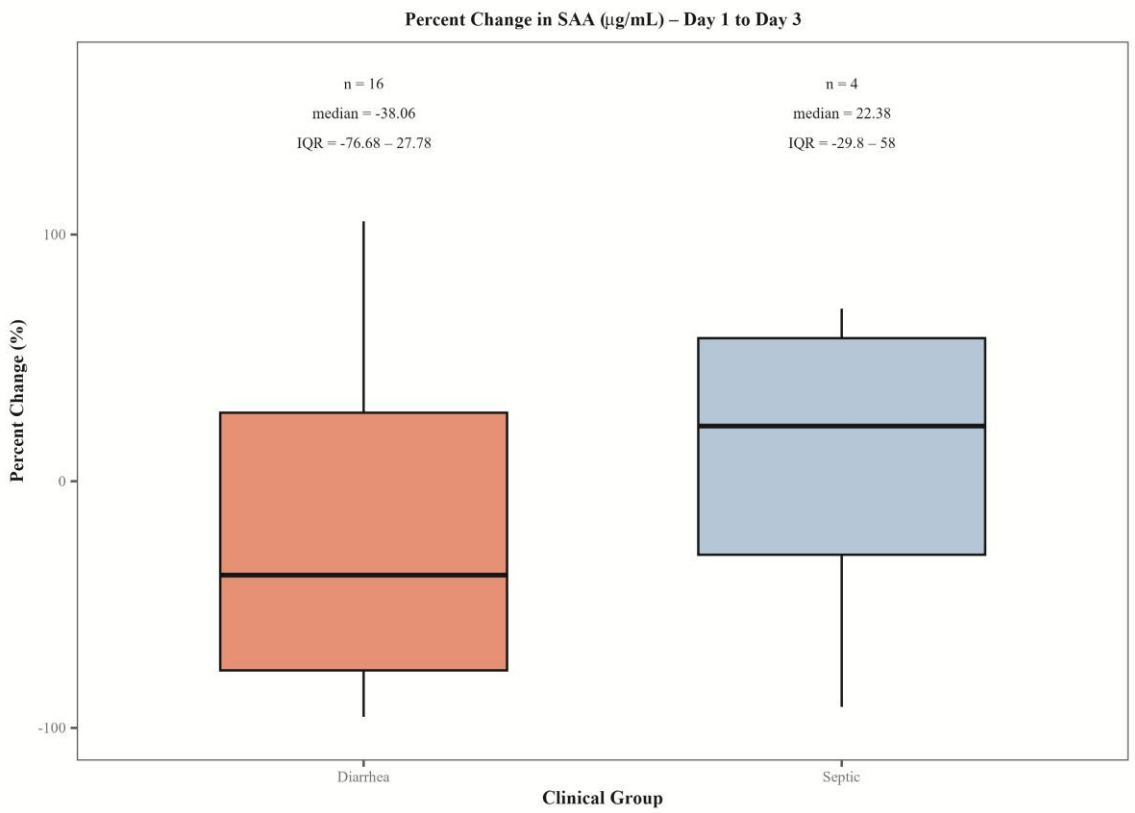


Figure 4.4. Percentage change on SAA from Day 1 to Day 3 on Diarrhea and Septic foals.

Objective 3

Evaluate if Septic and Diarrhea foals differ in the rate of SAA change from Day 1 to Day 2 and from Day 1 to Day 3.

There was no significant difference in the percentage change in SAA from Day 1 to Day 2 and from Day 1 to Day 3 between Septic and Diarrhea foals ($W = 24$, $p = 0.41$; and $W = 41$, $p = 0.44$, respectively).

Objective 4

Monitor pattern of SAA for foals with Diarrhea and Septic foals throughout their hospitalization.

Figure 4.5 shows the pattern of SAA for both sick groups. The Diarrhea group displays a high pattern on SAA that peaks at day 2 (median: 1377 µg/mL) and progressively decreases during the next days of hospitalization. The Septic group describes higher values than the Healthy group since intake with a peak on day 1 (median: 1122.5 µg/mL), showing more variability and a slower decline than the Diarrhea group. A notable reduction was present on both groups by day 6. Additionally, Table 12 displays SAA concentrations per day in both sick groups, with medians and Q1-Q3.

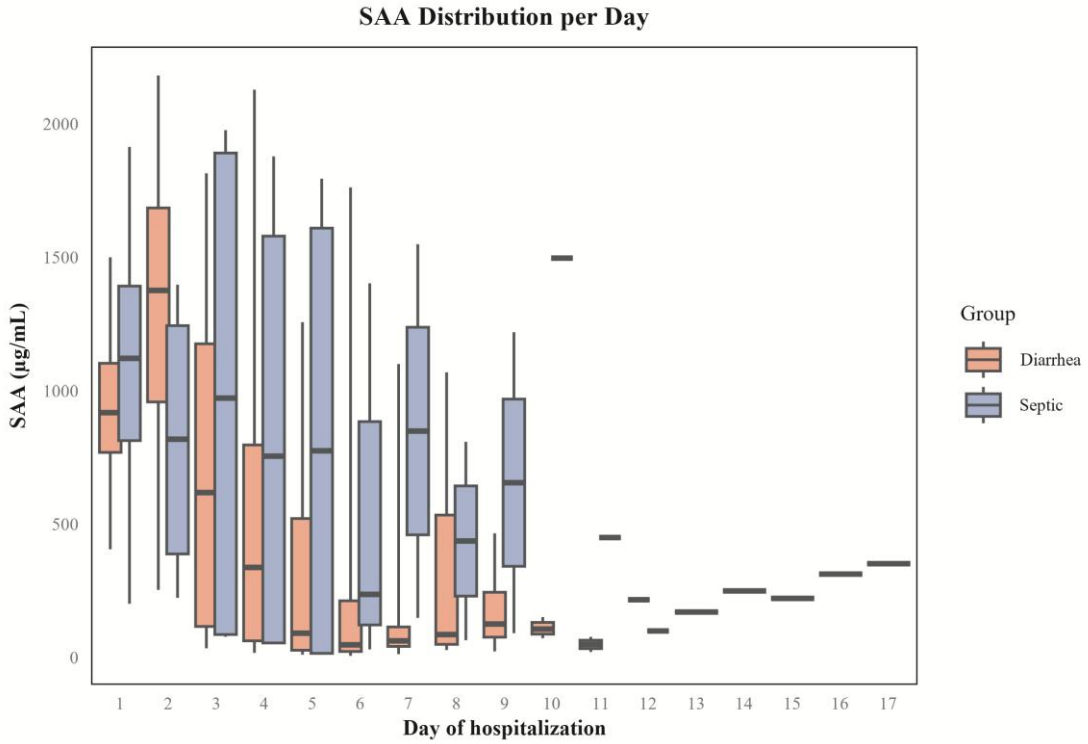


Figure 4.5. Serum Amyloid A concentrations (µg/mL) per day in Diarrhea and Septic foals through their hospitalization. Box and whisker plots display the median, interquartile range and outliers for each group.

Table 12. Serum amyloid A (median, Q1 – Q3) and number of foals per day during hospitalization, in Diarrhea and Septic foals.

Day	Diarrhea, median (Q1–Q3)	n	Septic, median (Q1–Q3)	n
1	919 (770–1104)	17	1122.5 (814.2–1393.2)	4
2	1377 (959–1686)	17	819.5 (389.5–1245)	4
3	619.5 (117.8–1177)	16	973.5 (87.5–1891.8)	4
4	339 (64–798)	17	756 (56–1580.5)	4
5	92.5 (28.8–522.2)	16	776 (17–1610.2)	4
6	49 (24–214)	13	238 (123.5–885.5)	3
7	64 (43–116)	9	850 (461–1239)	2
8	87.5 (50.8–535.2)	6	438 (231.5–644.5)	2
9	127 (77.8–246)	4	656.5 (343.2–969.8)	2
10	108 (89–133)	3	1498	1
11	50.5 (34.8–66.2)	2	451	1
12	218	1	101	1
13	172	1	—	—
14	251	1	—	—
15	223	1	—	—
16	314	1	—	—
17	353	1	—	—

Objective 5

Determine if SAA on admission is associated with blood culture result (gram positive vs gram negative vs no growth).

Figure 4.6 stratifies foals in 3 different groups according to the blood culture result: gram positive (n = 3, median: 986 µg/mL, IQR: 878-1182 µg/mL), gram negative (n = 8, median: 1060 µg/mL, IQR: 901- 1380.5 µg/mL) and no growth (n = 10, median: 855.5 µg/mL, IQR: 632.8 – 1092.8 µg/mL).

There is a significant overlap between the IQR in all groups, and there was no statistically significant difference between groups, $\chi 2 = 1.65$, df = 2 and p = 0.43.

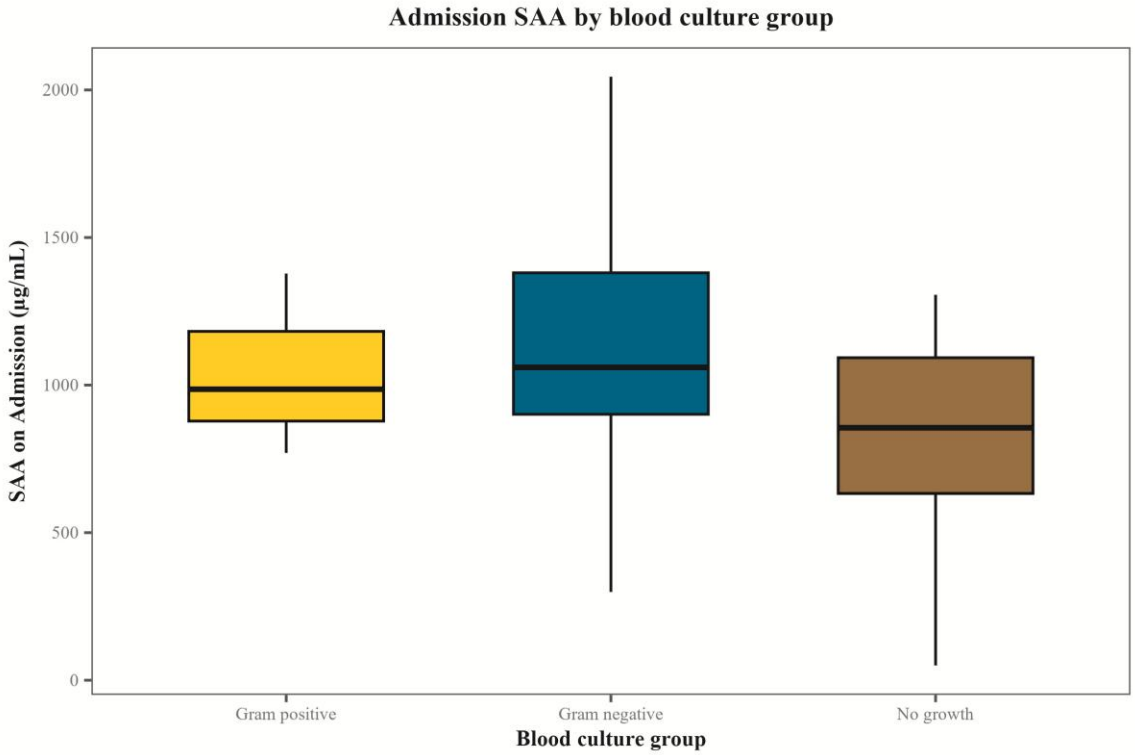


Figure 4.6. SAA concentrations on admission for Diarrhea and Septic foals stratified by blood culture result (gram positive = yellow, gram negative = teal and no growth = brown). Box and whiskers plot display medians and IQR.

Hypothesis 1

To evaluate if SAA will be higher on admission in foals with Diarrhea than in Septic or Healthy foals.

In the Healthy group (n = 14) median was 2 µg/mL, IQR 0-15 µg/mL and range 0-95 µg/mL; in the Diarrhea group (n = 17) median was 919 µg/mL, IQR 770-1104 µg/mL and range

299-1994 µg/mL and in the Septic group (n = 4) median was 1122 µg/mL, IQR 814-1393 µg/mL and range 50-2045 µg/mL.

There was a significant difference across all three groups ($\chi 2 = 24.45$, df = 2, $p < 0.0001$).

Both Diarrhea and Septic foals had significant differences compared to Healthy foals ($p < 0.001$ and p 0.002, respectively). No significant difference between Diarrhea and Septic foals ($p = 0.696$) was found. Results are illustrated in Figure 4.7 and Table 3 presents descriptive statistics.

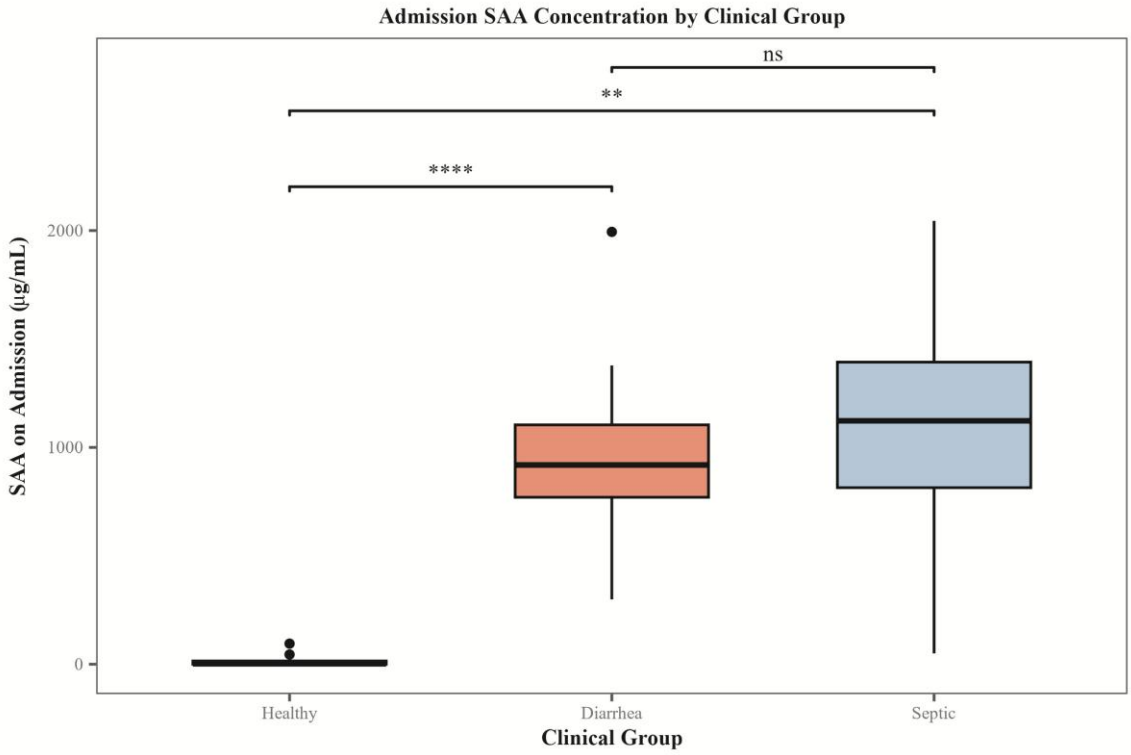


Figure 4.7. SAA concentrations on admission for Healthy (green), Diarrhea (red) and Septic (blue). Box and whiskers display median and IQR. Significance bars on the upper part show significant difference between the Healthy group compared with Diarrhea and Septic groups, but no between Diarrhea and Septic groups.

Hypothesis 2

Foals with a higher SAA on admission stay hospitalized longer.

There was an association with length of hospitalization and SAA concentration on admission in Diarrhea and Septic foals < 1 month old. The p value and ρ were 0.0052 and 0.59, respectively. These results indicate a positive correlation as noted in Figure 4.8, which supports our hypothesis.

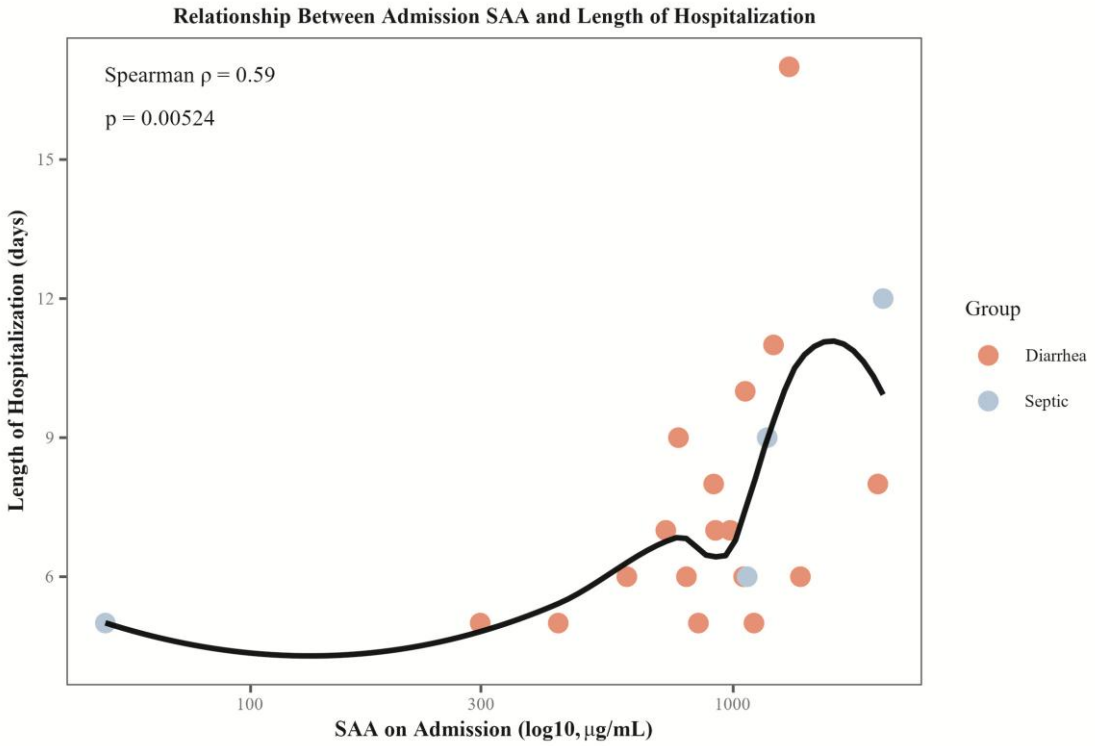


Figure 4.8. Scatterplot of SAA at admission in foals with Diarrhea and Septic.

A clinically relevant threshold at which SAA at admission could predict a prolonged length of hospitalization, and an optimal cutoff in our sample was determined as presented in Figure 4.9 with a Se of 82% and a Sp of 60%.

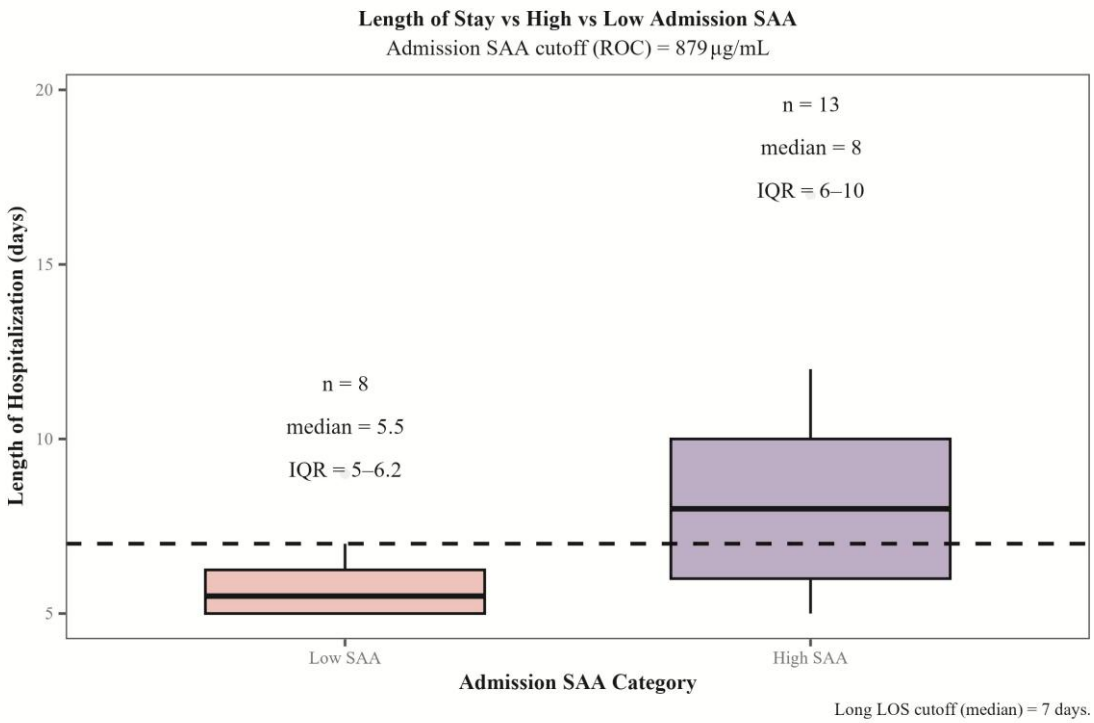


Figure 4.9. Boxplot of SAA on admission in Diarrhea and Septic foals where a cutoff for length of hospitalization was obtained with ROC.

Hypothesis 3

Foals with a higher peak SAA through hospitalization stay hospitalized longer.

There was a strong positive correlation between peak SAA concentration and length of hospitalization in Diarrhea and Septic foals < 1 month old with a p value < 0.001 $\rho = 0.84$, as noted in Figure 4.10, which supports our hypothesis.

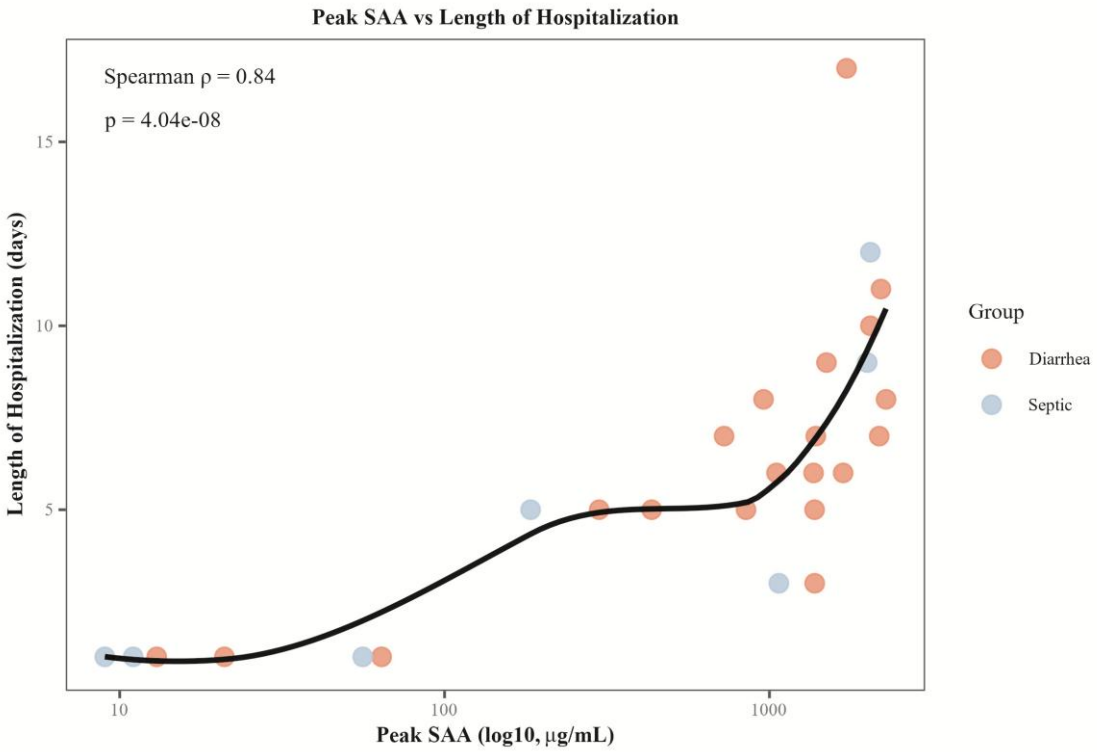


Figure 4.10. Scatterplot of peak SAA in foals with Diarrhea and Septic.

Chapter 5 - Discussion.

This thesis evaluated SAA concentration patterns in Healthy, Diarrhea and Septic neonatal foals with repeated sampling during hospitalization, its utility as a biomarker for diagnosis and evaluation of response to therapy for diarrhea, and its association with blood culture results and length of hospitalization.

The median concentrations of SAA in Healthy foals on each day was similar to multiple authors in the past, such as Barr and Nieman (24) in 2021 or Giguère *et al.*(57) who obtained a 0 μg/mL value, and Stoneham *et al.* who obtained a value of 2.20 μg/mL using latex agglutination immunoturbidimetric assay(48). The Healthy group maintained low SAA concentrations across all three days, which was expected.

Regarding the Diarrhea group, the current results are significantly higher than the median value presented by Hultén and Demmers (60), which was <0.5 μg/mL; or the median value from Satoh *et al.* of 110.30 $\pm$ 114.7 μg/mL (45). This discrepancy could be explained by differences in methodologic assays and sample type used. Another explanation could be that SAA takes 6-12 hours to increase and up to 48 hours to peak, so there is a possibility the concentrations were not at its peak at the moment of sampling (34). Currently there is no studies that utilized POC Stablelab to measure SAA in neonatal foals with diarrhea. Barr and Nieman used POC Stablelab to measure SAA in foals. Some foals in the sick-non septic group had enteritis; however, they did not specify that any foals had diarrhea (24).

In the Septic group, SAA during the first 3 days was between 819.5 to 1122.5 μg/mL. Considering the cutoff established by Barr and Nieman and Stoneham *et al.* of >100 μg/mL to

diagnose a foal as septic (24,48), the Septic group was consistent with that previously established cutoff.

One study found median in septic foals to be lower than the current study, for example a median of 114 μg/mL found by Barr and Nieman (24). In contrast to a median SAA concentration of 1079 μg/mL reported by Hoeberg *et al.* and a range of 834 to 1022 μg/mL reported by de Bruijn *et al.,* the current study's findings where more consistent with these later reports (23,59). There is a possibility that those differences are due to methodologic assays differences (23). De Bruijn *et al*. used POC Stablelab with whole blood similar to the current study, which may be the reason for the similar results (59).

There was a significant difference on day 1, 2, and 3 in SAA concentrations between the Diarrhea and Healthy groups, and between the Septic and the Healthy groups, with both sick groups having higher SAA concentrations compared to the Healthy group. Between both sick groups there was no significant difference.

Diarrhea foals could have enteritis, colitis or enterocolitis, which is a local inflammatory process that could increase SAA, or could cause translocation of bacteria to the blood and subsequent sepsis. Additionally, some of these foals were administered antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs) and intestinal adsorbents prior to hospitalization, which could have limited the ability to detect differences between groups.

During the first 3 days, the Diarrhea group had a peak on day 2 and the Septic group on intake (day 1). This could suggest that the foals in the Diarrhea group presented to the hospital in an earlier phase of the disease, since diarrhea is more easily recognizable than the non-specific

signs of sepsis. However, these patterns should be interpreted cautiously, since the Septic group was composed of only four foals.

Concerning the pattern of SAA for Diarrhea and Septic foals throughout their hospitalization, it was evident in Figure 4.5 that SAA decreased constantly and more rapidly in the Diarrhea group compared with the Septic group; however, the Diarrhea group had a median of 5 days and the Septic group of 3 days to be under the 100 μg/mL cutoff. This finding contradicts the data presented in Figure 4.5, likely because this finding is based on only three foals from the Septic group, excluding that in which SAA concentrations never returned to normal. Overall, in both groups the concentration decreased through hospitalization, which is likely related to response to treatment.

Regarding the blood culture results, the Healthy group had growth of multiple microorganisms that have been considered contaminants, such as *Bacillus* species and *Streptococcus* species, similar to Hacket *et al*. in which bacteremia is reported in apparently healthy foals (22). *Corynebacterium* species and *Cutibacterium acnes* have been named as contaminants by researchers in human medicine (19,20), which explains why multiple foals in the Healthy group were positive to them. Interestingly, some of the organisms can also be classified as pathologic microorganisms such as *Corynebacterium* species, *Streptococcus* α hemolytic, and *Bacillus* species as described in Wong *et al*. in 2018 (14). It is worth noting that some of our Healthy foals were positive to different *Acinetobacter* species, which according to van der Kolk *et al.* can be non-pathogenic microorganisms that are ubiquitous, present in soil, water and sediments (21). *Acinetobacter baumanni,* is considered a nosocomial microorganism that can be resistant to carbapenem; however, some reports do not associate it to disease in horses (21). In contrast, the Diarrhea and the Septic groups had positive blood cultures with bacteria that are typically

associated with pathology/sepsis and not contamination, such as *Streptococcus* species, *Listeria monocytogenes, Escherichia coli* and *Salmonella* species (19,20).

Regarding whether admission SAA concentration was associated with blood culture result (gram positive vs gram negative vs no growth), the current results indicated that there was no statistically significant difference between groups, which contradicts de Bruijn *et al.* who found a significant difference between admission SAA and positive blood culture (59), or Hultén and Demmers, who found a higher SAA in foals with a positive blood culture compared to the ones with negative blood culture (60). This could be explained due to small sample size, previous antibiotic treatment or presence of contaminants microorganisms in some of the positive blood culture, as discussed previously.

In the current study, the majority of the causative etiologic agents of diarrhea were either positive to *Clostridioides difficile* or *Clostridium perfringens*. This is consistent with the typical disease distribution in the author's hospitalized population. Conversely, *Clostridium perfringens* type A and *Clostridioides difficile* can be present even in healthy foals (1,7). One of the foals had *Clostridium perfringens* type C diarrhea, which is associated with hemorrhagic enteritis and high mortality. This foal did have multiple complications including presumptive disseminated intravascular coagulation (DIC), anorexia, repeated colic, bilateral jugular thrombosis, omphalitis, and pneumonia. This patient was discharged against medical advice with a guarded to grave prognosis (1,7). Samples from some of the Diarrhea foals, some were positive for genes for netF and cpe. NetF is a beta pore-forming toxin and cpe is a conjugative-plasmid that is constantly found with it. NetF causes cytotoxicity and is associated with necrotizing enterocolitis in foals by Mehdizadeh Gohari *et al.* (61,62). Additionally, netF prevalence has been studied in Ontario, and it was not found in young foals, which increases the likelihood of it being associated with disease

(63). Interestingly, one of those 3 foals positive to netF and cpe, presented with more complications other than diarrhea and sepsis including pneumonia, patent urachus, and thrombophlebitis.

Our hypothesis that SAA will be higher on admission in foals with Diarrhea than in Septic or Healthy foals was not supported, because even when there was significant difference between the Healthy group and both sick groups, there was no difference between the Diarrhea and Septic groups. This may be explained, in part, by the fact that many of the Diarrhea foals were likely septic also.

With our second hypothesis we found that foals with a higher SAA on admission stay hospitalized longer since there was an association with length of hospitalization and SAA concentration on admission in Diarrhea and Septic foals. An optimal cutoff in our sample was determined as 879 μg/mL with a Se of 82% and a Sp of 60%, to predict hospitalization $\geq$ 7 days. Although the Sp is not ideal, this cutoff is clinically useful as it will determine which foals likely will need prolonged hospitalization (>7 days). Some of the foals categorized as such may not actually require that length of hospitalization but that would be a positive outcome and benefit for owners of these foals.

This cutoff is similar to those that have been previously obtained to predict non-survival at 1250 μg/mL (23) or an admission cutoff of 419 μg/mL for growth in the blood culture (59). To the author's knowledge, this was the first time that a cutoff of SAA has been determined to know the length of hospitalization in hospitalized neonatal foals with diarrhea was obtained, which gives multiple clinical implications, such as being able to have more meaningful and informed conversations with owners on the day of admission regarding expectations of days in the hospital and financial implications. Finally, our hypothesis of foals with a higher peak SAA through hospitalization stay hospitalized longer was supported with a strong positive correlation between

peak SAA concentration and length of hospitalization in Diarrhea and Septic foals. This is consistent with SAA reflecting the magnitude of the inflammatory response, so foals with higher SAA have more inflammation and require longer hospitalization.

This study has several limitations. First, our sample size was small, specifically for the Septic foal, so our results should be considered cautiously, especially when comparing the Septic group. Future studies will benefit from increasing the sample size. Second, all of the samples came from the same geographic area and from referred foals, which could leave foals with mild diarrhea or clinical signs out of the study. In the future, a multicenter study could be considered. Third, the foals in the current study were <1 month old. It has been noted that foals peak SAA concentration occurs during the first 3 days of life in what is interpreted as a physiological increase. In our study, even when all foals were <1 month old, not all of them were the same age (Healthy foals had a higher median age than either Diarrhea or Septic foals). SAA concentrations may vary with age during the neonatal period, so a physiologic contribution to the higher SAA concentrations in the Septic and Diarrhea groups could be suggested. However, physiologic increase in SAA concentrations is typically less severe. However, in the future, decreasing the age range to <15 days or <7 days could be considered.

In conclusion, SAA on admission of the first 3 days of hospitalization was higher in foals with diarrhea and septic foals compared to healthy foals.

A progressive decline in SAA concentrations during hospitalization indicates its utility as a marker of treatment response.

Admission SAA concentrations >879 μg/mL and higher peak concentrations were associated with a longer length of hospitalization of >7 days. This suggests that SAA could be useful to help predict length of hospitalization in foals with diarrhea.

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Appendix A - Questionnaire

MARE'S QUESTIONNAIRE

Name: Patient ID:

Age:

Breed:

Function:

Was the mare under any treatment before foaling?

If yes: what was/were the treatment(s) and why?

When was her due date?

When did she foal (date)?

What was the foal's gestational age? (if known)

Is this her first foal? If not, how many times has she been pregnant?

Did she have any problems during gestation (placentitis, vulvar discharge, premature udder development or lactation, other unrelated to pregnancy)?

Was foaling attended?

Did she present with any problems during foaling (dystocia, red bag, other)?

Did she present with any problems after foaling (retention of the placenta, vulvar discharge)?

When was her last deworming administration?

Did the mare receive pre-foaling vaccinations approximately 1 month prior to parturition? When and which vaccinations?

Was the mare vaccinated for equine herpesvirus-1 during gestation? Approximately when?

Is there a history of a previous foal of this mare or a history on the farm of foal diarrhea? If so, was a pathogen identified?

FOAL'S QUESTIONNAIRE

Name: Patient ID:

Age on arrival: Breed: Sex:

Gestational age in days:

When was the foal born?

Time to stand:

Time for nursing:

Did the foal nurse colostrum?

Did the foal get any treatment postpartum?

When did the foal show clinical signs?

Description of clinical signs:

Treatments administered before arrival at the hospital:

Are there other animals with the same clinical signs?

IgG test was performed before arrival?

Were any other tests performed before arrival?

FOAL'S KSU INFORMATION FORMAT

Name: Patient ID:

Age: Breed: Sex:

Name of the mare:

Reason for hospitalization:

Physical examination on arrival

Weight:

Mentation:

General appearance:

HR and any associated changes:

RR and any associated changes:

Temperature:

Mucous membranes:

Eyes:

Lymph nodes:

GI sounds:

Umbilicus:

Joints:

Hydration status (% dehydration):

TESTS

Sepsis score:

Ultrasonography findings:

IgG snap test:

CBC:

Biochemistry:

Blood culture:

Collect fecal sample:

Equine neonate diarrhea PCR panel (send):

SAA 1:

SAA 2:

SAA 3:

SAA 4:

SAA 5:

SAA 6:

SAA 7:

Treatments administered during hospitalization:

Progress during hospitalization:

Complications during hospitalization:

Outcome (discharged or not, when was it discharge, if it was euthanized why and when):

Follow up after 7 days of discharge:

Any follow-up SAA measurements after discharge: