Serum and Synovial Fluid Serum Amyloid A Response in Equine Models of Synovitis and Septic Arthritis

Elsa K. Ludwig[1], R. Brandon Wiese[1], Megan R. Graham[1], Amelia J. Tyler[1], Julie M. Settlage[1], Stephen R. Werre[2], Christina S. Petersson-Wolfe[3], Isis Kanevsky-Mullarky[3], and Linda A. Dahlgren[1]

[1]Department of Large Animal Clinical Sciences and [2]Laboratory for Study Design and Statistical Analysis, Virginia-Maryland College of Veterinary Medicine, and [3]Department of Dairy Science, College of Agriculture and Life Sciences, Virginia Tech, Blacksburg, Virginia

Corresponding Author
Linda A. Dahlgren
205 Duck Pond Drive
Virginia-Maryland College of Veterinary Medicine
Virginia Tech
Blacksburg, VA 24061-0442
lad11@vt.edu

Submitted April 2016
Accepted April 2016

DOI:10.1111/vsu.12531

Objective: To investigate the serum and synovial fluid serum amyloid A (SAA) response in equine models of synovitis and septic arthritis and to compare handheld and validated immunoturbidometric assays for SAA quantification.
Study Design: Controlled, experimental study.
Animals: Healthy adult horses (n = 9).
Methods: Synovitis (n = 4) and septic arthritis (n = 5) were induced using lipopolysaccharide and *Staphylococcus aureus*, respectively, and serial serum and synovial fluid samples were collected. Serial synovial fluid cytology was performed for both models and synovial fluid from the septic arthritis model was submitted for bacterial culture. Serum and synovial fluid SAA were quantified by handheld test and immunoturbidometric assay. Cytologic and SAA data were compared within and between models (mixed model ANOVA) and results of SAA assays were compared using category-by-category analysis (weighted kappa coefficient).
Results: Synovial fluid total nucleated cell counts and total protein increased significantly following induction of both models. Serum and synovial fluid SAA remained normal in synovitis horses and increased significantly in septic arthritis horses. Serum SAA increased more rapidly than synovial fluid SAA. Agreement was 98% when SAA concentrations were low (<50 μg/mL) but the assays diverged when concentrations were greater than ~100 μg/mL. Overall, there was good category-by-category agreement between SAA assays (weighted kappa = 0.824).
Conclusion: Serum and synovial fluid SAA may be useful adjuncts in diagnosing septic arthritis in horses. SAA concentrations for the assays diverged and examination using a larger sample size is needed before direct numeric comparisons between the assays can be made.

Septic arthritis in adult horses occurs most commonly in association with traumatic wounds and can become life-threatening due to difficulties clearing established infections and development of degenerative changes associated with ongoing inflammation.[1,2] Only 56–81% of horses return to their original function following treatment for septic arthritis.[3,4] Early diagnosis is critical to rapid resolution of infection and inflammation to avoid ongoing cartilage degradation and osteoarthritis.

Septic arthritis is diagnosed primarily based on clinical signs and synovial fluid analysis. In severe cases, the diagnosis is unequivocal and characterized by marked elevations of synovial fluid total protein (TP) and total nucleated cell count (TNCC). However, differentiation between acute nonseptic inflammation and infection based on clinical signs and synovial fluid cytology alone can be challenging in some horses.[1,5–9] Horses with acute nonseptic inflammation can show mild to moderate lameness and there can be significant overlap in TNCC and TP in some horses with synovitis and septic arthritis.[5–9]

A rapid assay to confirm sepsis at the time of examination would be of value, especially for practitioners in the field without ready access to a laboratory.[1,2] Measurement of serum amyloid A (SAA) has been recently described for screening of systemic inflammation and infection in horses and could be used for screening synovial inflammation and infection.[10–14] SAA, the major acute phase protein in horses, increases systemically in response to injury, infection, and inflammation.[13,15,16] It is found in very low or undetectable concentrations (0.5–20 μg/mL) in the serum of healthy horses,[12,13,17,18] and is produced primarily in the liver.[13,19,20] SAA quickly rises in response to infection and inflammation, and can be used as a sensitive and reliable indicator of active inflammation in the horse.[13,15] The rapid peak at 36–48 hours and short half-life (24 hours) make SAA ideal for monitoring the progression of disease and response to treatment.[13,15,19]

Presented at the Veterinary Orthopedic Society Annual Conference, Big Sky, MT, March 2016.

A variety of disease states are reported to induce increased SAA concentrations in horses, including bacterial and viral infections, septic arthritis, surgery, gastrointestinal tract disease, reproductive disease, and local inflammation,[13,14] and data suggest that SAA may be an order of magnitude higher with bacterial infection vs inflammation.[13,16] SAA is produced locally within synovial joints[11,14,21]; however, information on SAA in equine joints is limited.[11,21] SAA is present in low or undetectable levels in normal equine synovial fluid,[10,12,22] does not increase in synovial fluid following repeated arthrocentesis,[10,12] and does increase in synovial fluid in response to sepsis.[12,14]

Equine SAA levels are commonly determined by immunoturbidometric assay using an automated chemistry analyzer and monoclonal anti-human SAA antibodies.[18] The assay is offered by a limited number of laboratories and thus, samples are usually frozen and shipped, delaying receipt of results. A handheld lateral flow immunoassay (handheld test) has recently become commercially available to measure SAA in equine whole blood or serum. The assay can be performed immediately following sample collection and produces results in 10 minutes. Results are read by visual categorical analysis or using a portable colorimetric reader (StableLab, Epona Biotech Limited, Sligo, Ireland). Data on use of this handheld test to measure SAA in equine synovial fluid are not available. The ability to determine serum or synovial fluid SAA using the handheld test could be used in conjunction with other diagnostics for septic arthritis.

The objectives of our study were to investigate the serum and synovial fluid SAA response in equine models of synovitis and septic arthritis and to compare SAA results of a new handheld test with those from a validated immunoturbidometric assay using the same equine models of acute synovitis and septic arthritis. We hypothesized that SAA in serum and synovial fluid from horses with septic arthritis would be significantly elevated compared to horses with synovitis. We also hypothesized there would be good agreement between the handheld test and the validated SAA immunoturbidometric assay in equine serum and synovial fluid.

MATERIALS AND METHODS

Study Design

Synovitis (n = 4) or septic arthritis (n = 5) were induced in 1 randomly assigned radiocarpal joint of adult horses free of orthopedic disease. The study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Biosafety Committee. Blood and synovial fluid were collected before and following model induction for synovial cytology and SAA analysis by a handheld test and a previously validated immunoturbidometric assay and compared across time, between models, and between SAA assays.

Experimental Horses

Nine healthy adult horses (3 mares, 6 geldings) were determined to be free of musculoskeletal disorders related to the carpal joints based on physical and lameness examinations. Horses had a mean (±SD) age of 16.2 (±5.1) years and weight of 479.7 (±38) kg. Breeds included Arabian or Arabian crosses (2), Paint (2), Tennessee Walking Horse (2), American Quarter Horse, American Saddlebred, and Thoroughbred (1 each). All horses had pain-free range of motion of the carpal joints, were evaluated at the walk and trot in a straight line and on circles, and carpal flexion tests were negative. Horses were housed at the Veterinary Teaching Hospital of the Virginia-Maryland College of Veterinary Medicine in 12′ × 12′ stalls for the duration of the study without forced exercise, fed free choice water and grass hay, and allowed a 24 hour acclimatization period prior to model induction.

On the day of model induction, a 14 gauge, 5.5 inch IV catheter (Abbocath®, Abbott Laboratories, Abbott Park, IL) was placed aseptically in one jugular vein with an extension set and 3-way stopcock attached. The catheter was sutured in place and maintained throughout the course of the study. Catheters were flushed with 10 mL heparinized saline (10 IU/mL heparin in 250 mL 0.9% saline solution) every 6 hours or following administration of medications to maintain catheter patency. Each horse underwent physical examination, including in-stall lameness evaluation, every 6 hours. Lameness was evaluated subjectively in the stall as the horse was walked and turned. If the horse became visibly lame in the stall, it received butorphanol (0.02 mg/kg IV and IM) every 4 hours until lameness was no longer observed. Originally, estimations of pain response based on the literature required that horses in the septic arthritis group receive phenylbutazone (2.2 mg/kg IV) at the time of model induction and every 12 hours thereafter until euthanasia. However, based on our experience with the first horse in the septic arthritis group, the IACUC protocol was amended such that the remaining 4 horses in the septic arthritis group received phenylbutazone (2.2 mg/kg IV) if they became visibly lame in the stall and every 12 hours until lameness was no longer observed or they were euthanatized at the end of the study, whichever came first. After final sample collection, each horse was euthanatized with pentobarbital sodium (86 mg/kg IV).

Synovitis and Septic Arthritis Models

Synovitis was induced by lipopolysaccharide (LPS) injection of 1 randomly selected (RANDBETWEEN function, Microsoft Excel, Microsoft Corporation, Redmond, WA) radiocarpal joint using a low dose selected based on published studies.[11,23–25] Lyophilized LPS (*Escherichia coli* strain 055:B5, Sigma-Aldrich Corp, St. Louis, MO) was diluted to 2.5 mg/mL in sterile saline, aliquoted, and stored at −20°C until use. Immediately prior to induction of synovitis (within 15 minutes), thawed aliquots of LPS were vortexed vigorously and serially diluted in sterile saline to 0.0125 ng/μL. The total dose of 0.5 ng LPS per radiocarpal joint was further diluted in 2 mL saline, vortexed, and drawn into a sterile syringe, sealed in a plastic bag, and stored on ice until injection. The low dose was selected based on the literature to induce changes in TP and TNCC similar to those

anticipated for the septic arthritis model yet avoid systemic signs of endotoxemia.[11,23–25]

Septic arthritis was induced using a previously described bacterial model.[6,26–28] A strain of *Staphylococcus aureus* isolated from a case of naturally occurring bovine mastitis and previously validated for use in a bovine model of mastitis was used to induce sepsis.[29] *Staphylococcus aureus* inoculum preparation was conducted in Biosafety Level 2 approved facilities approved by the Institutional Biosafety Committee. The day prior to model induction, the frozen *S. aureus* culture was streaked on a bovine blood agar plate and incubated for 24 hours at 37°C. A single colony of bacteria was cultured in trypticase soy broth for 6 hours at 37°C with shaking, bacteria were pelleted by centrifugation at 2,500 × *g* for 10 minutes at 4°C (IEC CL31R, Thermo Fisher Scientific, Pittsburgh, PA), washed twice with phosphate-buffered saline (PBS), and resuspended in PBS to achieve a concentration of approximately 1.5×10^4 colony forming units (CFU)/mL. Approximate concentration was based on a standard curve relating optical density to CFU for our bacterial isolate (Genesys 20 Spectrophotometer, Thermo Fisher Scientific, Pittsburgh, PA). Following preparation, the inoculation dose was brought up to a total injection dose of 2.5 mL in sterile PBS, transferred to a sterile syringe, sealed in doubled plastic bags, and stored on ice until injection. Intra-articular injection of the inoculation dose was performed within 30 minutes of dose preparation. To minimize variation from one bacterial inoculum preparation to another, horses were grouped such that 1–3 horses were injected on the same day from the same bacterial preparation (3 preparations total). CFU of the challenge inoculum were determined by delivering four 25 μL replicates of the final dilution to the surface of a trypticase soy agar plate, incubating for 18–24 hours at 37°C, and manually counting the number of colonies. Inoculation doses showed minimal variation between the 3 preparations (median, 1.62×10^4 CFU/joint; range, $1.29–1.76 \times 10^4$). The total dose of 1.5×10^4 CFU per joint was selected based on the published literature in the horse and on previous experience with this particular strain of bacteria in a bovine mastitis model.[6,26–29]

Model Induction and Sample Collection

Prior to model induction, 6 mL blood was collected from the IV catheter for baseline serum SAA concentration. Horses were then sedated with detomidine (0.01 mg/kg IV) and butorphanol (0.01 mg/kg IV) and the randomly selected radiocarpal joint clipped and prepared for arthrocentesis with povidone-iodine and alcohol using standard aseptic technique. With the carpus held in flexion by an assistant, a 20 gauge 1.5 inch hypodermic needle was placed in the dorsolateral synovial pouch and 4–6 mL synovial fluid collected and transferred to a blood tube containing EDTA to serve as the baseline control sample for each horse. Without removing the needle, the prepared dose of either LPS or *S. aureus* (depending on model) was injected using aseptic technique (and proper biosecurity precautions for *S. aureus*). Following injection, a light bandage consisting of a sterile nonstick pad, roll gauze, and elastic adhesive tape (Elastikon, Johnson & Johnson, New Brunswick, NJ) was applied over the arthrocentesis site to maintain cleanliness. A stack bandage of cotton sheets, gauze, and cohesive bandage was applied from the distal radius to the coronary band to control edema.

Timing of sample collection following model induction was customized for each model based on published information, real time evidence of changes in serum and synovial fluid SAA and synovial fluid TP and TNCC concentrations, and humane considerations of animal welfare based on degree of lameness. Because the models do not progress at the same rates, the collection times for each model are purposefully not identical so as to match sample collection with key changes in synovial fluid parameters. In addition to baseline samples, blood and synovial fluid were collected at 6, 12, 24, and 30 hours following LPS injection and at 12, 24, and 36 hours following *S. aureus* injection. Longer sampling periods were used for the first horse in each group until a known pattern of disease progression was established. Blood samples (6 mL) were collected from the IV catheter following removal of 10 mL discard sample to clear the catheter and extension set of stagnant blood diluted with heparinized saline. Serial radiocarpal synoviocentesis (4–6 mL) and bandaging were performed as described above. Bandages were maintained throughout the course of the study with replacement following each synoviocentesis.

Sample Processing

Following collection, clotted blood samples were centrifuged at 2,000 × *g* for 10 min at 22°C and serum collected and divided into 2 aliquots: 1 for immediate SAA analysis using the handheld test and 1 stored in a polypropylene tube at −20°C for later SAA analysis using the equine immunoturbidometric assay and the colorimetric reader for the handheld test.[15,18] Following collection, synovial fluid was divided into aliquots. One aliquot was submitted for cytology without centrifugation (Virginia Tech Animal Laboratory Services, Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA). Subjective assessment of synovial fluid color and viscosity were recorded and TP, TNCC, and white blood cell differential were quantified (ADVIA 2120 hematology analyzer, Siemens Healthcare Diagnostics, Inc, Tarrytown, NY). TP > 4.0 g/dL and TNCC > 30,000 cells/μL were used to confirm synovitis and septic arthritis models.[5] A second aliquot of synovial fluid was centrifuged as above to pellet cells and debris and the supernatant removed and further aliquoted for immediate SAA analysis using the handheld test or frozen as described above for SAA analysis using the immunoturbidometric assay and the colorimetric reader for the handheld test. To confirm induction of sepsis, a third synovial fluid sample (1 mL when TNCC and TP were highest) was transferred to a blood culture bottle (SIGNAL Blood Culture System, Oxoid Limited, Hampshire, UK) and submitted for bacterial culture and identification (Virginia Tech Animal Laboratory Services).

SAA Quantification

Quantification of SAA from fresh, centrifuged serum or synovial fluid was performed using a handheld SAA test

(StableLab, Epona Biotech Limited, Sligo, Ireland) according to the manufacturer's instructions. Briefly, 5 μL of serum or synovial fluid was measured using the supplied pipette, added to the supplied volume (~3.5 mL) of handheld test mix solution to dilute the sample, and gently inverted, as instructed. With the test cartridge placed on a flat surface, 4 drops of diluted sample were applied to the cartridge well of the test kit using the supplied dropper and the result read following the 10 minute wait period. Using the supplied StableLab reference card, the SAA concentration was estimated based on color intensity of the test band and assigned to 1 of 4 categories (0–15, >15–50, >50–200, and >200–1,000 μg/mL; Fig 1). All test cartridges were digitally photographed and the real time categorical SAA concentration used to monitor model progression along with synovial fluid cytology. Frozen serum and synovial fluid samples were batch shipped overnight on dry ice for SAA analysis via a previously validated equine immunoturbidometric assay (Acute Phase Protein Laboratory, Miller School of Medicine, Miami, FL). Confirmation of SAA concentrations using the handheld test was made on frozen aliquots using a colorimetric reader (SAA quantification range 0–3,000 μg/mL, StableLab, Epona Biotech Limited) to generate continuous data for statistical comparisons.

Figure 1 Reference card (left) and representative cartridge (right) for handheld serum amyloid A (SAA) test of serum from a septic arthritis horse 36 hours post-inoculation. Note the test band color (T) indicates a reference card SAA concentration of >200–1,000 μg/mL (intermediate), corresponding to 624 μg/mL by colorimetric handheld reader and 357.7 μg/mL on immunoturbidometric assay. C = Positive control band.

Data Analysis

Data were assessed for normality using normal probability plots. Data for weight, age, cytology, and immunoturbidometric SAA were normally distributed. TNCC were log transformed (base e) to stabilize the statistical model. Data are presented as mean (±SD) and geometric mean (95% CI) for normally distributed and TNCC data, respectively. Separately within synovitis and septic arthritis groups, the effect of time on cytology and immunoturbidometric SAA data were analyzed using mixed model ANOVA followed by Tukey's procedure for multiple comparisons for time points containing ≥4 data points. The linear model specified time as a fixed effect and horse as the random effect. The effect of time on continuous SAA data from the handheld test was assessed using Friedman chi-square test. Cytology and immunoturbidometric SAA data were also compared between synovitis and septic arthritis using mixed model ANOVA at 0, 12, and 24 hours when comparable times and ≥4 data points were available for both models. The linear model specified group (synovitis vs septic arthritis), time, and the interaction between group and time as fixed effects and horse nested within group as the random effect. To specifically compare groups at each time point, the slicediff option of proc glimmix was applied to the interaction between group and time. Only immunoturbidometric data were used to compare across time within models (synovitis and septic arthritis) and between models. Continuous data for the handheld test were skewed and are presented as median (range).

A preliminary assessment of agreement between paired continuous handheld and immunoturbidometric data was performed using a bias plot (values from the 2 assays for the same sample were plotted against each other). This plot was generated for only samples from the septic arthritis horses, as all values for samples from synovitis horses were essentially 0 (<2.5 μg/mL). Because the scatter plot showed obvious divergence between assays after a concentration of ~100 μg/mL, Bland-Altman analysis was not performed. In addition, continuous data from both the handheld and immunoturbidometric assays were converted to categorical data using the following categories (μg/mL) derived from the handheld reference card and relating to clinical relevance: 0–15 (normal), >15–50 (mild elevation), >50–200 (moderate elevation), and >200 (severe elevation). Category-by-category analysis was then performed to assess assay agreement using a weighted kappa coefficient. All data analysis was performed using commercial software (SAS Version 9.4, SAS Institute Inc, Cary, NC). Real time categorical data generated from the handheld test were used only for assessment of model progression and were excluded from data analysis in favor of the continuous handheld data. Categorical data are reported along with the continuous data from the same samples for transparency. Significance was set at $P < .05$.

RESULTS

Model Induction

As expected, variability existed between horses for both models of synovitis and septic arthritis but synovial fluid TP

Table 1 Mean ± SD synovial fluid cytology for horses (N) with lipopolysaccharide-induced synovitis

Time (hours)	N	Total protein (g/dL)	TNCC* (cells/μL × 10^3)	Neutrophil (%)	Lymphocyte (%)	Macrophage (%)
0	4	1.6 ± 0.5[a]	0.1 (0–0.7)[a]	5.3 ± 9.8[a]	41.2 ± 7.0[a]	52.5 ± 11.4[a]
6	4	3.6 ± 1.5[b]	52.0 (1.2–2,303.6)[a]	82.5 ± 16.4[b]	3.0 ± 2.8[b]	14.2 ± 14.3[b]
12	4	5.1 ± 0.7[b]	76.0 (18.6–310.2)[b]	80.5 ± 14.8[b]	3.8 ± 3.6[b]	15.8 ± 11.9[b]
24	4	4.7 ± 0.6[b]	31.9 (13.4–76.0)[b]	59.6 ± 23.9[b]	10.4 ± 10.2[b]	29.8 ± 14.8[b]
30	4	4.4 ± 0.5[b]	19.7 (4.3–90.9)[b]	56.2 ± 27.4[b]	10.8 ± 11.0[b]	33.0 ± 17.4[b]

*Total nucleated cell count, Geometric mean (95% CI).
[a]Means within a column with the same superscript are not significantly different.

>4.0 g/dL and TNCC >30,000 cells/μL were achieved for all horses in both models (Tables 1 and 2).[5] All septic arthritis horses had positive synovial fluid cultures for *S. aureus*.

Synovitis

Following induction of synovitis, the gross appearance of synovial fluid changed rapidly and become serosanguinous with reduced viscosity. Changes were moderate at 6 hours, but became marked by 12 hours and remained markedly abnormal for the duration of the study. No horses exhibited lameness in their stalls, all maintained normal physical examination parameters (temperature, pulse, and respiratory rate; TPR), and none had visible soft tissue swelling at the arthrocentesis sites. Synovial fluid TP increased by 6 hours, was highest at 12 hours, and remained elevated throughout the study (Table 1; $P < .001$). Synovial fluid TNCC and percent neutrophils were highest 12 hours following LPS injection and gradually decreased, remaining elevated at the end of the study (Table 1; $P < .001$ for both). Percent lymphocytes and macrophages decreased precipitously following induction of synovitis and remained decreased throughout the course of the study (Table 1; $P < .001$ for both).

Serum and synovial fluid SAA was 0 μg/mL at all sampling time points using the handheld test with both the reference card and the colorimetric reader (Table 2; $P = 1.0$ for both). Serum and synovial fluid SAA were measurable by immunoturbidometric assay but very low at all time points, and did not change over time (Table 2; $P = .480$ and .532, respectively).

Table 2 Serum and synovial fluid serum amyloid A (SAA) concentration for horses (N) with lipopolysaccharide-induced synovitis. SAA in synovial fluid or serum quantified with a commercial handheld test (Handheld, median [range]) and a validated immunoturbidometric assay (Immuno, mean ± SD) showed no differences over time

		Serum SAA (μg/mL)		Synovial fluid SAA (μg/mL)	
Time (hours)	N	Handheld	Immuno	Handheld	Immuno
0	4	0 [0–0]	0.2 ± 0.2	0 [0–0]	0.4 ± 0.3
12	4	0 [0–0]	0.4 ± 0.4	0 [0–0]	0.3 ± 0.4
24	4	0 [0–0]	0.6 ± 0.7	0 [0–0]	0.6 ± 1.0
30	2	0 [0–0]	–	0 [0–0]	–
48	2	0 [0–0]	–	0 [0–0]	–

Septic Arthritis

Gross changes in the synovial fluid after induction of septic arthritis were similar to those in the synovitis group, with the addition of fibrin present in the fluid of 2 horses at 24 and 36 hours. The first horse induced with septic arthritis received a prophylactic dose (2.2 mg/kg IV) of phenylbutazone at the time of model induction in anticipation of the increased pain associated with the septic arthritis model compared to synovitis and in accordance with the original IACUC protocol. Serial sampling revealed a delayed onset of synovial fluid changes compared to what was expected based on the literature and the horse did not show lameness in the stall. As a result, the 36 hour dose of phenylbutazone (2.2 mg/kg IV) was withheld until 66 hours post-induction when lameness became apparent at 66 hours. Analgesic administration and data collection for subsequent horses was adjusted accordingly and approved by the IACUC and expected synovial fluid changes were achieved. Horses with septic arthritis maintained normal physical examination parameters (TPR) and had no visible soft tissue swelling at the arthrocentesis sites. Two horses showed mild lameness in the stall 12 hours following model induction (occasional lame steps when turning), but remained weight bearing. The degree of lameness remained unchanged despite administration of phenylbutazone and butorphanol. Sampling end points were determined based on humane considerations.

Synovial fluid cytology parameters were not significantly different between synovitis and septic arthritis (Tables 1 and 3; TP $P = .997$; TNCC $P = .150$; % neutrophils $P = .213$; % lymphocytes $P = .395$; % macrophages $P = .334$). Septic arthritis synovial fluid TP increased by 12 hours and was highest between 36 and 48 hours (Table 3; $P < .001$). Synovial fluid TNCC and percent neutrophils were highest 24 hours following *S. aureus* injection and gradually decreased, remaining elevated at the end of the study (Table 3; $P < .001$ for both). Percent lymphocytes and macrophages decreased following induction of septic arthritis and remained decreased throughout the course of the study (Table 3; $P < .001$ and $P = .045$, respectively).

Serum SAA following induction of septic arthritis began to increase at 24 hours and was highest at 36 hours (Tables 4 and 5). Synovial fluid SAA began to increase more slowly than in serum, but was still highest at 36 hours (Tables 4 and 5). Once SAA concentrations increased above normal, serum concentrations were higher compared to synovial fluid. Immunoturbidometric SAA concentrations increased

Table 3 Mean ± SD synovial fluid cytology for horses (N) with *S. aureus*-induced septic arthritis

Time (hours)	N	Total protein (g/dL)	TNCC* (cells/μL × 10^3)	Neutrophil (%)	Lymphocyte (%)	Macrophage (%)
0	5	1.4 ± 0.5[a]	0.2 (0–0.3)[a]	18.5 ± 24.5[a]	37.0 ± 12.5[a]	43.5 ± 27.4[a]
12	5	4.1 ± 1.4[b]	42.4 (6.4–280.1)[b]	74.0 ± 24.3[b]	5.8 ± 5.9[b]	20.4 ± 25.2[ab]
24	5	5.8 ± 0.9[cd]	151.0 (100.9–226.1)[b]	89.4 ± 5.2[b]	4.4 ± 5.7[b]	6.2 ± 4.0[b]
36	4	6.0 ± 1.1[d]	110.4 (20.5–594.8)[b]	84.2 ± 9.9[b]	1.2 ± 2.5[b]	14.5 ± 7.8[ab]
48	2	6.6 ± 0.8	152.6	93.0 ± 2.8	1.5 ± 0.7	6.0 ± 1.4

*TNCC, Geometric mean [95% CI].
[a]Means within a column with the same superscript are not significantly different, time 48 excluded from analysis since N ≤ 4.

Table 4 Serum and synovial fluid serum amyloid A (SAA) concentration for horses (N) with *S. aureus*-induced septic arthritis quantified using a commercial handheld test (Handheld, median [range]) and a validated immunoturbidometric assay (Immuno, mean ± SD)

		Serum SAA (μg/mL)		Synovial fluid SAA (μg/mL)	
Time (hours)	N	Handheld	Immuno	Handheld	Immuno
0	5	0 [0–0][a]	5.4 ± 7.6[a]	0 [0–0][a]	2.3 ± 2.4[a]
12	5	0 [0–0][a]	1.4 ± 0.8[a]	0 [0–0][a]	1.1 ± 1.1[a]
24	5	115 [0–210.0][a]	111.9 ± 116.3[a]	0 [0–61][a]	27.3 ± 40.1[a]
36	4	663 [217–1434][b]	354.3 ± 46.4[b]	135 [60–555][b]	144.3 ± 114.5[b]
48	2	410 [210–610]	347.7 ± 18.6	75 [4–146]	86.7 ± 106.3

[a]Means within a column with the same superscript are not significantly different, time 48 excluded from analysis since N ≤ 4.

significantly over time in both serum ($P < .001$) and synovial fluid ($P = .007$; Table 4). Immunoturbidometric SAA concentrations were significantly increased in septic arthritis compared to synovitis at 24 hours in serum (0 hours $P = .882$; 12 hours $P = .977$; 24 hours $P = .005$) and synovial fluid (0 hours $P = .874$; 12 hours $P = .948$; 24 hours $P = .038$).

SAA concentrations for paired samples measured by the immunoturbidometric assay and the handheld colorimetric reader were similar below 50 μg/mL and diverged above ~100 μg/mL (Fig 2). When evaluated using category-by-category analysis there was good agreement between the two SAA assays (Fig 3; weighted kappa = .824).[30]

DISCUSSION

Serum and synovial fluid SAA were successfully measured using both the handheld test and immunoturbidometric assay, remained within normal limits in samples collected from our model of synovitis, and were significantly increased in samples from our model of septic arthritis. Septic arthritis serum SAA began increasing earlier (24 vs. 36 hours) and was higher than synovial fluid SAA. Concentrations obtained from the handheld test and the immunoturbidometric assay diverged above ~100 μg/mL although there was good overall agreement between the 2 assays for SAA in both serum and synovial fluid when agreement was assessed using category-by-category analysis. Our models of synovitis and septic arthritis both resulted in targeted synovial fluid TP and TNCC in all horses and had similar increases in TP and TNCC at the highest values.

Induction of synovitis using a range of dosages of intra-articular LPS has previously been reported.[11,23,24,31] The intra-articular dosage of 0.5 ng LPS per radiocarpal joint was selected for our study to induce cytology changes similar to acute synovitis[5] and those induced in the *S. aureus* model of septic arthritis. The low dose was selected as the lowest dose

Table 5 Numbers of samples by category for serum and synovial fluid serum amyloid A (SAA) concentration for horses (N) with *S. aureus*-induced septic arthritis quantified using the handheld reference card

		Serum SAA (μg/mL)				Synovial fluid SAA (μg/mL)			
Time (hours)	N	0–15	>15–50	>50–200	>200–1,000	0–15	>15–50	>50–200	>200–1,000
0	5	5	0	0	0	5	0	0	0
12	5	5	0	0	0	5	0	0	0
24	5	2	1	2	0	3	2	0	0
36	4	0	0	2	2	0	1	2	1
48	2	0	0	1	1	0	1	1	0

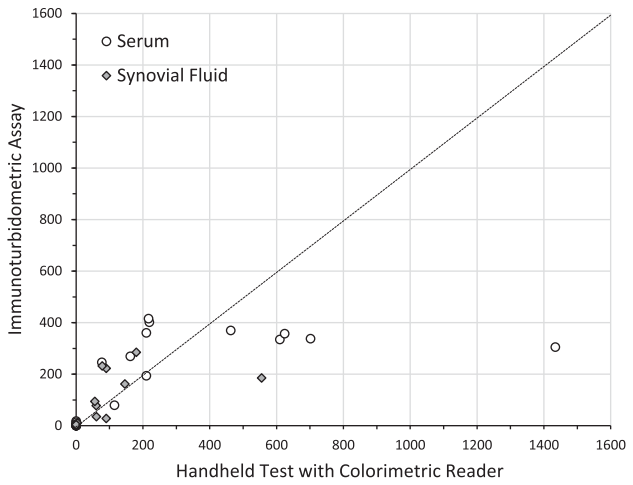

Figure 2 Simple bias plot for SAA concentrations (μg/mL) from paired serum and synovial fluid samples from 5 septic arthritis horses using the handheld test with colorimetric reader and immunoturbidometric assay. The dotted line represents line of equality.

reported to achieve the desired effect and to avoid systemic signs of endotoxemia that might confound data interpretation. Use of higher LPS dosages of 1–3 μg per joint resulted in increased serum and synovial fluid white blood cell counts and increased serum and synovial fluid SAA in a previous study.[11] However, the 4 horses receiving 1–3 μg LPS developed fever, tachycardia, severe lameness, and swelling of the joints following intra-articular LPS injection, which was deemed undesirable for our study.[11] In contrast, our synovitis horses had no change in physical examination parameters or lameness grades, nor did serum or synovial fluid SAA concentrations increase during synovitis, despite dramatic changes in synovial fluid cytology. Use of models for synovitis and septic arthritis do create limitations but they allow design of a controlled study with a known start of synovitis and bacterial contamination, both of which were necessary to complete our stated objectives. Based on our data, clinical studies can be designed to evaluate the SAA response in naturally occurring cases of synovitis and septic arthritis.

Staphylococcus aureus-induced septic arthritis has been previously reported in horses, but to our knowledge this is the first study to use a bovine *S. aureus* isolate.[6,26–28] In addition to increased synovial fluid TP and TNCC, positive synovial fluid bacterial cultures for *S. aureus* were obtained for all septic arthritis horses in our study. This is the largest experimental study to date reporting the measurement of SAA in equine synovial fluid. Significant increases in serum and synovial fluid SAA were detected by both the handheld test and immunoturbidometric assay.

Serum SAA ranges from 0.5 to 20 μg/mL in normal horses[12,13,22,32] and increases rapidly in response to infection and inflammation.[13,15,32] Marked difference in serum SAA between infection and inflammation have been used to help distinguish between infectious and noninfectious disease.[15,16,32,33] Results of our study are consistent with these published reports and confirm normal SAA in serum and synovial fluid during acute synovitis and marked increases during septic arthritis in the models used. The relatively higher concentrations in serum compared to synovial fluid are consistent with another recent report.[14] To our knowledge, this is the first in vivo septic arthritis study evaluating paired serum and synovial fluid SAA. We did not expect to see a temporal difference in serum and synovial fluid SAA responses and the delayed SAA increase in synovial fluid may not be ideal for the timely diagnosis of septic arthritis. However, early increases in serum SAA along with clinical signs and synovial fluid changes consistent with septic arthritis may enable a prompt diagnosis of septic arthritis by simple blood collection and SAA quantification in the field at the time of examination without the need for arthrocentesis, and facilitate timely referral. Synoviocentesis can be challenging in the field, whereas blood collection is commonplace. Used with other diagnostics, elevated serum SAA may support the diagnosis of septic arthritis. Based on our results, we recommend measuring SAA in serum and synovial fluid in addition to synovial fluid TP, TNCC, cytology, and bacterial culture.

The first septic arthritis horse received a prophylactic dose of phenylbutazone at the time of model induction, in expectation of pain and lameness. This horse had an unexpected delay in the serum and synovial fluid SAA response although it did reach SAA concentrations at 72 hours that were observed in the other septic arthritis horses at 36 hours. This first septic arthritis horse may have been slow to respond

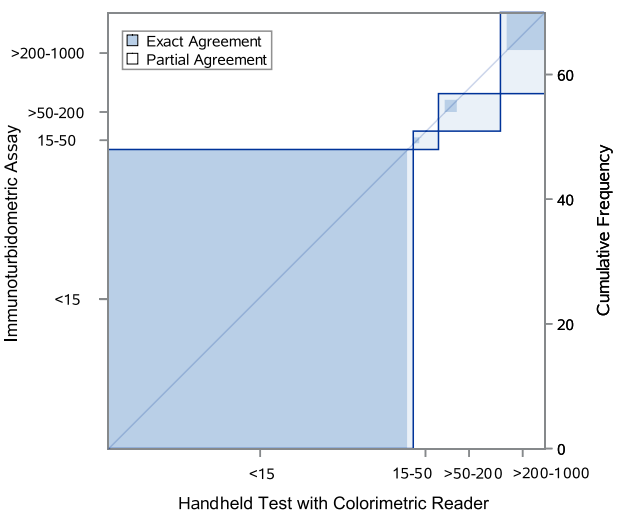

Figure 3 Plot showing category-by-category agreement for SAA concentrations between the handheld test the immunoturbidometric assay for all 70 (μg/mL) samples with pairwise data. The diagonal line represents perfect agreement. At low SAA concentrations (≤50 μg/mL) there was 98% agreement (49/50) between the handheld test with colorimetric reader and the immunoturbidometric assay for SAA concentration. Note the large area of dark blue shading indicates exact agreement. At SAA concentrations >50 μg/mL, 40% of samples had perfect agreement (8/20) and 60% had partial agreement (12/20), clustering along the line of perfect agreement.

to *S. aureus* inoculation or the anti-inflammatory medications may have dampened or delayed the SAA response.

The acute phase response following injury or infection results in activation of inflammatory mediators and cytokines[13,19,32,34] that induce synthesis of acute phase proteins, including the rapid production of SAA.[13,19,32] Nonsteroidal anti-inflammatory drugs (NASID) inhibit the cyclooxygenase (COX) pathway and therefore the synthesis of prostanoids and thromboxanes.[35–38] Phenylbutazone resulted in decreased synovial fluid white blood cell counts following induction of equine synovitis with intra-articular LPS compared to horses with induced synovitis not receiving phenylbutazone.[39] The effects of selective and nonselective NSAID on the human acute phase response have been reported.[40,41] People undergoing thoracotomy and treated with flurbiprofen, a nonselective NSAID, had lower concentrations of several acute phase reactants compared to people who did not receive flurbiprofen, leading the authors to suggest that anti-inflammatory medications may contribute to attenuation of the postoperative inflammatory response.[40] In people treated with COX-2 selective NSAID for rheumatoid arthritis, C-reactive protein, an acute phase protein, was not decreased. However, in the few treated with a non-selective COX inhibitor, C-reactive protein did decrease, leading the authors to speculate that there may be a relationship between the effects of COX-2 selective and non-selective inhibitors.[41]

The handheld SAA assay provided a convenient, rapid way to measure SAA. Based on results from our study using models of synovitis and septic arthritis in conjunction with clinical signs and synovial fluid analysis, an elevation in serum or synovial fluid SAA above normal concentrations may indicate synovial sepsis. However, a larger sample of clinical cases would be required to establish more distinct cut-off ranges for diagnosis. Based on our findings, serum SAA may serve as an earlier marker for sepsis than synovial fluid SAA. Early differentiation between aseptic synovitis and septic arthritis can allow more rapid initiation of therapy and improve the prognosis for soundness and survival. Serum or synovial fluid SAA is a promising adjunct to existing laboratory analyses in the diagnosis of septic arthritis and warrants further investigation in clinical cases of suspected synovial sepsis.

ACKNOWLEDGMENT

The authors thank Wendy Wark (Department of Dairy Science) for assistance with *S. aureus* preparation and StableLab (Epona Biotech Limited) for their generous donation of a portion of the handheld test kits. The study was funded by the Veterinary Memorial Fund, Virginia-Maryland College of Veterinary Medicine.

DISCLOSURE

The authors declare no conflicts of interest related to this report.

REFERENCES

1. Morton AJ: Diagnosis and treatment of septic arthritis. *Vet Clin North Am Equine Pract* 2005;21:627–649
2. Lugo J, Gaughan EM: Septic arthritis, tenosynovitis, and infections of hoof structures. *Vet Clin North Am Equine Pract* 2006;22:363–388
3. Schneider R, Bramlage L, Moore R, et al: A retrospective study of 192 horses affected with septic arthritis/tenosynovitis. *Equine Vet J* 1992;24:436–442
4. Wright I, Smith M, Humphrey D, et al: Endoscopic surgery in the treatment of contaminated and infected synovial cavities. *Equine Vet J* 2003;35:613–619
5. Steel CM: Equine synovial fluid analysis. *Vet Clin North Am Equine Pract* 2008;24:437–454
6. Tulamo R, Bramlage L, Gabel A: Sequential clinical and synovial fluid changes associated with acute infectious arthritis in the horse. *Equine Vet J* 1989;21:325–331
7. LaPointe JM, Laverty S, Lavoie JP: Septic arthritis in 15 Standardbred racehorses after intra-articular injection. *Equine Vet J* 1992;24:430–434
8. Bertone AL: Update on infectious arthritis in horses. *Equine Vet Educ* 199;11:143–152
9. McIlwraith CW, Billinghurst RC, Frisbie DD: Current and future diagnostic means to better characterize osteoarthritis in the horse-routine synovial fluid analysis and synovial fluid and serum markers. *Proc Am Assoc Equine Pract* 2001;47:171–179
10. Sanchez Teran A, Rubio-Martinez L, Villarino N, et al: Effects of repeated intra-articular administration of amikacin on serum amyloid A, total protein and nucleated cell count in synovial fluid from healthy horses. *Equine Vet J* 2012;44:12–16
11. Jacobsen S, Niewold TA, Halling-Thomsen M, et al: Serum amyloid A isoforms in serum and synovial fluid in horses with lipopolysaccharide-induced arthritis. *Vet Immunol Immunopathol* 2006;110:325–330
12. Jacobsen S, Thomsen MH, Nanni S: Concentrations of serum amyloid A in serum and synovial fluid from healthy horses and horses with joint disease. *Am J Vet Res* 2006;67:1738–1742
13. Jacobsen S, Andersen P: The acute phase protein serum amyloid A (SAA) as a marker of inflammation in horses. *Equine Vet Educ* 2007;19:38–46
14. Robinson CS, Singer ER, Piviani M, et al: Can serum amyloid A, D-Lactate or L-Lactate aid diagnosis of synovial sepsis in horses? *Vet Surg* 2015;44:E64
15. Belgrave RL, Dickey MM, Arheart KL, et al: Assessment of serum amyloid A testing of horses and its clinical application in a specialized equine practice. *J Am Vet Med Assoc* 2013;243:113–119
16. Pepys M, Baltz ML, Tennent GA, et al: Serum amyloid A protein (SAA) in horses: objective measurement of the acute phase response. *Equine Vet J* 1989;21:106–109
17. Hulten C, Tulamo R-M, Suominen M, et al: A non-competitive chemiluminescence enzyme immunoassay for the equine acute phase protein serum amyloid A (SAA)—a clinically useful inflammatory marker in the horse. *Vet Immunol Immunopathol* 1999;68:267–281
18. Jacobsen S, Kjelgaard-Hansen M, Hagbard Petersen H, et al: Evaluation of a commercially available human serum amyloid

A (SAA) turbidometric immunoassay for determination of equine SAA concentrations. *Vet J* 2006;172:315–319

19. Uhlar CM, Whitehead AS: Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem* 1999;265:501–523
20. McDonald TL, Larson MA, Mack DR, et al: Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. *Vet Immunol Immunopathol* 2001;83:203–211
21. Berg LC, Thomsen PD, Andersen PH, et al: Serum amyloid A is expressed in histologically normal tissues from horses and cattle. *Vet Immunol Immunopathol* 2011;144:155–159
22. Nunokawa Y, Fujinaga T, Taira T, et al: Evaluation of serum amyloid A protein as an acute-phase reactive protein in horses. *J Vet Med Sci* 1993;55:1011–1016
23. Palmer JL, Bertone AL: Experimentally-induced synovitis as a model for acute synovitis in the horse. *Equine Vet J* 1994;26: 492–495
24. Palmer JL, Bertone AL, Malemud CJ, et al: Biochemical and biomechanical alterations in equine articular cartilage following an experimentally-induced synovitis. *Osteoarthritis Cartilage* 1996;4:127–137
25. Williams LB, Koening JB, Black, B, et al: Equine allogeneic umbilical cord blood derived mesenchymal stromal cells reduce synovial fluid nucleated cell count and induce mild self-limiting inflammation when evaluated in an LPS induced synovitis model. *Equine Vet J*. [Epub ahead of print, 2015]. doi: 10.1111/evj.12477.
26. Brusie R, Sullins K, II NW, et al: Evaluation of sodium hyaluronate therapy in induced septic arthritis in the horse. *Equine Vet J* 1992;24:18–23
27. Whitehair KJ, Bowersock TL, Blevins WE, et al: Regional limb perfusion for antibiotic treatment of experimentally induced septic arthritis. *Vet Surg* 1992;21:367–373
28. Tulamo R, Bramlage L, Gabel A: The influence of corticosteroids on sequential clinical and synovial fluid parameters in joints with acute infectious arthritis in the horse. *Equine Vet J* 1989;21:332–337
29. Petersson-Wolfe C, Wolf S, Hogan J: Experimental challenge of bovine mammary glands with *Enterococcus faecium* during early and late lactation. *J Dairy Sci* 2009;92:3158–3164
30. Petrie A, Watson P: Additional techniques, in Petrie A, Watson P (eds): *Statistics for veterinary and animal science* (ed 3). Ames, IA, Wiley-Blackwell, 2013, pp 200–227
31. Hawkins D, MacKay R, Gum G, et al: Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6, and prostaglandin E2 values in horses. *Am J Vet Res* 1993;54: 379–386
32. Crisman MV, Scarratt WK, Zimmerman KL: Blood proteins and inflammation in the horse. *Vet Clin North Am Equine Pract* 2008;24:285–297
33. Hulten C, Sandgren B, Skiöldebrand E, et al: The acute phase protein serum amyloid A (SAA) as an inflammatory marker in equine influenza virus infection. *Acta Vet Scand* 1998;40: 323–333
34. Cray C, Zaias J, Altman NH: Acute phase response in animals: a review. *Comp Med* 2009;59:517–526
35. Higgins A, Lees P: The acute inflammatory process, arachidonic acid metabolism and the mode of action of anti-inflammatory drugs. *Equine Vet J* 1984;16:163–175
36. Lees P, Higgins A: Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. *Equine Vet J* 1985;17:83–96
37. Lees P, Landoni M, Giraudel J, et al: Pharmacodynamics and pharmacokinetics of nonsteroidal anti-inflammatory drugs in species of veterinary interest. *J Vet Pharmacol Ther* 2004;27: 479–490
38. Tobin T, Chay S, Kamerling S, et al: Phenylbutazone in the horse: a review. *J Vet Pharmacol Ther* 1986;9:1–25
39. Morton AJ, Campbell NB, Redding WR, et al: Preferential and non-selective cyclooxygenase inhibitors reduce inflammation during lipopolysaccharide-induced synovitis. *Res Vet Sci* 2005; 78:189–192
40. Esme H, Kesli R, Apiliogullari B, et al: Effects of flurbiprofen on CRP, TNF-alpha, IL-6, and postoperative pain of thoracotomy. *Int J Med Sci* 2011;8:216–221
41. Tarp S, Bartels EM, Bliddal H, et al: Effect of nonsteroidal antiinflammatory drugs on the C-reactive protein level in rheumatoid arthritis: a meta-analysis of randomized controlled trials. *Arthritis Rheum* 2012;64:3511–3521