Serum amyloid A increases following routine vaccination of healthy adult horses

Cassandra M. Baumgarten, DVM, MS; Katherine M. Delph Miller, DVM, MS, DACVIM*; Elizabeth G. Davis, DVM, PhD, DACVIM; Laurie A. Beard, DVM, MS, DACVIM; Christopher A. Blevins, DVM, MS; Margo Wottowa, DVM; Melissa Hill, DVM; Robert L. Larson, DVM, PhD, DACT, DACVPM, DACAN

Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS

*Corresponding author: Dr. Delph Miller (kdelph@vet.k-state.edu)

OBJECTIVES
To measure the effect of routine vaccination on serum amyloid A (SAA) concentration in apparently healthy horses. We hypothesized that routine vaccination would increase SAA in healthy horses.

ANIMALS
21 apparently healthy client-owned horses and 15 Kansas State University College of Veterinary Medicine–owned horses.

METHODS
In experiment 1 (n = 8 horses), a blinded, randomized, prospective, crossover study was performed. Horses were either vaccinated (rabies, tetanus, West Nile, Eastern and Western equine encephalomyelitis, equine herpesvirus-1/-4, influenza) or administered saline, and SAA was measured at 6, 12, and 24 hours and daily until day 10 with a commercial lateral-flow immunoassay. In experiment 2 (n = 28 horses), a prospective, observational study measured SAA after vaccination at 12 and 24 hours and daily until day 10. A linear mixed-effect model with repeated measures over time blocked by horse tested the effect of treatment on SAA. A repeated-measures correlation tested the correlation between SAA and temperature.

RESULTS
Over time, vaccinated horses had increased model-adjusted SAA compared to unvaccinated horses without clinical evidence of adverse reaction (P < .01). In experiment 1, the model-adjusted SAA after vaccination peaked on day 2 (median, 1,872 µg/mL; IQR, 1,220.8 to 2,402.5 µg/mL) and returned to normal (< 20 µg/mL) by day 9 (median, 6 µg/mL; IQR, 0.8 to 23.5 µg/mL) after vaccination. In experiment 2, vaccinated horses had increased SAA over time; temperature and SAA were not correlated (P = .78).

CLINICAL RELEVANCE
Results of this study indicated that routine vaccination results in increased SAA concentration and provided evidence for a period of convalescence following vaccination. Measuring SAA for 10 days following vaccination cannot be used as an indicator of illness.

Keywords: acute-phase protein, serum amyloid A, vaccination, equine, horse

Received April 10, 2024
Accepted June 5, 2024
Published online July 3, 2024
doi.org/10.2460/javma.24.04.0244
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groups. As reported, evaluation of the inflammatory response involved measurement of several variables, WBC concentration increased most rapidly, with concentrations increasing after 9 hours, whereas, iron, SAA, and fibrinogen were significantly increased by 24 hours. The ISCOM vaccine resulted in higher SAA and fibrinogen concentrations than the vector vaccine. The authors concluded the differences were due to the ISCOM adjuvant, which is highly immunogenic and could account for the more intense acute-phase response. The decline of SAA concentration to its normal prevaccination level did not occur within the 4-day study period. Another study by Duran et al described peak SAA concentrations (1,365.9 ± 141.4 mg/L for horses) on day 1 after vaccination with an inactivated equine herpesvirus-1/-4 (EHV-1/-4) vaccine. Serum amyloid A elevation was not correlated with the intensity of the antibody response. The decline of SAA concentration to the normal prevaccination level was not reported by Duran et al within the 21-day study period. Another study performed by Skipper and Pusterla investigated SAA after vaccination in relation to West Nile virus (WNV) antibody titer. The vaccine administered contained antigens for eastern and western equine encephalomyelitis (EEE/WEE), WNV, and tetanus. Skipper and Pusterla reported elevated SAA concentration at 72 hours after vaccination (median, 554 µg/mL; IQR, 113 to 784 µg/mL). There was a significant difference in median peak SAA concentration in horses that developed an adverse vaccine reaction compared to those that did not. The other studies did not report increased SAA in association with adverse vaccine reactions. There was no significant association between peak SAA concentration and fold change in WNV antibody titer. There was no reported timing of when SAA concentrations returned to normal after vaccination.

Based on these studies, peak SAA concentration is reported to occur 1 to 3 days after vaccination, in which vaccine reactions may or may not influence the level in SAA response after vaccination. There are no reports of when SAA concentrations return to normal after vaccination. No study has investigated the response in SAA concentration following routine vaccination with all core antigens and common risk-based antigens.

The objective of the current study was to measure the effect of routine vaccination (rabies, tetanus, WNV, EEE/WEE, EHV-1/-4, and EIV) on SAA concentration using a commercial lateral-flow immunoassay in healthy horses. We hypothesized that routine vaccination would increase SAA concentration in healthy horses compared to unvaccinated control horses for a specified period of time.

**Methods**

**Animals**

Apparently healthy client-owned horses (n = 21) and Kansas State University College of Veterinary Medicine–owned horses (15) were used for this study. Informed client consent was obtained for client-owned horses. Eight horses were included in experiment 1 (6 Quarter Horses, 1 warmblood, and 1 draft), with a mean age of 14 years (range, 2 to 27 years) and sex distribution of 5 mares, 3 geldings, and 0 stallions. Twenty-eight horses were included in experiment 2 (16 Quarter Horses, 5 warmbloods, 2 Paint Horses, 1 half-Arabian, 1 Arabian, 1 Thoroughbred, 1 Quarter Horse–Thoroughbred cross, and 1 draft), with a mean age of 17 years (range, 4 to 27 years) and sex distribution of 16 mares, 11 geldings, and 1 stallion. In experiment 1, 62.5% (5/8) of the horses included were 15 years of age or older. In experiment 2, 71.4% (20/28) of the horses included were 15 years of age or older. Fifteen years or older was chosen as a cutoff for a comparison of geriatric horses versus younger horses and because pituitary pars intermedia dysfunction (PPID) is more likely to be diagnosed in horses over 15 years of age.

All horses were maintained on farm under routine management practices. Consistent with study enrollment protocols, owners were expected to avoid transportation and strenuous exercise, report administration of any anti-inflammatories or antibiotics, and report any concerns during the 10-day study period.

**Ethical approval**

Approval for this project was granted by the Kansas State University Institutional Animal Care and Use Committee (protocol No., 4547; modification No., 4547.1).

**Inclusion criteria**

Horses were included with known vaccine history to ensure this was not a primary series, but horses were not excluded based on any previous vaccine protocol or brand used. Horses were considered healthy based on physical examination and interpretation of baseline bloodwork (CBC, serum biochemistry, fibrinogen, and SAA) performed by the veterinary investigators (CMB, CAB, KMD) not more than 10 days prior to vaccine or control administration. The normal reference range for SAA was < 20 µg/mL, based on in-laboratory–derived reference intervals. Comorbidities with no known effect on SAA, such as navicular disease, were included in the study. There were 8 horses included in experiment 1 and 28 horses included in experiment 2.

**Exclusion criteria**

Horses were excluded from the study if they were determined “unhealthy” by bloodwork (CBC, chemistry, and SAA) and physical examination findings. Foals, pregnant mares, Miniature Horses, donkeys, and mules were excluded, as these signalments have been reported to influence SAA concentration. Horses were eliminated from the study if they experienced a known cause of increase to SAA within 24 hours prior to the experiment or during the study period. This included transportation, strenuous exercise, medical intervention during the study period, systemic illness or injury that necessitated need for administration of anti-inflammatory or antimicrobials, development of an active episode of equine asthma, or diet change. Due to abnormal SAA values at time point 0, 2 horses...
were excluded from experiment 1, and 3 horses were excluded from experiment 2.

**Study design**

**Experiment 1**

Experiment 1 (n = 8) was a blinded, randomized, prospective, crossover study. Horses were randomly assigned to vaccinated or placebo control groups by a computerized random number generator. Horses received injections of vaccine or placebo. Prior to vaccination, a whole blood sample (3 mL) was collected into a purple-top EDTA tube via jugular venipuncture by means of a 20-gauge vacutainer needle, and the horses were evaluated at each time point after injection (0, 6, 12, and 24 hours and 2, 3, 4, 5, 6, 7, 8, 9, and 10 days). After the initial study period, there was a minimum 2-week washout interval between studies, and then the study was repeated with horses receiving alternate treatment of vaccine and placebo. Clinicians evaluating horses after injection (vaccine/placebo) were blinded to subject treatment.

Data recorded at each time point included physical examination findings (mentation, temperature, heart rate, respiratory rate, mucous membranes, capillary refill time, gastrointestinal sounds, and digital pulses) and vaccine site reactions (abscess, swelling, pain to palpation, stiff movement, or any other abnormal activity/behaviors). Signalment (age, breed, sex) was also recorded. Rectal temperature was measured with a digital thermometer.

**Experiment 2**

In experiment 2 (n = 28), a prospective, observational study was performed in which horses were routinely vaccinated. Prior to vaccination, a whole blood sample (3 mL) was collected into a purple-top EDTA tube via jugular venipuncture by means of a 20-gauge vacutainer needle. Physical examination findings including rectal temperature were evaluated at each time point (0, 12, and 24 hours and 2, 3, 4, 5, 6, 7, 8, 9, and 10 days). Data recorded at each time point were the same as experiment 1.

**Vaccination and placebo**

Horses in the study received a single dose (1 mL, IM, left neck) of an inactivated EHV-1/-4 and EIV vaccine (Vetera 2XP with CARBIMMUNE adjuvant) and a single dose (1 mL, IM, right neck) of rabies, tetanus, WNV, and EEE/WEE vaccine (CORE EQ INNOVATOR with MetaStim adjuvant). Vaccines were administered per American Association of Equine Practitioners guidelines for core and common risk-based vaccines as a component of each horse’s routine preventative care management and according to veterinarian recommendations. As part of experiment 1, horses received 0.9% sodium chloride injection (1 mL, IM, right and left neck each) as a placebo treatment.

**Laboratory analysis**

Both experiments involved collection of a whole blood sample (3 mL) at time 0, 12, and 24 hours and then 2, 3, 4, 5, 6, 7, 8, 9, and 10 days. Additionally, only in experiment 1, whole blood was collected at 6 hours after vaccination. Serum amyloid A expression was measured on only EDTA-anticoagulated whole blood using a previously validated point-of-care (POC) lateral-flow membrane-based immunoassay (Stablelab EQ-1 Handheld Reader; Epona Biotech Ltd) by trained personnel per manufacturer instructions and within 12 hours of sample collection. For the POC tests in experiment 1, all the tests were from the same batch. For the POC tests in experiment 2, the tests were from multiple batches, but it was attempted to use a single batch for a single horse’s serial measurements of SAA to decrease interbatch variability. For each horse, a sample of EDTA-anticoagulated whole blood underwent a CBC via an automated analyzer performed by the Kansas State Veterinary Diagnostic Laboratory and a slide examination, performed by the clinical pathologists at the Kansas State Veterinary Diagnostic Laboratory on days 0, 4, and 7 after injection. Fibrinogen was measured with the heat precipitation method. Blood was analyzed for CBC within 24 to 48 hours of collection and refrigerated as needed.

**Statistical analysis**

The changes in SAA, WBC count, fibrinogen, and temperature were described in relation to time after vaccination (experiment 1 and experiment 2). All statistical analyses were conducted in RStudio (RStudio, version 4.0.3; Posit). A linear mixed-effect model with repeated measures over time blocked by horse was used to test the effect of treatment on SAA after vaccination (experiment 1). A repeated-measures correlation was used to test the correlation between SAA and body temperature after vaccination (experiment 2). Additionally, the SAA of horses ≥ 15 years old and that of horses < 15 years old were compared via a linear mixed model. A cutoff of P ≤ .05 was used to classify differences as statistically significant.

**Results**

**Experiment 1**

Over time, vaccinated horses had an increased model-adjusted SAA expression profile compared to unvaccinated horses (P < .01; Figure 1).

![Figure 1—Median serum amyloid A (SAA) after vaccination (experiment 1). Vaccinated horses are in blue, and unvaccinated control horses are in red. Bars indicate interquartile range.](image-url)
Model-adjusted SAA after vaccination peaked on day 2 (median, 1,872 µg/mL; IQR, 1,220.8 to 2,402.5 µg/mL). Model-adjusted SAA after vaccination was within the reference interval by day 9 (median, 6 µg/mL; IQR, 0.8 to 23.5 µg/mL). There was no change in model-adjusted SAA after placebo administration ($P > .05$).

There was variation observed between horses: monophasic and biphasic trends in SAA response after vaccination, degree of SAA elevation, and timing of return to normal after vaccination (Figure 2). In experiment 1, monophasic elevation in SAA occurred in 4 of 8 horses and biphasic elevation occurred in 4 of 8 of horses.

**Experiment 2**

The model-adjusted SAA concentration of vaccinated horses differed over time ($P < .01$; Figure 3). Consistent with findings in experiment 1, model-adjusted SAA after vaccination peaked on day 2 (median, 1,303 µg/mL; IQR, 941 to 1,741.5 µg/mL). Model-adjusted SAA after vaccination returned to the

![Figure 2](image-url) - Serum amyloid A after vaccination in individual vaccinated horses (experiment 1). Each different color represents an individual horse’s SAA pattern after vaccination.

![Figure 3](image-url) - Box-and-whisker plot of SAA concentration after vaccination (experiment 2). The middle line indicates the median value, the box indicates the 25th to 75th percentiles, the whiskers indicate the 5th and 95th percentiles, and the single data points outside these bounds indicate outliers. On the x-axis, D stands for day after vaccination.
reference interval by day 9 (median, 9.5 µg/mL; IQR, 0 to 20.5 µg/mL).

Variation was observed between horses: monophasic elevation in SAA occurred in 57.1% (16/28) of horses, and biphasic elevation in SAA occurred in 42.9% (12/28) of horses (Figure 3). Initial elevation (> 20 µg/mL) in SAA after vaccination was observed by 12 hours (0.5 day) in 71.4% (20/28) of horses and between 12 and 24 hours (1 day) in 28.6% (8/28) of horses. Serum amyloid A peaked between days 2 and 6, with a variable degree in peak SAA elevation (SAA, 387 to 2,539 µg/mL): 3.6% (1/28) of horses had peak SAA on day 1 after vaccination, 50% (14/28) of horses had peak SAA on day 2 after vaccination, 32.1% (9/28) of horses had peak SAA on day 3 after vaccination, 10.7% (3/28) of horses had peak SAA on day 4 after vaccination, 3.6% (1/28) of horses had peak SAA on day 6 after vaccination. The day after vaccination when SAA returned to normal (within reference range, < 20 µg/mL) was variable between horses (between day 3 and day 10): 3.6% (1/28) of horses returned to normal by day 3 after vaccination, 10.7% (3/28) of horses returned to normal by day 6 after vaccination, 10.7% (3/28) of horses returned to normal by day 7 after vaccination, 14.3% (4/28) of horses returned to normal by day 8 after vaccination, 39.3% (11/28) of horses returned to normal by day 9 after vaccination, and 7.1% (2/28) of horses returned to normal by day 10 after vaccination. Serum amyloid A returned to normal by day 10 in 85.7% (24/28) of horses. Serum amyloid A did not return to normal within the 10-day sampling period in 14.3% (4/28) of horses. Of the horses with SAA above normal on day 10, 50% (2/4) had peak SAA on day 2, 25% (1/4) had peak SAA on day 3, and 25% (1/4) had peak SAA on day 6. Of the horses with SAA above normal on day 10, the median day to peak SAA was 2.5, and the median number of days to return to normal was 7.5. Of the horses with SAA that had returned to normal by day 10, the median day to peak SAA was 2, and the median number of days to return to normal was 6. There was no difference in the number of days to return to normal between horses with SAA that had returned to normal by day 10 and horses with SAA above normal on day 10 (P = .16).

While there was an overrepresentation of horses ≥ 15 years of age (20/28 [71.4%]) in experiment 2, there was no difference in peak SAA after vaccination between horses that were distinguished by age < 15 years old compared to ≥ 15 years old (median, 1,280 and 1,729 µg/mL, respectively; P > .05). In experiment 2, the median WBC counts at days 0, 4, and 7 were 6,750, 6950, and 7,250 WBCs/µL, respectively, and the median fibrinogen concentrations at days 0, 4, and 7 were 250, 400, and 300 mg/dL, respectively. The WBC count and fibrinogen concentrations showed no change after vaccination (P > .05 each).

Fever (temperature ≥ 38.4 °C) was observed in 78.6% (22/28) of horses after vaccination. Temperature peaked (38.5 to 39.8 °C) at 12 hours (0.5 days) in 81.8% (18/22) of horses after vaccination. Temperature peaked (38.4 to 39.2 °C) at 24 hours (1 day) in 18.2% (4/22) of horses after vaccination. Temperature returned to normal by day 1 in 54.5% (12/22) of horses and by day 2 in 45.5% (10/22) of horses after vaccination. In experiment 2, based on repeated-measures correlation, body temperature and SAA concentration were not correlated ($R^2 = 0.0009; P = .78$).

No concerns were appreciated in other physical examination parameters (mentation, heart rate, respiratory rate, mucous membranes, capillary refill time, gastrointestinal sounds, and digital pulses). Mild dermal edema was noted at the injection site of some horses, but no adverse effects of vaccination were noted in any horse in the study.

Discussion

Routine vaccination against core and common risk-based diseases induced a prominent increase in SAA concentration in healthy adult horses compared to horses receiving placebo. These results are consistent with other studies performed to monitor the acute-phase response after vaccination. Previously reported trends observed in SAA concentration after vaccination included an initial increase at 24 hours after vaccination, with peak concentration observed at 48 to 72 hours after vaccination. In other studies, not all horses’ SAA concentrations returned to normal within the study’s sampling period (4 days), or the SAA concentrations’ return to normal were not reported. The study reported here is the first in horses to follow SAA concentrations for 10 days and found that 85.7% of horses’ SAA returned to normal (within reference range, < 20 µg/mL) by day 10.

The SAA response differed markedly between individual horses. The individual variation of SAA response was consistent with other studies evaluating SAA after vaccination. This seems to be consistent across species and has been shown in cattle, mice, and humans. There is some speculation that high interindividual variation in immune response may be genetic, as some individuals’ immune response can be categorized as hyper- or hyporesponsive. Other factors that may have an effect on SAA response include age, breed, and gender. Skipper and Pusterla reported that age had a significant effect on SAA concentration, with a lower SAA concentration in healthy adult horses compared to horses 1 to 15 years of age vs ≥ 15 years old. There is some evidence that升高 individual variation in acute-phase proteins in healthy animals; however, responses to some inflammatory conditions have shown alterations in acute-phase proteins in adult animals. Our study population was predominately Quarter Horses. Breed-related differences have not been reported in horses; however, other studies investigating SAA response after vaccination were of more heterogenous populations that reported similar trends in SAA following vaccination. Likely, one can conclude breed does not affect SAA concentrations in horses after vaccination. It is also possible there would be reduced variance with a larger population of horses.

The results of this study also show that measuring SAA for diagnostic purposes following vaccination cannot be used as an indicator of illness within at least 4–6 days.
9 days because 14.3% (4/28) of horses in our study did not return to normal by day 10. Further investigation into a larger population of horses following SAA concentration for a longer sampling period is required to determine the length of time required for some horses' SAA value to return to normal. In this study, a postvaccination fever was observed in 78.6% of horses that were otherwise clinically normal with no adverse vaccine reactions. Clinically normal was defined as eating, drinking, and other physical examination parameters within normal limits. Peak temperatures were seen at 12 to 24 hours after vaccination. These changes in SAA and temperature after vaccination are significant when evaluating horses for illness. Within the described postvaccination time period, elevated SAA for 10 days after vaccination and fever for 12 to 24 hours after vaccination cannot be used as indicators of illness. This provides evidence to clinicians that, when evaluating a patient's clinical status after vaccination, they should not rely on 1 or 2 clinical parameters but rather should evaluate the whole patient. Additionally, elevated SAA and temperature after vaccination indicate an inflammatory response to vaccination. The prolonged time period that SAA remained elevated despite the horse's temperature returning to normal provides support to allow for a period of convalescence after vaccination before travel and intense exercise are undertaken. It has been previously shown that strenuous exercise itself can cause suppression of the immune system. Folsom et al demonstrated that vaccinated ponies that underwent strenuous exercise for 5 days had a decreased lymphoproliferative response specific to EIV compared to rested, vaccinated ponies. Additionally, all ponies in this study were experimentally infected with EIV. Of the 4 exercised, vaccinated ponies, 3 showed clinical signs of influenza and tested positive for EIV antigen in nasal secretions, compared to none (0/4) of the rested, vaccinated ponies. Since transport by road has been demonstrated to cause an increase in plasma cortisol as well, it is possible that travel may cause immune suppression. Since the SAA remained elevated for a prolonged time period following vaccination in the current study, it would be recommended to avoid anything that may suppress an immune response during that time period of inflammatory response.

There was no significant change observed in fibrinogen values after vaccination. This differed from Andersen et al, who found a significant increase in plasma fibrinogen concentrations after vaccination. The difference in results from the current study was likely because fibrinogen concentrations were measured more frequently in the study performed by Andersen et al (9, 24, 48, 72, and 96 hours after vaccination), in comparison to the current study protocol that measured fibrinogen on postvaccination days 1, 4, and 7. Similarly, there were no significant changes observed in total WBC count or leukocyte count after vaccination in the current study. This also differed from Andersen et al, who found a significant increase in WBC count following vaccination with the ISCOM vaccine, in comparison to the live recombinant vector vaccine. The difference could be attributed to more frequent sampling in the Andersen et al study. However, the variation in response in SAA, fibrinogen, and WBC count observed by Andersen et al between the ISCOM vaccine and live recombinant vector vaccine could be attributed to the variation in type of vaccine or adjuvant. Future studies could be aimed at investigating the effect of monovalent versus multivalent vaccine protocols or the effect of adjuvant type on SAA concentrations.

There were several limitations to our study. Although health status was determined by no abnormalities on physical examination and bloodwork, diseases such as equine asthma, equine PPID, and equine metabolic syndrome were not fully ruled out. However, clinical signs of an active severe equine asthma episode (cough, increased respiratory rate, effort, or nasal discharge) were not detected on physical examination. There is conflicting evidence to show that equine asthma may influence SAA concentrations. However, it is important to consider that a developing episode of equine asthma may have influenced SAA concentrations in horses that had elevated SAA concentrations extended past the 10-day sampling period. The effect of PPID on SAA concentration has not been evaluated. However, it is important to consider that horses with equine PPID are susceptible to secondary infections, and immunosuppression is suspected to play a role. This may have had an effect on peak SAA concentration and elevated SAA concentration beyond the 10-day sampling period. The effect of equine metabolic syndrome on SAA concentration has not been investigated; however, obesity in mice and humans has been associated with an increase in acute-phase proteins. Body condition scores were not discriminatory in our inclusion or exclusion criteria. However, this may have influenced SAA concentrations. Another limitation of the study was variation in season in which horses were vaccinated. Horses were vaccinated in accordance with American Association of Equine Practitioners guidelines for core and common risk-based "spring" vaccines. Horses were vaccinated between February and July of 2021 and 2022. There are no published reports on the effect of season on insect bites on SAA concentrations. Horses vaccinated in the spring and summer months were exposed to higher levels of insects. However, these are normal environmental conditions for horses. The lateral-flow membrane-based immunoassay used in this study has been previously validated for use in horses. Our study measured SAA concentration using only the Stablelab POC assay, without comparison to a turbidimetric immunoassay (TIA). Previous studies comparing this lateral-flow membrane-based immunoassay to the previously-validated human and veterinary TIA found acceptable accuracy and precision with SAA concentrations. While we did not use a gold standard TIA for measuring SAA, using the lateral-flow membrane-based immunoassay is clinically relevant and is common in practice. However, sampling error due to machine error could have been accounted for by implementing the use of 2 readers for each sample at each time point into the study design, thus increasing the accuracy of SAA value at each time point after vaccination. Outlier SAA values or individuals that did not follow the monophasic trend in SAA concentrations.
values could have been attributed to machine error. Schwartz et al.\(^6\) discussed the linearity and precision of this POC lateral-flow membrane-based immunosay and reader and described acceptable accuracy and precision in equine plasma with SAA concentrations up to at least 1,000 μg/mL, but low interbatch precision at high concentrations.\(^{10}\) To increase precision and accuracy of the SAA values, duplicate testing could have been performed on each sample at each time point.

In conclusion, the results of our study indicated that routine vaccination results in an increased SAA concentration. This may provide evidence that, when advising owners considering travel or competition for their horse, practitioners should recommend the horse undergo a period of convalescence following vaccination. Further studies investigating the effect of travel or competition on antibody response are required. Measuring SAA for 10 days following vaccination cannot be reliably used as an indicator of illness.

**Acknowledgments**

The authors would like to thank Ms. Kara Smith (Kansas State University Department of Clinical Sciences) for procuring supplies for this study.

**Disclosures**

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

**Funding**

Funding for this study was provided by the Kansas State University Department of Clinical Science.

**ORCID**

K. M. Delph Miller https://orcid.org/0000-0003-0259-7210

**References**


**ORCID**

K. M. Delph Miller https://orcid.org/0000-0003-0259-7210