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Short Communication

Whole-Blood Validation of a New Point-of-care Equine Serum Amyloid A Assay

Bruno Karam [a], Siddra Hines [b], Lauren Skipper [a], Nicola Pusterla [c, *]

[a] The William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis, CA
[b] Veterinary Medical Research and Development, Pullman, WA
[c] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA

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ABSTRACT

Serum amyloid A (SAA) is considered a major acute phase protein (APP) in horses. Serum amyloid A stall-side assays are commercially available to assess the inflammatory response of patients with various infectious and noninfectious conditions. The objective of this study was to determine the analytical performance of a new point-of-care (POC) assay for the measurement of SAA in whole blood and plasma of horses. One hundred and sixty blood samples were collected from 60 horses at various time points after immunization with an equine core vaccine. Analytical validation of the SAA POC assay included the measurement of SAA in whole blood and plasma, assessment of linearity and precision, and comparison of the SAA POC results with those obtained with a validated turbidimetric immunoassay (TIA). The SAA POC assay yielded similar results in whole blood and plasma ($P > .05$), and the results were positively correlated with the TIA ($R^2 = 0.964$). The assay displayed solid linearity throughout the detection range of $\leq$ 20 to 3,000 μg/mL ($R^2 = 0.984$) with inter-assay and intra-assay coefficients of variation ranging from 7.8% to 13.3% and 5.7% to 12.0%, respectively. The new SAA POC assay was able to reliably measure SAA in both whole blood and plasma. Similar to previously validated assays, the new SAA POC assay is a valuable tool to investigate the inflammatory response in various clinical diseases of horses.

1. Introduction

Serum amyloid A (SAA) is the only acute phase protein (APP) to be considered a major APP in all domestic mammals, as well as humans, and it is the only known major APP in horses [1]. Serum amyloid A has been advocated as an objective biochemical index of disease activity in a number of different equine inflammatory processes [2–5], and the research applications of SAA in the equine field are expanding at a fast pace. Multiple platforms that measure SAA are commercially available. However, none of these platforms use equine-specific SAA as the basis of the test [6–8]. Fortunately, antihuman SAA antibodies, which comprise the basis of most commercially available SAA assays for equids, have shown cross-reactivity with equine SAA [9]. The recent introduction of lateral flow immunoassays (LFIAs) for the stall-side measurement of SAA has enabled equine practitioners to assess the inflammatory response of horses in real time, to initiate timely treatment, and to monitor the inflammatory response over time. In the United States of America, three point-of-care (POC) SAA tests are commercially available and as of yet only one of them has undergone a thorough independent validation [7]. Validation of such POC SAA assays is essential as equine practitioners are recognizing the value of SAA testing to help support acute infections, monitor response to treatment, and assess health [5] and many clinical research studies are ongoing. The objective of this study was to evaluate the analytical performance of a new LFIA for the measurement of equine SAA as a POC assay, including validation with whole blood.

2. Materials and Methods

Study material was composed of 160 blood samples (whole blood and plasma) collected from 60 horses at various time points

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* Corresponding author at: Nicola Pusterla, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616.
E-mail address: npusterla@ucdavis.edu (N. Pusterla).

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after immunization with an equine core vaccine (Vetera EWT + WNV, Boehringer Ingelheim Vetmedica, Inc., Duluth, GA, USA). Power calculation determined the inclusion of 60 horses to be able to observe at least a 20% difference in the magnitude of SAA. Recent literature has shown that vaccinating horses with a routine combination vaccine induces an APP response with significant increase of SAA from baseline [10]. Procedures were approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC protocol number 21616).

The POC SAA assay used for this study is an LFIA recently introduced to the USA market (VMRD, Pullman WA, USA). The manufacturer's recommendations were followed for the testing of whole blood and plasma. Briefly, 29 μL of plasma was mixed with 21 μL of plasma diluent to achieve a plasma/buffer diluent ratio of 1.4:1.0. The dilution of plasma or serum is important to achieve a comparable volume distribution as in whole blood (60% plasma and 40% cellular components). Thereafter, whole blood or plasma/buffer diluent was collected using a sampling capillary preset to collect a volume of 5 μL. The filled capillary was then inserted into the sealed dilution tube and mixed thoroughly. Three drops of the resulting solution were applied into the sample well of the cartridge. After an incubation of 10 minutes, the results were read using a handheld reader. Each SAA test box included a calibration card to be attached to the reader to provide lot-specific calibration settings. The results were reported quantitatively in μg/mL, and the testing range was 21 to 3,000 μg/mL. The instrument reported results from 0 to 20 μg/mL as ≤ 20 μg/mL and results above 3,000 μg/mL as > 3,000 μg/mL.

The analytical validation of the SAA POC included the measurement of SAA in whole blood and plasma, the assessment of linearity and precision, and the comparison of the SAA POC results with those obtained with a validated turbidimetric immunoassay (TIA). One hundred and sixty paired EDTA whole blood and EDTA plasma samples taken at various time points after vaccination (0, 24, 48, 72, 96, and 168 hours postvaccine administration) were used to include a wide range of SAA concentrations and allow comparison of SAA values between whole blood and plasma. The samples were run in parallel within 1 hour of collection. Although the SAA POC assay predominantly uses whole blood, agreement between blood and plasma was first determined because further validation and comparison of the results with the TIA were performed on plasma. From these samples, a high SAA pool of 10 plasma samples with individual values ranging from 2,500 to 3,000 μg/mL was created to assess linearity of the assay. A total of 13 dilutions (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, and 1.25%) were performed using this high SAA plasma pool. The dilutions were tested in triplicate to evaluate the linearity across the working range, including an additional pool of 10 samples with values ≤ 20 μg/mL.

To assess precision of the assay, six pools of 10 plasma samples each within various concentration ranges were created (≤20, 21–200, 201–1,000, 1001–2,000, 2001–3,000, and > 3,000 μg/mL). Intra-assay variation or repeatability was determined by testing each of the six plasma pools 15 times on the same day. Inter-assay variation or reproducibility was determined using the same plasma pools and testing them once daily for 15 days. Finally, 110 plasma samples spanning a wide SAA range were selected (20 plasma samples with SAA values ≤ 20 μg/mL, 20 samples with SAA values 21–200 μg/mL, 20 samples with SAA values 201–1,000 μg/mL, 20 samples with SAA values 1,001–2,000 μg/mL, 15 samples with SAA values 2,001–3,000 μg/mL, and 15 samples with SAA values > 3,000 μg/mL) to be measured with a validated TIA for SAA (LZ-SAA, Eiken Chemical Co., Tokyo, Japan). The plasma samples were shipped frozen to the University of Miami Comparative Pathology Laboratory (Miami, FL, USA) for testing.

All statistical analyses were performed using commercial software (Stata 14, Version 14, College Station, TX, USA), and statistical significance was set at $P < .05$. Data were tested for normality using the Shapiro–Wilk test. A paired *t*-test was used to assess differences in SAA concentrations between whole blood and plasma measured via the POC SAA and between the POC SAA assay and the TIA using plasma. Simple linear regression was used to compare whole blood and plasma results measured via the POC SAA assay and between observed and expected results for the linearity study. Linear regression was also used to compare the SAA results between the POC SAA assay and the TIA. Mean, standard deviation, and coefficient of variation (CV) were calculated for each SAA plasma pool for the precision study.

3. Results

Most of the horses displayed normal SAA values before vaccine administration (0–20 μg/mL) with a rapid increase in the first 72 hours after vaccination and a normalization of SAA values by 168 hours after vaccination (data not shown). The SAA concentrations between the 160 paired whole blood and plasma samples were positively correlated ($R^2 = 0.940$) with no significant differences ($P = .05$; Figure 1). Seventy-four whole blood samples gave higher SAA values (mean difference 196 μg/mL, range 4–1,227 μg/mL) than plasma samples. Thirty-five whole blood samples had SAA values lower (mean difference 106 μg/mL, range 4–492 μg/mL) than the values obtained with plasma samples. Fifty-one samples gave similar results between the two sample types, with values being either ≤ 20 or > 3,000 μg/mL. The POC SAA showed great linearity over the testing range ($R^2 = 0.984$). The intra-assay and inter-assay CVs ranged from 7.8% to 13.3% and from 5.7% to 12.0%, respectively (Table 1). The 110 samples selected to be tested via the SAA TIA were positively correlated with the POC SAA assay ($R^2 = 0.964$). When classified by various SAA ranges determined with the POC SAA assay, the overall agreement between the POC SAA assay and the SAA TIA was 80% (Table 2).

4. Discussion

In recent years, SAA has gained in popularity in equine medicine. It is often used to assess the presence and magnitude of inflammation, help establish early treatment protocols, support health in performance horses at risk of developing infections, and monitor response to treatment. Initial SAA testing platforms were time-consuming, required laboratory-based settings, and results were often delayed because of shipping and processing time [9]. With the introduction of stall-side POC SAA assays and the ability to

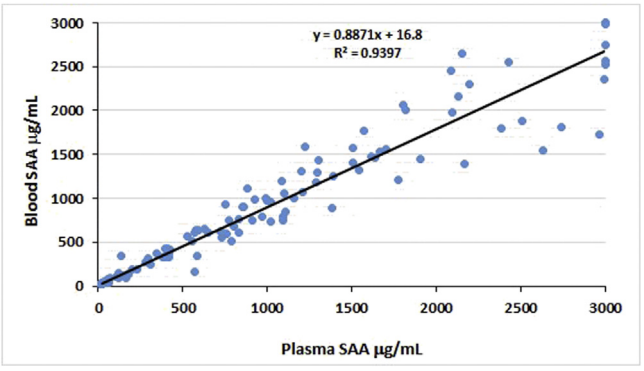

Fig. 1. Linear regression analysis showing the comparison of the SAA POC test results in 160 paired whole blood and plasma samples.

Table 1
Intra-assay and inter-assay variation expressed as mean and standard deviation (SD) and coefficient of variation for the POC SAA assay using 20 replica of pools of equine plasma samples at various SAA ranges.

Precision	SAA Range (μg/mL)	SAA Mean and SD (μg/mL)	Coefficient of Variation (%)
Intra-assay	≤ 20	0 ± 0	0
	21–200	113.8 ± 8.6	7.8
	201–1,000	684.1 ± 64.9	9.8
	1,001–2,000	1,416.8 ± 182.2	13.3
	2,001–3,000	2,601.7 ± 215.3	8.6
	> 3,000	0 ± 0	0
Inter-assay	≤ 20	0 ± 0	0
	21–200	95.3 ± 11.0	12.0
	201–1,000	668.9 ± 62.3	9.7
	1,001–2,000	1,437.3 ± 139.4	10.0
	2,001–3,000	2,710.0 ± 149.1	5.7
	> 3,000	0 ± 0	0

Abbreviations: POC, point-of-care; SAA, serum amyloid A.

measure SAA in real time, equine practitioners can detect the presence and severity of inflammatory conditions, especially acute infectious diseases [4,5]. In the United States of America, there are three commercially available stall-side POC assays for the measurement of equine SAA (StableLab SAA, Epona Biotech, Sligo, Ireland; EquiCheck, Accuplex Diagnostics Ltd., Maynooth, Ireland; Serum Amyloid A Test, VMRD, Pullman, WA, USA), one of which has previously undergone a thorough independent validation [7]. Before adopting a new testing platform, it is important to perform a method and instrument validation to guarantee that the new test performs in accordance with the manufacturer's claims. Proper assay validation requires following guidelines for quality assurance and method comparison, which have been outlined by the Assurance and Laboratory Standards committee of the American Society for Veterinary Clinical Pathology [11]. Although the aim of this study was to evaluate the analytical performance of a new SAA POC assay, it should be noted that there is no true gold standard for the measurement of equine SAA, as all tests use cross-reactive antibodies and absolute quantitation of native equine SAA has not yet been achieved.

To gain acceptance in the equine veterinary field, a POC SAA assay requires various characteristics such as user-friendliness, clinical accuracy, and acceptable analytical performance. The POC SAA test and reader used in the present study were easy to use and only required a few simple handling steps. Furthermore, the use of a sample capillary to collect the appropriate blood volume increased convenience and abolished any pipetting steps. Analytical performance of the POC SAA test focused on determining the agreement of the SAA results between whole blood and plasma, establishing linearity and precision, and comparing the results with a different validated diagnostic platform. The testing of SAA in whole blood and plasma at the same time yielded comparable results. The study results between whole blood and plasma were not statistically different; however, whole blood yielded overall higher SAA concentrations compared with plasma, with twice as many samples exhibiting positive bias in whole blood compared with samples with a negative bias. The study results are in contrast with the recent validation of a different equine LFIA, which showed significant differences between the two sample types with a proportional negative bias observed in whole blood sample with SAA concentrations > 500 μg/mL [7]. The observed and reported differences in SAA concentrations between whole blood and plasma/serum emphasize the need to use the same sample type when performing serial measurements on the same patient. The concentrations used to assess linearity included pooled samples with SAA values close to the limits of detection and various dilutions covering the detection range of the test. The POC SAA assay was linear within the manufacturer's recommended reportable range of ≤ 20–3000 μg/mL. The estimation of precision was determined through short- and long-term replication studies including six levels with 15 replicates for each level. The intra-assay and inter-assay CVs along the detection range of the POC SAA assay ranged from 7.8% to 13.3% and 5.7% to 12.0%, respectively. Although there are no laboratory standards for equine SAA, the established precision results are in agreement with a study validating a TIA for equine SAA [9].

The limitation of establishing linearity and precision using equine plasma for SAA concentrations is that results may not necessarily be directly translated to whole blood, which is considered the sample of choice when testing horses. However, it is impractical to determine analytical performance of the POC SAA assay using whole blood because of inherent challenges of pooling,

Table 2
Agreement of 110 plasma samples between the POC SAA assay and the SAA TIA covering various arbitrarily chosen SAA ranges determined with the POC SAA assay.

TIA SAA (μg/mL)						
	≤ 20	21–200	201–1,000	1,001–2,000	2,001–3,000	> 3,000
POC SAA (μg/mL)						
≤ 20 (n = 20)	19	1	0	0	0	0
21–200 (n = 20)	0	18	2	0	0	0
201–1,000 (n = 20)	0	2	16	2	0	0
1,001–2,000 (n = 20)	0	0	3	16	1	0
2,001–3,000 (n = 15)	0	0	0	6	8	1
> 3,000 (n = 15)	0	0	0	0	3	12

Abbreviations: POC, point-of-care; SAA, serum amyloid A; TIA, turbidimetric immunoassay.

storing, and diluting such samples, as well as the lack of available techniques for reference testing of whole blood. Based on the excellent overall agreement between whole blood and plasma SAA concentrations, validation using equine plasma was an acceptable sample surrogate for whole blood in the present study. The authors were unable to perform a method comparison study because of the lack of available equine-specific standard reference material. Therefore, the results of the POC SAA assay and SAA TIA assay cannot be used to establish systematic error, rather assess agreement of the same samples between two different methodologies for the testing of SAA. The study results are in agreement with a previous study showing an acceptable agreement between the LFIA and the TIA SAA assay [7]. The testing of 110 samples across the detection range showed great overall agreement between the two platforms with similar SAA kinetics in individual horses. The kinetics of SAA measured in this study reflect a biological response to an inflammatory stimulus (i.e., vaccine administration), similar to previous reports [10,12]. The data also point to the value of repeat testing of SAA to monitor the temporal inflammatory response using the SAA POC assay.

In conclusion, the POC SAA assay validated in this study showed acceptable analytical performance throughout the reported detection range of $\leq$ 20 to 3,000 μg/mL. It was able to consistently monitor the SAA response postvaccine administration in 60 horses using both whole blood and plasma. The SAA POC assay was consistently able to distinguish equine samples with normal SAA concentrations from samples with elevated SAA at low, intermediate, and high SAA range. Although SAA is a sensitive marker of inflammation, it is important to remember that elevated SAA cannot be associated with a specific disease process. As an adjunct laboratory tool, SAA has to be evaluated in combination with patient's history, physical parameters, and additional diagnostic tests. The new SAA POC assay is a valuable, easy to perform, and reliable test that will help characterize the inflammatory response of horses with various clinical conditions, monitor health status in horses at risk of developing infections, and monitor the temporal response to treatment.

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