Check for updates

Received: 8 December 2023 | Revised: 25 March 2024 | Accepted: 9 April 2024

DOI: 10.1111/vcp.13355

Veterinary Clinical Pathology
An International Journal of Laboratory Medicine
WILEY

BRIEF COMMUNICATION

Performance of an automated immunoturbidimetric assay for bovine serum amyloid A

Lise N. Nielsen | Mette B. Petersen | Nynne Capion | Jo F.-H. Lundsgaard | Asger L. Jensen

Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

Correspondence
Lise N. Nielsen, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Dyrlaegevej 16, 1870 Frederiksberg C, Denmark.
Email: lini@sund.ku.dk

Funding information
Danish Cattle and Milk Levi fund

Abstract

Background: Acute phase proteins are a group of vital constituents of the innate immune system, which may also serve as circulatory biomarkers of inflammation. The major acute phase protein serum amyloid A (SAA) is a reliable and sensitive biomarker in cows, allowing for rapid detection of inflammatory disease. A multispecies automated immunoturbidimetric assay (VET-SAA, Eiken) has been validated for horses, dogs, and cats, and it has been used to measure SAA concentrations in bovine samples.

Objectives: The aim of the present study was to perform an analytical validation of the VET-SAA immunoturbidometric assay based on monoclonal antihuman SAA antibodies for the measurement of SAA in clinical samples from cows.

Methods and Results: The validation included an assessment of imprecision, inaccuracy, and detection limit, as well as an evaluation of the overlap performance, using banked serum from healthy and sick cows with or without inflammatory disease. Intra- and interassay variation ranged from 0.91% to 2.9% and 2.5% to 5.8%, respectively. The assay was performed with acceptable accuracy within a clinically relevant range of SAA, although minor signs of inaccuracy were detected. Overlap performance was acceptable, with the VET-SAA assay able to differentiate between healthy cows and cows with inflammatory and noninflammatory conditions. The automated VET-SAA assay is considered acceptable for the measurement of SAA in cows.

KEYWORDS
acute phase response, biomarker, cattle, inflammation

Assessment of the acute phase response by measuring acute phase proteins has a long tradition in veterinary clinical pathology.[1,2] In cattle, the major acute phase proteins are haptoglobin and serum amyloid A (SAA),[3] which have been investigated in a range of conditions, including inflammatory, respiratory, and metabolic diseases.[4] One practical limitation for the clinical application of SAA measurements is associated with the measurement methods, as they were mostly based on ELISAs.[3] In horses, SAA can be used to evaluate the progression of disease and response to treatment due to the speed with which SAA increases and decreases in response to inflammation.[5] It would be highly valuable to be able to rapidly detect and closely follow inflammatory disease and response to treatment in cattle in a similar manner. Currently, little has been published about SAA markers for cattle

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2024 The Authors. *Veterinary Clinical Pathology* published by Wiley Periodicals LLC on behalf of American Society for Veterinary Clinical Pathology.

in clinical practice that possess this quality. For SAA to become a clinically useful, routinely used biomarker in cattle, a validated, cost-effective automated assay is necessary. A new multispecies automated SAA assay (VETSAA, Eiken Chemicals Co, Ltd., Tokyo, Japan) has become available. The assay has previously been validated in horses,[5] dogs,[6] and cats,[7] and it has been used in experimental studies in cattle.[8–10] Besides a single short report examining plasma samples in cows with mastitis,[11] little information regarding performance characteristics for measuring SAA in clinical bovine serum has been reported. Thus, the aim of the present study was to examine and provide additional data to the analytical performance of this automated VETSAA assay in serum from cows by assessing imprecision, inaccuracy, limit of detection, limit of blank, effects of potential interference due to hemolysis and lipemia, and an initial overlap performance between clinically healthy cows and cows with different inflammation-related and noninflammation-related diseases. The study was approved by the local Ethical and Administrative Committee (2022-018) at the Department of Veterinary Clinical Sciences, Taastrup, University of Copenhagen, Denmark.

Serum amyloid A was measured using an immunoturbidimetric assay (VET-SAA, Eiken Chemical Co., Japan) based on monoclonal rat antihuman SAA antibodies and implemented using the automated clinical chemistry analyzer CH Atellica Solution (Siemens Healthineers, Ballerup, Denmark) with a provided calibration curve and control material from the manufacturer. The setup included an automated 1:5 preanalysis dilution using Atellica buffer solution with 50 µL of serum and a reflex dilution of 1:15 when the analysis measured results above 200 mg/L.

Banked surplus serum kept at −80°C and collected between January 2018 and September 2022 from healthy, inflammatory, or noninflammatory diseased cows were used to assess the level of detection, imprecision, and inaccuracy of the VET-SAA assay. All cows underwent clinical examination and routine hematology and biochemistry analysis. At the discretion of the attending veterinarian, additional diagnostic procedures, such as diagnostic imaging, were included to achieve a final diagnosis. Cows were selected for this study based on their clinical presentation, hematology, and whether their final diagnoses were considered inflammatory or noninflammatory. A $p < .05$ was considered statistically significant.

The performance study was performed between January 2023 and June 2023.

1 | IMPRECISION

Imprecision was assessed by intra- (within the same day) and interassay (between days) coefficients of variation (CV) of mean and SD from 10 repeated measurements of three serum pools[12] from the cows representing low (approximately 10 mg/L), intermediate (approximately 70 mg/L), and high concentrations (approximately 130 mg/L) of SAA. Pools were produced by mixing three to four serum samples from cows, and the samples were kept at −80°C and went through a total of 2–3 freeze–thaw cycles. Intra- and interassay variation ranged from 0.91% to 2.9% and 2.5% to 5.8%, respectively (Table 1).

2 | INACCURACY

Inaccuracy was assessed indirectly by investigating linearity under dilution[12] of two dilution curves.

A sample with high concentration of SAA (150 mg/L) was diluted with Atellica buffer solution to obtain sample volume percentages of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%.

To account for potential matrix effect, a second dilution curve (modified spike-and-recovery analysis) was performed with a sample of high concentration of SAA (150 mg/L) being diluted into a sample of low concentration of SAA (9.1 mg/L) and estimated sample volume percentages of 0%, 20%, 40%, 60%, 80%, and 100% were obtained.

The curves were assessed using Passing-Bablok regression and a Cusum test in MedCalc Statistical Software version 22.013 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023) to determine whether data deviated significantly from the applied model. The assay showed no significant deviation of the slope from 1 (1.046, 95% CI 0.942–1.125), but with a Y-intercept not including 0 (−13.70, 95% CI −20.21 to −5.39) (Table 2). This inaccuracy was confirmed by a direct plot of observed and expected SAA measurements (Figure 1), visualizing the tendency of the VET-SAA to slightly underestimate the SAA concentration during dilution. Modified spike-and-recovery analysis showed a high level of recovery (79.5%–100%), with a single measurement showing a lower recovery of 75.2%.

3 | LIMIT OF BLANK AND LIMIT OF DETECTION

Limit of blank (LoB),[12] that is, the highest apparent analyte concentration expected to be found when replicates of a blank

TABLE 1 Intraassay and interassay variation in determination of serum amyloid A (SAA) concentrations in bovine serum samples.

Comparison	Number of repeats	Mean (mg/L)	SD	CV (%)
Intraassay				
Low conc.	10	9.1	0.27	2.9
Medium conc.	10	70	0.64	0.9
High conc.	10	139	3.1	2.2
Interassay				
Low conc.	10	9.3	0.54	5.8
Medium conc.	10	69	1.9	2.8
High conc.	10	134	3.4	2.5

Abbreviations: conc, concentration; CV, coefficient of variation; SD, standard deviation.

1939165x, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/vcp.13355 by Det Kongelige, Wiley Online Library on [03/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Analytical inaccuracy of serum amyloid A (SAA) concentrations assessed by dilution (buffer).

Passing-Bablok regression	
y-intercept (95% confidence interval)	−13.70 (−20.21, −5.39)
Slope (95% confidence interval)	1.046 (0.9417, 1.250)
Residual SD (RSD) (±1.96 RSD)	3.46 (−6.77, 6.77)
Cusum test for linearity	No significant deviation from linearity ($P = .28$)

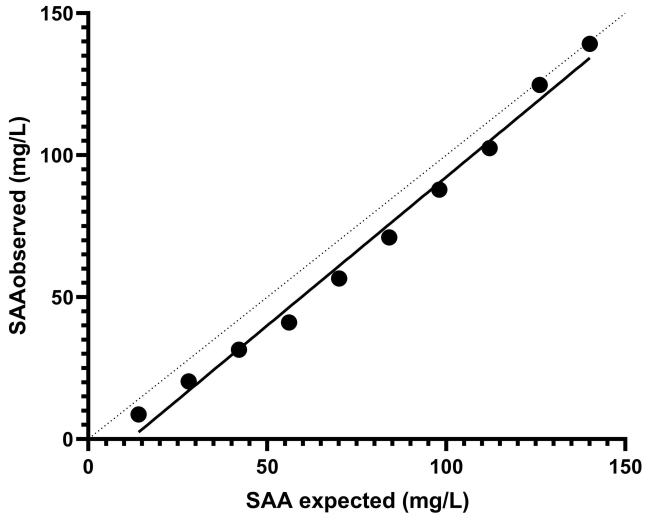

FIGURE 1 Linearity under dilution of a bovine serum pool with a high concentration of serum amyloid A (SAA). The line of best fit (regression) is indicated by the solid line, and the line $x = y$ (perfect agreement) by the dashed line. Minor signs of inaccuracy were noted, with a tendency of the VET-SAA to slightly underestimate the SAA concentration during dilution.

sample containing no analyte and calculated as $LoB = mean_{blank} + 1.645(SD_{blank})$, was determined by 20 analyses using Atellica CH buffer solution (CH diluent, Siemens Healthineers, Erlangen, Germany). LoB was 1.01 mg/L (mean 0.5 mg/L; SD 0.31 mg/L).

The limit of detection (LoD)[13] is the lowest analyte concentration that can reliably be differentiated from the LoB, and it can be estimated by $LoD = LoB + 1.645(SD_{low\ concentration\ sample})$. Using the SD from Table 1 for measurements of low SAA values (i.e., SD 0.27 and 0.54 mg/L for intra- and interassay variation, respectively), the LoD ranged between 1.45 and 1.90 mg/L.

4 | HEMOGLOBIN AND LIPID INTERFERENCE

Potential interference of hemolysis and lipemia was examined according to the CLSI guidelines.* Briefly, a solution of hemolysed bovine erythrocytes was produced by the osmotic shock procedure. Stock concentration of hemoglobin was determined using the ADVIA 2021i (Siemens Healthineers, Ballerup, Denmark). To investigate for the effects of lipemia, a commercial lipid solution (Kabiven (200 g/L), Fresenius Kabi, Bad Humburg, Germany) was purchased. A hemoglobin solution, lipid solution, and control in the form of ionized water were added to cow serum pools with initial concentrations of 10.8, 34.1, and 158.7 mg/L SAA (stock hemoglobin concentration 6.4 g/L, final hemoglobin concentrations in each pool of 1.0, 2.5, and 4.9 g/L), or 15.8 and 48.0 mg/L SAA (stock lipid concentration 200 g/L, final lipid concentration of 1.0, 2.5, and 10 g/L) and measured in quintuple. Acceptance was set at ±10%.[5]

Lipids produced a negative bias in samples with low SAA concentrations (Table 3).

Interference was acceptable for lipids in samples at moderate SAA concentrations and for hemoglobin at low, moderate, and high SAA concentrations (Table 3).

5 | OVERLAP PERFORMANCE

Being a marker of the acute phase response to inflammation,[1,2] SAA values are expected to be higher among cows with inflammatory diseases compared with healthy cows and cows with noninflammatory diseases. Thus, to evaluate the overlap of assay performance for the detection of systemic inflammation, samples from clinically healthy cows ($n = 23$), cows with noninflammatory diseases ($n = 19$), and cows with moderate to severe inflammatory disease ($n = 22$) were included from patients previously presented at the Large Animal Teaching Hospital at University of Copenhagen, Denmark.

Cows with noninflammatory disease were diagnosed with left displaced abomasum (LDA) with ketosis ($n = 10$) and without ketosis,[3] rumen atony ($n = 1$), osteochondrosis ($n = 1$), uterine torsion ($n = 1$), abdominal fat necrosis ($n = 2$), and reproduction problems ($n = 1$). Cows with moderate-to-severe inflammatory disease were diagnosed with carpal arthritis ($n = 1$), uterine rupture with a necrotic calf ($n = 1$), peritonitis ($n = 5$), lymphangitis, thrombophlebitis and pneumonia ($n = 1$), double sole and white line abscess ($n = 1$), suppurative mastitis ($n = 5$), traumatic reticuloperitonitis ($n = 3$), penetrating abomasal ulcer with peritonitis ($n = 1$), endometritis and vaginitis ($n = 1$), sepsis ($n = 1$), trauma with necrosis ($n = 1$), and enterocolitis ($n = 1$). Using the D'Agostino-Pearson test for Normal distribution in MedCalc, data from healthy cows and cows with noninflammatory diseases were not normally distributed ($P < .0001$ and $p = .0006$, respectively), whereas data from cows with inflammatory diseases were normally distributed ($P = .1179$). Thus, to test if SAA differed between groups of cows, the Kruskal-Wallis test and post hoc Dunn test were applied to a significance level of $p < .05$.

As expected, cows with inflammatory diseases had significantly higher SAA levels than healthy cows, and cows with

*Clinical Laboratory Standard Institute (CLSI), approved guidelines, *Interference testing in clinical chemistry*, EP07, 3rd edition and Supplemental tables for *interference testing in clinical chemistry*, EP37, 1st edition, Wayne, PA, 2019.

1939165x, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/vcp.13355 by Det Kongelige, Wiley Online Library on [03/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Measured SAA and observed/expected ratios following the addition of interfering substances to bovine samples with low, moderate, and high SAA concentrations, compared with controls with the addition of ionized water.

							Measured SAA (mg/L)			
Interferent	Interferent Conc.	Low obs.	Low estim.	O/E[b] (%)	Moderate obs.	Moderate estim.	O/E[b] (%)	High obs.	High estim.	O/E[b] (%)
Hemoglobin	Blank	10.8		-	34.1		-	158.7		-
	1.0 g/L	10.3	10.5	98.5	32.3	32.2	100.4	165.0	165.9	99.5
	2.0 g/L	10.0	10.4	96.3	32.0	32.1	99.6	160.2	166.6	96.1
	4.9 g/L	9.6	10.1	95.6	30.1	31.6	95.2	149.0	156.4	95.3
Lipid	Blank	15.8	-	-	48.0	-	-	-	-	-
	1.0 g/L	13.6	14.8	92.4	46.2	46.1	98.7	-	-	-
	2.5 g/L	14.1	15.8	89.0[a]	44.7	46.1	97.0	-	-	-
	10 g/L	14.1	15.4	91.3	44.7	43.6	102.6	-	-	-

Note: Observed values were measured from samples spiked with interference diluted in diluent, whereas the expected values were measured from samples all spiked with an equal volume of diluent alone. Both observed and expected values were measured in quintuples. Expected values not shown.

Abbreviations: Conc, concentration; Estim, estimated; Obs, observed; SAA, serum amyloid A.

[a]Bias above acceptable limit of ±10%.

[b]Observed/expected (%).

noninflammatory conditions ($P < .0001$), with median concentrations and 25th–75th percentiles in the three groups of 54.5 mg/L (43.1–110.9), 5 mg/L (5.0–7.7), and 9.6 mg/L (5.0–14.5), respectively (Figure 2). The levels of these concentrations of SAA in cows with or without inflammatory disease were compatible with previous experimental and clinical studies examining cows with or without mastitis.[10,11] Although minor differences in detection limits, CVs or concentration of SAA in sick cows were present scrutinizing these studies, the current findings suggest that the assay performs well both in serum or plasma using three different automatic biochemical analyzers. No significant difference was found between healthy cows and cows with noninflammatory disease ($P = .5$). Two of the cows in the noninflammatory disease group had higher SAA concentrations (>20 mg/L) than expected (Figure 2). These two cows presented with LDA, and one of the two had concurrent ketosis and diarrhea. Increased concentrations of SAA in blood have been observed in in some cows with both left-abdominal displacement[14] and ketosis[15] earlier, which in these two cases, also was supported by the concurrent slight to moderately increased fibrinogen concentrations (fibrinogen of 9.7 and 14.5 g/L, respectively). In addition, three cows in the inflammatory group had an SAA concentration below 22 mg/L. These cows suffered from peritonitis, traumatic reticuloperitonitis, and mastitis. It is possible that these cows were in a slightly more chronic stage of their disease, which was also supported by fibrinogen concentrations of 21–40 g/L.

The present study showed acceptable performance of the VET-SAA assay when used to measure SAA in serum from cows. The observed CV (0.9%–5.8%) met the quality requirement of CV <12.5% reported by Kiemle et al (2022) for horses 18. We used this quality goal as no quality requirements are available on the desired intra- and interassay variation for bovine SAA assays. While the level

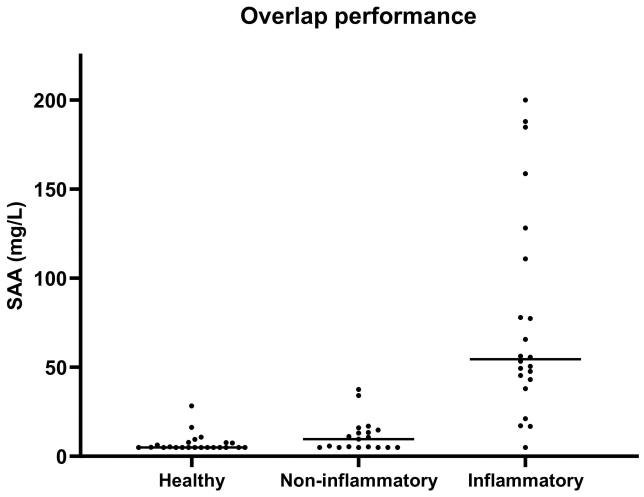

FIGURE 2 Bovine serum concentrations of serum amyloid A (SAA) determined in three different groups. Cows with inflammatory disease had significantly higher SAA levels than those in other groups ($P < .0001$).

of intra- and interassay variability was good and comparable with previously reported imprecisions,[10,11] a small systematic inaccuracy was identified. Similarly, a low level of inaccuracy has been found in horses and dogs[16] but not in cats[7] and may be prescribed to the assay's reactivity against bovine SAA, given that the VET-SAA assay uses a heterologous antibody and a human calibrator. Unfortunately, it was not possible to perform an agreement study, as there currently is no gold standard bovine SAA assay readily available. The effect of freezing and thawing was not investigated, but studies looking at SAA results in dogs and people showed that SAA was highly stable during room temperature exposure and repeated freeze/thaw cycles.[17,18]

1939165x, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/vcp.13355 by Det Kongelige, Wiley Online Library on [03/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Future studies examining a larger population of inflammatory or septic cows could reveal the true biological behavior of SAA in this species. Finally, a large population of well-characterized healthy cows is needed to determine the reference interval for SAA in cows.

ACKNOWLEDGMENTS

The authors wish to acknowledge Technicians Birgitte Klitgaard and Frida Schroeder for assistance with laboratory analyses, Anne Kirstine Rømer Krogh for scientific discussions, and The Danish Milk and Cattle Levy funds for funding the study.

CONFLICT OF INTEREST STATEMENT

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

ORCID

Lise N. Nielsen https://orcid.org/0000-0002-3988-0979
Mette B. Petersen https://orcid.org/0000-0001-7195-2103
Asger L. Jensen https://orcid.org/0000-0003-2048-9221

REFERENCES

1. Eckersall PD, Conner JG. Bovine and canine acute phase proteins. *Vet Res Commun*. 1988;12(2–3):169-178. doi:10.1007/BF00362798
2. Petersen HH, Nielsen JP, Heegaard PM. Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res*. 2004;35(2):163-187. doi:10.1051/vetres:2004002
3. Cray C. Acute phase proteins in animals. *Prog Mol Biol Transl Sci*. 2012;105:113-150. doi:10.1016/B978-0-12-394596-9.00005-6
4. El-Deeb W, Elsohaby I, Fayez M, Mkrtchyan HV, El-Etriby D, ElGioushy M. Use of procalcitonin, neopterin, haptoglobin, serum amyloid A and proinflammatory cytokines in diagnosis and prognosis of bovine respiratory disease in feedlot calves under field conditions. *Acta Trop*. 2020;204:105336. doi:10.1016/j.actatropica.2020.105336
5. Jacobsen S, Vinther AM, Kjelgaard-Hansen M, Nielsen LN. Validation of an equine serum amyloid a assay with an unusually broad working range. *BMC Vet Res*. 2019;15(1):462. doi:10.1186/s12917-019-2211-3
6. Bulgarelli C, Ciuffoli E, Troia R, Goggs R, Dondi F, Giunti M. Apolipoprotein A1 and serum amyloid A in dogs with sepsis and septic shock. *Front Vet Sci*. 2023;10:1098322. doi:10.3389/fvets.2023.1098322
7. Waugh EM, Haining H, Harvie J, Ridyard AE, Eckersall PD. Validation of an automated immunoturbidimetric assay for feline serum amyloid A. *BMC Vet Res*. 2022;18(1):359. doi:10.1186/s12917-022-03456-5
8. Otsuka M, Nishi Y, Tsukano K, Tsuchiya M, Lakritz J, Suzuki K. Sequential changes in hepatic mRNA abundance and serum concentration of serum amyloid A in cattle with acute inflammation caused by endotoxin. *J Vet Med Sci*. 2020;82(7):1006-1011. doi:10.1292/jvms.19-0629
9. Shinya U, Yamato O, Iwamura Y, et al. Usefulness of measuring serum amyloid A concentration in Japanese black cattle in clinical practice. *Vet Sci*. 2023;10(8):528. doi:10.3390/vetsci10080528
10. Franco-Martinez L, Munoz-Prieto A, Contreras-Aguilar MD, et al. Changes in saliva proteins in cows with mastitis: a proteomic approach. *Res Vet Sci*. 2021;140:91-99. doi:10.1016/j.rvsc.2021.08.008
11. Otsuka M, Sugiyama M, Ito T, Tsukano K, Oikawa S, Suzuki K. Diagnostic utility of measuring serum amyloid a with a latex agglutination turbidimetric immunoassay in bovine mastitis: comparison with haptoglobin and alpha 1 acid glycoprotein. *J Vet Med Sci*. 2021;83(2):329-332. doi:10.1292/jvms.20-0550
12. Jensen AL, Kjelgaard-Hansen M. Diagnostic test validation. In: Weiss DJ, Wardrop KJ, eds. *Schalm's Veterinary Haematology*. 6th ed. Wiley-Blackwell; 2010.
13. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev*. 2008;29(Suppl 1):S49-S52.
14. Guzelbektes H, Sen I, Ok M, Constable PD, Boydak M, Coskun A. Serum amyloid A and haptoglobin concentrations and liver fat percentage in lactating dairy cows with abomasal displacement. *J Vet Intern Med*. 2010;24(1):213-219. doi:10.1111/j.1939-1676.2009.0444.x
15. Du X, Chen L, Huang D, et al. Elevated apoptosis in the liver of dairy cows with ketosis. *Cell Physiol Biochem*. 2017;43(2):568-578. doi:10.1159/000480529
16. Kiemle J, Hindenburg S, Bauer N, Roecken M. Comparison of a point-of-care serum amyloid A analyzer frequently used in equine practice with 2 turbidimetric immunoassays used in human and veterinary medicine. *J of Vet Diagn Invest*. 2022;34(1):42-53. doi:10.1177/104063872011056029
17. Behling-Kelly E, Haak CE, Carney P, Waffle J, Eaton K, Goggs R. Acute phase protein response and changes in lipoprotein particle size in dogs with systemic inflammatory response syndrome. *J Vet Intern Med*. 2022;36(3):993-1004. doi:10.1111/jvim.16420
18. Gao J, Ulvik A, McCann A, Ueland PM, Meyer K. Microheterogeneity and preanalytical stability of protein biomarkers of inflammation and renal function. *Talanta*. 2021;223(Pt 1):121774. doi:10.1016/j.talanta.2020.121774

How to cite this article: Nielsen LN, Petersen MB, Capion N, Lundsgaard J-H, Jensen AL. Performance of an automated immunoturbidimetric assay for bovine serum amyloid A. *Vet Clin Pathol*. 2024;00:1-5. doi:10.1111/vcp.13355

1939165x, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/vcp.13355 by Det Kongelige, Wiley Online Library on [03/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License