

Systematic evaluation supports the use of ELISA for quantification of calprotectin in equine feces, a first step toward noninvasive quantification of intestinal inflammation in horses

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OBJECTIVE

To optimize and evaluate methods for the detection of the inflammatory biomarkers myeloperoxidase (MPO) and calprotectin (CP) in equine feces by ELISA.

ANIMALS

Healthy horses (n = 28) and horses with intestinal inflammation (n = 10).

METHODS

Feces were suspended in buffer to create fecal supernatant. Serum and fecal supernatant were analyzed using ELISA kits validated for the detection of MPO and CP in equine serum. Assay validation steps included intra- and interassay variability (coefficient of variation [CV]), dilution linearity, spike recovery, and sample type correlation. Variations in sample handling protocols (centrifugation speed, extraction buffer, and filtration) were evaluated.

RESULTS

17 paired fecal and serum samples were used for initial analysis (10 healthy horses, 7 colitis). Previously reported sample handling protocols resulted in detectable MPO and CP but poor CV, linearity, and spike recovery. There was a linear correlation between serum and fecal samples for CP but not MPO. There was a significant difference between the concentration and CV of alternative sample handling protocols for CP and MPO, with improved CV for CP (2.1% to 18.6%) but not MPO (14.4% to 53.4%). Processing fresh feces with a fecal extraction buffer and filtration of supernatant resulted in the best CV (0.5% to 3.8%) and recovery (45% to 64%) for CP. Detection of MPO was inconsistent regardless of method.

CLINICAL RELEVANCE

There are few reliable diagnostic modalities for inflammation of the equine large colon. Findings support quantification of CP in equine feces using the described ELISA kit and protocol. With additional study to establish reference interval and clinical utility, the fecal inflammatory biomarker CP may allow for noninvasive quantification of intestinal inflammation in horses.

Keywords: colitis, horse, assay validation, manure, inflammation

Despite the clinical implications and frequent occurrence of inflammatory conditions of the large intestine in horses, available diagnostic modalities are limited. Endoscopy and biopsy are hampered by accessibility. Ultrasonography is useful for diagnosing large colon pathology,¹ but detectably increased

wall thickness may be delayed² and only a small portion of the colon may be visualized, which may not accurately reflect global intestinal health. Fecal occult blood tests and fecal albumin/hemoglobin measurements have been proposed for the diagnosis of right dorsal colitis but are neither sensitive nor specific for that condition.³⁻⁵ Thus, there is a need to evaluate novel diagnostic markers of intestinal inflammation that can be used clinically in equine patients.

Fecal inflammatory biomarkers are a direct product of intestinal inflammation and enter the feces

Received April 15, 2024

Accepted June 20, 2024

Published online July 9, 2024

doi.org/10.2460/ajvr.24.04.0110

when mucosal barrier function is lost.⁶ In human patients, quantitative measurements of inflammatory biomarkers in the feces are used in the diagnosis and monitoring of conditions such as ulcerative colitis and Crohn disease, decreasing the need for repeated invasive procedures.⁷ Specifically, myeloperoxidase and calprotectin are promising biomarkers for intestinal inflammation but have not been systematically examined for their potential diagnostic utility in horses with inflammatory intestinal disease.

Myeloperoxidase (MPO) is released from neutrophil primary granules during acute inflammation.⁸ In humans, fecal MPO levels correlate with clinical and endoscopic response to treatment in ulcerative colitis and Crohn disease.^{9,10} In horses, tissue-specific elevated MPO levels have been associated with inflammation in synovial sepsis, experimentally induced colitis, reactive airway obstruction, and endometritis.^{11–15} Increased plasma and peritoneal MPO levels have been associated with acute colic.^{16,17}

Calprotectin (CP) is expressed by activated neutrophils, monocytes, and infiltrating macrophages.^{18,19} A strong correlation has been shown between CP concentration and active inflammation in the human gut,^{20,21} with good diagnostic accuracy for inflammatory bowel disease.²² In horses, mucosal CP expression has been associated with neutrophil infiltration and inflammatory response in large colon tissues in ischemia-reperfusion models.²³

Multiple equine-specific assays for MPO are commercially available and have been reported for use in various biological fluids (plasma,^{16,17} synovial fluid,¹⁵ bronchoalveolar lavage fluid,¹⁴ and peritoneal fluid¹⁶). Of these applications, a true assay validation has only been reported for the measurement of MPO in equine blood.²⁴ A single study²⁵ has reported increased fecal MPO in healthy horses administered phenylbutazone. An equine-specific CP assay is commercially available for the detection of CP in equine serum. However, none of the available equine-specific assays have been validated for the detection of MPO or CP in horse feces.

The aim of this study was to develop a reliable method for the detection and quantitation of MPO and CP in equine feces using commercially available ELISA kits. Equine-specific ELISA kits for MPO and CP designed for use with equine serum were systematically evaluated using feces. Evaluation steps included determining intra- and interassay variability, dilution linearity, and spike recovery. We hypothesized that MPO and CP would be detected in equine feces with precision and accuracy within inherent biological variability and within a similar range of quantitation as described for equine serum.

After initial validation procedures were unrewarding, we evaluated alterations in preanalytical sample processing and repeated validation procedures. Additional a posteriori hypotheses identified over the course of the study included that processing of fresh rather than frozen fecal samples would result in more consistent fecal supernatant (Methods—Evaluation of supernatant processing protocols) and that filtration of fecal supernatant and use of a

custom fecal extraction buffer (FEB) would improve assay reliability (Methods—Extraction buffer comparison). These studies do not follow a tidy path, and practical limitations prevented complete validation procedures for every protocol variation. However, we felt it was important to report all modifications and available data to inform future method development for other fecal biomarkers or in other species.

Methods

Fecal samples were obtained from university-owned and client-owned horses that were either clinically healthy (normal physical examination and no history or clinical signs of gastrointestinal disease) or had large colon inflammation (defined as measurable right dorsal colon mural thickness ≥ 5 mm on an abdominal ultrasound). Complete physical examinations were performed prior to sample collection (university-owned horses) or at the time of hospital admission (clinical cases) by study personnel or the attending clinician. All study procedures were approved by the IACUC (protocol #20115), and informed owner consent was obtained when samples were collected from client-owned animals.

Initial systematic validation

Subject identification and evaluation

Samples were collected from healthy horses ($n = 10$) and horses with colitis ($n = 7$). Both healthy horses and horses with clinical signs of colitis were included to obtain samples with a range of endogenous fecal MPO and CP. However, as the objective of the study was to evaluate assay performance, the precise health status of the horses was not relevant to the experimental objectives. Specific utilization of the 17 samples after screening ELISA is described under Systematic Evaluation Steps.

Sample collection and analysis

Fecal samples were collected from a freshly voided pile (observed defecation) or by rectal palpation by an investigator wearing a lubricated sleeve and stored on ice (< 1 hour) until aliquoted for long-term storage at -80°C . The maximum duration of sample storage was 6 months prior to analysis.

Samples were thawed at room temperature prior to processing. As described by Richardson et al,²⁵ a 500-mg aliquot from each fecal sample was homogenized in 1 mL PBS by vortexing. Samples were centrifuged at 10,000 $\times g$ at 4°C for 5 minutes, and the supernatant was removed and used for assays. Based on pilot samples, fecal supernatant was diluted with PBS in a 1:3 ratio before analysis to bring expected concentrations within the linear detection range of the assay. Samples were analyzed using commercially available ELISA kits for equine MPO and equine CP (MyBiosource Inc) according to manufacturer protocols. The linear range for both assays was 6.25 to 200 ng/mL; accounting for sample dilution, this equates to a range detection from 25 to 800 ng/mL. Absorbance was detected at 450 nm (SpectraMax iD3; Molecular Devices).

Systematic evaluation steps

An initial screening of fecal samples was performed to identify samples with low, medium, and high levels of MPO and CP. Based on the measured MPO and CP concentrations, samples were selected for continued analysis. Assay evaluation consisted of the following steps²⁶:

1. Intra-assay variation: Three samples, 1 in each category (low, < 100 ng/mL; medium, 100 to 400 ng/mL; and high, > 400 ng/mL), were assayed 10 times each on the same plate to determine within-run repeatability.
2. Interassay variation: Aliquots of the same 3 samples (low, medium, and high concentrations) were assayed in duplicate, repeated on 5 separate days using 5 separate plates to determine between-run repeatability.
3. Dilution linearity: Using a sample with known high MPO and CP concentration, 5 serial dilutions were made such that the lowest expected concentration was near the lower end of the standard curve. Dilutions were assayed in duplicate on a single plate, concentrations were averaged, and the expected concentration was compared to the measured concentration.
4. Spike recovery: Fecal supernatant from 5 individuals with previously determined low or medium concentrations of endogenous MPO and CP were divided into 4 aliquots each. Aliquots of each sample were spiked with an equivalent amount of manufacturer-provided standard within the linear range of the assay (100, 50, and 25 ug/mL) or measurand-free diluent. Samples were analyzed in duplicate on a single plate. Expected and measured concentrations were compared to determine the detector response obtained from the analyte added to and extracted from the biological matrix.

Evaluation of supernatant processing protocols

Subject identification

Fresh fecal samples were obtained from a group of 4 clinically healthy university-owned horses for evaluation of fecal supernatant preparation protocols.

Sample collection and analysis

Feces were collected from a freshly voided pile and stored on ice for < 1 hour until processing. As for the initial validation experiment (Initial systematic evaluation—Sample collection and analysis), for the original protocol (OP) feces were frozen neat in multiple aliquots and stored at -80°C until the day of analysis. Prior to analysis, samples were thawed at room temperature, and a 500-mg aliquot from each fecal sample was homogenized in 1 mL PBS by vortexing. Samples were centrifuged at $10,000 \times g$ at 4°C for 5 minutes, and the supernatant was removed and used for assays.

The first protocol change (NP1) was to decrease the speed of the centrifuge. Samples were processed identically to the OP until centrifugation. NP1 samples were centrifuged at $600 \times g$ at 4°C for 5 minutes,

and the supernatant was removed and used for assays. The second protocol change (NP2) was to prepare supernatant from fresh feces. On the day of collection, a 5-g aliquot of feces was homogenized in 10 mL PBS by vortexing, Samples were centrifuged at $10,000 \times g$ at 4°C for 5 minutes. Aliquots of supernatant were stored at -80°C until analysis. Feces/fecal supernatant were allowed to thaw at room temperature prior to analysis by ELISA as described for the initial validation experiment. Abbreviated systematic evaluation (see Systematic evaluation steps) was performed to determine which protocol modifications to pursue further. Samples from each of the 4 horses were assayed in duplicate, repeated on 4 separate days to allow the calculation of intra- and interassay variability. Two samples were randomly selected for analysis of dilution linearity, comparing samples prepared by the OP and NP2. For each sample, a series of 5 serial dilutions was made such that the lowest expected concentration was near the low end of the standard curve. Dilutions were assayed in duplicate on a single plate, and the expected concentration was compared to the measured concentration. Statistical analyses are described for all experiments under Statistical analysis.

Extraction buffer comparison

Subject identification

Fresh fecal samples were obtained from a second cohort of 17 university- and client-owned horses ($n = 14$ healthy and $n = 3$ colitis) for the evaluation of fecal supernatant preparation protocols.

Sample collection and analysis

Feces were collected from a freshly voided pile and stored on ice for < 1 hour until processing. Two buffers were compared: 1X PBS and FEB. PBS was prepared by 1:10 dilution of 10X PBS (Fisher Scientific) with deionized water. FEB consisted of 8.0 pH TrisEDTA (Sigma-Aldrich) with 10 mmol/L calcium chloride (Sigma-Aldrich) and 0.25 mmol/L thimerosal (Sigma-Aldrich).¹⁹ After collection, 2 5-g aliquots of feces were placed in conical tubes with 10 mL buffer: 1 aliquot was combined with 1X PBS and the other with FEB. Samples were homogenized by vortexing and then centrifuged at $1,000 \times g$ at 4°C for 20 minutes. Aliquots of fecal supernatant were stored at -80°C until analysis.

Samples were prepared with 4 methods: PBS unfiltered, PBS filtered, FEB unfiltered, and FEB filtered. Fecal supernatant was allowed to thaw at room temperature prior to analysis. For filtered conditions, fecal supernatant was filtered with $0.22 \mu\text{m}$ low protein-binding syringe filters (Merck Millipore). Fecal supernatant was diluted with the appropriate buffer in a 1:3 ratio before analysis. Samples were assayed in duplicate using commercially available ELISA kits as described under Initial systematic evaluation, Sample collection and analysis.

One sample of low endogenous concentration was prepared with all 4 methods and divided into 4 aliquots each. Three aliquots of each sample were spiked with standard at 3 concentrations within the

linear range of the assay (200, 100, and 50 ug/mL MPO; 100, 50, and 25 ug/mL CP). All additions were equal in volume, and an equivalent volume of measurement-free diluent was added to the fourth aliquot to account for dilution. Spiked and neat samples were analyzed in duplicate in a single run. Expected and measured concentrations were compared to determine the detector response obtained from the analyte added to and extracted from the biological matrix to determine the behavior of the analyte in solvent.

The remaining systematic evaluation steps were completed for CP measurement using the FEB-filtered samples. Two samples with high endogenous CP concentration were randomly selected for analysis of dilution linearity. For each sample, a series of 4 serial dilutions was made such that the lowest expected concentration was near the lower limit of quantitation. Dilutions were assayed in duplicate on a single plate, and the expected concentration was compared to the measured concentration. Three samples (low, medium, and high concentration) were assayed 8 times on a single plate for the assessment of intra-assay variability and in duplicate across 5 plates to assess interassay variability.

Statistical analysis

Optical density data were preprocessed in Excel (Microsoft Corp), and mycurvefit.com (MyAssays Ltd) was used to generate the model that best fit the standard curve for each plate: exponential for MPO and symmetric sigmoidal (4pL) for CP. Further analysis was performed in R, version 4.2.1²⁷ using RStudio, version 2022.12.0 (Posit). Figures were created using the package ggpubr, version 0.5.0.²⁸ The measured concentration of analyte in each sample was calculated from the optical density and standard curve. The actual concentration was calculated by multiplying the measured concentration by the dilution factor. The actual concentration was used for the remainder of the analysis; summary data are presented as median (quartile 1, quartile 3).

Initial systematic evaluation

Intra- and interassay variances were calculated from the means and standard deviations and expressed as the coefficients of variation (CV) using the package matrixStats, version 0.61.0.²⁹ As there is no recommended allowable total error for equine MPO or CP, the precision was considered acceptable if $CV \leq 15\%$.^{30,31} The Pearson correlation was used to assess the linearity of the relationship between the measured and expected concentration for dilution and spike series. The expected values, percentage of recovery, and proportional systematic error were calculated based on the measured and expected concentration of the spiked samples, with 80% to 120% recovery considered acceptable.²⁶ For spike recovery analysis, a Pearson $R < 0.975$ suggested the presence of analytical error.³² Random and systematic error were assessed by an F test and the Welch two-sample t test, respectively. Where R was ≥ 0.9 , the association between the measured and expected concentration was determined by

Passing-Bablok regression using the package mcr, version 1.3.3.³³

Supernatant processing protocols

Intra-assay CV, interassay CV, and dilution linearity were assessed as described for Initial systematic validation. Measured concentrations of CP and MPO were compared between protocols by the Friedman test and post hoc Wilcoxon rank-sum test with Bonferroni correction.²⁹⁻³¹

Extraction buffer comparison

Intra-assay CV, interassay CV, spike recovery, and dilution linearity were assessed as described for Initial systematic validation.^{32,33} Measured concentration, CV, and percentage of recovery were compared between protocols by the Friedman test and post hoc Wilcoxon rank-sum test with Bonferroni correction.^{29-31,33}

Results

Initial systematic evaluation

On initial screening, CP was detected within the linear range of the kit in all 17 samples, with a median concentration of 359.2 (76.6, 450.8) ng/mL and a range of 28.7 to 508.8 ng/mL. MPO was only detected within or above the linear range of the kit in 5 samples, with a median concentration of 120.5 (87.5, 174.2) ng/mL and range of 41.6–567.0 ng/mL.

Interassay CVs were above the acceptable limit of 15% for all samples, whereas intra-assay CV were $< 15\%$ for half of the samples evaluated (range, 0.8% to 58.7%; **Supplementary Table S1**). Interassay CV could not be calculated for the medium concentration for MPO because 4 of 5 replicates were below the low end of the standard curve. The dilution series resulted in a linear relationship between measured and expected concentrations for MPO ($R = 0.99$; $P = .001$) but not for CP ($R = 0.61$; $P = .27$). Passing-Bablok regression suggested fair agreement between the expected and observed MPO concentrations, with moderate constant error (intercept, 26.0; 95% CI, -0.65 to 142) but minimal proportional error (slope, 1.2; 95% CI, 0.81 to 1.4).

For spike recovery series, 1 sample was removed from MPO analysis due to concentrations well above the linear range of the assay (> 200 ng/mL). Recovery of CP was generally poor and highly variable, with most samples measuring lower than the unspiked sample, $-209.8\% \pm 212.8\%$. Recovery of MPO was also poor, $13\% \pm 35.8\%$. There was a significant linear correlation between the observed and expected values for both MPO ($R = 0.970$; $P < .001$) and CP ($R = 0.788$; $P < .001$); however, $R < 0.975$ suggests the presence of analytical error (**Figure 1**). There was no significant difference in variance between the observed and expected values for CP ($P = .553$) or MPO ($P = .72$), suggesting no contribution of random error to the measurements. There was a significant difference in the means for CP ($P < .001$), suggesting a component of systematic error in the measurement. There was no significant difference in the means between the observed and expected values for MPO ($P = .913$).

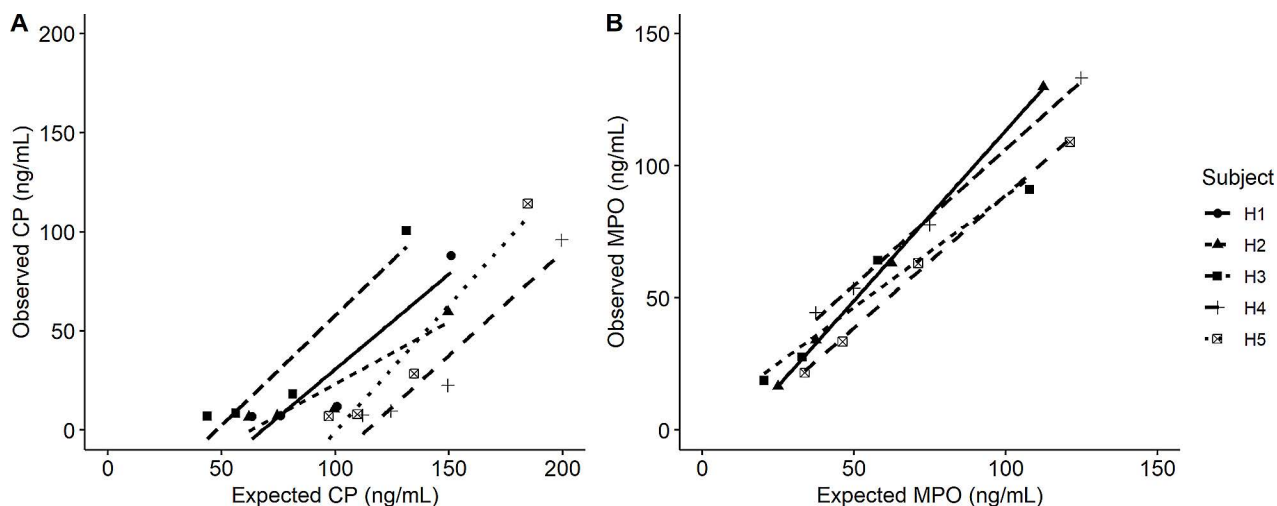


Figure 1—Scatterplot showing expected versus observed concentration of (A) calprotectin (CP) and (B) myeloperoxidase (MPO) spike recovery series. Shape and line type reflects initial starting sample (individual horses H1 to H5), lines reflect within-sample linear regression.

Evaluation of supernatant processing protocols

On initial screening, CP was detected within the linear range of the kit in all horses under all protocols. For MPO, there were samples below the low end of the standard curve for all protocols: 7 for OP, 4 for NP1, and 4 for NP2. There was a significant difference in measured concentration between protocols for CP ($P < .001$) and MPO ($P < .001$) (**Figure 2**). For CP, there was a significant difference between all protocols: OP versus NP1 ($P < .001$), OP versus NP2 ($P < .001$), and NP1 versus

NP2 ($P = .03$). For MPO, there was a significant difference between OP and both NP1 ($P < .001$) and NP2 ($P = .001$) but no difference between NP1 and NP2 ($P = .5$).

Intra-assay coefficients of variation were variable across horses and protocols (**Supplementary Table S2**). For CP, CVs were lowest for NP1 (range, 2.1% to 18.5%) and lower for NP2 (range, 8.9% to 24.4%) than OP (range, 12.9% to 27.2%). For MPO, there was no clear trend of lower CV for any protocol but rather apparent variation between horses, with a range of 6.8% to 53.4%.

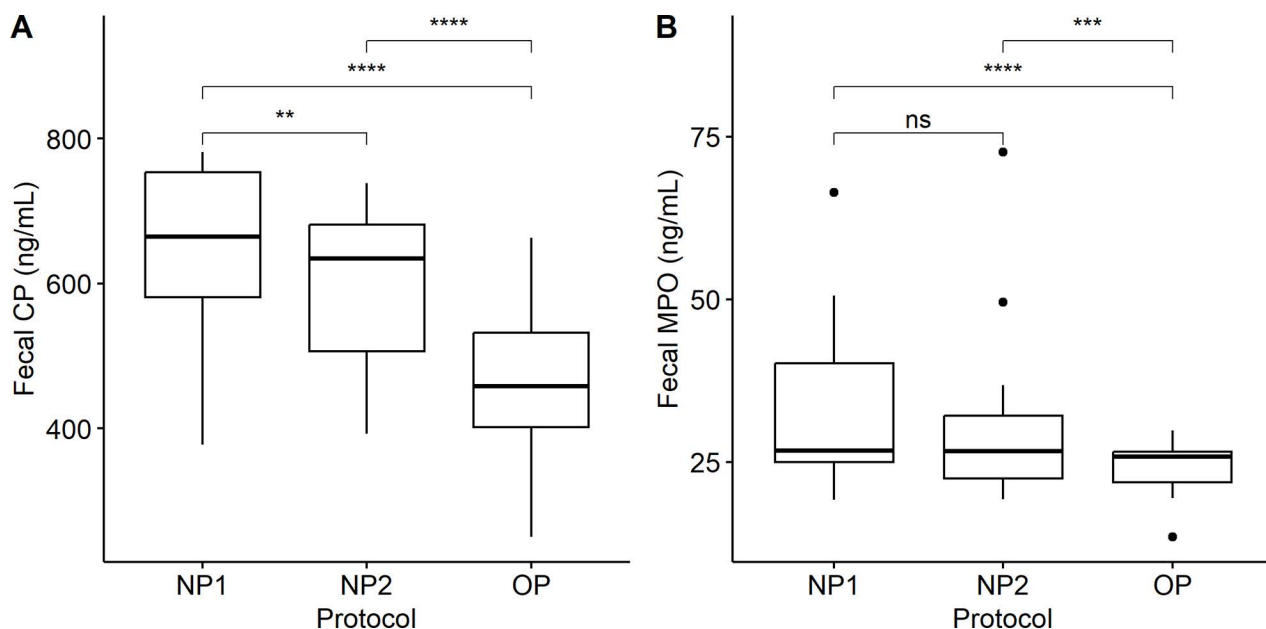


Figure 2—Boxplots showing measured concentration of (A) calprotectin (CP) and (B) myeloperoxidase (MPO) from fecal samples prepared using 3 protocols (frozen feces spun at 10,000 X *g* original protocol [OP], frozen feces spun at 600 X *g* first protocol change [NP1], and fresh feces spun at 10,000 X *g* second protocol change [NP2] prior to freezing for storage). Lower and upper box boundaries represent the 25th and 75th percentiles (first and third quartiles), with the middle line indicating the median. Lower and upper whiskers reflect the 10th and 90th percentiles, and outliers are represented by circles. ** $P < .01$, *** $P < .001$, and **** $P < .0001$. ns = $P \geq .05$.

When CP dilution curves were compared between protocols, there was a stronger linear relationship for dilution series resulting from NP2 (horse 1: $R = 0.98$, $P = .004$; horse 4: $R = 0.93$, $P = .02$) than for OP (horse 1: $R = -0.94$, $P = .02$; horse 4: $R = -0.92$, $P = .03$). Notably, there was an inverse linear relationship between samples for OP, wherein measured concentration was higher in the more dilute samples (**Figure 3**). For MPO, OP resulted in dilution series that were at or below the low end of the standard curve. There was a significant linear relationship for the dilution series for both horse 1 ($R = 0.99$; $P < .001$) and horse 4 ($R = 0.95$; $P = .01$).

Extraction buffer comparison

CP was detected in all samples, whereas MPO measurements were below the lower limit of detection for all samples under all 4 methods (**Figure 4**). There was a significant difference between all preparation methods other than PBS unfiltered and FEB filtered for CP and PBS unfiltered and PBS filtered for MPO. However, as all measured values of MPO were below the limit of detection, the significance of statistical analysis is limited, and results should not be overinterpreted. For CP, within-subject CVs were less than 15% for all samples under all methods; there was a significant effect of method on within-subject

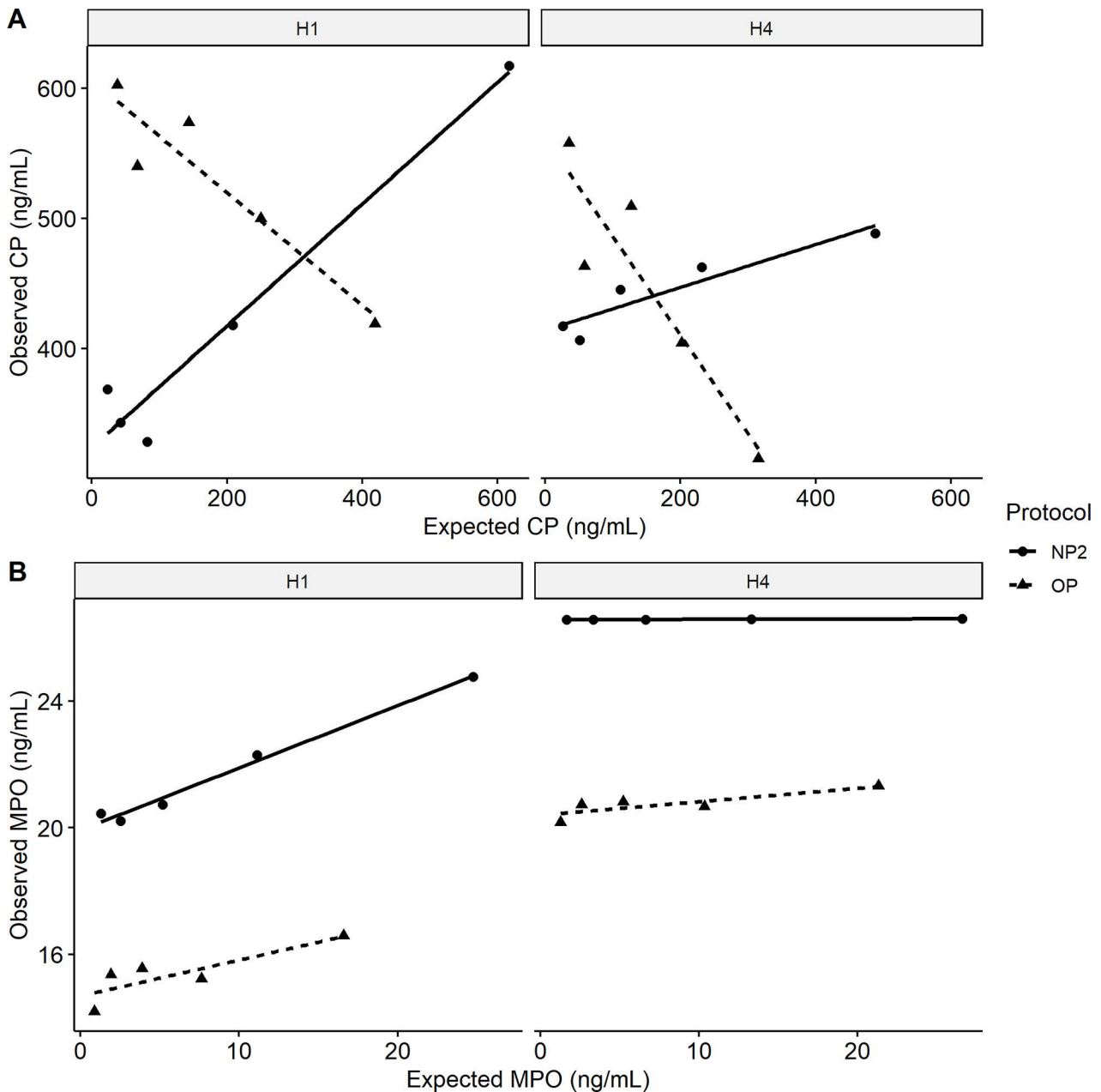


Figure 3—Scatterplot showing the observed versus expected concentrations of (A) calprotectin (CP) and (B) myeloperoxidase (MPO) using fecal samples from 2 horses (H1 and H4) and following 2 protocols (original protocol [OP], triangle and dashed line; second protocol change [NP2], circle and solid line). Linear regression lines represent the linear relationship between the expected and measured values.

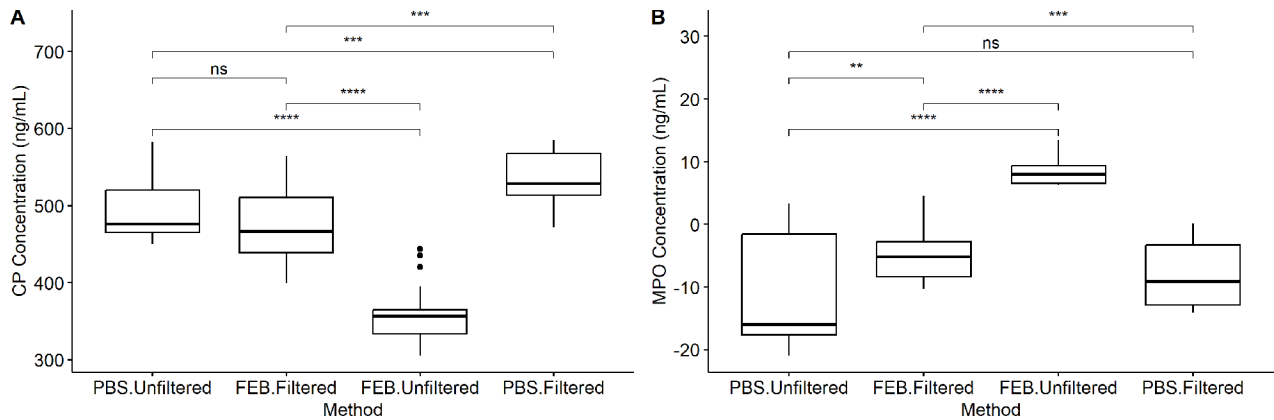


Figure 4—Boxplots showing measured concentration of (A) calprotectin (CP) and (B) myeloperoxidase (MPO) from fecal samples prepared using 2 buffers (1X PBS or fecal extraction buffer [FEB]), both filtered and unfiltered. Significance values reflect post hoc Wilcoxon rank-sum test with Bonferroni correction. ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

CV ($P = .04$), attributed to a significant difference between FEB filtered ($2.9\% \pm 2.9\%$) and FEB unfiltered ($5.5\% \pm 4.1\%$; $P = .03$) (Figure 5).

There was a significant linear correlation between expected and observed concentrations for spike recovery series for both CP and MPO across all methods. Equal variance and no significant difference in

means between expected and observed concentrations suggests that there was not random or systematic error in the measurements. Passing-Bablok regression indicated that the FEB-filtered method resulted in the best agreement between expected and observed concentrations of both CP and MPO (Table 1). The percentage of recovery was greater

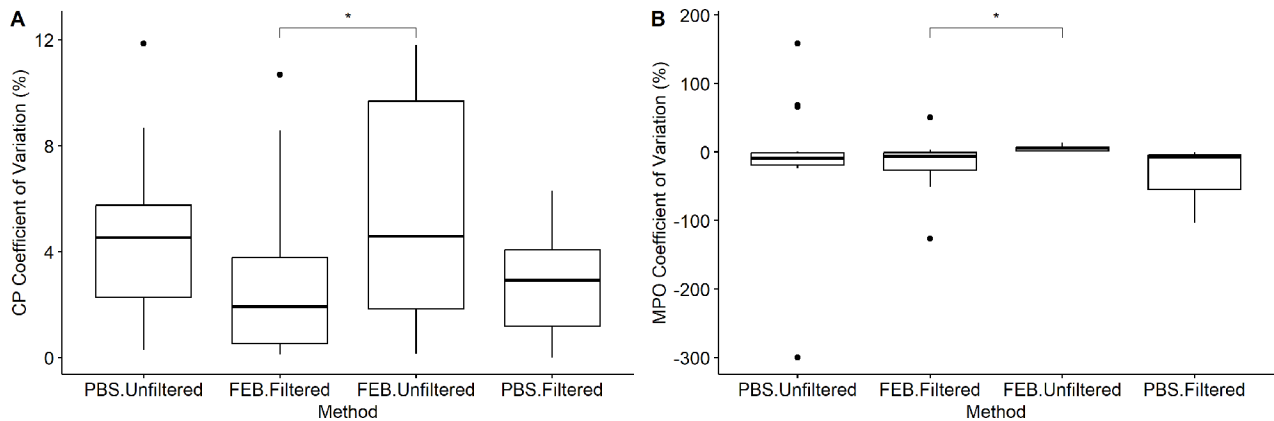


Figure 5—Boxplots showing calculated within-assay coefficient of variation of (A) calprotectin (CP) and (B) myeloperoxidase (MPO) from fecal samples prepared using 2 buffers (1X PBS or fecal extraction buffer [FEB]), both filtered and unfiltered. Significance values reflect post hoc Wilcoxon rank-sum test with Bonferroni correction. * $P < .05$.

Table 1—Results of spike recovery analysis for calprotectin (CP) and myeloperoxidase (MPO) measurement by ELISA for fecal samples prepared by 4 methods: 1X PBS or fecal extraction buffer (FEB), filtered or unfiltered.

Protocol		Pearson correlation	F test	t test	Passing-Bablok regression	Intercept (95% CI)	Slope (95% CI)
		R	P value	P value	P value		
CP	PBS unfiltered	0.988	.01	.2	.6	4.21 (3.41 to 11.26)	0.49 (0.23 to 0.75)
	PBS filtered	0.980	.01	.4	.7	6.58 (-0.67 to 12.46)	0.58 (0.1 to 0.79)
	FEB unfiltered	0.973	.03	.8	.9	3.67 (-12.38 to 8.89)	0.76 (0.31 to 1.17)
	FEB filtered	0.992	.008	.7	.7	0.86 (-12.19 to 3.67)	1.19 (0.9 to 1.55)
MPO	PBS unfiltered	0.968	.03	.2	.6	-5.34 (-96.56 to 0.75)	1.51 (1.05 to 3.01)
	PBS filtered	0.967	.03	.1	.5	-9.38 (-119.98 to 0.56)	1.9 (1.14 to 3.7)
	FEB unfiltered	0.972	.03	.1	.5	-14.24 (-110.35 to 0.55)	2.22 (1.09 to 3.69)
	FEB filtered	0.994	.006	> .9	> .9	1.25 (-17.18 to 12.81)	1.02 (0.62 to 1.21)

For the diagnostic test, $R < 0.975$ suggests the presence of analytical error; a two-sample t test was used to assess systematic error, and an F test was used to assess random error.

overall for MPO compared to CP, although initial MPO concentration was below the lower limit of the standard curve, which may have skewed the results. For CP, the percentage of recovery was greatest with the FEB-filtered method, whereas MPO had a greater percentage of recovery with FEB unfiltered and PBS filtered.

Further validation steps for CP were completed using only the FEB-filtered method as initial results showed low within-subject CV, acceptable agreement between expected and measured concentrations for spike recovery samples, and a greatest percentage of recovery. For the same samples assayed multiple times on 1 plate and across multiple days, within-assay CV ranged from 5.8% to 7.9% for the measured samples, whereas between-assay CV was between 2% and 22%. There was a significant linear relationship between expected and observed concentrations for serial dilutions of 2 samples ($R = 0.9$, $P = .04$; $R = 0.93$, $P = .02$). Passing-Bablok regression suggested good agreement between expected and observed concentrations, with no significant constant or proportional error (intercept, 0.12; 95% CI -5.4 to 2.8; slope, 0.93; 95% CI, 0.89 to 1.0)

Discussion

Although initial systematic evaluation using the sample preparation method previously described in one report²⁵ of equine fecal MPO ELISA produced inconsistent results, the findings of this study ultimately suggest that CP ELISA, using samples prepared with FEB consisting of TrisEDTA, thimerosal, and calcium chloride and filtered as described above, is reliable for quantification of CP in equine feces. However, the reliability of the MPO ELISA cannot be established based on these data, suggesting that the commercially available MPO ELISA is not suitable for the measurement of MPO in equine feces.

In the initial systematic evaluation, findings of high intra- and interassay variability and poor spike recovery suggest that the commercially available ELISA kits assessed in this study were not reliable for use with equine feces using the described protocol. Notably, poor recovery of exogenous spike into fecal samples suggests that components of the fecal matrix interfere with either the extraction of MPO and CP from the biosolids or the performance of the ELISA. Matrix interference is an important consideration when using ELISA for the analysis of biological samples.³⁴ Interference may manifest as either falsely decreased or increased measurements of analyte concentrations. Possible sources of interference from the fecal matrix include the interaction of bile (or other) salts with assay reagents, the effect of sample pH on assay reactions, mechanical interference of particulate matter, and the interaction of endogenous proteases with the measurand or assay reagents. It is also possible that there is crossreactivity between other endogenous proteins and the CP or MPO antibody, including competition for antibody binding, masking of antibody binding, or sequestration of antigen.

Due to poor performance during the initial evaluation, we assessed several alternative processing protocols related to the processing/preparation of fecal samples. Reducing the centrifugation speed and creating supernatant from fresh fecal samples rather than previously frozen resulted in greater recovery of both MPO and CP and improved interassay CV compared to the OP. It is possible that centrifugation at 10,000 X *g* caused inconsistent sedimentation of CP and MPO proteins within the fecal pellet, whereas centrifugation at 1,000 X *g* resulted in more protein within the supernatant. Additionally, some reports in other species describe fresh sample processing and storage of fecal supernatant rather than storage of frozen neat feces. Subjectively, the water content and consistency of feces change after freezing, with frozen samples having less moisture and more inconsistent texture. Processing fresh manure to create fecal supernatant prior to storage did result in fecal supernatant that was more consistent in gross appearance between samples (similar in color and opacity). However, dilution linearity was poor, and interassay CV was above the desired limit even with the modified protocol. These findings suggested continued matrix interference.³⁴ This prompted the evaluation of supernatant filtration and the use of a FEB reported in the human literature.¹⁹ These modifications resulted in acceptable values for intra- and interassay variation, dilution linearity, and spike recovery for CP but not for MPO.

The range of endogenous CP measured in the samples prepared using the FEB-filtered method was 399.3 to 564.1 ng/mL. Of the samples used for this portion of the study, there were no samples that measured with high endogenous CP. Additional study is needed to determine the normal range of endogenous CP and evaluate assay performance outside of that range. Future work including a larger cohort of horses with better clinical characterization of colonic health is warranted but is beyond the scope of this study.

The limitations of this study include a relatively small sample size and sampling on a convenience basis. Unexpected challenges in the initial planned assay validation procedures resulted in the development of a posteriori hypotheses that were then tested before arriving at the final protocol. Additionally, test validation would ideally include a method comparison.²⁶ However, there is no established gold standard test for equine fecal MPO or CP and no specific ante-mortem test for intestinal inflammation in horses. Complete clinical characterization of health status was not performed as the objective of this study was only to evaluate assay performance. Descriptions of horses as "healthy" and "clinical colitis" were included only to describe the source of samples used to obtain a range of endogenous MPO and CP concentration. Future work to compare fecal CP levels to the degree of neutrophilic inflammation in large colon tissues (by histopathology) should be considered. Additionally, further work in a large population of horses with and without intestinal inflammation is needed to assess the diagnostic utility of fecal CP measurement using the described protocol.

These initial evaluation steps suggest that the described protocol, using FEB with filtration, can reliably measure CP in equine feces. Further work is needed to determine the expected range of CP concentration in the feces of normal horses and to compare feces from healthy horses to those with large intestine inflammation. If we can demonstrate that fecal CP discriminates between healthy horses and those with large intestine inflammation, additional validation steps, such as comparison to histologic evidence of inflammation, should be considered.

Acknowledgments

None reported.

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 American Quarter Horse Foundation (AQHF #C0302).

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