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ORIGINAL ARTICLE

# The use of serum paraoxonase-1 to assess inflammation in horses with colitis

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## Abstract

**Background:** Paraoxonase-1 (PON-1) has been suggested as a marker of inflammation and oxidative stress in horses and could potentially be used for prognostication in horses with colitis.

**Objectives:** Assessment of PON-1 in horses with colitis and comparison of two methods.

**Methods:** Serum PON-1 was measured by two methods (paraoxon and p-nitrophenyl acetate) in 161 horses with colitis and 57 controls. Follow-up samples obtained during hospitalization were available from 106 horses with colitis. The two methods were compared.

**Results:** Serum PON-1 was significantly lower in horses with colitis than in healthy horses ($P < .0001$ for both methods) as well as in nonsurvivors compared with survivors ($P = .0141$ [paraoxon-based method] and $P < .0001$ [p-nitrophenyl acetate-based method]), but with marked overlap between groups. PON-1 activity did not change parallel to a change in inflammatory status in response to treatment when assessed at admission and in up to seven follow-up samples. Admission PON-1 activity could not reliably classify horses as survivors or nonsurvivors, with sensitivity and specificity ranging between 53.1% and 72.9%. Results from the two methods were comparable.

**Conclusions:** Both methods reliably measured serum PON-1 activity. Significant differences in PON-1 activity were found between healthy horses and horses with colitis and between survivors and nonsurvivors. However, PON-1 activity varied considerably within groups. Both the proposed reference intervals as well as alternative cutoff values resulted in suboptimal diagnostic and prognostic performance, and the use of serum PON-1 in horses with colitis thus seems to add little to existing diagnostic and prognostic markers.

KEYWORDS
colitis, equine, inflammation, method comparison, oxidative stress, paraoxonase-1

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## 1 | INTRODUCTION

Paraoxonase-1 (PON-1) is an antioxidant enzyme synthesized in the liver.[1] Serum PON-1 activity decreases during oxidative stress and in response to inflammation, and PON-1 is thus a negative acute phase protein.[2] Oxidative stress occurs due to the imbalance between the production of reactive oxygen species (ROS) and the availability of antioxidants, and it is linked to inflammation. Excess of ROS can oxidize biomolecules or structurally modify proteins and genes, which may trigger signaling cascades that elicit inflammation.[3] Phagocytes recruited to the site of inflammation may produce further ROS and cause oxidative stress and tissue injury.[4] Conditions characterized by inflammation and oxidative stress have been shown to be accompanied by reduced serum activity of PON-1 in several species, including laboratory animals,[2] dogs,[5–7] and dairy cows.[8] Decreased serum PON-1 activity has been demonstrated in a small study of horses naturally infected with *Theileria equi* accompanied by increased ROS production[9] as well as in horses with severe inflammation after intravenous infusion of lipopolysaccharide[10] and in horses with inflammation of different etiology and varying severity.[11] The latter study found that horses with low PON-1 activity at admission were more likely to have systemic inflammatory response syndrome (SIRS) and a negative outcome, whereas normal PON-1 activity at admission did not exclude neither SIRS nor a negative outcome.[11]

In horses, acute inflammation of the colon (colitis) is a common and potentially fatal disease, which is characterized by severe systemic inflammation[12] as well as cardiovascular compromise with a high mortality rate.[13] Affected horses present with colic, depression, fever, diarrhea, and signs of dehydration.[13] Profound hematologic and biochemical changes have been demonstrated, with increased packed cell volume (PCV), normal to low total plasma protein (TPP) caused by enteric loss of protein, leukopenia with a left shift, and very high serum amyloid A (SAA) concentrations.[13,14] Treatment of colitis is aggressive and often very costly. It is, therefore, highly relevant to predict outcomes, as this can be used to guide clinical decision-making and help identify horses with grave prognoses, where euthanasia is warranted in lieu of treatment to avoid unnecessary suffering.

There is limited knowledge regarding the potential of PON-1 as a biomarker in horses, and the aim of the study was, thus, to assess changes in serum PON-1 activity in horses with acute colitis and to evaluate its ability to differentiate survivors from nonsurvivors. The activity of PON-1 was assessed with two enzymatic methods, a paraoxon-based method and a p-nitrophenyl acetate-based method, and the results were compared.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals, samples, and files

Serum samples from 177 horses admitted with acute colitis and 76 healthy horses admitted for elective surgery (such as castration or arthroscopy) were available. All serum samples were retrieved from the biobank of the Large Animal Teaching Hospital (LATH), University of Copenhagen. Samples from horses admitted between January 1, 2015 and September 30, 2022, were included as described below.

Relevant data were retrieved from the medical files of the horses. These included results of clinical, hematologic, and biochemical analyses. Demographic data (age, weight, sex, and breed) were recorded, and breeds were grouped into "warmblooded horses" (Warmbloods, Standardbreds, Thoroughbreds, Arabians, Western breeds) and "coldblooded horses" (Icelandic horses, ponies, draught horses), similar to what has been done in other studies.[15]

#### 2.1.1 | Healthy horses

The 76 healthy control horses were included if they had no clinical signs of disease and if hematology and biochemical analyses did not show changes indicating inflammatory disease. The healthy control horses were admitted in 2015 ($n=19$), 2019 ($n=20$), 2020 ($n=17$), and 2022 ($n=20$), respectively, and these samples were also used for evaluation of the effect of storage on serum PON-1 activity.

#### 2.1.2 | Horses with acute colitis

Horses with acute colitis were included if they had at least one blood sample taken (the admission sample) and if they were diagnosed with acute colitis, enterocolitis, or typhlocolitis as the primary disease process (hereafter denoted "colitis"). The diagnosis was based on clinical examination (rectal temperature, heart rate [HR], respiratory rate, borborygmi, nasogastric reflux, and rectal palpation findings), results of abdominal ultrasound, results of blood analyses (WBC, SAA, TPP, PCV, serum iron, plasma fibrinogen, and blood lactate), and on surgery and necropsy reports, when available. Horses were included if they had increased wall thickness of the colon or caecum (>0.3 cm) on ultrasound[16] without signs of strangulation and/or if they had acute diarrhea (either at admission or developing within the first 48 h of hospitalization). Horses diagnosed at surgery or necropsy were always included.

Horses were excluded if they were <1 year old, had rupture of the gastrointestinal tract at admission or if they were euthanized at the owner's request. To ensure that the disease process was inflammatory, horses were excluded if they had low SAA concentrations at admission (<30 mg/L before spring 2022, <75 mg/L hereafter) that did not increase (>800 mg/L before spring 2022, >400 mg/L hereafter) during hospitalization. Two different SAA cutoff levels were used, as the hospital changed the SAA analysis method in the spring of 2022, resulting in changes of reference interval (RI) and concentration range.

From a subset of the horses with colitis ($n=106$), additional serum samples were obtained during hospitalization for monitoring purposes. These were available for PON-1 assessment, yielding horses with 1 ($n=37$), 2 ($n=34$), 3 ($n=25$), 4 ($n=7$), 5 ($n=1$), or 7 ($n=2$) follow-up sample(s) obtained 1–18 days after admission.

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## 2.2 | Sample collection and laboratory analyses

Blood was sampled into vacutainer tubes (BD A/S, Albertslund, Denmark) containing either no anticoagulant, EDTA (for hematology), or citrate (for fibrinogen measurements). Blood samples were stored at 5°C immediately after collection and until further processing, which took place within a maximum of 72 h after collection. Tubes used for the preparation of serum for the LATH biobank were allowed to clot before centrifugation at 1800×*g* for 10 min at room temperature, and serum was immediately placed at −20°C. Samples for determination of lactate were collected in heparinized syringes and analyzed immediately as described below.

Hematologic analyses (WBC, PCV) were obtained by an automated analyzer (ADVIA 2120 analyzer, Siemens Healthcare Diagnostics Inc, USA). Serum SAA was measured with an automated immunoturbidometric assay (LZ-SAA, Eiken Chemical Co., Japan).[17] During the final 6 months of the project period, serum SAA was assessed with a different automated immunoturbidometric assay from the same manufacturer (VET-SAA, Eiken Chemical Co., Japan).[18] Serum iron was measured by colorimetric spectrophotometry (ADVIA 1650, Bayer A/S, Lyngby, Denmark). Plasma fibrinogen concentration was obtained with a prothrombin-based assay using HemosIL RecombiPlastin (ACL Top 500, ILS, Denmark). Heparinized blood was used for lactate measurement on a blood-gas analyzer (Radiometer ABL 725, Radiometer Medical ApS, Denmark).

### 2.2.1 | Measurement of serum PON-1 activity

Serum PON-1 activity was assessed with two spectrophotometric methods; a paraoxon-based method previously validated for horses,[19] and a p-nitrophenyl acetate-based method, which has been validated in dogs,[20] and pigs.[21]

The paraoxon-based method is described in detail by Feingold et al.[2] Briefly, a 6 µL sample was incubated at 37°C with 89 µL distilled water and 100 µL mixture buffer (glycine buffer 0.05 mM), pH 10.5 containing 1 mM paraoxon-ethyl >90% purity (Sigma-Aldrich) and 1 mM $CaCl_2$. The hydrolyzation rate of paraoxon to p-nitrophenol was measured by the increase in absorbance at 504 nm using the molar extinction coefficient of 18 050 $L \times mol^{-1} \times cm^{-1}$. The activity of PON-1 was expressed as U/mL and defined by the formation of 1 nmol p-nitrophenol/minute under the before-mentioned conditions.

The p-nitrophenyl acetate-based method was performed as described by Tvarijonaviciute et al.[20] The method was originally described by Haagen et al[22] but slightly modified by removing the substrate from the working reagent buffer and preparing it in water as a separate starting reagent remaining colorless.[23] This modification was made as p-nitrophenyl acetate is subject to spontaneous hydrolysis, grossly apparent by the yellow color of the reagent. The prepared starting reagent was added to initiate the kinetic reaction. Briefly, 63 mg of p-nitrophenyl acetate was dissolved in 10 mL of methanol, and hereafter, 1 mL of this mixture was added to 20 mL of distilled water. Five microliters of serum sample were added to 307 µL buffer containing 50 mM Tris and 1 mM $CaCl_2$ (pH 8.0) together with the before-mentioned mixture containing 2.5 mM p-nitrophenyl acetate. The increase in absorbance was read at 405 nm at 37°C, and the PON-1 activity was expressed as U/mL, where 1 U equals the hydrolyzation of 1 µmol phenyl acetate per minute. The molar extinction coefficient used to calculate the rate of hydrolysis was 14 000 $M^{-1} \times cm^{-1}$.

Analyses took place from December 2022 to January 2023, that is, from 8 years to 3 months after samples were obtained and frozen.

## 2.3 | Statistical analysis

Statistical analyses were made by using Graph PadPrism9 (GraphPad Software, California, USA) and MedCalc Statistical Software version 20.218 (MedCalc Software Ltd., Ostend, Belgium). Statistical significance was defined as $P < .05$.

A comparison of the two different methods was performed by Deming regression analysis and visualized by a Bland–Altman plot. To be able to perform Deming regression and construct a Bland–Altman plot with results of the two methods, data had to be normalized as activity ranges differed between methods. Normalization was performed as

$$Z = \frac{Y - \mu}{\sigma},$$

where $Z$ denotes the value of the standardized scale, $Y$ denotes the value of the natural scale, whereas $\mu$ denotes the population mean, and $\sigma$ denotes the standard deviation of the population.[24]

To assess the effect of storage at −20°C, PON-1 activity in healthy horses was compared between years (2015, 2019, 2020, and 2022) by Kruskal–Wallis analysis, and Dunn's multiple comparisons test was performed post hoc when significant results were obtained.

Demographic data were compared between healthy controls and horses with colitis using a Mann–Whitney test (continuous variables, i.e., age and weight) or a chi-square test of independence (nominal variables, ie, sex and breed).

The distribution of serum activity of PON-1 was assessed by the D'Agostino-Pearson test and visual inspection of residual plots, which showed that PON-1 activity did not follow a Gaussian distribution, and nonparametric analyses were therefore used.

Descriptive statistics were used for data presentation. Differences in PON-1 activity between groups (healthy and colitis or survivors and nonsurvivors) and differences in PON-1 activity between survivors and nonsurvivors on Day 1 and Day 2 from admission were investigated using the Mann–Whitney test.

Proportion of healthy horses as well as horses with colitis having PON-1 activity below the lower limit of the proposed RI of 38.1 U/mL for the paraoxon-based method[19] and 2.5 U/mL (unpublished data, José J. Céron) for the p-nitrophenyl acetate-based method were assessed. Horses were classified as true positive

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(TP) = horses with colitis and PON-1 activity below RI, false positive (FP) = healthy horses with PON-1 activity below RI, true negative (TN) = healthy horses with PON-1 activity above the RI and false negative (FN) = horses with colitis and PON-1 activity above the RI. The ability of PON-1 activity to predict outcome was assessed. Nonsurviving horses were classified as "positive" and the surviving horses as "negative." To assess the diagnostic and prognostic capacity of PON-1, TP, TN, FP, and FN were used to calculate sensitivity, specificity, and the positive and negative predictive values (PPV and NPV, respectively).

The discriminatory ability of PON-1 to correctly categorize survivors and nonsurvivors was assessed using a receiver operating characteristics curve (ROC curve). The area under the curve (AUC) and 95% confidence interval were calculated. The optimal cutoff value for PON-1 was determined using the Youden index.

The correlation between serum PON-1 activity on one side and levels of inflammatory markers (WBC, SAA, fibrinogen, iron) or parameters reflecting disease severity (HR, PCV, blood lactate) on the other side was assessed using Spearman's correlation analysis. Only data from admission were used for the correlation analyses.

## 3 | RESULTS

### 3.1 | Effect of storage

PON-1 activity in samples from the healthy controls admitted in 2015 was significantly lower than in the healthy controls admitted in 2019, 2020, and 2022 and hence all samples from 2015 from both healthy controls ($n = 19$) and horses with colitis ($n = 16$) were excluded from further analyses, resulting in a study population of 57 healthy horses and 161 horses with colitis.

### 3.2 | Method comparison

Serum PON-1 activity for all samples ($n = 445$) was included in the method comparison. Serum activity of PON-1 determined with the two methods correlated significantly ($r = .91$, $P < .0001$). The Deming regression of method comparison revealed no proportional disagreement (slope [95% confidence interval] was 1.00 [0.96, 1.05]), and there was no constant error (Y-intercept [95% confidence interval] was 0.0002 [−0.04, 0.04]) (Figure 1A). The Bland–Altman plot did not reveal systematic bias (Figure 1B).

### 3.3 | Study population

The demographic data of the included horses are described in Data S1.

Horses with colitis were categorized according to the anatomical location of colitis. Some horses had more than one location of the gastrointestinal tract affected. The ascending colon was affected in 121 horses (survivors: $n = 102$, nonsurvivors: $n = 19$), the descending colon was affected in 6 horses (survivors: $n = 3$, nonsurvivors: $n = 3$). The cecum was affected in 1 surviving horse, and both the cecum

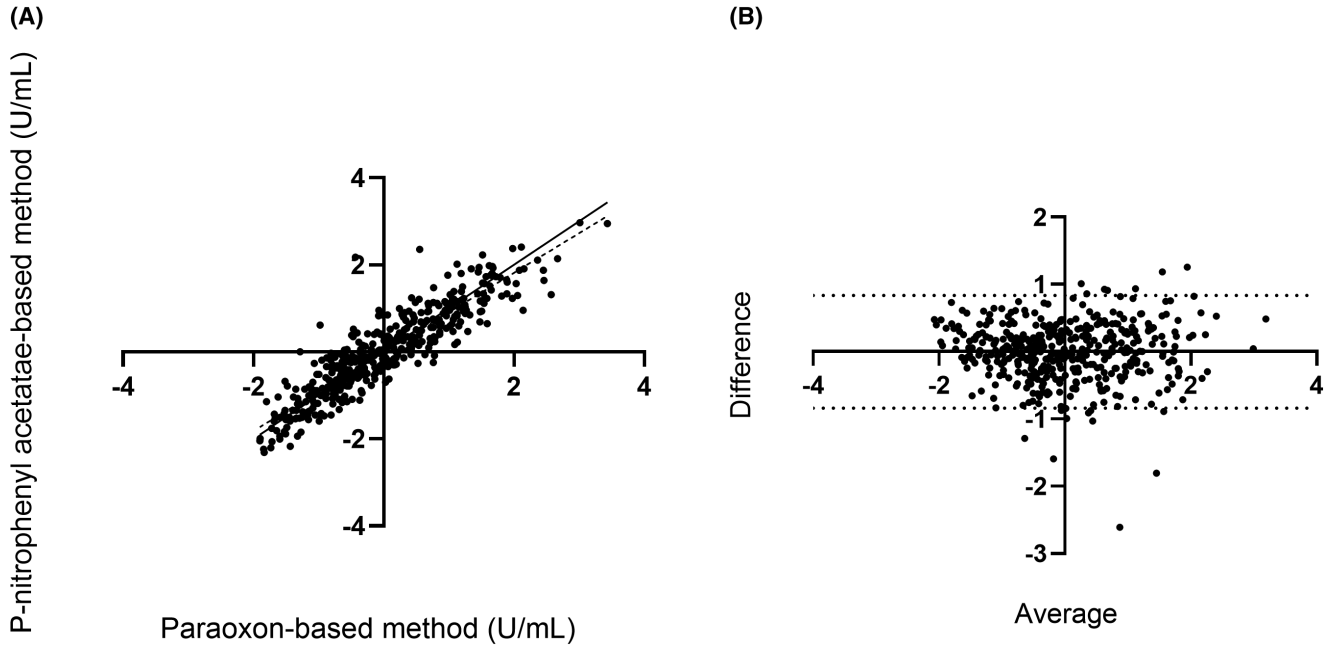


FIGURE 1 Comparison of the paraoxon-based and the p-nitrophenyl acetate-based methods for measuring serum paraoxonase-1 activity. Data were normalized in order to obtain comparable results. (A) Analysis methods were strongly and positively correlated ($p < .0001$) with no inaccuracy. The solid line represents the Deming regression line, whereas the dashed line represents a simple linear regression line. (B) The Bland–Altman plot visualizes agreement between the two enzymatic methods with no systematic bias, and the dashed lines represent the 95% confidence interval.

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and colon were affected in 37 horses (survivors: $n=10$, nonsurvivors: $n=27$).

Horses with colitis had abnormal vital parameters at admission, including (median, min–max range) hypo- or hyperthermia (rectal temperature = 38.1, 34.2–40.7), tachycardia (HR = 60, 26–100), and tachypnea (respiratory rate = 20, 8–80). Horses had varying degrees of cardiovascular compromise, as they presented with (median, min–max range) increased hematocrit (PCV = 46.0, 24.0%–80.0%), hypo- or hyperproteinemia (TPP = 54.2, 26.1–76.3 g/L), and elevated blood lactate (2.9, 0.5–26.5 mmol/L). At admission, 62 horses had increased wall thickness (>0.3 cm) of caecum and/or colon, whereas 28 horses had ultrasound findings subjectively classified as abnormal (increased intestinal wall thickness [not measured], intestinal wall edema, and/or liquid intestinal contents). Sixty-two horses did not have abdominal ultrasonography performed or had inconclusive findings, and nine horses had normal abdominal ultrasound findings at admission.

In many cases, the etiology of colitis remained undetermined. *Clostridium* spp. was identified in 22 horses (survivors: $n=16$, nonsurvivors: $n=6$) with clostridium toxin A/B identified in one nonsurviving horse. Also, coronavirus was detected in 12 surviving horses, larval cyathostominosis in 3 horses (survivors: $n=2$, nonsurvivors: $n=1$) and *Salmonella* spp. in 2 nonsurviving horses. Accumulation of sand was found in 21 horses (survivors: $n=14$, nonsurvivors: $n=7$), and NSAIDs and antibiotics were assumed to be the cause of the colitis in 12 horses (survivors: $n=7$, nonsurvivors: $n=5$).

## 3.4 | Serum PON-1 activity in healthy horses and horses with colitis

In healthy horses, serum PON-1 activity median (min-max range, interquartile range (IQR) defined as the mid spread difference [IQR = Q3–Q1]) was 77.5 U/mL (11.3–112.6, 23.0) and 2.36 U/mL (1.2–3.0, 0.3) at admission measured with the paraoxon- and the p-nitrophenyl acetate-based method, respectively. In horses with colitis, serum PON-1 activity at admission was 44.7 U/mL (3.8–147.0, 30.9) and 1.6 U/mL (0.6–3.4, 0.7) measured with the paraoxon- and the p-nitrophenyl acetate-based method, respectively (Figure 2A,B). The difference between horses with colitis and healthy controls was statistically significant when measured with the paraoxon as well as the p-nitrophenyl acetate-based method ($p<.0001$ for both).

## 3.5 | Serum PON-1 activity in survivors and nonsurvivors

Serum PON-1 activity was significantly lower in nonsurvivors than survivors at admission with both methods ($p=.0141$ for the paraoxon-based method and $p<.0001$ with the p-nitrophenyl acetate-based method). Nonsurvivors had serum PON-1 activity of median (min–max range, IQR) 38.6 U/mL (3.8–147.0, 37.7) and 1.4 U/mL (0.6–3.4, 0.7), while survivors had 49.4 U/mL (8.8–118.0, 32.5) and 1.7 U/mL (0.6–3.1, 0.6) when measured with the paraoxon- and the p-nitrophenyl acetate-based method, respectively (Figure 2C,D).

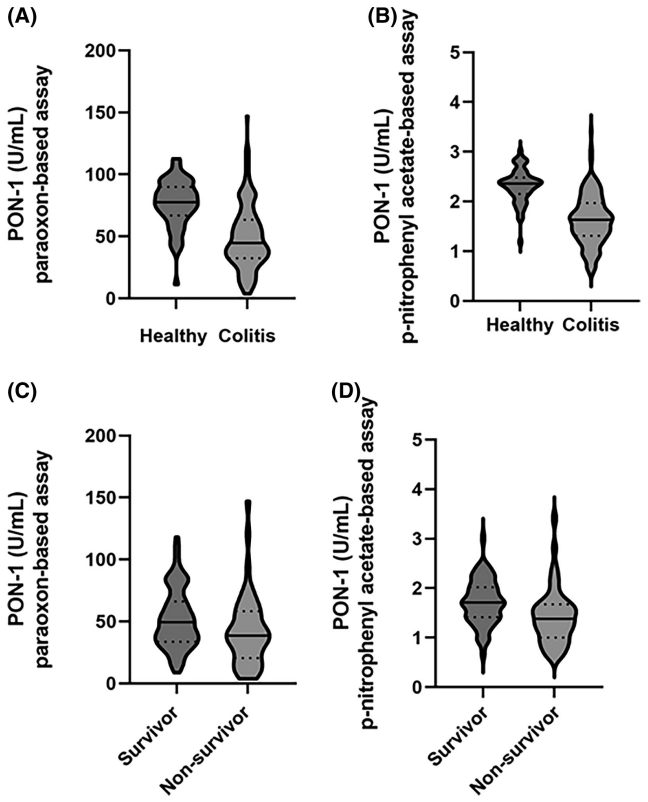


**FIGURE 2** Serum activity of paraoxonase-1 (PON-1) was measured at admission in 57 healthy horses and 161 horses with colitis. Solid line = median, dotted lines = 25% and 75% quartiles, ends of violin represents min and max. Serum PON-1 activity differed significantly between healthy controls and horses affected by colitis (A: paraoxon-based method, $P<.0001$, B: p-nitrophenyl-based method, $P<.0001$). Serum PON-1 activity differed significantly when horses with colitis were grouped according to outcome (survivor, nonsurvivor) (C: paraoxon-based method, $P=.0141$, D: p-nitrophenyl-based method, $P<.0001$).

## 3.6 | Changes in serum PON-1 activity over time

One hundred and six horses had one or more follow-up blood samples (median = 2, range 1–7) obtained during hospitalization. Samples were obtained depending on the responsible clinician's assessment of the individual horse between Day 1 and Day 18 after admission (Data S2). There was no obvious pattern of change in serum PON-1 activity over time, neither in horses responding to treatment (survivors) nor in horses not responding to treatment (nonsurvivors) (Figure 3).

As nonsurvivors had visually lower PON-1 activity than survivors in the first days after admission (Figure 3), this crucial time period was further investigated. PON-1 activity in survivors and nonsurvivors was compared on Day 1 ($n=38$ and $n=5$, respectively) and Day

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2 ($n=33$ and $n=7$, respectively) after admission, but no significant differences were found using either method (Data S4).

### 3.7 | Diagnostic capacity of PON-1 activity

At admission, 57/218 and 200/218 horses had PON-1 activity below the lower limit of the RI of the paraoxon- (38.1 U/L) and the p-nitrophenyl acetate-based method (2.5 U/mL), respectively. The diagnostic ability (sensitivity, specificity) in terms of categorizing horses as healthy or diseased based on the PON-1 activity at admission is shown in Table 1.

### 3.8 | Prognostic capacity of serum PON-1 activity

To further analyze the ability of PON-1 to discriminate nonsurvivors from survivors, ROC curves were plotted (Figure 4A,B). Diagnostic strength (AUC) was statistically significant, but poor to moderate (paraoxon-based method AUC = 0.622, 95% CI 0.542–0.697, $P=.014$; p-nitrophenyl acetate-based method AUC = 0.696, 95% CI 0.618–0.766, $P<.0001$). The best threshold (cutoff) for distinguishing nonsurvivors from survivors with the paraoxon-based method was 45.9 U/mL, with a sensitivity of 66.7% and specificity of 53.1%. Eighty-four horses with colitis had serum activity of PON-1 below this threshold at admission, 31 of these were nonsurvivors, corresponding to 36.9% being correctly categorized. With the p-nitrophenyl acetate-based method, the best threshold was 1.6 U/mL, yielding a sensitivity of 72.9% and a specificity of 60.2%. Seventy-eight horses had serum activity of PON-1 below this threshold at admission, 34 of these were nonsurvivors, corresponding to 43.6% being correctly categorized. The PPV and NPV were calculated using the proposed RIs for both methods (Table 1).

### 3.9 | Correlation between PON-1 serum activity and inflammatory and disease severity markers

Using both methods, serum PON-1 activity had a weak and negative statistically significant correlation with serum SAA concentration as well as HR and blood lactate. The correlations between serum PON-1 and PCV were weak and negative, but only statistically significant when using the p-nitrophenyl acetate-based method. The correlations between serum PON-1 activity and plasma fibrinogen, serum iron, and WBC were not significant (Table 2).

## 4 | DISCUSSION

As hypothesized, serum PON-1 activity was significantly lower in horses with colitis than in healthy horses and in nonsurvivors compared with survivors. However, there was substantial overlap in serum PON-1 activity between groups, and the discriminatory ability (differentiation between survivors and nonsurvivors) was overall poor, although statistically significant. Taken together, the results show that the biomarker potential of PON-1 in horses with colitis is limited, even in our patient group with very severe systemic disease.

Oxidative stress is characterized by increased intracellular levels of ROS,[25] and as several studies have suggested that PON-1 is a marker of oxidative stress,[8,9,11,26] a very clear and demarcated decrease in PON-1 activity was anticipated in horses with colitis, a

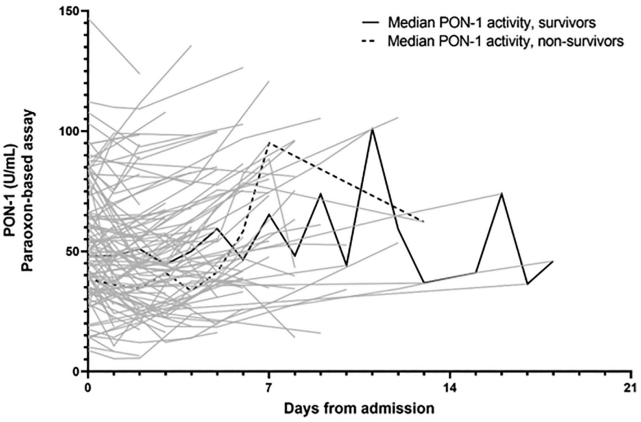


FIGURE 3 Changes in serum paraoxonase-1 (PON-1) activity during hospitalization in 161 horses admitted with acute colitis. Follow-up samples were obtained in 106 horses with varying intervals depending on the responsible clinician's assessment of the individual horse. Thin gray lines represent each individual horse, the solid black line represents the median PON-1 activity in survivors, and the dotted black line represents the median PON-1 activity in nonsurviving horses. See Data S3 for results obtained by the p-nitrophenyl acetate-based method and for illustrations of PON-1 activity over time in horses with low PON-1 activity at admission.

TABLE 1 Sensitivity (Se) and specificity (Sp) were calculated for the included horses according to disease status (healthy or colitis) and outcome (survivors or non-survivors) using both the paraoxon and the p-nitrophenyl acetate-based method. The positive predictive value (PPV) and negative predictive value (NPV) methods were estimated for outcomes. Two by two tables are shown in Data S5.

| | Disease | | Outcome | | | |
|---|---|---|---|---|---|---|
| | Se% | Sp% | Se% | Sp% | PPV% | NPV% |
| Paraoxon-based method | 34.8 | 98.2 | 45.8 | 79.4 | 38.6 | 83.9 |
| P-nitrophenyl acetate-based method | 97.5 | 24.8 | 95.8 | 9.4 | 23.0 | 88.9 |

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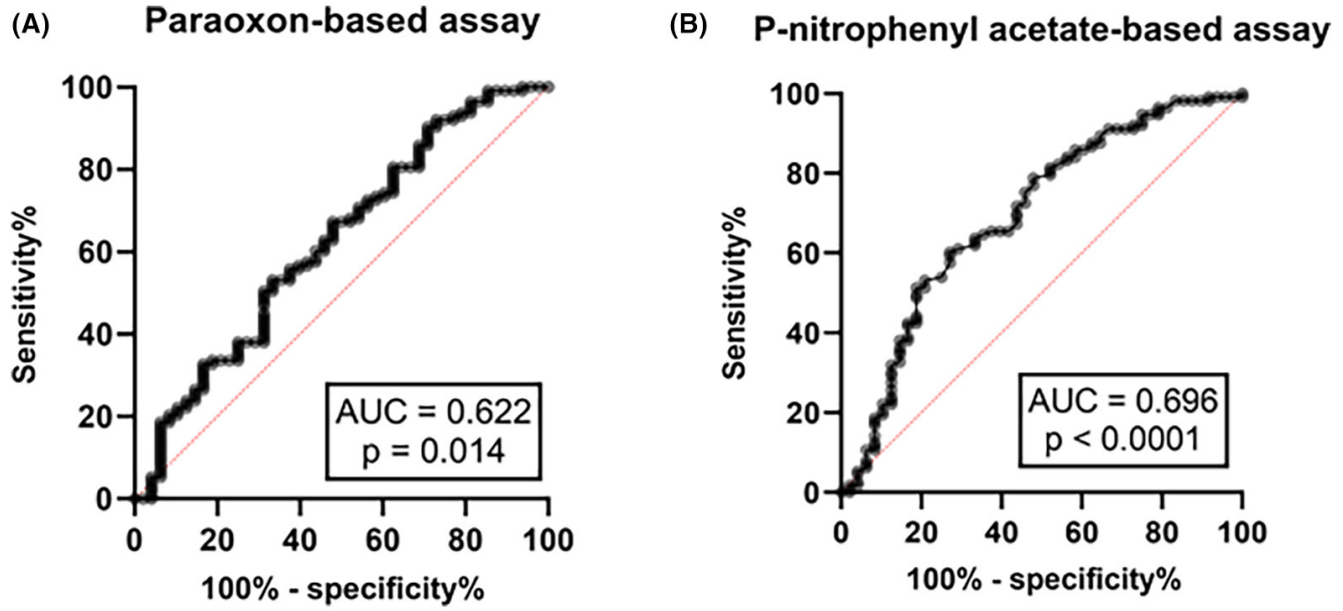


FIGURE 4 Receiver operating curve for diagnostic ability of paraoxonase-1 activity determined by the paraoxon-based method (A) and the p-nitrophenyl acetate-based method (B) in serum obtained at admission from 161 horses with colitis with the purpose of categorizing survivors and nonsurvivors. AUC, area under curve.

TABLE 2 Correlation between serum paroxonase-1 (PON-1) activity and levels of other inflammatory markers (serum amyloid A [SAA], fibrinogen, iron, and white blood cell count [WBC]) as well as commonly used severity markers (heart rate [HR], blood lactate, and packed cell volume [PCV]) in admission blood samples from 161 horses with colitis.

| | Spearman correlation coefficient | |
|---|---|---|
| Correlations (X:Y) | Paraoxon-based method | P-nitrophenyl acetate-based method |
| Serum SAA: serum PON-1 | −0.169 ($p = .034$) | −0.194 ($p = .015$) |
| Serum fibrinogen: serum PON-1 | 0.044 ($p = .587$) | 0.102 ($p = .207$) |
| Serum iron: serum PON-1 | 0.072 ($p = .369$) | 0.042 ($p = .599$) |
| WBC: serum PON-1 | −0.033 ($p = .679$) | −0.110 ($p = .165$) |
| Heart rate: serum PON-1 | −0.263 ($p = .001$) | −0.300 ($p < .001$) |
| Blood lactate: serum PON-1 | −0.215 ($p = .006$) | −0.252 ($p = .001$) |
| PCV: serum PON-1 | −0.133 ($p = .094$) | −0.219 ($p = .005$) |

disease accompanied by profound inflammation and hence oxidative stress.[14,27] Inflammation and production of ROS are present in humans with inflammatory abdominal diseases.[3,4] In patients with Crohn's disease, serum activity of PON-1 was decreased, and there was a good correlation between disease activity and excellent diagnostic capacity, as PON-1 differentiated patients with Crohn's disease from healthy individuals with a sensitivity of 93.6% and a specificity of 91.3%.[28]

There are only a few studies investigating PON-1 in horses, including differences in PON-1 activity in healthy and diseased horses. In horses infected with *T. equi*, PON-1 activity was significantly lower (45% reduction) than in healthy horses.[9] In contrast, infection with *Leptospira* spp. did not seem to affect serum PON-1 activity.[29] Ruggerone et al investigated PON-1 activity in 58 sick horses with a variety of clinical conditions classified as SIRS-positive and SIRS-negative. PON-1 activity in serum did not differ between horses in the two groups.[11] However, when analyses were restricted to mares, SIRS-positive horses had significantly lower serum PON-1 activity than SIRS-negative.[11]

Our study comprised a large and well-defined group of horses ($n = 161$) with severe inflammation and oxidative stress, which is the optimal setup to make the first investigations into the diagnostic performance of a biomarker.[30] Despite this, there was substantial variation in serum PON-1 activity in horses with colitis, with activity ranges of 3.8–147.0 U/mL and 0.6–2.8 U/mL using the paraoxon- and the p-nitrophenyl acetate-based methods, respectively. These ranges encompass almost the entire RI for healthy horses, which obviously weakens the diagnostic potential.

Studies in humans have suggested that several factors unrelated to disease may influence serum PON-1 activity, including genetics,[31] sex,[32] age,[33] ethnicity,[34] and lifestyle such as diet.[35] Few studies in animals have investigated possible confounders, and sex, age, and breed have been shown to influence the activity of PON-1 in laboratory animals, horses, and dogs.[6,11,19,36] These factors may have influenced the results of our and previous studies. While RIs can take such factors into account, it does complicate the interpretation of measured levels and limit their usefulness when clinicians have to consider the age, sex, and breed of the horse under investigation.

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There was no apparent consistent change in serum PON-1 activity in response to treatment, neither in horses that responded to treatment (resulting in reduced inflammation) nor in nonsurvivors that did not respond to treatment. The expected response pattern of a negative acute phase reactant should have an initial decrease and subsequent increase in surviving horses responding to treatment, as has been shown for other equine negative acute phase reactants, for example, iron.[37] Changes in PON-1 activity in response to changes in inflammatory activity have previously been investigated in a small group of horses ($n=6$ mares) with experimentally induced endotoxemia.[10] Plasma PON-1 activity was significantly lower 48–120 h after intravenous injection of 30 ng/kg lipopolysaccharide and returned to preinjection level at 120 h. However, PON-1 activity levels overlapped considerably at all sampling points, that is, under noninflammatory conditions as well as in the early and late phase of the experimental inflammation.[10]

To perform well, it is highly desirable that biomarkers have a fast response and the ability to mimic the course of disease since they are used for the assessment of disease progression, resolution, and severity.[38] However, the change in PON-1 activity over time did not correspond to the clinical status of the patients, and even though there was a statistically significant difference in activity between survivors and nonsurvivors at the time of admission, there was substantial overlap between those groups. This suggests that neither point samples nor repeated assessments of PON-1 can predict the outcome of acute colitis in horses, corresponding to previous findings in horses, where repeated measurements of PON-1 activity did not predict outcome.[11] In humans, PON-1 has been shown to correlate with disease activity and severity in patients with sepsis,[39,40] where low PON-1 activity was found to be related to mortality.[40] However, species differences may exist, as no consistent correlation between PON-1 activity and disease severity or activity has been found in veterinary species. In a study of canine leishmaniasis, low PON-1 activity was suggested to be an indicator of severe disease; however, severely affected dogs also had normal PON-1 activity at admission.[5] Similarly, a study in dogs with inflammatory diseases found that the activity of PON-1 only decreased significantly in dogs with very high plasma concentrations of the positive acute phase reactant C-reactive protein (CRP) and that PON-1 activity and CRP concentration correlated only weakly.[6]

In this study, PON-1 activity did not correlate strongly with any of the investigated known inflammatory markers (SAA, serum iron, serum fibrinogen, and WBC) or with clinical severity markers (lactate, HR, PCV) previously shown to be of prognostic value in horses with colitis.[14] Poor correlation may be attributed to differences in the speed of change of the markers under investigation. A previous study demonstrated a significant decrease in serum PON-1 activity 48 h after experimental induction of inflammation by intravenous injection of lipopolysaccharide,[10] which could explain poor correlation with fast-reacting biomarkers such as WBC and serum iron, but not with SAA, which is known to peak 48–72 h after induction of inflammation.[41] It has been speculated that horses experience less oxidative stress than other species during inflammation,[10,11] but oxidative stress has been shown to be present during colitis in horses.[42] A study by El-Deep et al. showed good to excellent correlation between oxidative stress markers (nitric oxide, superoxide dismutase, and total antioxidant concentration) and an array of inflammatory biomolecules, including SAA, haptoglobin, and proinflammatory cytokines in horses with colitis,[42] and the absence of oxidative stress does not seem to explain the lack of correlation between serum PON-1 activity and inflammatory markers.

While serum PON-1 activity was statistically different between survivors and nonsurvivors, the ROC curves showed that the ability of PON-1 to correctly classify survivors and nonsurvivors was poor, with sensitivity and specificity ranging from 53.1% to 71.2% for the two methods, resulting in misclassification of a large proportion of horses when using the cutoff values calculated based on the Youden index. When using the proposed RIs, the PPV of serum PON-1 activity was poor (38.6 and 23.0% for the paraoxon- and p-nitrophenyl acetate-based method, respectively) for the identification of nonsurvivors. Sensitivity for the p-nitrophenyl acetate-based method was excellent, that is, both horses with colitis and nonsurvivors were likely to have serum PON-1 activity below the RI, while sensitivity was poor for the paraoxon-based method, similar to results from a previous study using the same method.[11] Contrarily, the paraoxon-based method exhibited high specificity (98.2% and 78.4%, respectively), so horses with serum activity of PON-1 above the RI were likely to be healthy and survive. This was in contrast to the p-nitrophenyl acetate-based method, which had very low specificity for identifying horses with colitis and prognosticating outcomes (24.6 and 9.4%, respectively).

As results obtained by the two different methods are highly correlated with no apparent bias, the diagnostic performance is highly dependent on the appropriateness of the cutoff value used. High specificity is desired when predicting nonsurvival to ensure that horses categorized as nonsurvivors truly belong to this group, as misclassification may result in the euthanasia of a horse, which truly would have survived. When the proposed RIs were used, 35 (61.4%) and 154 (77.0%) horses were misclassified as nonsurvivors (FP) using the paraoxon- and the p-nitrophenyl acetate-based method, respectively. To mitigate this issue, for example, by increasing specificity to at least 90%, the resulting cutoff values would be decreased to 25.4 U/mL and 1.2 U/mL using the paraoxon- and the p-nitrophenyl acetate-based method, respectively. These new cutoff values would reduce FP to 12 horses for both methods corresponding to 48.0% for the paraoxon-based method and 41.4% using the p-nitrophenyl acetate-based method. However, the increased specificity would be at the expense of the sensitivity, resulting in increased FN and more horses being incorrectly categorized as survivors.

A study by Rossi et al in dogs with leishmaniasis found similar results with suboptimal diagnostic performance, as the serum activity of PON-1 failed to predict the outcome.[5] Ruggerone et al. investigated sick horses classified as either SIRS positive or SIRS negative

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and did not find any difference in PON-1 activity between survivors or nonsurvivors. Sensitivity was low (38.9%), but specificity high (90.0%), and the likelihood of nonsurvival was 3.89 times higher than the likelihood of survival in horses with admission PON-1 activity below the RI. They also found that horses with low PON-1 at admission were likely to be SIRS-positive and nonsurvivors, similar to the results of our study. However, the group of nonsurvivors included horses with both low PON-1 activity as well as horses with normal PON-1 activity at admission, and the authors concluded that the results only partly supported a role for PON-1 as a diagnostic marker in horses.[11]

The paraoxon-based method of assessing PON-1 has been validated in horses,[19] whereas the p-nitrophenyl acetate-based method has not but has been used in cats,[43] dogs,[20] and pigs.[21] Our results showed that the two methods had excellent agreement and no bias, suggesting that also the p-nitrophenyl acetate-based method is useful for measuring PON-1 activity in equine samples. As the activity range varies between the methods, different RI must be used, and an equine-specific RI must be established for the p-nitrophenyl acetate-based method.

Our study has some limitations. The stability of PON-1 activity in serum samples during storage is not entirely clear. A previous study found significantly decreased activity of PON-1 in equine serum samples after storage for 3 months at −20°C,[44] whereas another study found the activity of PON-1 to be significantly increased in canine serum samples after storage for 6 months at −20°C. In contrast to this, the activity of PON-1 in human serum samples was found to be very stable, with a calculated half-life of 9.5 years when stored at −20°C.[45] We found significantly lower PON-1 activity in serum samples obtained from healthy horses and stored for 8 years at −20°C than in healthy control serum stored for 4, 3, and <1 year prior to analysis, necessitating exclusion of all horses (healthy and colitis) from 2015. Other limitations of this study are mainly related to those inherent to the retrospective study design. While this study design made it possible to investigate the biomarker in a large and clinically relevant group of animals, it has the disadvantage of the lack of control over parameters that may have affected the PON-1 levels, for example, preadmission duration of colic, treatment, etiology of colitis, and lack of standardization of the follow-up sampling. Furthermore, it would have been of interest to include other markers of oxidative stress to allow a better assessment of the relationship between PON-1 activity and oxidative stress.

## 5 | CONCLUSIONS

Serum activity of PON-1 differed significantly between healthy horses and horses with colitis as well as between survivors and nonsurvivors. However, PON-1 activity varied considerably between groups, and no apparent change in PON-1 activity was seen over time in either horses responding to treatment (survivors) or in horses that did not respond to treatment (nonsurvivors). Using the proposed RIs as well as alternative cutoff values resulted in suboptimal diagnostic performance and little prognostic ability (as sensitivity and specificity ranged between 53.1% and 72.9%, resulting in misclassification of horses). In conclusion, the use of serum PON-1 measurements in horses with colitis thus seems to add little to existing diagnostic and prognostic markers. The paraoxon-based and the p-nitrophenyl acetate-based methods had excellent agreement, and both may be used for the quantification of serum PON-1 activity in horses.

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## CONFLICT OF INTEREST STATEMENT

None.

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