



# Detection of pathogens in blood or feces of adult horses with enteric disease and association with outcome of colitis

Jamie J. Kopper<sup>1</sup>  | Jaclyn A. Willette<sup>1</sup> | Clark J. Kogan<sup>2</sup> | Alexis Seguin<sup>3</sup> | Steven R. Bolin<sup>4</sup> | Harold C. Schott II<sup>1</sup> 

<sup>1</sup>Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, Michigan, USA

<sup>2</sup>Department of Mathematics and Statistics, Washington State University, Pullman, Washington, USA

<sup>3</sup>IDEXX Laboratories, Inc, Westbrook, Massachusetts, USA

<sup>4</sup>Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan, USA

## Correspondence

Jamie J. Kopper, Department of Veterinary Clinical Sciences, Iowa State University, Ames, IA 50010, USA.

Email: jkopper@iastate.edu

## Abstract

**Background:** Rates of detecting  $\geq 1$  potential enteric pathogens (PEP) or toxins (PEP-T) in feces, blood, or both of horses  $\geq 6$  months of age with enteric disease and impact of multiple detections on outcome of horses with colitis has not been reported.

**Objective:** To determine detection rates of PEP/PEP-T in feces, blood, or both of horses with enteric disease and effect of detecting multiple agents on outcome of horses with colitis.

**Animals:** Thirty-seven hundred fifty-three fecal samples submitted to IDEXX Laboratories and 239 fecal and blood samples submitted to Michigan State University's Veterinary Diagnostic Laboratory (MSUVDL).

**Methods:** Retrospective evaluation of PEP/PEP-T testing results was performed to determine rates of detection of 1 or more PEP/PEP-T. Impact of detecting multiple agents on outcome was assessed in 239 horses hospitalized for colitis.

**Results:** One or more PEP/PEP-T was detected in 1175/3753 (31.3%) and 145/239 (60.7%) of samples submitted to IDEXX Laboratories and MSUVDL, respectively. In a hospitalized cohort, survival to discharge was lower (76%) in horses with 1 agent, compared to horses with either no (88%) or multiple (89%) agents. There was no difference ( $P = .78$ ) in days of hospitalization between horses with 0 (1–17), 1 (1–33), and  $> 1$  positive (1–20) result. There was no difference in cost of hospitalization ( $P = .25$ ) between horses with 0 (\$2357, \$1110–15 553), 1 (\$2742, \$788–11 005), and  $> 1$  positive (\$2560, \$1091–10 895) result.

**Conclusions and Clinical Importance:** Detection rates of PEP/PEP-T in horses with colitis vary with cohorts and tests performed. Detection of more than 1 PEP or PEP-T did not affect outcome.

## KEYWORDS

*Clostridiodes difficile*, coinfection, diarrhea, *Neorickettsia risticii*, salmonella

**Abbreviations:** AE, Qiagen elution buffer; BG, brilliant green; bp, base pairs; CDT, *C. difficile* binary toxin; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; GI, gastrointestinal; MSU, Michigan State University; OD, optical density; PaLOC, pathogenicity locus; PEP, potential enteric pathogen; PEP-T, potential enteric pathogen toxin; PHF, Potomac Horse Fever; rDNA, ribosomal deoxyribonucleic acid; VDL, Veterinary Diagnostic Laboratory.

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## 1 | INTRODUCTION

Colitis, often manifested as an acute onset of diarrhea, is a life-threatening disease in horses and determining the cause of colitis can be challenging.<sup>1-3</sup> As an example, an etiologic diagnosis was established in only 7.7% of horses with colitis at a veterinary teaching hospital in 1 report.<sup>4</sup> Determining a microbial cause of colitis and other enteric diseases is desirable to institute therapies directed against specific pathogens and to allow appropriate biosafety measures to be instituted, based on pathogen-specific concerns for disease transmission to other horses, contamination of the environment, and zoonotic potential.

Pathogens including *Salmonella* spp.,<sup>2,5</sup> *Neorickettsia risticii*,<sup>2,5</sup> toxin producing strains of *Clostridiodes difficile* and *Clostridium perfringens*,<sup>4,6-16</sup> and more recently coronavirus<sup>17-19</sup> are well-recognized agents of acute colitis in adult horses. Infection with other pathogens, including rotavirus,<sup>10,20</sup> *Lawsonia intracellularis*,<sup>21-23</sup> *Rhodococcus equi*,<sup>24</sup> *Aeromonas* spp.,<sup>25</sup> *Eimeria* spp.,<sup>26</sup> and *Cryptosporidium parvum*<sup>27,28</sup> as well as infestation with internal parasites<sup>29,30</sup> can also cause enteric disease in equids, with some pathogens more commonly causing disease in younger equids. Isolation of any of these agents from feces of equids with enteric disease leads to establishment of a definitive etiologic diagnosis by many clinicians. Although this conclusion can be accurate for some pathogens (ie, *N. risticii*), cause and effect when 1 or more potential enteric pathogens (PEP) or their toxins (PEP-T) is detected is not always clear. As molecular diagnostic tools (ie, real-time PCR) have progressed to allow more economic screening for multiple PEP and PEP-T in fecal samples, higher rates of PEP and PEP-T detection have been achieved and, in some samples, multiple positive results provide support for infection by more than 1 PEP.<sup>31,32</sup> However, multiple positive results can also cause confusion when developing treatment and biosecurity plans. As an example, a recent systematic review of 23 studies comparing PCR fecal diagnostic panels to conventional fecal testing in humans with diarrhea found that molecular tests consistently yielded a higher percentage of positive results than conventional tests; however, the study concluded that it was unclear whether or not the additional positive test results were clinically relevant.<sup>33</sup>

The objective of this retrospective study was to determine the rate of detecting 1 or more PEP or PEP-T in horse fecal and blood samples. Further, in a cohort of 239 horses hospitalized at Michigan State University's Veterinary Medical Center (MSUVMC) for treatment of colitis, we hypothesized that detection of more than 1 PEP or PEP-T would be associated with a poorer outcome (lower survival rate), longer hospitalization, and greater treatment cost in comparison to horses in which either no or only a single PEP or PEP-T was detected.

## 2 | MATERIALS AND METHODS

### 2.1 | Case selection

Results of 3753 enteric disease fecal real-time PCR panel submissions to IDEXX Laboratories between January 1, 2010 and December 31, 2014 for horses ( $\geq 6$  months of age) were reviewed. Submission

forms were often incomplete and evaluation of the reason(s) for sample submission could not be evaluated. In addition, medical records for horses  $\geq 6$  months of age admitted to MSUVMC between July 1, 2007 and September 1, 2014 for treatment of acute colitis, that also had complete results for the Michigan State University Veterinary Diagnostic Laboratory (MSUVDL) enteric disease panel, were reviewed. Data retrieved from medical records included signalment, presenting complaint, survival to discharge, duration of hospitalization, cost of treatment, and results of enteric disease testing. Enteric disease panel testing methodology was consistent at MSUVDL during the study period.

### 2.2 | IDEXX Laboratories enteric disease panel

#### 2.2.1 | Real-time PCR assays

A panel consisting of 10 hydrolysis probe-based real-time PCR assays was used to test nucleic acid extracts of 3753 equine fecal samples for *Salmonella* spp., *N. risticii*, *L. intracellularis*, *R. equi*, *Cryptosporidium* spp., coronavirus, rotavirus, *C. difficile* toxin A and B genes (*tcdA* and *tcdB*), and *C. perfringens* alpha toxin gene, as previously described.<sup>32</sup> Real-time PCR was performed with 7 quality controls including PCR positive controls, PCR negative controls, negative extraction controls, a DNA preanalytical quality control targeting the host *ssr rRNA* (18S *rRNA*) gene complex, a RNA preanalytical quality control targeting the host *ssr rRNA* gene complex, an internal positive control spiked into the lysis solution, and an environmental contamination monitoring control. Fecal samples were collected and shipped at 4°C and total nucleic acid was extracted using previously published protocols.<sup>34</sup> Enteric disease panel testing methodology was consistent during the study period.

### 2.3 | In hospital data collection

#### 2.3.1 | MSUVDL enteric disease panel

The MSUVDL enteric disease panel tested whole blood and fecal samples by PCR for *N. risticii*, feces by enzyme-linked immunosorbent assay (ELISA) for *C. perfringens* and *C. difficile* toxins, and feces for *Salmonella* spp. by an enriched bacterial culture.

#### 2.3.2 | PCR assay for *N. risticii*

Whole blood collected into sterile, evacuated tubes containing ethylenediaminetetraacetic acid and fecal samples collected into sterile containers were tested. DNA was extracted using commercially available kits (QIAGEN, Valencia, California) following manufacturer instructions with the exception that the final step for DNA elution was performed using 75  $\mu$ L of the elution (AE) buffer instead of the

recommended 200  $\mu$ L. A nested PCR assay targeting the 16S ribosomal deoxyribonucleic acid (rDNA) gene and producing a first round amplicon of approximately 599 base pairs (bp) and a second round amplicon of approximately 529 bp was performed as described.<sup>35</sup> Gel electrophoresis of the second round PCR product was performed using a 1.5% agarose gel and ethidium bromide staining for detection of DNA.

### 2.3.3 | Detection of *C. difficile* and *C. perfringens* toxins

A single commercially available ELISA was used to detect toxins A and B produced by *C. difficile* (TECHLAB, Blacksburg, Virginia) and enterotoxin produced by *C. perfringens* (TECHLAB, Blacksburg, Virginia). Both ELISAs were performed according to the manufacture's recommendations with either freshly collected fecal samples or with fecal samples that had been stored for  $\leq$ 48 hours at 4°C. The optical density (OD) of each well was assessed with a microplate reader at 450 nm within 10 minutes of applying the stop solution. Negative samples were defined as those that had a spectrophotometer reading with an OD450 less than 0.12.

### 2.3.4 | Salmonella fecal culture

Fecal samples submitted for isolation of *Salmonella* spp. were directly plated onto MacConkey and brilliant green (BG) agar plates and incubated for 24 hours at 35 to 37°C. An approximately 1 g sample was also inoculated into 10 mL of selenite broth and, after incubation for 24 hours at 35 to 37°C, the broth was subcultured onto BG agar plates. Lactose-negative colonies on agar plates were tested for agglutination with *Salmonella* spp. somatic antigen polyvalent antisera, and colonies positive for agglutination were further confirmed to be *Salmonella* spp. by use of an automated biochemical identification

system. *Salmonella* spp. isolates were serotyped by means of antigen testing, using a panel of antisera directed against somatic and flagellar salmonella antigens.

## 2.4 | Data analysis

Rates of detection are expressed as percentages of positive results for 1 or more PEP or PEP-T from each laboratory. Pearson correlation coefficients and associated *P*-values were computed for all pairwise PEP and PEP-T presence/absence outcome variables. Dependence of survival to discharge on detection of 1 or more PEP or PEP-T for hospitalized horses was assessed with a chi-square test. To compare duration and cost of hospitalization between horses with 0, 1, or > 1 positive enteric disease panel result, both parameters were first assessed for normality with the Shapiro-Wilk test, and subsequently analyzed using a Kruskal-Wallis test. All statistical analysis were performed using either R<sup>36</sup> or SigmaStat (Systat Software, San Jose, California).

## 3 | RESULTS

### 3.1 | IDEXX Laboratories enteric disease panel results

Of 3753 fecal samples submitted to IDEXX Laboratories for enteric disease panel testing from 2010 to 2014, 31.3% yielded a positive result with 23.5%, 6.2%, 1.3%, and .3% having 1, 2, 3, or 4 positive results, respectively. Percentage of positive results were similar for all years (Table 1). Detection of both *C. difficile* toxin A and B genes (*tcdA* and *tcdB*) from the same sample was the most strongly correlated result. Additional pairwise comparisons were also significantly associated but correlation coefficients were low (.11 or less; Table 2).

**TABLE 1** Detection rates (%) for each potential enteric pathogen (PEP) or potential enteric pathogen toxins (PEP-T), rate of detection of multiple agents, and total number of fecal samples collected from horses  $\geq$ 6 months of age by year for samples submitted to IDEXX Laboratories

%	2010	2011	2012	2013	2014	All years
<i>Clostridiodes difficile</i> toxin A gene ( <i>cdtA</i> )	3.6	3.0	3.8	2.3	4.0	3.3
<i>Clostridiodes difficile</i> toxin B gene ( <i>cdtB</i> )	3.2	2.9	3.6	2.5	3.9	3.2
<i>Clostridium perfringens</i> alpha toxin	0.7	1.0	9.3	7	8.2	5.2
<i>Neorickettsia risticii</i>	5.4	3.6	4.2	4.4	2.2	4.0
<i>Salmonella</i> spp.	6.2	3.9	5.0	6.8	6.6	5.7
<i>Lawsonia intracellularis</i>	2.1	2.1	0.9	1.5	0.5	1.4
<i>Rhodococcus equi</i>	1.0	1.8	0.6	0.8	0.6	1.0
Equine coronavirus	5.4	8.2	6.0	5.4	5.3	6.1
Rotavirus	1.5	2.5	2.7	1.8	3.4	2.4
<i>Cryptosporidium</i> spp.	11	12	7.8	5.9	6.6	8.7
Overall PEP or PEP-T detection rate	28	33	33	30	32	31.2
Detection rate of multiple agents	8.0	7.2	8.7	7.5	8.0	7.9
Total # of submissions	276	559	665	914	1339	3753

**3.2 | MSUVDL enteric disease panel results**

Of 239 horses admitted to MSUVMC for treatment of acute colitis from 2007 to 2014 that had blood and feces submitted to MSUVDL for enteric disease panels, 60.7% (n = 145) of samples yielded positive results. Of these, 117 yielded 1 positive result, 26 yielded 2 positive results, and 2 yielded 3 positive results (Table 3).

**3.3 | Outcome of horses with colitis**

Overall, 197 of 239 (82%) horses survived to hospital discharge. Survival was lowest for horses from which 1 PEP or PEP-T was detected, as compared to horses from which either no pathogen or multiple pathogens were detected (Table 4). No difference was detected in duration of hospitalization ( $P = .78$ ) or cost of hospitalization ( $P = .25$ ).

**TABLE 2** Correlation coefficients ( $\rho$ ) and  $P$ -values for codetection of potential enteric pathogens in horses  $\geq 6$  months of age by IDEXX Laboratories

$\rho$ $P$ -value	cdtA	cdtB	<i>C. perf</i>	Coronavirus	PHF	<i>Salmonella</i> spp.	<i>Lawsonia</i> spp.	<i>R. equi</i>	Rota	Crypto
<i>C. diff</i> A	1	<b>.92</b>	<b>.07</b>	-.02	-.02	-.09	-.01	-.02	-.02	0
		<.0001	<.0001	.22	.24	<.0001	.68	.30	.24	.89
<i>C. diff</i> B	<b>.92</b>	1.0	<b>.08</b>	-.03	-.02	-.09	-.01	-.02	-.02	-.01
	<.0001		<.0001	.10	.25	<.0001	.69	.30	.25	.65
<i>C. perf</i>	<b>.07</b>	<b>.08</b>	1.0	-.01	<b>.11</b>	<b>.09</b>	<b>.06</b>	0	-.01	-.01
	<.0001	<.0001		.38	<.0001	<.0001	<b>.0002</b>	.99	.44	.55
Coronavirus	-.02	-.03	-.01	1.0	-.05	-.03	<b>.03</b>	-.01	0	0
	.21	.10	.38		<b>.003</b>	.05	<b>.04</b>	.51	.88	.80
PHF	-.02	-.02	<b>.11</b>	-.05	1.0	0	-.01	-.02	-.01	.01
	.24	.25	<.0001	<b>.003</b>		.90	.61	.28	.48	.56
<i>Salmonella</i>	<b>.09</b>	<b>.09</b>	<b>.09</b>	-.03	0	1.0	<b>.07</b>	0	-.01	-.02
	<.0001	<.0001	<.0001	.05	.89		<.0001	.88	.59	.36
<i>Lawsonia</i>	-.01	-.01	<b>.06</b>	<b>.03</b>	-.01	<b>.07</b>	1.0	-.01	0	-.01
	.68	.69	<b>.0002</b>	<b>.04</b>	.61	<.0001		.53	.92	.40
<i>R. equi</i>	-.02	-.02	0	-.01	-.02	0	-.01	1.0	.02	<b>.05</b>
	.30	.30	.99	.51	.28	.88	.92		.15	<b>.002</b>
Rota	-.02	-.02	-.01	0	-.01	-.01	0	.02	1.0	-.03
	.24	.25	.44	.88	.48	.59	.92	.15		.05
Crypto	0	-.01	-.01	0	.01	-.02	-.01	<b>.05</b>	-.03	1.0
	.89	.65	.55	.80	.56	.36	.40	<b>.002</b>	.05	

Note: *Clostridioides difficile* toxin A and B genes are denoted as cdtA and cdtB, *Clostridium perfringens* enterotoxin gene is denoted as *C. perf*, *Neorickettsia risticii* is denoted as PHF (Potomac Horse Fever), *Rhodococcus equi* is denoted as *R. equi*, rotavirus is denoted as Rota and *Cryptosporidium* spp. is denoted as Crypto. Codetections with  $P < .05$  are in bold font.

**TABLE 3** Detection rates (%) for each potential enteric pathogen (PEP) or potential enteric pathogen toxins (PEP-T), rate of detection multiple agents, and total number of fecal/blood samples collected from horses  $\geq 6$  months of age with acute colitis by year for samples submitted to Michigan State University Veterinary Diagnostic Laboratory

%	2007	2008	2009	2010	2011	2012	2013	2014	All years
<i>Clostridioides difficile</i> toxin ELISA	12	13	5.2	12	29	19	27	14	16.4
<i>Clostridium perfringens</i> toxin ELISA	41	19	5.2	7.7	20	19	0	0	14.0
<i>Neorickettsia risticii</i> (blood or feces)	41	40	53	46	43	22	18	39	37.8
<i>Salmonella</i> spp.	12	8.5	0	0	2.9	5.4	0	3.6	4.1
Overall PEP or PEP-T detection rate	82	70	63	50	69	51	36	50	61.0
Detection rate of multiple agents	24	11	0	0	23	14	9.1	7.1	11.7
Total # of submissions	17	47	38	26	35	37	11	28	239

Note: A single ELISA was used to detect *C. difficile* toxin A and B and *C. perfringens* enterotoxin.

**TABLE 4** Outcome (survival to discharge), median days of hospitalization, and median cost of treatment (US\$) for 239 horses  $\geq 6$  months of age presented to Michigan State Veterinary Medical Center for treatment of colitis from which either no, 1, or more than 1 potential enteric pathogen or potential enteric pathogen toxins were detected on the enteric disease panel submitted to the Michigan State University Veterinary Diagnostic Laboratory

	0 positive	1 positive	>1 positive	P value
Number of horses	94	117	28	na
Survival to discharge (%)	88% <sup>a</sup>	76% <sup>b</sup>	89% <sup>a</sup>	.04
Days of hospitalization median (range)	4 (1-17)	4 (1-33)	4 (1-30)	.78
Cost of hospitalization (\$) median (range)	2357 (1110-15 353)	2742 (788-11 005)	2560 (1091-10 895)	.25

Note: Values with different superscript letters are different ( $P < .05$ ).

between the 3 groups. For horses from which only 1 pathogen was detected, there was no difference in survival ( $P = .52$ ) between horses that tested positive for *Salmonella* spp. (8/10, 80%), *N. risticii* (71/90, 79%), *C. difficile* toxins (31/37, 84%), or *C. perfringens* enterotoxin (22/34, 82%).

## 4 | DISCUSSION

This study found substantial variation in detection of PEP and PEP-T from feces/blood collected from horses with enteric disease between 2 laboratories, ranging from 31% for detection of 1 or more agents in feces using a panel of real-time PCR tests to a 61% detection rate using multiple tests of feces and blood in a group of horses admitted to a hospital for treatment of acute colitis. This difference is likely due to several factors including case selection, time of year, region of the country, and tests performed. For example, samples submitted to IDEXX Laboratories were collected from horses with varying complaints (eg, fever of undetermined origin, diarrhea, or other suspected GI disease) from across the United States, while samples submitted to MSUVDL were collected from hospitalized horses with colitis in the Midwest. The discrepancy in positive test result rates can further be explained by differences in study populations which can have notable effects on test sensitivity, specificity, and positive and negative predictive values.<sup>37</sup> Samples evaluated by MSUVDL were collected from horses admitted to a tertiary care facility for treatment of acute colitis. Detection of *N. risticii* was common in Midwest horses hospitalized for treatment of colitis in summer months. Further, the 2 diagnostic laboratories performed different tests to detect PEP or PEP-T. IDEXX Laboratories's enteric disease panel uses real time PCR to detect genes of PEP and PEP-T. Although a sensitive testing methodology, detection of genetic material does not indicate that viable organisms are present or that transcription of toxin genes are "turned on" and producing toxins. In contrast, MSUVDL uses an ELISA to detect *C. difficile* and *C. perfringens* toxins in fecal water. If toxins are detected, toxin genes must be present. Similarly, MSUVDL performed enriched bacterial culture methods to detect viable *Salmonella* spp. which also allowed performance of antimicrobial susceptibility testing and serovar determination. Thus, appropriate interpretation of a positive result requires an understanding of test methodology and must always be considered in light of clinical findings of the animal

(or environment) sampled. Next, regardless of the enteric disease panel used to detect PEP and PEP-T, a large percentage of samples still yielded negative results. Although negative results are frustrating for both clinicians and animal owners, they serve as a reminder that management factors (eg, diet, exercise, travel, etc), intestinal parasites, and other disorders (eg, inflammatory bowel disease, neoplasia, etc) need to be considered during evaluation of horses with enteric disease. In addition, negative results could also be attributable to emerging pathogens and limitations of current testing. For example, several horses with acute colitis attributed to *N. risticii* infection in Ontario had negative PCR results in blood and feces using conventional primers for *N. risticii*.<sup>38</sup>

Detection of more than 1 PEP or PEP-T in horses hospitalized for colitis was not associated with a poorer outcome (lower survival), longer hospitalization, and greater treatment cost in comparison to horses in which either no or only a single agent was detected. Horses from which only 1 PEP or PEP-T was detected were less likely to survive to discharge (76%). However, survival of horses from which more than 1 agent was detected (89%) was not different from horses with negative enteric panel testing results (88%). Further, poor outcome (nonsurvival) of horses with colitis might be more likely be a consequence of development of complications (ie, laminitis with *N. risticii*) than lack of resolution of enteric disease. Thus, comparison of survival rates based on number of PEP and PEP-T detected might have limited relevance.

In addition to reporting detection rates for 1 or more PEP and PEP-T using equine enteric disease testing panels, another impetus for our study was the challenge of test interpretation when multiple positive results are reported for sick horses. As an example, multiple pathogens were detected in 42% of 51 Thoroughbred foals ( $\leq 17$  weeks of age) with enteric disease.<sup>32</sup> However, a single PEP or PEP-T was also detected in 37% of a comparison group of 50 healthy foals, with "coinfections" detected in 11% of healthy foals. Although the rate of detection of multiple agents was higher in sick than in healthy foals, there was no impact on survival as only 1 foal with enteric disease did not survive. Further, a high rate of pathogen detection in healthy foals supports the concept that detection might not be the same as infection. Similar findings were reported by another group in 2016, which found coinfections in 46% of foals with diarrhea and 33% of foals without diarrhea.<sup>39</sup> Of note, since both groups used molecular based techniques to identify nucleic acids associated with

PEP and PEP-T, codetection would be a more appropriate term than coinfection. At MSUVMC we have found a number of horses with colitis due to *N. risticii* to also have positive ELISA results for *C. difficile* or *C. perfringens* toxins. This finding led to speculation that disruption of normal enteric flora during infection with *N. risticii* could lead to upregulation of toxin production by *Clostridium* spp. Further, if resident *Clostridium* spp. started to produce toxins during clinical disease due to *N. risticii*, these toxins could pose a further insult to the intestinal barrier, prolonging disease course and increasing case fatality rate. Codetection of *N. risticii* and *Clostridium* spp. toxins also raised the question of whether addition of metronidazole to the treatment regimen should be considered, although the latter drug could also have adverse effects. Although neither a significant association of detecting both *N. risticii* and *Clostridium* spp. toxins nor an increased risk of death with detection of more than 1 PEP or PEP-T was found in our cohort of horses with colitis, these questions remain unanswered.

When clinicians submit samples for enteric disease panels, which is typically a more economic approach than a barrage of individual tests, it is important to understand the information that might be returned. For example, detection of a *Salmonella* spp. by real-time PCR does not provide further information that can be obtained from a positive fecal bacterial culture result. Specifically, the latter can be used to determine a *Salmonella* spp. serovar and antimicrobial susceptibility profile, important data for investigation of suspected nosocomial or zoonotic spread of salmonellosis. Recognizing these limitations, IDEXX Laboratories has added a *Salmonella* spp. fecal culture for all PCR positive tests without additional charge. Next, detection of *Clostridium* spp. toxin DNA by real-time PCR is different than detection of toxins in fecal water by ELISA. The former only indicates that the potential for toxin production exists while the latter documents presence of toxin in feces. The use of a technical cut-off for reporting positive results based on crossing point by in the real-time PCR run at IDEXX Laboratories might improve the correlation between detection of the toxin gene and the production of toxins but is not expected to uniformly predict active toxin production. Further, detection of a PEP that is not commonly considered a pathogen in nonneonate or weanling horses (eg, *L. intracellularis*, *Cryptosporidium* spp., *R. equi*, or rotavirus) can result in confusion, and possibly lead to unnecessary or inappropriate treatment. Thus, it is imperative that test results are interpreted in light of signalment, history, and other clinical findings.

Detection of PEP and PEP-T in this study by both laboratories was higher than the rate of 7.7% previously reported in 1 study.<sup>4</sup> This low detection rate was reported as unpublished data from a retrospective study of 156 horses and information regarding fecal testing was not provided. In the cases that were subsequently studied prospectively by these authors, 22% and 19% of horses hospitalized for colitis tested positive for *C. difficile* and *C. perfringens* toxins, respectively, by ELISA.<sup>4</sup> These results are similar to our findings in hospitalized colitis patients in which 15% (36/239) and 14% (34/239) had positive ELISA results for *C. difficile* and *C. perfringens* toxins, respectively, of which 5% (10/239) tested positive for both *C. difficile* and *C. perfringens* toxins. Finding both *C. difficile* toxin A and B was the most common codetection in feces tested by real-time PCR by IDEXX

Laboratories. Other codetections had significant *P*-values (Table 2), but only moderate to weak correlation coefficients make clinical importance uncertain.

Enteric disease associated with *C. difficile* is most commonly recognized in association with antimicrobial therapy in both human patients and equids.<sup>40</sup> Previously, *C. difficile* was not considered a component of the normal GI flora in horses; however, a 2011 study identified *C. difficile* by fecal culture in 8% of horses from a healthy population.<sup>41</sup> Toxin production by *C. difficile* is necessary for clinical disease but only 75% of *C. difficile* isolates produce toxins,<sup>42,43</sup> and therefore up to 25% of *C. difficile* cultured from horse feces might not be relevant to clinical disease. Results of this study were based either on detection of *C. difficile* toxins A and B DNA via real-time PCR or actual toxins via a commercially available ELISA, not culture results. Detection of clostridial toxin(s) in feces is generally accepted as a positive result (not a laboratory error),<sup>44,45</sup> but degradation of toxins after collection of feces can cause false-negative results<sup>41</sup> that could lead to underestimation of infection rates.<sup>44,45</sup> Additionally, current tests only screen for presence of *C. difficile* toxins A and B, however, other toxins (binary toxin) might also be important in causing disease. Failure to test for all potential toxins could lead to decreased ability to identify horses with *C. difficile* associated colitis. Similarly, *C. perfringens*  $\beta$ 2- and NetF-toxigenic strains have also been identified in feces of horses with enteric disease; thus, as with *C. difficile* associated colitis, *C. perfringens* associated colitis might still be underdiagnosed.<sup>46-48</sup>

The IDEXX Laboratories enteric disease panel tests feces for the presence of *L. intracellularis*, *Cryptosporidium* spp., *R. equi*, and rotavirus, agents which, although associated with disease in foals, generally do not cause clinical signs in horses  $\geq 6$  months of age. In fact, *Cryptosporidium* spp. was the most common PEP detected in feces of horses  $\geq 6$  months of age over all years, yet the clinical importance of this finding is unknown. Previous studies have found *C. parvum* in equine perinatology units<sup>27</sup> and *Cryptosporidium* spp. oocysts have been identified in up to 50% of equine fecal samples submitted to a diagnostic laboratory for fecal floatation.<sup>28</sup> While implications of identifying *Cryptosporidium* spp. in horses  $\geq 6$  months of age remains unknown, detection could be of concern for zoonotic disease.<sup>27</sup> Detection of PEP that appear to be nonpathogenic in horses  $\geq 6$  months of age could result in confusion and unnecessary, and perhaps detrimental, treatment with antimicrobial agents.

This retrospective study had several limitations. First, clinical complaints prompting fecal sample submission for the enteric disease panels offered by IDEXX Laboratories and outcome data were not available. Although one would assume that enteric disease panels were performed on fecal samples collected from horses with signs of GI disease, this might not always have been the case (eg, fever of undetermined origin). Further, lack of outcome data from the IDEXX Laboratories submissions makes interpretation of the clinical importance of detecting 2 or more PEP or PEP-T impossible. Second, performing a single fecal bacterial culture or PCR for identifying *Salmonella* spp. might have led to underestimation of either fecal shedding of *Salmonella* spp. or clinical salmonellosis, as it generally



recommended that 3 or more fecal samples should be tested to improve sensitivity of detecting *Salmonella* spp. in equine feces.<sup>49,50</sup> Finally, our review of medical records of horses with colitis at MSUVMC did not clearly reveal whether nonsurvival was due to ongoing enteric disease or development of complications. Consequently, whether detection of more than 1 PEP and PEP-T affected the severity of colitis was unable to be accurately determined.

In conclusion, we found substantially different detection rates for PEP and PEP-T between the 2 enteric disease panels, most likely due to different study populations tested. Finding both *C. difficile* toxins A and B was the most common codetection with enteric disease testing of horses  $\geq 6$  months of age; however, this finding likely indicated dual toxin production by the same organism, rather than infection with separate organisms. Contrary to our hypothesis, detection of more than 1 PEP or PEP-T was not associated with increased death, longer hospitalization, or a greater cost of treatment; however, this conclusion was based on a small cohort of horses and limitations of medical records. Although submission of a real-time PCR enteric disease panel testing for multiple potential pathogens and toxins at 1 time can be a more economic approach to detect PEP and PEP-T in horses with GI disease, results must be interpreted in light of signalment and clinical signs, particularly when positive results are returned for PEP with unknown clinical importance in horses  $\geq 6$  months of age. Further, there are limitations of detecting only PEP or PEP-T DNA, as compared to finding a *Clostridium* spp. toxin by ELISA or isolating a *Salmonella* spp. by bacterial culture. Next, it warrants emphasis that finding more than 1 PEP or PEP-T in feces should be termed “codetection” rather than “coinfection” and careful assessment of the horse is required to determine if all PEP or PEP-T detected warrant treatment. In addition, detection of a PEP or PEP-T does not necessarily imply causation, as some PEPs and PEP-Ts can be detected in apparently healthy horses. Finally, despite casting a wider net to identify PEP and PEP-T by performing enteric disease panels, a substantial number of samples yielded negative results. Although some negative results may be attributed to limitations of testing methodology, this finding also supports the concept that enteric disease in individual horses is multifactorial in origin and finding a definitive etiological cause can be elusive. Nevertheless, testing for PEP and PEP-T remains an important diagnostic tool, especially for investigation of outbreaks of enteric disease.

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#### CONFLICT OF INTEREST DECLARATION

M. Alexix Seguin is an employee of IDEXX Laboratories, Inc. The remaining authors do not have any conflict of interest to declare.

#### OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

#### INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

#### HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

#### ORCID

Jamie J. Kopper  <https://orcid.org/0000-0001-6829-7224>

Harold C. Schott II  <https://orcid.org/0000-0002-7728-5409>

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