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Performance of commercial PCR assays to detect toxigenic *Clostridioides difficile* in the feces of puppies

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Abstract

Clostridioides difficile is an important enteric pathogen that causes significant morbidity and mortality in humans. With community-acquired infections on the rise, it is important to identify reservoirs of the pathogen. Companion animals can be asymptomatic carriers of *C. difficile* and may therefore represent a reservoir, but epidemiological studies of *C. difficile* within the pet-owner unit are needed, along with validated methods to detect *C. difficile* in both people and animals. The goal of this study was to assess the performance of commercial qPCR assays and a multiplex PCR for *C. difficile* compared to toxigenic culture. These assays were tested on up to 103 fecal samples from puppies, a population in which the prevalence of *C. difficile* is the highest. The sensitivities, specificities, positive predictive values and negative predictive values were respectively 84.2%, 87.7%, 61.5%, and 95.9% for the Cepheid GeneXpert; 66.7%, 66.7%, 29.6%, and 90.9% for the DiaSorin Simplexa; and 94.4%, 85.0%, 65.4%, and 98.1%, for the multiplex qPCR. The agreement was highest between the GeneXpert and the multiplex PCR (90.1% agreement, with a kappa statistic of 0.77). For diagnostic purposes, the positive predictive values of the assays were low. However, the high sensitivities of the assays could render them useful for epidemiologic purposes.

KEYWORDS
Clostridioides difficile, dogs, feces, PCR, validity

1 | INTRODUCTION

Clostridioides difficile is a spore-forming anaerobic, Gram-positive bacillus that is the leading cause of antibiotic-associated and nosocomial diarrhea in humans and a significant enteric pathogen in many species of animals. With community-acquired infections on the rise (Eyre et al., 2013; Khanna et al., 2012), there is increasing interest in identifying reservoirs of the pathogen. Companion animals were considered potential reservoir species as early as 1983 (Borriello et al., 1983), and the prevalence of toxigenic *C. difficile* in canine feces has been reported to range from 1.2% to 40% (Alvarez-Perez et al., 2017; Clooten et al., 2008; Orden et al., 2017; Sokolow et al., 2005; Stone et al., 2016). While no studies have demonstrated the direct transmission of *C. difficile* between pets and their owners, one study found that two of nine (22%) cats and two of five (40%) dogs belonging to owners who had experienced *C. difficile* infection (CDI) carried *C. difficile* isolates with identical pulsed-field gel electrophoresis profiles as their owners, suggesting that transmission between people and their pets may occur (Loo et al., 2016). However, more epidemiological studies of the distribution of *C. difficile* in pet owners and their pets are required to further

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explore this hypothesis, and validated methods to detect the pathogen in both people and their pets are necessary.

While anaerobic culture remains the gold standard for detecting *C. difficile*, it is not widely available, requires specialized equipment, and is labor-intensive, technically difficult, and time consuming (Dionne et al., 2013; Kufelnicka & Kirn, 2011). Moreover, isolation protocols validated for use in human stool may be less effective in canine stools where competing microbial communities may differ from those found in human feces (Tenover et al., 2011). Indeed, Blanco et al. (2013) showed that conventional culture protocols yielded many false negatives in swine. Antigen enzyme immunoassays (EIA), which detect *C. difficile* toxins in stool and are more often used in clinical veterinary medicine, have been shown to have poor sensitivity in people (Planche et al., 2008), dogs (Chouicha & Marks, 2006), and swine (Keessen et al., 2011), and are therefore of limited utility for epidemiological purposes.

In contrast, nucleic-acid amplification tests for *C. difficile*, which are used routinely in human medicine (Burnham & Carroll, 2013), have been found to be highly sensitive and specific in swine (Avbersek et al., 2011) and cattle (Houser et al., 2010). These assays generally detect toxin genes and are therefore useful for differentiating toxigenic from nontoxigenic strains. Moreover, cycle thresholds (Ct) in qPCR assays have been found to be highly correlated with bacterial load (Dionne et al., 2013). The PCR Ct value is inversely related to the quantity of target nucleic acid in the sample, with lower Ct values reflecting higher bacterial load. Thus, colonized animals (i.e., asymptomatic carriers) could potentially be distinguished from infected animals (i.e., clinically diseased) (Chachaty et al., 1993; Dionne et al., 2013). Commercial qPCR assays are used routinely in human medicine; however, their performance in the stool of companion animals is unknown. The aim of this study was to assess the performance of commercial qPCR assays for *C. difficile* relative to toxigenic culture and to a multiplex qPCR validated for use in canine feces. If the performance of these assays is high in both human and canine feces, their use can be recommended for epidemiological studies of *C. difficile* within the pet-owner unit.

2 | MATERIALS AND METHODS

2.1 | Samples

Fecal samples were obtained from pet owners bringing their puppies to the pediatric service of our veterinary hospital and from breeders who collected fecal samples from their puppies and shipped them on ice overnight to the laboratory. Puppies (<6 months of age) were chosen instead of adult dogs because, as in other species, the youngest animals have the highest prevalence of *C. difficile* (Alvarez-Perez et al., 2015; Buogo et al., 1995; Perrin et al., 1993) and are therefore most likely to represent a potential reservoir. All animals were healthy at the time of sampling. Institutional Animal Care and Use (IACUC) approval was deemed unnecessary by the IACUC of the University of Pennsyl-

vania, as freshly voided fecal samples were routinely collected by the owners/breeders.

After collection, samples were split into sterile cryogenic vials. One aliquot was processed for culture within 24 hr, while others were stored at -80°C and processed subsequently in batch for the qPCR analyses. Frozen samples were thawed only once prior to processing.

2.2 | Anaerobic culture and toxigenic testing

Upon arrival at the microbiology laboratory, 0.5 g of formed fecal sample was mixed with 0.5 mL of 100% ethanol. The mixture remained for 60 min at room temperature before being inoculated on Cycloserine-cefoxitin fructose modified agar (CCFA) (Remel™) or *Clostridium difficile* Selective Agar (BBL™) and Columbia Naladixic Acid (CAN) agar (Thermo Fisher Scientific Remel Products). Inoculated plates and broth were incubated in BD Gas-Pak™ EZ container systems with BD BBL™ CO_2 generators and BD BBL™ Gas Pak™ anaerobic CO_2 indicators (Franklin Lakes, NJ) at 36 ± 2°C under anaerobic growth conditions for 7 days and checked for growth every other day. Suspect colonies were identified and isolated. Isolates were confirmed to be *C. difficile* by Maldi-TOF identification and/or RapID ANA II System (Thermo Fisher Scientific Remel Products).

Confirmed isolates of *C. difficile* were inoculated into Brain Heart Infusion (BHI) broth and/or cooked meat broth to induce toxin production. The broth was incubated anaerobically at 36 ± 2°C for 48 hr then centrifuged at $13{,}400 \times g$ for 2 min. The supernatant was collected, and a 50 μL liquid specimen was tested by TechLab *C. difficile* Tox A/B II EIA (TechLab). Indeed, while not recommended as a standalone test to detect *C. difficile*, EIAs can be useful in differentiating toxigenic organisms from non-toxigenic organisms when used on pure cultures (She et al., 2009).

2.3 | Commercial qPCR assays

The GeneXpert *C. difficile*/Epi tcdB qPCR assay (Cepheid) and the Simplexa *C. difficile* Universal Direct (DiaSorin) are two commercially available assays in the United States that detect the *tcdB* (toxin B) gene directly in a stool sample. The GeneXpert assay also detects the binary toxin genes (*cdtA* and *cdtB*) and has a callout for ribotype NAP1/B1/027.

Fecal samples were analyzed with these assays according to the manufacturer's protocol on a GeneXpert Infinity System and a Liaison MDX System, respectively. Both assays are DNA-extraction less and are run for 40 thermal cycles. Cycle threshold (Ct) values for each sample were recorded for both assays. According to the manufacturer, the sensitivity and specificity of the Simplexa assay in human stool compared to direct toxigenic culture are 90.1% (95% CI: 83.8–94.1%) and 93.0% (91.0–94.5%), respectively, while those for the GeneXpert assay are 93.5% (95% CI: 90.3–95.9%) and 94.0% (95% CI: 92.9–95.0%), respectively.

TABLE 1 Oligonucletides used for the multiplex qPCR

Prime/Probe	Sequence 5'-3'	Amplicon size (bP)
tcdA-F	TTCAAGCAGAAATAGAGCACTC	166
tcdA-R	TATCAGCCCATTGTTTTATGTATTC	
tcdA-probe	FAM-TCACTGACTTCTCCACCTATCCATACAA-BHQ	
tcdB-F	GGTATTACCTAATGCTCCAAATAG	86
tcdB-R	TTTGTGCCATCATTTTCTAAGC	
tcdB-probe	HEX-ACCTGGTGTCCATCCTGTTTCCCA-BHQ	

2.4 | Multiplex qPCR

This assay was adapted from the method described by Houser et al. (2010) for the detection of the *C. difficile* gene *tcdB* (toxin B). This assay also detects the gene *tcdA* encoding toxin A, but only the results for *tcdB* will be presented here. The sequences of the primers and probes (IDT) are listed in Table 1. Briefly, DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions. Then, 5 μL DNA was added to 20 μL master mix consisting of tcdA and tcdB forward and reverse primers (final concentration of 500 nM of each tcdA primer and 300 nM of each tcdB primer), respective probes at a final concentration of 200 nM each and 12.5 μL Taq-Man Universal PCR master mix (Thermo Fisher Scientific). A set of primers and probe (VetMax Xeno Internal Positive Control (IPC)-LIZ assay; Thermo Fisher Scientific) was also added to the assay to detect an exogenous DNA control (VetMax Xeno IPC DNA; Thermo Fisher Scientific), which was spiked into all the samples during the extraction process to monitor for the presence of PCR inhibitors and confirm the efficiency of sample preparation and extraction. The assay was run on the Applied Biosystems 7500 fast real-time PCR System (Thermo Fisher Scientific) with the following temperature cycle conditions: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min.

2.5 | Data analysis

The sensitivity, specificity, positive predictive value, and negative predictive value of the commercial and multiplex assays relative to detection of toxins from culture by EIA were calculated. Additional sensitivity analyses were performed to assess whether the performance of the qPCR assays changed when considering a negative gold standard to be a toxin-negative result versus a culture-negative result. Percent agreement and Cohen kappa statistics were calculated to compare the qPCR assays. All calculations were performed in Stata (StataCorp, College Station, TX).

3 | RESULTS

A total of 103 canine fecal samples were collected from puppies ranging in age from 3 to 12 weeks of age. Seventy-six of the samples were

from 13 different litters of pre-weaned puppies, and 27 samples were obtained from weaned privately owned puppies. Thirty-seven (35.9%) samples were *C. difficile* culture-positive, including 19 (51.4%) toxigenic and 18 (48.6%) non-toxigenic strains.

3.1 | Commercial qPCR assays

A total of 100 samples were successfully analyzed using the GeneXpert assay (3 were dropped because of error). The sensitivity, specificity, positive predictive value, and negative predictive value of the GeneXpert assay was 16/19 (84.2%), 71/81 (87.7%), 16/26 (61.5%), and 71/74 (95.9%), respectively (Table 2). The three culture/toxin-positive samples that tested negative on qPCR had Ct values of 37.4, 37.9, and 38.2, which were very close to the threshold set by the manufacturer for declaring a positive (37.0). In fact, if the threshold for declaring a positive was increased by 2 cycles (i.e., 39), the sensitivity of the assay increased to 100% while the specificity decreased to 76.5%. Of the 10 samples that tested positive on qPCR but were culture or toxin-negative, six (60%) were culture negative and four (40%) were culture positive/toxin negative. No particular trend in cycle threshold was identified for these samples, with values ranging from 28.5-36.9. None of the samples tested positive for the binary toxin, and none had a presumptive callout for ribotype 027.

A total of 92 samples were successfully analyzed using the DiaSorin assay (3 were dropped because of reading error, insufficient sample was available to run the remaining 9). The sensitivity, specificity, positive predictive value, and negative predictive value of the DiaSorin assay were 10/15 (66.7%), 50/75 (66.7%), 10/35 (29.6%), and 50/55 (90.9%), respectively (Table 2).

3.2 | Multiplex qPCR

A total of 80 samples were analyzed using the multiplex qPCR assay (2 dropped because of reading error, insufficient sample was available to run the remaining 19). The sensitivity, specificity, positive predictive value, and negative predictive value of the multiplex assay for *tcdB* was 17/18 (94.4%), 51/60 (85.0%), 17/26 (65.4%), and 51/52 (98.1%), respectively (Table 2). Of the nine samples that tested positive on PCR for the *tcdB* gene but negative on toxigenic culture, four (44.4%) were culture-positive but toxin-negative.

TABLE 2 Validity of PCR assays for *C. difficile* relative to toxigenic culture

Assay	Cepheid GeneXpert®		DiaSorin Universal Direct		Multiplex PCR – *tcdB*	
	Gold standard results					
PCR Results	Culture and toxin positive	Culture negative OR toxin negative	Culture and toxin positive	Culture negative OR toxin negative	Culture and toxin positive	Culture negative OR toxin negative
PCR-positive (n)	16	10	10	25	17	9
PCR-negative (n)	3	71	5	50	1	51
Sensitivity (%)	84.2		66.7		94.4	
Specificity (%)	87.7		66.7		85.0	
Positive predictive value (%)	61.5		29.6		65.4	
Negative predictive value (%)	95.9		90.9		98.1	

TABLE 3 Agreement between different PCR assays for *C. difficile* in canine feces

Assay	Percent agreement/kappa statistic	
DiaSorin Universal Direct	–	
Cepheid GeneXpert®	63.6 / 0.18	–
Multiplex PCR Toxin B	72.2 / 0.39	90.1 / 0.77
	DiaSorin Universal Direct	Cepheid GeneXpert®

3.3 | Sensitivity analyses

To account for the reduced sensitivity of the EIA, we repeated these analyses considering a negative result by the gold standard method to be a culture-negative result rather than a negative culture or a toxin-negative culture-positive result. The sensitivity, specificity, positive predictive and negative predictive values were respectively 76.9%, 77.0%, 54.0%, and 90.5% for the GeneXpert; 54.3%, 80.0%, 63.3%, and 73.3% for the DiaSorin; and 80.8%, 85.7%, 72.4%, and 90.6% for the multiplex qPCR.

3.4 | Agreement between qPCR assays

The agreement between the DiaSorin and the GeneXpert assays was poor, at 63.6% agreement with a kappa statistic of 0.18 (Table 2). The agreement between the GeneXpert and the multiplex PCR for *tcdB* was 90.1%, with a kappa statistic of 0.77 (Table 3), indicating substantial agreement (Cohen, 1960). The agreement between the DiaSorin assay and the multiplex qPCR was 72.2% with a kappa statistic of 0.39, indicating fair agreement (Cohen, 1960) (Table 3),).

4 | DISCUSSION

We demonstrated that commercially available tests to detect *C. difficile* toxins have varying performance in canine feces. The GeneXpert assay had relatively high sensitivity and specificity, though still markedly lower than the performance in human stool, which is high in both infected symptomatic and colonized asymptomatic patients (Bai et al., 2017; Nissle et al., 2016). Interestingly, if the cycle threshold at which a positive result was declared was increased, the sensitivity and negative predictive values would have risen to 100%, suggesting that this assay could potentially be useful for epidemiological studies in canine populations if used "off-label." The performance of the DiaSorin assay was significantly lower in canine feces than in human stool, and lower than that of the GeneXpert assay in canine feces. It is unclear why this was the case. However, this finding is consistent with findings of another study, where a commercial qPCR was found to have much lower sensitivity and specificity in swine feces than in human feces (Keessen et al., 2011). It has been suggested that the reduced performance of these tests in animal feces could be due to inhibitors in the animal feces that interact with the DNA or interfere with the polymerase (Keessen et al., 2011), but further research is needed to test this hypothesis.

Discordant results were found relative to the gold standard. Notably, there were many more samples that were toxigenic culture-negative and PCR-positive than vice-versa, almost half of which were culture-positive but toxin-negative. There are several potential explanations for this finding. The organisms could have been dead or experienced a sub-lethal injury that precluded production of viable spores for the recovery phase of culture. The poor sensitivity of the EIA may have limited detection of toxin; however, the reduced performance of the qPCR assays when considering only *C. difficile* culture-negative results shows that the added information provided by the EIA is valuable, even if incomplete. It is also possible that the sensitivity of the gold standard (i.e., toxigenic culture) is actually lower than that of PCR (Gumerlock et al., 1991), especially given the lack of consensus on best methods for recovery of *C. difficile* in culture (Burnham & Carroll, 2013). In our study, samples underwent alcohol shock prior to incubation to select for clostridial spores in the feces, but other methods have been described, including heat shock and broth enrichment (Arroyo et al., 2005; Blanco et al., 2013). In another study, investigators were able to successfully culture only 25 of 33 (75.8%) PCR-positive canine fecal samples (Stone et al., 2016), further suggesting that PCR may be more sensitive than anaerobic culture in canine feces.

The positive predictive values of the assays were variable, ranging from 29.6% to 65.4%. This means that, at best, there is a 65.4% chance that a sample that tests positive truly contains *C. difficile*. For clinical diagnostic purposes, this is likely to be too low, especially if the assay were to be used in a population where the prevalence of *C. difficile* is lower. The high sensitivities of the assays render them more useful for epidemiological purposes

A limitation of our study was the inability to run all 103 samples for all assays due to insufficient quantities of fecal material from every animal. The different performances of the assay could be due in part to the differences in samples used. However, because there was only a difference of 8 samples between the total number run for each assay, we expect the effect on performance to be minimal. Because we chose to use a gold standard (culture + toxin detection using EIA) that was slightly less sensitive than other reference gold standards such as the cytotoxin neutralization assay (She et al., 2009), the performance of the PCRs assays might be different than what we would have found using a different gold standard method. However, other studies have found no effect of the gold standard assay on the performance of PCR assays; therefore, we believe the overall findings and trends of our study to be valid and relevant. Finally, because our population consisted exclusively of puppies, the results may not be generalizable to more heterogeneous canine populations. However, the results demonstrated that GeneXpert assay and the multiplex qPCR can be used for epidemiological studies of *C. difficile* within the pet-owner unit.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

AUTHOR CONTRIBUTIONS

E.A. contributed to conceptualization, data curation, formal analysis, investigation, methodology, validation, and review and editing of the manuscript. A.B. contributed to the investigation and methodology. D.J.K. contributed to conceptualization, investigation, methodology, validation, and review and editing of the manuscript. L.E.R. contributed to conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, writing, review, and editing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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