Detecting *Cryptosporidium* in Stool Samples Submitted to a Reference Laboratory

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Abstract. When considering methods of detecting *Cryptosporidium* in patient samples, clinical and public health laboratories have historically relied primarily on microscopy. However, microscopy is time intensive and requires trained personnel to accurately identify pathogens that are present. Even with skilled analysts, the parasitemia level has the potential to fall below the level of detection. In addition, public health laboratories do not always receive specimens in fixatives that are compatible with the desired microscopic method. Antigen-based and molecular methods have proven to be effective at identifying *Cryptosporidium* at low levels and require less training and hands-on time. Here, we have developed and validated a real-time polymerase chain reaction (RT-PCR) laboratory-developed test (LDT) that identifies *Cryptosporidium hominis* and *Cryptosporidium parvum*, and also includes detection at the genus level to identify additional species that occasionally cause disease in humans. Results of the molecular test were compared with those obtained from modified acid-fast microscopy, immunofluorescent microscopy, an antigen-based detection rapid test, and a commercial gastrointestinal panel (GI panel). Of 40 positive samples, microscopy and antigen-based methods were able to detect *Cryptosporidium* in only 20 and 21 samples, respectively. The GI panel detected 33 of the 40 positive samples, even though not all specimens were received in the recommended preservative. The LDT detected *Cryptosporidium* in all 40 positive samples. When comparing each method for the detection of *Cryptosporidium*, our results indicate the LDT is an accurate, reliable, and cost-effective method for a clinical public health reference laboratory.

INTRODUCTION

The protozoan parasite *Cryptosporidium* causes the intestinal disease cryptosporidiosis in humans. Cryptosporidiosis symptoms typically include watery diarrhea, dehydration, and weight loss, and may last for up to 2 weeks. Infections are spread by consuming or having contact with contaminated water, consuming contaminated food, or close contact with people or animals that are infected. In North America, swimming pools and other recreational waters are common sources of infection for multiple reasons; the oocysts are passed in large numbers by infected individuals, are immediately infectious, hardy in the environment, insensitive to chlorine levels maintained in pools, too small (4–6 µm diameter) to be removed by typical filtration systems, and are able to cause an infection when as few as 10 organisms are ingested.^{1,2}

In 2014, 8,682 cases of cryptosporidiosis were reported to the CDC. However, diagnosis is likely to be significantly underreported, and estimates of the number of actual episodes of illness due to *Cryptosporidium* in the United States are as high as 748,000.^{3,4} From 1995 to 2004, the average incidence was slightly more than one case per 100,000 people, with a range of 0.9–1.4. In 2005, however, this number started to increase following a jump in the number of reported cases, leading to a new higher steady-state incidence rate of almost three cases per 100,000 people.⁵ Examples of contact with contaminated water that contributed to the increase in reported cases include an outbreak in a New York spray park in 2005,⁶ contaminated swimming pools in Utah,⁷ a man-made lake in Texas in 2008,⁸ and contaminated drinking water in a municipality in Oregon in 2013,⁹ which sickened 28% of the community. Increased awareness due to outbreaks such as these, as well as advances in testing methods, could have

also contributed to the higher reported incidence rates. Given that only a small percentage of cryptosporidiosis cases are believed to be reported, efforts to detect outbreaks earlier are likely to benefit from greater awareness of and more sensitive testing for this intestinal parasite.

Diagnosis by microscopic examination of acid-fast stained slides has been the gold standard, and although it is inexpensive, it is also labor intensive and lacks sensitivity compared with other methods. Immunofluorescent microscopy is more sensitive but still labor intensive. Antigen detection is simple to perform using rapid diagnostic tests, but their performance has been variable.^{10–13} More recently, FDA-approved multiplex panels that detect a variety of gastrointestinal pathogens, including *Cryptosporidium*, have become available, and these are now widely used.^{14,15} The commercial assays are easy to perform but can be costly. Laboratory-developed tests (LDTs), including real-time PCR (RT-PCR) assays,¹⁶ are less expensive and easily designed, as the genomes for *Cryptosporidium hominis*¹⁷ and *Cryptosporidium parvum*¹⁸ have been sequenced. However, not all laboratories have the capacity to develop or validate LDTs, and the required instrumentation is expensive. A complicating factor for reference laboratories is that samples are received in a variety of fixatives. Thus, the choice of testing method is likely to depend on several factors including available resources, workload, and the population being tested.

As a provider of clinical public health reference testing in New York State, the Wadsworth Center Parasitology Laboratory confirms the presence of *Cryptosporidium* in patient specimens submitted from hospitals and commercial laboratories. All specimens are evaluated by modified acid-fast staining and direct fluorescent antibody (DFA) immunomicroscopy; however, for approximately 25% of submitted specimens, *Cryptosporidium* cannot be identified by microscopy. In this study, we developed a LDT that specifically targets *C. parvum* and *C. hominis* and includes degenerate genus-level primers and a probe to detect other species of *Cryptosporidium*, which occasionally cause disease in

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humans. Because microscopy was anticipated to be the least sensitive method, the focus was on specimens that were negative when tested by acid-fast staining and immunofluorescent microscopy. After routine analysis, specimens were tested by the LDT as well as a rapid immunoassay for antigen detection and a gastrointestinal panel (GI panel). In addition to the microscopy-negative samples, 20 microscopy-positive specimens were included in the study to verify the specificity of the classical methods. For the purposes of comparison, a composite reference was used such that a specimen was considered a true positive if two or more methods yielded a positive result or the presence of *Cryptosporidium* could be confirmed by DNA sequencing.

MATERIALS AND METHODS

Stool sample selection and concentration. Samples were received over a 5-month period from February to March; a total of 92 specimens were tested by all methods. Stool samples were concentrated using Fecal Parasite Concentrator Kits (Evergreen Scientific, Buffalo, NY). Briefly, 1–2 mL of homogenized stool in fixative or transport medium was poured into the provided test tube, and three drops of 20% Triton X-100 were added along with 1 mL of CitriSolv (Thermo Fisher Scientific, Hanover Park, IL). The tube was filled with 10% formalin, the centrifuge tube strainer was screwed in place, and the unit was rocked at 180° for 30 seconds. Once the liquid portion had all reached the centrifuge tube, then the unit was disassembled, capped, and centrifuged at 1,500 RPM for 10 minutes to pellet the debris and any parasites. The supernatant was poured off, and the pellet was resuspended in a small amount of 10% formalin. The stool was then applied to clean slides, in an area about the size of a dime, and left to dry overnight in preparation for modified acid-fast and DFA staining.

Staining and microscopy. After fixing the dried specimens in methanol for 10 seconds and air-drying, modified acid-fast staining (Remel, San Diego, CA) was performed as per manufacturer's instructions. Briefly, slides were placed in fuchsin stain for 5 minutes and in decolorizer for 1 second, and counterstained for 4 minutes. Between each step, the slides were rinsed briefly with deionized water. Slides were allowed to air-dry before viewing at 1000× using bright field microscopy. All of the sample on the slide was reviewed and determined positive if the staining, size, and morphology of organisms present matched with that of a typical *Cryptosporidium* oocyst.

Direct fluorescent antibody staining was performed on the supplied slides using a *Cryptosporidium/Giardia* detection kit (Meridian, Cincinnati, OH) according to the manufacturer's instructions. One drop of the detection reagent and one drop of the counterstain were added to the dried stool specimen and allowed to incubate for 30 minutes in a humid chamber at room temperature, in the dark. Excess stain was removed from the slide with wash buffer, a drop of mounting medium was added to each sample, and a cover slip was applied to each slide. The entire sample applied to the slide was visualized under 200× magnification using the fluorescein isothiocyanate filter. Samples were determined positive if apple-green staining with the correct pattern and size for *Cryptosporidium* oocysts were visible.

Stock solution preparation. To determine the limits of detection for the GI panel and the LDT, a stock of *C. parvum* at a concentration of 1×10^6 oocysts/mL (Bunch Grass Farm,

Drury, ID) was spiked into phosphate-buffered saline (PBS) and stool. The stool, which had previously been determined free of all parasites, was preserved in the alcohol-based fixative Total-Fix. One milliliter of the oocyst stock was spiked into 9 mL of PBS or stool and serially diluted to a concentration of one oocyst/mL, and then washed and extracted as described in the following text.

DNA extraction. For clinical samples, 1 mL of homogenized stool in fixative or transport medium was centrifuged in a 1.5-mL tube for 5 minutes at full speed ($20,500 \times g$), and the supernatant was discarded. The pellet was washed two times with 1 mL of PBS to remove any residual fixative and resuspended with 1 mL of NucliSENS easyMAG Lysis Buffer (Biomerieux, Durham, NC). The sample was transferred to Lysing Matrix E tubes (MP Biomedicals, Solon, OH), briefly vortexed, and placed on a 75°C heat block for 15 minutes. Samples were homogenized in a FastPrep instrument (Thermo Fisher Scientific, Hanover Park, IL) for 45 seconds at speed setting 6.0, and then centrifuged at $20,500 \times g$ for 5 minutes to pellet debris. DNA was extracted using a standard stool extraction protocol on the easyMAG automated DNA extractor (Biomerieux). Briefly, in one vessel, 800 μ L of stool supernatant, 1.2 mL NucliSENS Lysis Buffer, 140 μ L magnetic silica (Biomerieux), and 10 μ L of an internal extraction control were combined, extracted using Protocol Specific B 2.0.1, and eluted in 100 μ L.

PCR amplification. Sequences for primers and probes for each PCR reaction are provided in Table 1. Real-time reactions were performed using a QuantaBio 5X ToughMix (QuantaBio, Beverly, MA) containing hot start DNA polymerase, stabilizers, deoxyribonucleotide triphosphates (dNTPs), and $MgCl_2$. All cycling reactions were carried out on a ViiA7 RT-PCR system (Applied Biosystems, Foster City, CA) using the 96-well block under standard conditions beginning with a 10-minute 95° denaturation step followed by 45 cycles of 95° for 15 seconds and 60° for 1 minute. A volume of 10 μ L of extracted DNA was tested in a final reaction volume of 25 μ L; all specimens were tested in duplicate. A second set of duplicate reactions was tested using 1 μ L of extracted DNA to test for inhibition.

Reactions for nested conventional PCR were performed in duplicate with a final volume of 50 μ L containing 0.5 μ M primer, dNTPs at 200 μ M each, 1 $\times$ Phire Reaction Buffer containing $MgCl_2$, 1 μ L of Phire Hot Start II DNA Polymerase (Thermo Scientific), and 10 μ L of DNA. When the 18S rRNA gene was targeted, the conventional thermal cycling conditions were 98°C for 30 seconds followed by 35 rounds of 98°C for 5 seconds, 55.5°C (primary) and 61.6°C (secondary) for 5 seconds, 72°C for 15 seconds, and a final extension at 72°C for 1 minute. When the GP60 gene was targeted, the conventional thermal cycling conditions were 98°C for 30 seconds followed by 35 rounds of 98°C for 5 seconds, 65.1°C (primary) and 54.4°C (secondary) for 5 seconds, 72°C for 15 seconds, and a final extension at 72°C for 1 minute. Products were visualized on a 2% agarose gel stained with ethidium bromide. Products that were of the expected size (800 bp) were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions before being sequenced by the Wadsworth Center Applied Genomics Technology Cluster.

Positive amplification controls for the RT-PCR assay were designed using genus-level primers and amplifying 1) patient specimens previously determined to be positive for *C. hominis* and *Cryptosporidium* sp. and 2) *C. parvum* DNA extracted

TABLE 1
Primers and probes

Organism	Type	Primers and probe	Target	Reference
<i>C. parvum</i> and <i>C. hominis</i>	Real-time primers	F—TTA ATG TAA CTC CAG CTG AAT TCT TTT TC R—GGA GTT CAG ATT CTT TAA TTT AAT CTA TCA TTT AAT	Gene of unknown function	Ref. 22 23
<i>C. hominis</i>	Probe	P—Cy5/ATT TAT CTC TTA CTT CGT GGC GGC G/IBRQ		
<i>C. parvum</i>	Probe	P—TexRd/ATT TAT CTC TTC GTA GCG GCG/BHQ2	<i>Cryptosporidium</i> oocyst wall protein	This study
<i>Crypto. spp</i>	Real-time primers	F—TGY CCN CCN GGN TTY GTN GAY R—YGG NGG RCA YTC BGG RTT NGG WGG RGC P—FAM/CAG GCA TWG/ZEN/TRA AYG CAA CAC AAT CTC T/IBFQ		
<i>Crypto. spp.</i>	Conventional (nested)	F1—TTC TAG AGC TAA TAC ATG CG R1—CCC ATT TCC TTC GAA ACA GGA F2—GGA AGG GTT GTA TTT ATT AGA TAA AG R2—CTC ATA AGG TGC TGA AGG AGT A	18S	Ref. 25
<i>Crypto. parvum/</i> <i>hominis</i>	Conventional (nested)	F1—ATG AGA TTG TCG CTC ATT ATC R1—TTA CAA CAC GAA TAA GGC TGC F2—TCC GCT GTA TTC TCA GCC R2—GGA AGG AAC GAT GTA TCT	GP60	Ref. 24 25

C. hominis = *Cryptosporidium hominis*; *C. parvum* = *Cryptosporidium parvum*.

from oocysts isolated from cows (Bunch Grass Farm). Control plasmids were constructed using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, ligation of appropriate PCR products into the pCR4-TOPO vector was followed by transformation into One Shot competent *Escherichia coli* cells (Invitrogen). The plasmid was then purified using the QIAGEN Plasmid Mini Kit (Qiagen) and stored at -20°C . To test for inhibition, an internal control plasmid¹⁹ was added to patient specimens after the heating and vortexing step, immediately before automated extraction. In addition, a no template control, which contained no added DNA, a negative extraction control (NEC), and a positive extraction control (PEC) were included in every RT-PCR assay. The PEC included the internal inhibition control plasmid, which was used both to monitor extraction efficiency and as a comparator for amplification from the internal control plasmid added to patient samples. The matrix of the NEC and PEC consisted of previously determined negative stool extracted in tandem with all patient samples to ensure no contamination occurred during handling or DNA extraction.

Commercial GI panel. Samples were tested with a commercially available panel (FilmArray, BioFire, Salt Lake, UT) which includes bacterial, viral, and parasitic pathogens. As the GI panel is only intended for use with stool samples in Cary-Blair transport medium, samples preserved in fixatives were washed as described earlier and resuspended in 1 mL PBS followed by processing according to the manufacturer's instructions. Briefly, hydration solution was first injected into the pouch, and then, sample buffer and 200 μL of sample were added to the sample/buffer injection tube, which was inverted three times and allowed to sit at room temperature for 5 seconds before being injected into the pouch. The pouch was loaded into the instrument where the sample undergoes extraction, nucleic acid purification, two rounds of PCR, DNA melt curve analysis, and result interpretation.

Rapid immunoassay assay. All samples were tested using the antigen detection rapid immunoassay *Giardia/Cryptosporidium* Quik Chek (G/CQC) in accordance with the manufacturer's instructions. Briefly, a preserved stool sample (100 μL) was added to a tube containing 400 μL of diluent and one drop of conjugate. Five hundred microliters of the diluted

sample-conjugate mixture were transferred to the sample well of the cartridge and incubated at room temperature for 15 minutes. Wash buffer and two drops of substrate were added to the reaction window, and results were read after 10 minutes.

RESULTS

Preservative and demographics. Samples were received in a variety of fixatives, as shown in Table 2. Preservation in formalin has been the standard for clinical parasitology, and most specimens were received in either 10% formalin (45.7%) or sodium acetate formalin (SAF) (23.9%). The age of patients ranged from 10 months to 91 years, although adults aged 22–65 years comprised 43.5% of the patient population (Table 3). For specimens where the gender of the patient was reported (86%), more specimens came from male patients (60.8%) than female patients.

Microscopy. Microscopic examination consisted of both modified acid-fast staining and DFA analysis. A sample was considered positive by microscopy if either technique displayed organisms with the staining characteristics and morphological features of *Cryptosporidium*. Of the 92 samples, microscopy detected 20 positive samples; 19 were positive using both methods and one sample was positive only by modified acid-fast staining. Among the remaining 72 specimens, an additional 15 were positive by two or more methods, and five were positive by one method and confirmed by conventional PCR and sequencing. This resulted in 40 true-positive samples, 51 true-negative samples, and one

TABLE 2
Fixative type for submitted patient samples

Fixative	Number of samples (%) [*]
10 % formalin	42 (45.7)
Sodium acetate formalin	22 (23.9)
Total Fix	9 (9.8)
Cary-Blair	4 (4.3)
Polyvinyl alcohol	2 (2.2)
ProtoFix	1 (1.1)
ParaPak clean vial	1 (1.1)
Fixative type not available	11 (12.0)

^{*} Percentage of 92 samples.

TABLE 3
Age distribution

Age (years)	Number of specimens (%) [*]
0–1	1 (1.1)
1–5	16 (17.4)
6–21	15 (16.3)
22–65	40 (43.5)
> 65	8 (8.7)
Unknown	12 (13.0)

^{*} Percentage of 92 samples.

specimen for which the status could not be resolved, leaving 91 samples (Figure 1, Supplemental Table 1).

Laboratory-developed test. Because molecular methods have been shown to be more sensitive than microscopy and antigen detection,²⁰ we developed an LDT for *Cryptosporidium*. The LDT was designed to specifically detect *C. parvum* and *C. hominis*, as these species are known to cause most cryptosporidiosis cases.²¹ The assay also was designed to detect *Cryptosporidium* at the genus level such that infections caused by other species could be detected. For the *Cryptosporidium* genus-level arm of the assay, the *Cryptosporidium* oocyst wall protein (COWP) gene was targeted, and primers and probes were designed with degeneracies in the sequence to detect as many species as possible (Supplemental Figure 1). The species-specific primers were designed based on previously published assays and target a gene of unknown function.^{22,23} Forty specimens were positive in the LDT assay, and these were also determined to be positive by the composite reference. As expected, most specimens contained either *C. parvum* ($n = 24$, 60%) or *C. hominis* ($n = 9$, 22.5%). Seven specimens (17.5%) were detected only by the genus-level COWP portion of the assay. To rule out the possibility that the LDT was incorrectly amplifying DNA from other organisms in DNA extracted from stool samples, alternate primers were used to amplify the 18S and GP60 genes of *Cryptosporidium* for the seven samples detected at the genus level.^{24,25} Sequencing of the 18S gene identified chipmunk genotype in three specimens, but four could not be identified further. These four samples, however, were confirmed as *Cryptosporidium* positive using other methods; one of the four samples was positive by all other methods, including microscopy, two were positive by the G/CQC immunoassay, and one was positive by the GI panel.

Giardia/Cryptosporidium Quick Chek. The G/CQC antigen-based test was able to detect 21 of the 40 (52.5%) true-positive samples and was concordant with all other methods when true negatives were analyzed ($n = 51$). Thus, the sensitivity of the G/CQC compared with the composite reference was 52.5%, and the specificity was 100%. G/CQC

detected *Cryptosporidium* in five samples that were missed by microscopy but failed to detect the parasite in four specimens that were positive by both modified acid-fast staining and DFA. All of the 19 false-negative specimens were positive when tested by the LDT. Twelve were identified as *C. parvum*, five as *C. hominis*, and two as other *Cryptosporidium* species. Thirteen of the samples were also positive in the GI panel, five were confirmed by sequencing, and one was positive via both microscopy methods. When tested using the LDT, samples that were positive by the antigen-based test had a lower average cycle threshold (Ct) value of 28.9 than the false negatives, where the average Ct value was 32.4. The G/CQC immunoassay provides optimal results when specimens are tested within 72 hours of collection. The specimens in this study were collected and tested before being submitted to the reference laboratory for confirmation or additional testing. Therefore, the test would not have been performed within that optimal window.

Gastrointestinal panel. The GI panel originally identified 32 of the 40 specimens that were determined to be true positives and all 51 samples that were negative by the composite reference. Testing was repeated on the eight true positives that yielded negative results in the GI panel; of these samples, seven samples again yielded negative results on that platform. One sample gave a positive result on repeat testing. Taking the repeat testing into account, the GI panel had a sensitivity of 82.5% and a specificity of 100% compared with the composite reference.

Looking at the false-negative samples, one was positive by microscopy, by LDT, and by sequencing; and one was positive by both the G/CQC immunoassay and the LDT (Table 4). Five samples were positive by the LDT and confirmed by sequencing to verify the species. All of the specimens with false-negative results were preserved in 10% formalin, which is not a validated specimen type for the GI panel. Interestingly, 17 of the specimens that gave a true-positive result in the GI panel were also preserved in either 10% formalin or SAF. Seven specimens that gave positive results with the GI panel were preserved in Total-Fix, four were unpreserved, and for five specimens, the preservative was unknown.

Limits of detection and specificity. The limit of detection (LOD) was determined for both the LDT and GI panel by spiking PBS and stool with known amounts of *C. parvum* oocysts and testing each concentration in duplicate. The overall LOD for the two assays was comparable. When spiked into PBS, both the GI panel and LDT were able to detect *Cryptosporidium* at a concentration of 100 oocysts/mL. When oocysts were spiked into stool, the GI panel detected oocysts at a concentration of 100 oocysts/mL and the LDT detected *C. parvum* oocysts in duplicate at 1,000 oocysts/mL and in one of two samples at concentrations of both 100 and 50 oocysts/mL.

Because *C. hominis* oocysts are not commercially available, the positive-control plasmid bearing the *C. hominis* target was used to test the LOD for this species in the LDT. When spiked into stool, the *C. hominis* plasmid was detected at a concentration of 4,000 copies/mL. As one oocyst contains four sporozoites, the LODs for *C. parvum* and *C. hominis* are comparable. Because the targets of the GI panel are not known, the LOD for *C. hominis* could not be assessed using that platform.

To evaluate the specificity of the LDT, DNA from a panel of 69 organisms including bacteria, viruses, and other parasites

		Microscopy		G/CQC Antigen Detection		GI Panel		LDT RT-PCR	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
		Pos (40)	Neg (51)	Pos (40)	Neg (51)	Pos (40)	Neg (51)	Pos (40)	Neg (51)
Composite Reference	Pos (40)	20	20	21	19	33	7	40	0
	Neg (51)	0	51	0	51	0	51	0	51

FIGURE 1. Results obtained by each method compared with the composite reference.

TABLE 4
Sequence analysis of discrepant samples

Sample	Gastrointestinal panel result	Laboratory-developed test real-time PCR result (average Ct value)	Sequence match	Other test method
2	(-)	<i>C. parvum</i> (34.3)	<i>C. parvum</i>	Microscopy (+)
65	(-)	<i>C. parvum</i> (40.2)	<i>C. parvum</i>	NA
66	(-)	<i>C. parvum</i> (34.8)	<i>C. parvum</i>	NA
77	(-)	<i>C. parvum</i> (36.0)	<i>C. parvum</i>	NA
83	(-)	<i>C. parvum</i> (29.7)	<i>C. parvum</i>	NA
86	(-)	<i>C. hominis</i> (38.8)	<i>C. hominis</i>	NA
87	(-)	<i>Cryptosporidium</i> sp. (33.9)	NA	G/CQC

C. hominis = *Cryptosporidium hominis*; *C. parvum* = *Cryptosporidium parvum*.

were evaluated for cross-reactivity (Table 5). Organisms tested included those causing similar gastrointestinal symptoms, pathogens likely to be found in stool specimens, and additional parasites. All samples were negative, except those containing *Cryptosporidium*.

Analysis of discordant samples. To resolve results for the seven specimens that were positive by LDT and negative by the GI panel, each was retested by both molecular methods to confirm original results, and the RT-PCR products of the LDT were sequenced to confirm the presence of *Cryptosporidium* DNA. Six of the seven samples returned sequences confirming the presence of *Cryptosporidium* DNA. Although the seventh sample did not yield readable sequence, the presence of the target organism was confirmed by a positive result using the antigen-based G/CQC test (Table 4).

DISCUSSION

Although microscopy has long been the gold standard for the detection of *Cryptosporidium*, more sensitive molecular-

based methods are now available. We tested 91 stool specimens that were submitted to the public health laboratory to confirm the presence of *Cryptosporidium*. Of those, 20 were positive based on microscopy, another 20 were positive when tested by other methods, and 51 were negative in all assays. Of the 40 samples determined positive by the composite reference, most were found positive by a molecular method and in some cases, by the LDT alone (Figure 2). The LDT described here identified 20 specimens that were negative when tested by microscopy but determined to be positive by the composite reference. The average Ct value for those specimens when tested by the LDT was 31.7, compared with an average Ct value of 29.2 for the 20 samples that were positive by microscopy. Similarly, as noted earlier, specimens that yielded a false-negative result by G/CQC had a higher Ct value than specimens that were positive by G/CQC. Although the number of samples in each group was low, the results suggest that specimens with a low parasite burden may be missed by the G/CQC immunoassay. These results are consistent with previous studies^{26,27} which indicate microscopy and G/CQC lack sensitivity compared with nucleic acid amplification-based testing.

Although amplification-based testing has better sensitivity, inhibition can be a problem when performing PCR on stool

TABLE 5
Specificity of the laboratory-developed test

Organism	Number of species tested	Result
<i>Aeromonas</i> spp.	11	Negative
Adenovirus	1	Negative
<i>Babesia microti</i>	1	Negative
<i>Blastocystis hominis</i>	1	Negative
<i>Campylobacter</i> spp.	3	Negative
<i>Clostridium difficile</i>	1	Negative
<i>Clostridium sordellii</i>	1	Negative
Coxsackievirus	3	Negative
<i>Cryptosporidium hominis</i>	1	Positive
<i>Cryptosporidium parvum</i>	1	Positive
<i>Cryptosporidium</i> sp.	1	Positive
<i>Cyclospora cayetanensis</i>	1	Negative
<i>Entamoeba histolytica</i>	1	Negative
Enterovirus	4	Negative
<i>Escherichia coli</i>	8	Negative
<i>Giardia lamblia</i>	1	Negative
HIV	2	Negative
<i>Leishmania</i> spp.	2	Negative
Microsporidia	1	Negative
Norovirus	2	Negative
<i>Plasmodium</i> spp.	4	Negative
<i>Plesiomonas shigelloides</i>	1	Negative
<i>Salmonella enteritidis</i>	1	Negative
<i>Shigella</i> spp.	2	Negative
<i>Staphylococcus aureus</i> (Ent A-E)	5	Negative
<i>Trypanosoma brucei</i>	1	Negative
<i>Vibrio</i> spp.	7	Negative
<i>Yersinia enterocolitica</i>	1	Negative

FIGURE 2. Distribution of positive samples by method.

TABLE 6
Cost of an assay

Detection method	Approximate cost (\$)
Microscopy	7.50
<i>Giardia/Cryptosporidium</i> Quik Chek	11.50
Laboratory-developed test	15.00
Gastrointestinal panel	130.00

samples, which are inherently variable. In the LDT described here, inhibition was evaluated in two ways. First, the inhibition of sample amplification was monitored by adding an internal inhibition control plasmid to the patient sample, and PEC and Ct values were compared after amplification. Second, for each patient sample, a 1:10 dilution of the extracted DNA was tested to dilute the effect of any possible inhibitors. In two cases, a sample tested at the 1:10 dilution gave duplicate positive results, whereas the undiluted sample gave one positive and one negative result. This indicates that in a small percentage of samples, amplification was inhibited and inhibition could be overcome by diluting the DNA extract.

We also compared the results of the LDT to a commercially available GI panel. All of the samples that yielded discordant results had been preserved in 10% formalin, which is not approved for this assay. Interestingly, the GI panel was able to successfully detect *Cryptosporidium* in 17 samples that had been preserved in a formalin-based fixative. Although the LDT performed well with formalin-fixed specimens in this study, we have observed (data not shown) that the Ct values for formalin-fixed stool increased with longer storage times. The results suggest an interference with extraction, inhibition of DNA amplification, or both. Although each formalin-preserved sample processed in this study was washed two times with PBS to remove as much fixative as possible, it is unknown how long each sample was stored in formalin before being washed. Cary-Blair transport medium and preservatives that are alcohol-based are preferred when performing molecular analysis.¹⁸ The compatibility of these fixatives with molecular analysis was also evident in this study. Both the LDT and the GI panel produced a positive result for all samples that were submitted in Total-Fix or Cary-Blair transport medium.

For samples that gave discordant results when comparing the LDT and the GI panel, conventional PCR and sequencing were performed to confirm the presence of *Cryptosporidium* DNA. For six of the seven samples, sequencing verified the results of the LDT. For one sample, an interpretable sequence could not be obtained; however, the G/CQC antigen-based test was consistent with the presence of *Cryptosporidium* (Table 4). Of note, this sample had been stored in formalin, potentially contributing to a negative result on the GI panel and the inability to obtain readable DNA sequence.

Another consideration for reference or clinical laboratories is the cost of performing an assay. In Table 6, we show the cost of each assay, excluding labor. Microscopy and G/CQC are the least expensive, although personnel costs for microscopy can be high as it is labor intensive. Additionally, microscopy and G/CQC were not as sensitive. The LDT is slightly more expensive than microscopy or G/CQC at approximately \$15 per sample, including DNA extraction and PCR. The LDT is affordable even when performed in duplicate, as there is only a modest increase in cost for the additional PCR reagents. The cost of a single GI panel is approximately eight-fold higher at

\$130 per sample, at the time of study. When comparing personnel costs for the LDT and GI panel, although the sample preparation for the LDT can be time consuming, DNA extraction and amplification can be completed for approximately 45 samples in an 8-hour workday. By comparison, the GI panel requires less hands-on specimen preparation time. However, a single instrument has the maximum capacity of processing one sample at a time and is limited to roughly 7–8 samples in a day.

Some limitations of this study were due solely to the study being performed by a public health reference laboratory. For example, the optimal time to perform an antigen-based test is within 72 hours of collection. At a reference laboratory, this is not always feasible, as some submitters may hold and batch samples to send multiple specimens to the laboratory at once. Also, the fixative used to collect a sample may not be compatible with the testing algorithm the laboratory has established. Molecular methods are most compatible with a fixative that is ethanol-based or with a specimen that is unpreserved. However, most stool samples for parasitology testing are still collected in a formalin-based fixative, which does not allow for optimal performance of the LDT or the GI panel. In addition, to properly perform DFA microscopy analysis, a polyvinyl alcohol fixative is not recommended because of the high levels of background that can be generated. As a reference laboratory, we must adapt and perform each test to the best of our capabilities given the specimens we receive.

The LDT described here is a method that laboratories can use to accurately and reliably test for *Cryptosporidium* in patient stool samples. It has proven to be a sensitive, specific, and cost-efficient option that allows for fast and easy result interpretation. In addition, the LDT can be used in a high-throughput situation, such as an outbreak. The assay specifically detects *C. parvum* and *C. hominis*. Other species can be detected by the genus-level primers, although the sensitivity is decreased because of degeneracy in the primers and probes. An added benefit of the LDT is that the extracted DNA can be genotyped to aid in source tracking and epidemiological investigations.

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