Application of a qPCR Assay with Melting Curve Analysis for Detection and Differentiation of Protozoan Oocysts in Human Fecal Samples from Dominican Republic

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Abstract. A quantitative polymerase chain reaction assay with melt curve analysis (qPCR-MCA) was applied for the detection of protozoan oocysts in 501 human fecal samples collected in Dominican Republic. Samples were subjected to qPCR using universal coccidia primers targeting 18S rDNA to detect oocysts followed by MCA to identify oocyst species based on amplicon melting temperature. Putative positive samples were also tested by conventional PCR and microscopy. *Cystoisospora belli* (×3), *Cryptosporidium parvum* (×3), *Cryptosporidium hominis* (×5), *Cryptosporidium meleagridis* (×1), *Cryptosporidium canis* (×1), and *Cyclospora cayetanensis* (×9) were detected by qPCR-MCA and confirmed by sequencing. This assay consistently detected 10 copies of the cloned target fragment and can be considered more efficient and sensitive than microscopy flotation methods for detecting multiple species of oocysts in human feces. The qPCR-MCA is a reliable protozoan oocyst screening assay for use on clinical and environmental samples in public health, food safety and veterinary programs.

INTRODUCTION

Oocyst-producing protozoa known as coccidia are a large and relatively diverse subclass of the Apicomplexan phylum of parasites that infects virtually all vertebrate hosts. Some members of this group have significant veterinary and public health implications, especially related to agriculture, food safety, and economics. Diagnosis of the infection is typically by detection of oocysts in fecal samples of the definitive host using traditional microscopy techniques, which are labor-intensive, lack sensitivity and specificity, and require parasitology expertise to identify pathogenic coccidian species. In humans, gastrointestinal illnesses caused by *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Cystoisospora belli* are of significant concern, particularly for the elderly, young children, and immunocompromised individuals.[1] There are several diagnostic limitations for the detection of *C. cayetanensis*. It is not included in routine ova-parasite fecal examinations, oocysts are shed intermittently in low numbers, and they have poor uptake of many histological stains.[2] Both *C. cayetanensis* and *C. belli* are probably underreported because of, in part, these factors.[3]

Existing molecular methods for detecting *Cyclospora* can overcome some of the limitations of traditional methods; however, most cannot distinguish between *Cyclospora* and closely related non-zoonotic *Eimeria* spp., which may lead to false-positive results.[4] Therefore, a reliable and specific assay for detection of *Cyclospora* and other common protozoan oocysts of human health importance, such as *Cryptosporidium* spp. and *C. belli*, would be a valuable diagnostic tool. We recently reported on the development of a real-time quantitative polymerase chain reaction assay with melting curve analysis (qPCR-MCA) using a universal coccidia primer cocktail for use in detection and differentiation of a wide range of coccidia species of public and veterinary health concern.[5] Using genomic DNA (gDNA) from pure isolates of coccidia, we showed that *C. cayetanensis*, *Cryptosporidium parvum*, *Cryptosporidium muris*, *Toxoplasma gondii*, *Eimeria bovis*, *Eimeria acervulina*, *Cystoisospora suis*, and *Sarcocystis cruzi*

could be identified by unique melting curves and differentiated based on a specific melting temperature (Tm). However, the assay had not been validated for use on field matrices, such as food, water, or feces, where PCR inhibitors and background DNA are commonly present and could interfere with detection and identification of species. The objectives of the present study were to assess the suitability of the qPCR-MCA assay for use in screening human fecal samples for a variety of protozoan oocysts of public health importance and determine the occurrence of these parasites in the Santiago region of Dominican Republic.

MATERIALS AND METHODS

Fecal samples. A total of 501 human fecal samples was collected from patients ages 20 days to 89 years (age information not available for all samples) at nine different hospitals, clinics, or medical centers in the north central region of Dominican Republic. The samples were mixed with two volumes of 2.5% potassium dichromate and stored at 4°C until used. As part of a training initiative in Dominican Republic, a portion of each sample was initially screened by flotation microscopy (as below) for parasites before being shipped to Canada for additional analysis. The first 103 samples were analyzed by flotation microscopy followed by qPCR-MCA. Because of the laborious requirement of microscopic examination, the remaining 398 samples were initially analyzed by qPCR-MCA, and any samples found positive were then examined by flotation microscopy for visualization of the parasites. Oocysts of *E. papillata* were used routinely as controls for the DNA extraction procedure and preparation of the qPCR standard curve. *E. papillata* oocysts had been propagated by passage in mice and isolated from feces by washing and straining with water followed by flotation with sucrose. The oocysts were sporulated (72%) and stored in 2.5% potassium dichromate at 4°C until use.

Microscopy and parasite recovery. Fecal samples (140) were prepared for microscopy examination using a modified sucrose flotation method.[6] Briefly, approximately 2 mL washed fecal suspension were mixed with a sucrose solution (specific gravity of 1.27) to fill a 16 × 100-mm glass tube and form a convex meniscus, which was covered with a coverslip

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to form a seal, and centrifuged at room temperature for 10 minutes at $300 \times g$ using a swing-out rotor. The coverslip was removed onto a standard microscope slide and examined for helminth eggs at $100\times$ magnification and protozoan oocysts at $200\times$ magnification in a hash tag pattern. Material under the coverslip was then collected by placing the slide and coverslipin a 50-mL plastic centrifuge tube with 15 mL 0.1% Tween-H_2O and soaking for at least 5 minutes at room temperature to dissolve the sucrose solution. After soaking, slides and coverslips were rinsed with an additional 35 mL 0.1% Tween-H_2O, and then discarded. Any parasites were concentrated by centrifugation ($2,060 \times g$ for 15 minutes) and stored at 4°C. Some fecal samples (26) were selected for an additional examination using a $ZnSO_4$ (specific gravity of 1.18) flotation method specifically for detection of non-oocyst protozoa, such as *Giardia* and *Entamoeba*,[7] based on clinical information provided by sample providers in Dominican Republic. Enough $ZnSO_4$ solution was mixed with 2 mL fecal suspension to fill a 16×100-mm glass tube and form a convex meniscus, which was covered with a coverslip to form a seal, and then, it was centrifuged at room temperature for 5 minutes at $260 \times g$ using a swing-out rotor. Coverslips were removed 10 minutes after centrifugation was complete, placed on a slide with a droplet of iodine, and examined as above. Parasites observed were collected from glass coverslips and slides as above.

DNA extraction. DNA was extracted from slide wash sediments and *E. papillata* controls using a QIAamp DNA Micro Kit (Qiagen, Mississaugua, Canada) with modifications as described previously.[8] DNA extraction directly from fecal samples was performed using a QIAamp DNA Stool Mini Kit (Qiagen) with the following modifications to the manufacturer's protocols. (1) Approximately 1–2 mL fecal suspension were washed three times with milli-Q H_2O by centrifugation ($20,000 \times g$ for 15 minutes) to remove potassium dichromate. The remaining 200 µL fecal pellet was mixed with 1.4 mL ASL buffer (Qiagen) and subjected to eight cycles of freezing in liquid nitrogen for 1 minute followed by thawing in a 95°C water bath for 1 minute. (2) The sample was then incubated with 20 µL proteinase K (20 mg/mL; Qiagen) for 18 hours overnight at 56°C. (3) The lysed suspension was centrifuged at $20,000 \times g$ for 3 minutes to pellet stool particles, and 1.4 mL supernatant were transferred to a clean 2-mL tube and treated with the provided InhibitEX tablet to remove inhibitors according to the manufacturer's (Qiagen) protocol. (4) Resulting lysate was incubated with 200 µL Buffer AL (Qiagen) for 10 minutes at 70°C followed by purification through QIAamp microcolumns (Qiagen) according to the manufacturer's protocol and elution with 35 µL AE buffer (Qiagen). A negative control of only reagents and a positive control of 10^4 *E. papillata* oocysts were included in each batch of samples for DNA extraction. For the qPCR standard curve, DNA was extracted from 10-fold serial dilutions of purified *E. papillata* oocysts (10^5–10^1), which were enumerated by triplicate readings using a McMaster chamber.

Plasmid controls. Plasmid DNA from a selection of coccidia species was run on each qPCR plate along with test samples. The melting curves of test samples were visually compared with the coccidia plasmid DNA controls to identify the species, which was then confirmed by sequencing of the qPCR products. To generate the plasmid DNA controls, gDNA from *E. bovis*, *E. tenella*, *E. necatrix*, *C. cayetanensis*, *T. gondii*, *C. belli*, *C. parvum*, *C. meleagridis*, and *C. hominis*

was amplified with the universal coccidia primer cocktail, cloned, and purified as below for positive samples. Purified plasmid DNA was digested with Sca1 (New England Biolabs, Pickering, Canada) overnight at 37°C with supplied NEBuffer according to the manufacturer's instructions. The digested plasmid DNA was analyzed by gel electrophoresis, and linearized plasmid fragments were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). The plasmid DNA was quantified by spectrophotometer (Nanodrop 2000c; Thermo Scientific, Wilmington, DE) and diluted to 0.05 ng/µL for use as routine controls in the qPCR assay; 10-fold dilutions of plasmid DNA from *C. cayetanensis* were also prepared to be equivalent to 10^5–10^0 copies of the small subunit ribosomal DNA (SSU rDNA) fragment. Six replicates of each copy number dilution were analyzed by qPCR-MCA to determine assay sensitivity as described below.

qPCR amplification. All qPCR-MCA assays were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules). The reaction mix was loaded into clear-well PCR plates (catalogue no. HSP9611; Bio-Rad) and contained 1× SsoFast EvaGreen Supermix (Bio-Rad), 400 nM Crypto-F, Crypto-R, Cyclo-F, Cyclo-R, and Sarco-R,[5] 800 nM Toxo-F,[5] 100 ng/µL bovine serum albumin (Sigma-Aldrich, Oakville, Canada), 2 µL DNA template, and sterile dH_2O to a final volume of 25 µL. Standard curves for all qPCR runs consisted of DNA from diluted *E. papillata* oocysts (10^5–10^1). The qPCR cycling conditions were as follows: 2 minutes at 98°C followed by 40 cycles of denaturing at 98°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds. Data collection was enabled at the extension step. The melt curve protocol followed with 10 seconds at 95°C and then 10 seconds each at 0.2°C increments between 62°C and 95°C. Data collection was enabled at each increment of the melt curve. Melt and standard curves were generated by the CFX Manager Software (version 2.0; Bio-Rad) as described previously.[5] Oocyst-positive samples were identified by comparison of the test sample melt curves with the melt curves of the protozoan controls included in each run. Previously published conventional PCR assays targeting internal transcribed spacer 2 (ITS-2), 5.8S + ITS-2, and *Cryptosporidium* oocyst wall protein (COWP) regions[8–10] were used for additional confirmation of the positive samples of *C. cayetanensis*, *C. belli*, and *Cryptosporidium* spp., respectively.

Cloning and sequence analysis. Test samples that were positive for protozoan oocysts by qPCR-MCA (i.e., melt curve shape and Tm matched a positive control in the run) were cloned and sequenced to confirm species identification. First, qPCR products were analyzed by gel electrophoresis and purified with the Qiaquick Gel Extraction Kit (Qiagen). The purified products were subjected to an A-tailing reaction, which consisted of 4 µL qPCR product, 5 units GoTaq Flexi DNA polymerase (Promega, Madison), 0.2 mM dATP, 5× GoTaq reaction buffer, and dH_2O to a final volume of 10 µL incubated at 70°C for 15 minutes. The A-tailed products were then ligated into plasmid cloning vector pGEM-T Easy (Promega) according to the manufacturer's instructions. Plasmid DNA was purified using the QIAprep Miniprep Kit (Qiagen) and sequenced in both directions using the T7 and SP6 primers (Promega). Forward and reverse sequences were assembled using Clone Manager software (version 9.0; Sci-Ed Software, Cary), and the vector and primer sequences were removed. Nucleotide sequences were compared with the

GenBank collection of sequences using BLASTN and identified based on the degree of sequence identity.

RESULTS

During the first phase of the project, human fecal samples were first examined microscopically for parasites, and the DNA extracted from the slide washes or directly from the feces was tested by qPCR-MCA. Of 103 samples examined, oocysts of *Cystoisospora* spp. (×2) and *Cryptosporidium* spp. (×1) and eggs of *Trichuris* spp. (×1) were observed by microscopy (Figure 1 and Table 1). Using qPCR-MCA, *C. belli* was detected and confirmed by sequencing and conventional PCR for both samples, which were detected by microscopy. The *Cryptosporidium* sample observed by microscopy could not be confirmed by qPCR-MCA. Another sample that was positive for *C. hominis* by qPCR was confirmed by sequencing and conventional PCR amplification of the COWP gene fragment (Table 1). Figure 2 shows representative melt curve graphs for both the protozoan oocyst plasmid controls and the isolates detected in this study. The qPCR-MCA could consistently detect 10 copies of the cloned *C. cayetanensis* SSU rDNA plasmid DNA fragment. When plotted along the standard curve consisting of serially diluted gDNA from 10^5 *E. papillata* oocysts, the quantification cycle (Cq) values for all coccidian-positive samples were equivalent to a range of 0.5–5 oocysts.

During the second phase of the project, DNA was extracted directly from washed human fecal samples and screened for oocysts using qPCR-MCA. Samples positive for protozoan oocysts by qPCR-MCA were then examined by microscopy for visualization of oocysts. Of 398 samples examined during this phase, *Cryptosporidium* (×9), *Cyclospora* (×9), *Cystoisospora* (×1), and three unknown protozoa (melting curves did not match any of the available controls; Tm = 81.6°C for two samples and Tm = 84°C for the third sample) were detected by qPCR-MCA (Table 1). *Cryptosporidium* oocysts were observed by microscopy in all nine samples identified by qPCR-MCA. *Cyclospora* oocysts were observed by microscopy in two of nine samples identified by qPCR-MCA. Sequencing confirmed the positive protozoan oocyst samples as *C. hominis* (×4), *C. parvum* (×3), *C. canis* (×1), *C. meleagridis* (×1), and *C. cayetanensis* (×9) (Figure 2 and Table 1). Conventional PCR assays confirmed eight of nine positive *Cryptosporidium* spp. and five of nine *C. cayetanensis* samples. Three samples with melt curves in the Tm range of protozoan oocysts were sequenced and identified as *Adelina bambarooniae* (two samples; 97% identity by BLAST analysis), which is a coccidian
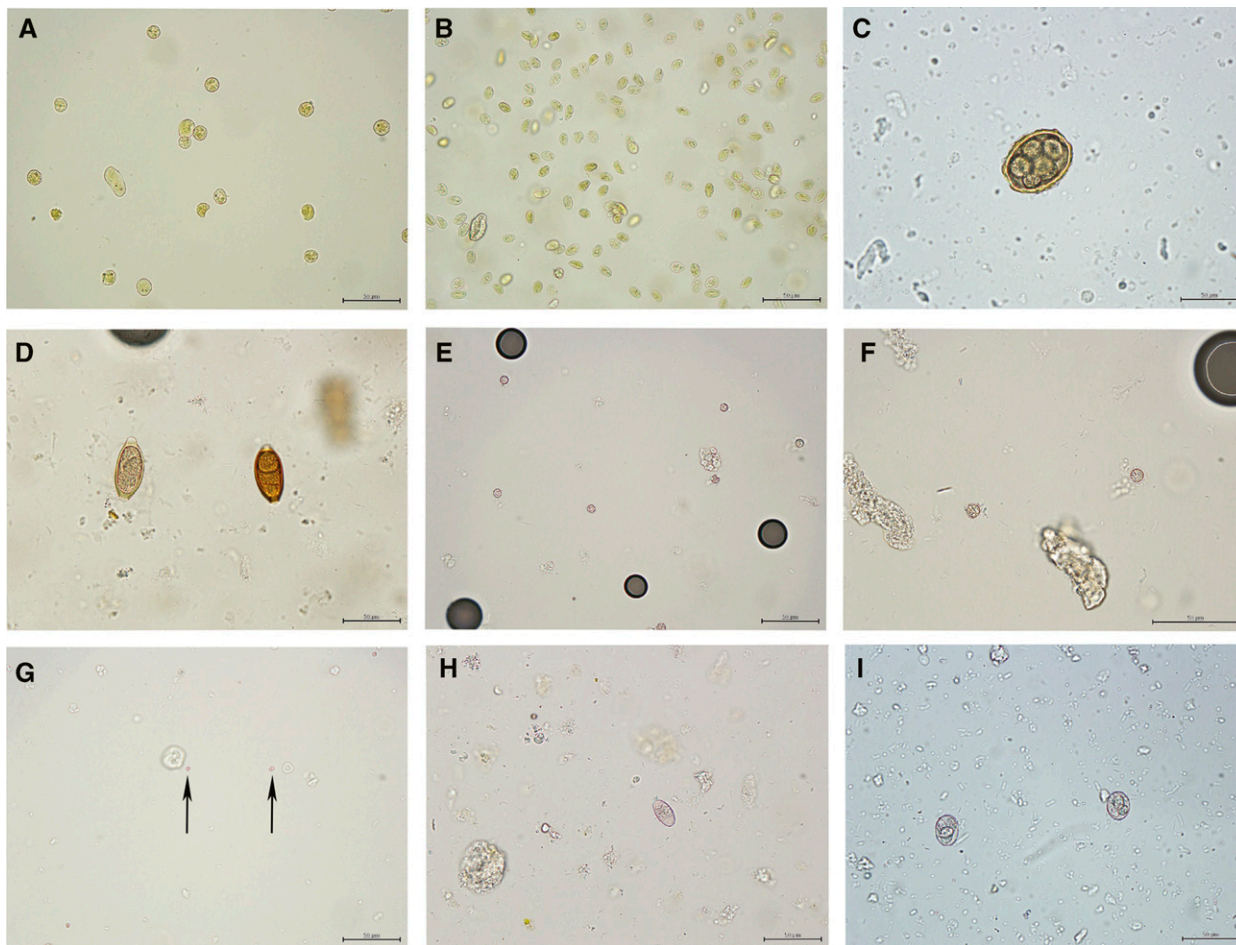

FIGURE 1. Representative photos of parasites observed in human fecal samples collected in Dominican Republic: (**A**) *Entamoeba* cysts stained with iodine, (**B**) *G. duodenalis* cysts stained with iodine, (**C**) *A. lumbricoides* egg, (**D**) *T. trichiuria* egg, (**E** and **F**) *C. cayetanensis* oocysts, (**G**) *Cryptosporidium* oocysts, (**H**) *C. belli* oocysts, and (**I**) *E. papillata* oocysts (control sample). Scale bars: 50 μm. Crown Copyright c 2012. Published by AJTMH. All rights reserved.

Parasite detected	Detection method			Species identified (no.) and confirmed by sequencing	Mean Tm (°C; SEM)
	Flotation microscopy (N = 140)	qPCR-MCA (N = 501)	PCR (N = 22)		
Cryptosporidium	10	10	9	*C. hominis* (5)	78.38 (0.02)
				C. parvum (3)	78.53 (0.06)
				C. canis (1)	78.80 (0)
				C. meleagridis (1)	78.45 (0)
Cyclospora	2	9	5	*C. cayetanensis* (9)	84.19 (0.06)
					87.53 (0.08)
Cystoisospora	3	3	2	*C. belli* (3)	84.33 (0.07)

parasite that infects cane beetles, and *Baldinia anauniensis* (one sample; 96% identity), which is a dinoflagellate.

In addition to those samples examined by Sheather's flotation for visual confirmation of oocysts detected by the qPCR-MCA, 39 samples were selected for examination by microscopy (13 samples using Sheather's flotation for hel-minth eggs and 26 samples using ZnSO₄ for other protozoa) based on clinical information provided by sample providers in Dominican Republic. From these selected samples, *Trichuris* (×2), *Ascaris* (×1), *Giardia* (×6), and *Entameoba* cysts (×10) were identified based on morphological features (Figure 1).
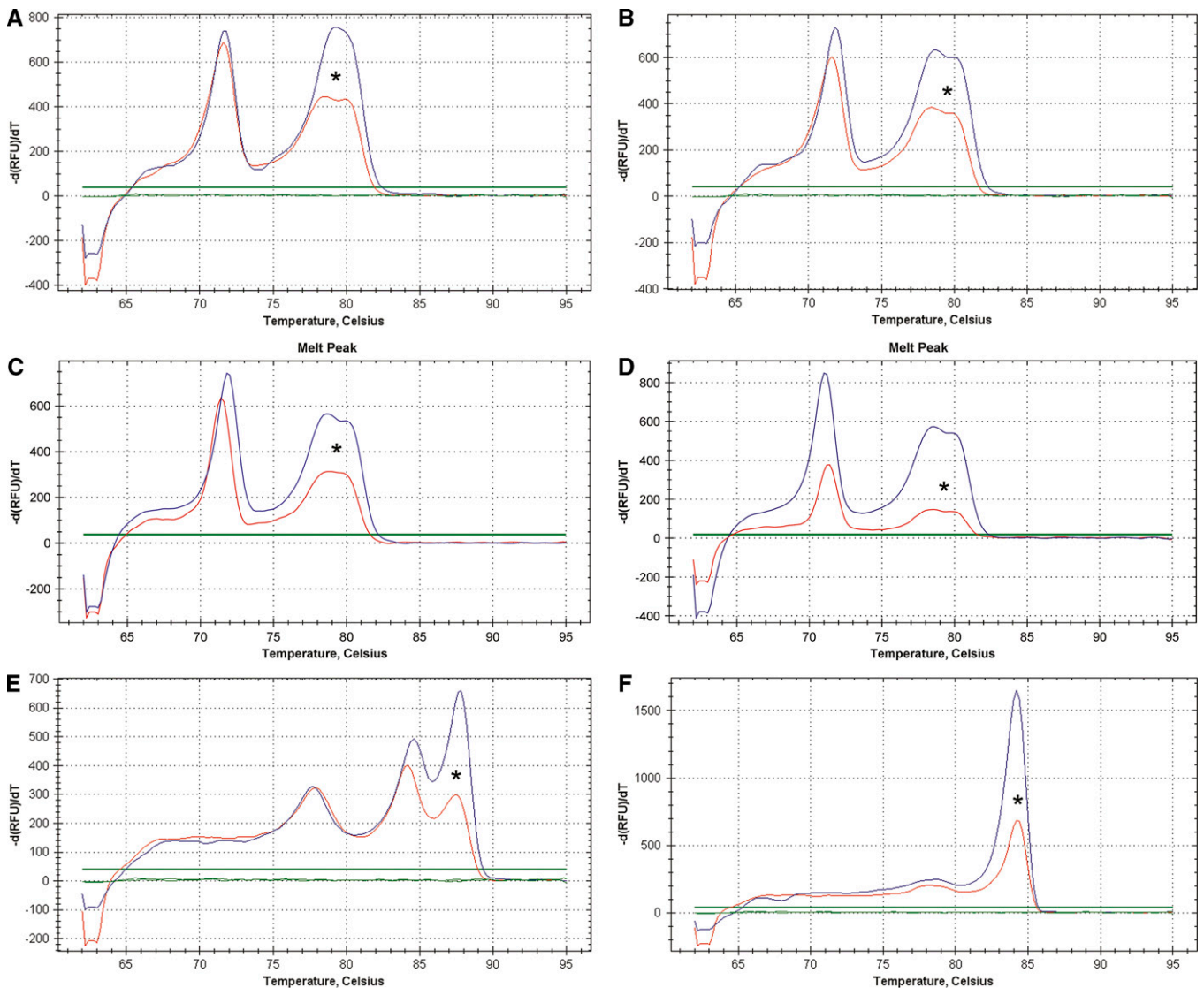

FIGURE 2. Representative melt curve graphs for (**A**) *C. hominis*, (**B**) *C. parvum*, (**C**) *C. canis*, (**D**) *C. meleagridis*, (**E**) *C. cayetanensis*, and (**F**) *C. belli*. Melt curve graphs from plasmid DNA controls are unmarked, and those graphs from field isolates identified in this study are marked with an asterisk. Temperature (°C) is displayed on the *x* axis, and −d(RFU)/dT is displayed on the *y* axis. Crown Copyright c 2012. Published by AJTMH. All rights reserved.

DISCUSSION

The primary aim of this study was to show the application of the qPCR assay with MCA for detection and identification of protozoan oocysts in human fecal samples. We selected Latin America as a source for the samples, because some areas within this region are known to be endemic for protozoan parasites, such as *Cryptosporidium* and *C. cayetanensis*.[2] The study was performed on samples from Dominican Republic, where there is little information about the occurrence of protozoan oocysts. In developing countries where molecular diagnostic instrumentation and expertise are limited, microscopy remains the gold standard for detection of protozoa in humans. Limitations of microscopic methods can include intermittent shedding and need to examine multiple fecal samples, inconsistent staining of oocysts, time required for oocysts to be sporulated for taxonomic identification, and need for parasitology experience to distinguish oocysts from fecal debris.[2] Although several commercial immunofluorescent antibody (IFA) kits are available for *Cryptosporidium* spp., there are currently none for *C. cayetanensis* or *C. belli*. Because of the limited amount of fecal material provided in this study, we opted to perform a simple sucrose flotation to screen all fecal samples for parasites by microscopy followed by qPCR-MCA in the first phase of the project. In the second phase, we screened fecal samples first using qPCR-MCA followed by microscopy for oocyst visualization.

The most recent report on the occurrence of helminths and protozoa in Dominican Republic was published in 1981, and it indicated a high prevalence of *A. lumbricoides* (13.1%), *T. trichiura* (35.8%), and *E. coli* (24.8%) in rural sugar plantation employees.[11] *G. duodenalis* was less frequently detected (0.7%). The present study showed that the prevalence of these helminth parasites was lower, possibly because of the inclusion of both urban and rural populations, improvements in parasite control programs, and improvements in sanitation and hygiene practices. However, *Giardia* and *Entamoeba* were detected more frequently in this study. Reports of *Cyclospora*, *Cryptosporidium*, and *Cystoisospora* from Dominican Republic relate primarily to travel-associated cases of infection[12,13]; thus little published information is available for the prevalence in the local population.

The qPCR-MCA assay was as sensitive and specific as microscopic analysis for the detection of *C. belli*. One fecal sample that was presumed positive for *Cryptosporidium* oocysts by microscopy was not confirmed by qPCR-MCA. Because of the small amount of fecal material available, it was not possible to perform an additional microscopy method (such as IFA) to detect *Cryptosporidium* spp. in this study; therefore, it is possible the putative oocysts observed were, in fact, plant material or yeast and as a result, not amplified by the qPCR-MCA assay. Another sample that was negative by microscopy was positive by qPCR-MCA and confirmed by sequencing and conventional PCR amplification. The qPCR-MCA is likely more sensitive than flotation microscopy; the estimated recovery efficiency of the Sheather's flotation method is ~60% for *Cryptosporidium* oocysts.[14] Because the qPCR-MCA assay is performed on DNA extracted directly from a separate aliquot of feces, it is possible that, because of inadequate sample mixing or low oocyst number, the results do not concur. An additional seven cases of *C. cayetanensis* were identified by qPCR-MCA and confirmed by sequencing,

but they were not seen on microscopy or all conventional PCR assays. In subclinical cases, *C. cayetanensis* is shed intermittently and in low numbers, and therefore, examination of only one sample by microscopy may not have been sensitive enough to detect the oocysts. It is also possible that the conventional PCR assay selected for confirmation was not sensitive enough to detect these *C. cayetanensis*-positive samples. All negative controls performed as expected during qPCR-MCA analysis, and therefore, the likelihood of false positives caused by cross-contamination is low.

We have shown that, by using a universal cocktail of seven primers, DNA from virtually any coccidia species can be amplified and then identified by melt curve shape and Tm.[5] The SSU rDNA region targeted by the primer cocktail was selected, because it is highly variable within the subclass, which allows for many coccidia species to be differentiated based on their Tm, and the identification can be confirmed by sequencing of the PCR products. Primers to amplify this region, originally designed by Morgan and others,[15] have also been modified by others and used in real-time PCR to detect and differentiate *Cryptosporidium* spp.[16] The melt temperatures determined for the *Cryptosporidium* species detected in this study differ by a small margin of 0.27° to 0.42° and therefore, would require high-resolution melt curve analysis for reliable species identification. An additional benefit to using a broad-specificity universal primer set is that unknown, emerging, or opportunistic coccidian pathogens may be discovered if this assay was used for difficult-to-diagnose cases or with immunocompromised patients. This type of extended-range approach was used by Murphy and others[3] to identify *C. belli* in an immunocompromised patient with persistent diarrhea of unknown cause.

The potential disadvantage of a broadly specific assay is that non-specific aberrant peaks are often observed when amplifying DNA from fecal or environmental samples. However, the qPCR-MCA allows these peaks to be distinguished from true positives by their Tm (typically less than 78°C) and shape (irregular) as well as comparison of melt peaks for test samples with well-characterized control samples included in each run. In this study, three samples displayed melt curves in the Tm range of coccidia but were not an exact match to any controls. The closest BLAST match for two of the samples indicated *Adelina* spp., which is a coccidian parasite of invertebrates that is not known to be zoonotic; it is likely a transient gut organism, or it may have been present as an opportunistic dweller if the host was immunocompromised. The DNA sequence from the other sample identified it as a dinoflagellate, a flagellate protist. The amplification of dinoflagellates and occasionally, fungi (another protist) suggests that the specificity of the primers used in the qPCR-MCA is not strictly coccidia-specific. This finding is supported by earlier rDNA studies showing a close phylogenetic relationship among some phyla of the diverse group of protists, including coccidia, dinoflagellates, piroplasms, and ciliates,[17] However, when appropriate controls are used in each run, these non-specific amplicons are easily identified and may be ruled out by sequencing.

An estimate of the number of oocysts detected per sample was based on a standard curve consisting of serially diluted DNA from 10^5 *E. papillata* oocysts. Sporulated *Eimeria* oocysts have eight haploid sporozoites and are thought to have approximately 140 copies of the rRNA gene,[18] whereas

Cryptosporidium has four sporozoites and may have as few as 5 copies of the gene.[19] Thus, the amount of target DNA available per oocyst is much greater for *Eimeria* and other species that have eight sporozoites, such as *C. belli*. We could consistently detect 10 copies of the *C. cayetanensis* SSU rDNA plasmid DNA fragment, which may be equivalent to less than one oocyst, depending on the number of gene copies per organism and the sporulation stage of the oocyst. The *Cryptosporidium* and *Cyclospora* oocyst numbers extrapolated from the *Eimeria* standard curve will be underestimated, and the assay sensitivity for these species may be reduced, because they inherently have less target 18S rDNA. For instance, using a standard curve consisting of serially diluted DNA from 10^5 *C. parvum* oocysts, the Cq values ranged from 22 to 36 for 10^5–10^1 oocysts (data not shown). A similarly constructed standard curve using *E. papillata* would have a Cq range of 17–30 for 10^5–10^1 oocysts, a difference of about five to six cycles (about 2 logs) compared with *C. parvum*. Thus, using an *Eimeria* standard curve to estimate numbers of *Cryptosporidium* and *Cyclospora* oocysts is not ideal. However, it is not practical to run more than one standard curve on every plate, and therefore, *E. papillata* was selected for this study, because it can be easily propagated in mice, and a fresh source of oocysts may be available. As a result, the oocyst quantification aspect of this assay is not entirely accurate; however, because it was designed to target multiple coccidia species, its power is not in quantification but species differentiation and identification.[5]

There are several multiplex real-time PCR assays targeting diarrhea-causing parasitic protozoa, including *G. duodenalis*, *Cryptosporidium* spp., and *E. histolytica*[20–22]; however, this qPCR-MCA assay is the only published method for detection, differentiation, and identification of a wide range of protozoan oocyst species that are important for human and animal health. The current method requires only a single channel real-time instrument and does not use probes, and therefore, it is simpler and may be more cost-effective to run than other multiplex real-time PCR assays used for diarrhea-causing protozoa. Although the qPCR-MCA assay can differentiate to the genus and species level by Tm alone in many cases,[5] distinguishing some species (such as *Cryptosporidium*) may require equipment and software capable of high-resolution melt curve analysis or sequencing to confirm results. The Tm values reported here using a qPCR mix containing EvaGreen dye differ from those values that we reported previously using SYBR Green qPCR chemistry.[5] However, the unique double-peak melt curve profile previously observed for *C. cayetanensis* was retained, enabling effective differentiation between *Cyclospora*, *Cystoisospora*, and *Eimeria* species. The distinctive melt curve profiles observed for all species detected here, including the dual peak for *Cyclospora*, are the result of differences in nucleotide sequence composition, length, and G-C composition. It is important that gDNA or plasmid DNA from suitable controls be included in each qPCR run for comparison with test samples, because Tm values can also vary based on qPCR reagent chemistry, amplification instrument, and DNA concentration in addition to the expected variation because of nucleic acid composition.

We have shown the qPCR-MCA assay's use for simultaneous detection and identification of multiple protozoan oocysts species in human feces. The extraction of DNA directly from washed fecal samples followed by qPCR-MCA is more efficient than and at least as sensitive as standard microscopy flotation methods, which are laborious and lack specificity. This study shows the use of the qPCR-MCA as a reliable screening tool for oocyst detection and identification for use in clinical and environmental samples in public health, food safety, and veterinary programs.

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