Am. J. Trop. Med. Hyg., 76(4), 2007, pp. 713–717
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MULTIPLEX REAL-TIME PCR ASSAY FOR DETECTION OF *ENTAMOEBA HISTOLYTICA, GIARDIA INTESTINALIS,* AND *CRYPTOSPORIDIUM* SPP.

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Abstract. *Entamoeba histolytica, Giardia intestinalis,* and *Cryptosporidium* spp. are not only three of the most important and common diarrhea-causing parasitic protozoa, but they often have similar clinical presentations. Microscopic diagnosis of these parasites is neither sensitive nor specific. Recently, more specific and sensitive alternative molecular methods (polymerase chain reaction [PCR] and antigen detection tests) have been introduced for all three of these parasitic infections. The use of these molecular diagnostic tests in routine diagnostic laboratories is still limited. In this study, we developed a multiplex real-time PCR assay for the simultaneous detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. in one reaction using species-specific probes. This assay was evaluated using clinical specimens and was found to be quite sensitive and specific. The reagents used in this multiplex PCR assay can also be used for detection of these parasites individually. The use of this real-time PCR multiplex assay in developing countries at present will have limited scope for routine diagnosis because the cost will be high for a single test, although in the developed world, the test could see immediate application.

INTRODUCTION

The World Health Organization (WHO) ranks diarrheal disease as the second most common cause of morbidity and mortality in children in the developing world.[1] Many studies have been conducted in various geographic sites to identify the etiology of these diarrheal illnesses and to formulate a composite picture for estimating their global burden.[2] The etiological agents of diarrhea include viruses, bacteria, and parasites.[3] Among parasites, *Entamoeba histolytica, Giardia intestinalis,* and *Cryptosporidium* spp. are considered to be the most common and important.[4–6] Amebic colitis and liver abscess are caused by infection with the enteric protozoan parasite *E. histolytica*. This parasite has recently been separated using modern diagnostic techniques from the nonpathogenic parasite *E. dispar,* which is more common and identical in appearance to *E. histolytica*.[7–9] The WHO estimates that ~50 million people worldwide suffer from invasive amebic infection each year, resulting in 40,000–100,000 deaths annually.[7–9] *G. intestinalis* (synonyms: *Giardia lamblia* and *Giardia duodenalis*) is the most common protozoan infection of the intestinal tract worldwide. *G. lamblia* is considered as one of the main non-viral causes of diarrhea in developed countries.[10] Cryptosporidiosis is a frequent cause of diarrheal disease in humans. In developing countries, *Cryptosporidium* spp. infections occur mostly in children younger than 5 years of age, with a peak in children younger than 2 years of age.[11,12] In immunodeficient humans, especially individuals with HIV/AIDS, cryptosporidiosis can be associated with chronic, potentially life-threatening diarrhea.[13]

Entamoeba histolytica, G. intestinalis, and *Cryptosporidium* spp. are not only three of the most important and common diarrhea-causing parasitic protozoa, but they often have similar clinical presentations.[3] Microscopic diagnosis of these parasites is neither sensitive nor specific. Recently, more specific and sensitive alternative molecular methods (polymerase chain reaction [PCR] and antigen detection tests) have been introduced for all three of these parasitic infections.[13–17] Sensitivity and specificity of these molecular based methods are quite good. However, the incorporation in a routine diagnostic laboratory of these parasite-specific methods for diagnosis of each of the respective infections is time consuming and increases the cost of a stool examination. Therefore, a more convenient diagnostic method is needed. Recently, real-time PCR, a new methodology that uses fluorescent labels to enable continuous monitoring of amplicon (PCR product) formation throughout the reaction, has recently been adapted to detect these parasites and reported in the literature.[18–21] In this study, a Taqman-based multiplex real-time PCR assay has been designed and evaluated to diagnose these three important protozoan parasites in one reaction. Results from this multiplex real-time PCR assay were compared with a gold standard diagnosis (defined as positive by both antigen detection and a standard singleplex PCR).

MATERIALS AND METHODS

Stool samples. One hundred twenty-nine stool samples were selected in which *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. were detected by the antigen detection tests and confirmed by specific PCR assays.[18,22,23] Of these 129 samples, 19 stool samples were positive only for *E. histolytica* by both antigen detection test and *E. histolytica*–specific PCR, and 23 *E. histolytica*–positive stool samples were co-infected with the other two parasites (21 with *G. intestinalis* and 2 with *Cryptosporidium* spp.). Twenty-nine stools samples were positive only for *G. intestinalis* by both antigen detection test and PCR. Four *G. intestinalis*–positive stool samples were co-infected with *Cryptosporidium* spp. Twenty-five stool samples were positive only for *Cryptosporidium* by both antigen detection test and PCR. Twenty-nine stool samples were negative for all three enteric protozoa by the antigen detection tests and PCR assays. There were no samples containing all three protozoan parasites. The TechLab *E. histolytica* II test (designed to detect specifically *E. histolytica*), *Giardia* II test, and *Cryptosporidium* test were used for detection of these three protozoan parasites in stool samples according to the manufacturer's instructions.

All antigen detection tests for these three protozoan parasites were procured from TechLab, Inc., Blacksburg, Virginia.

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Extraction of DNA from fecal specimens. For *E. histolytica* and *G. intestinalis,* 0.2 g of stool sample was used for extraction of DNA. The specimens were washed twice with sterile phosphate-buffered saline and centrifuged for 5 minutes at 14,000 rpm. For the QIAamp method, the stool pellet was subjected to six freeze-thaw cycles in liquid nitrogen and a 95°C water bath. DNA was extracted using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions except that the suspension was incubated in the kit's stool lysis buffer at 95°C, and a 3-minute incubation with the InhibitEX tablets was used. The DNA was eluted in 0.2 mL of AE buffer (supplied with the QIAGEN kit). Isolation of DNA from the *Cryptosporidium*-positive stool samples used a modification of the procedure. After diagnosis of *Cryptosporidium* by the antigen detection test, ~1 g of stool sample was concentrated using a modified ether-phosphate-buffered saline sedimentation technique and separated by density gradient centrifugation, and the oocysts were sonicated five times on the ice bath.[24] After sonication, DNA was extracted according to the method described above for *E. histolytica* and *G. intestinalis.*

Primers and probes. The primers and Taqman probes for *E. histolytica* (accession no. X64142) and *G. intestinalis* (accession no. M54878) were designed on small subunit ribosomal RNA gene.[18,25] The primers and Taqman probes for *Cryptosporidium* spp. were designed on *Cryptosporidium* Oocyst Wall Protein (COWP; accession no. AF248743).[20] The amplified targets were 134, 62, and 151 bp for *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp., respectively. All primers and Taqman probes used in this study were purchased from Eurogentec (Seraing, Belgium) (Table 1).

Singleplex real-time PCR assays. The amplification reactions for the singleplex real-time PCR assays were performed in a volume of 25 μL with Qiagen master mix (containing 100 mmol/L KCL; 40 mmol/L Tris-HCL, pH 8.4; 1.6 mmol/L deoxynucleoside triphosphate; iTaq DNA polymerase [50 units/mL], 2 mmol/L $MgCl_2$), with an additional 3 mmol/L $MgCl_2$ added. For *E. histolytica* and *G. intestinalis,* 0.4 μmol/L of each primer (Eh-f, Eh-r primers, and 0.08 μmol/L Eh-YYT probe for *E. histolytica;* Gd-80F, Gd-127R primers and 0.08 μmol/L Gd-FT probe for *G. lamblia*) and 1.5 μL of the extracted DNA were used in each reaction. For *Cryptosporidium* spp., 0.8 μmol/L of each primer (Cp-583F, Cp-733R primers) and 0.4 μmol/L Cp-TRT probe for *Cryptosporidium* spp. and 3 μL of the extracted DNA were used in each reaction. Amplification consisted of 3 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C for all three individual programmed. Fluorescence emitted at 530, 490, and 575 nm was measured for *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp., respectively.

Multiplex real-time PCR assay. Amplification reactions were performed in a volume of 25 μL with Qiagen master mix (containing 100 mmol/L KCL; 40 mmol/L Tris-HCL, pH 8.4; 1.6 mmol/L deoxynucleoside triphosphate; iTaq DNA polymerase [50 units/mL], 2 mmol/L $Mgcl_2$) and an additional 3 mmol/L $MgCl_2$ also added; 0.4 μmol/L of each Eh-f, Eh-r primers and 0.08 μmol/L Eh-YYT probes for *E. histolytica,* 0.4 μmol/L of each Gd-80F, Gd-127R primers and 0.12 μmol/L of Gd-FT probes for *Giardia,* and 1 μmol/L of each Cp-583F, Cp-733R primers and 0.5 μmol/L of Cp-TRT probes for *Cryptosporidium* and 3 μL of the DNA sample were used in each reaction. Amplification consisted of 3 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. Amplification, detection, and data analysis were performed with the i-Cycler real-time detection system (BioRad). Fluorescence was measured during the annealing step of each cycle. The ramping of the machine was 3.3°C/s in every step. Fluorescence at 530, 490, and 575 nm was measured for *E. histolytica, G. intestinalis,* and *Cryptosporidium,* respectively.

RESULTS

Analytical sensitivity and specificity. The analytical sensitivity of the developed multiplex real-time PCR assay for detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. was evaluated using cultured trophozoites of *E. histolytica, G. intestinalis,* and oocysts of *Cryptosporidium* spp. that were serially diluted in phosphate-buffered saline. The detection limit for the multiplex real-time PCR was 1 trophozoite of *E. histolytica* per extraction (100 μL), 10 trophozoites of *G. intestinalis* per extraction, and 100 oocysts of *Cryprosporidium* per extraction (Figure 1). The detection limit of singleplex PCR assays for these parasites was similar to the multiplex PCR assay. The multiplex real-time PCR assay and singleplex real-time PCR assays were specific for detection of *E. histolytica, Cryptosporidium* spp., and *G. intestinalis* because they were negative when DNA was introduced from

TABLE 1

Oligonucleotide primers and probes for real-time PCR assay for the simultaneous detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp.

Target organism: name of primers and probes	Oligonucleotide sequence (5′–3′)
E. histolytica	
Eh-f	AAC AGT AAT AGT TTC TTT GGT TAG TAA AA
Eh-r	CTT AGA ATG TCA TTT CTC AAT TCA T
Eh-YYT	**YYT**-ATT AGT ACA AAA TGG CCA ATT CAT TCA-**Dark Quencher**
G. intestinalis	
Gd-80F	GACGGCTCAGGACAACGGTT
Gd-127R	TTGCCAGCGGTGTCCG
Gd-FT	**FAM**-CCCGCGGCGGTCCCTGCTAG-**DDQ1**
Cryptosporidium spp.	
Cp-583F	CAA ATT GAT ACC GTT TGT CCT TCT G
Cp-733R	GGC ATG TCG ATT CTA ATT CAG CT
Cp-TRT	**Texas Red**-TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA T-**DDQ2**

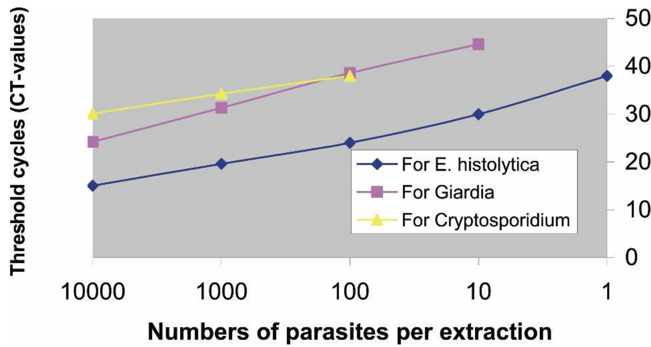

FIGURE 1. Analytical sensitivity of the multiplex real-time PCR assay for detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. This figure appears in color at www.ajtmh.org.

other parasites and bacteria, including *E. dispar, E. moshkovskii, B. hominis, V. cholerae,* and *E. coli.*

Comparison of multiplex real-time PCR assay and singleplex real-time PCR assays with the "gold standard" diagnosis on stool samples. The stool samples that were used for this evaluation included 42 positive for *Entamoeba histolytica,* 54 positive for *Giardia intestinalis,* 31 positive for *Cryptosporidium* spp., and 29 negative for all three parasites by the "gold standard" diagnosis (Table 2). Among the 42 *E. histolytica*–positive samples, 21 samples were also positive for *G. intestinalis,* whereas 2 stool samples were also positive for *Cryptosporidium* spp. There were four stool samples that were positive for both *G. intestinalis* and *Cryptosporidium* spp. Results of Taqman probe–based multiplex and singleplex PCR assays developed in this study for detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. compared with the "gold standard" diagnosis (both antigen detection test and PCR test positive) are shown in Tables 3–5.

Compared with the "gold standard" diagnosis, the multiplex real-time PCR assay had a sensitivity of 86% (36/42), whereas the singleplex PCR assay had a sensitivity of 95% (40/42) for detection of *E. histolytica.* Specificity of both multiplex and singleplex real-time PCR assays was 98% and 99%, respectively (Table 3). For *G. intestinalis* infection, the multiplex real-time PCR assay had a sensitivity of 89% (48/54), and the singleplex real-time PCR assay had a sensitivity of 91% (49/54) compared with the gold standard diagnosis. Specificity of both multiplex and singleplex PCR assays was

TABLE 2

Stool samples diagnosed according to "gold standard" diagnosis and singleplex and multiplex real-time PCR assays

Organisms	"Gold standard" diagnosis	Singleplex real-time PCR	Multiplex real-time PCR
E. histolytica only	19	20	19
G. intestinalis only	29	27	28
Cryptosporidium spp.	25	22	21
E. histolytica and *G. intestinalis*	21	19	17
E. histolytica and *Cryptosporidium* spp.	2	3	2
Cryptosporidium spp. and *G. intestinalis*	4	4	5
Negative for all three parasites	29	34	37
Total	129	129	129

TABLE 3

Results of multiplex and singleplex real-time Taqman-based PCR assays for detection of *E. histolytica* in stool specimens compared with "gold standard" (positive by antigen detection test and by another *E. histolytica*–specific PCR)

	Multiplex real-time PCR assay		Singleplex real-time PCR assay	
"Gold standard diagnosis"	Positive	Negative	Positive	Negative
Positive	36	6	40	2
Negative	2	88	1	86
	38	91	41	88

97% and 99%, respectively (Table 4). Compared with the gold standard diagnosis, the multiplex real-time PCR assay had a sensitivity of 90% (38/31), whereas the singleplex real-time PCR assay had a sensitivity of 94% (29/31). Specificity of both multiplex and singleplex PCR assays for detection of *Cryptosporidium* was 100% (Table 5).

The semiquantitative nature of the multiplex real-time PCR assay and singleplex real-time PCR assays may allow for the estimation of the parasite load in the different clinical samples. When the stool samples were positive only for *E. histolytica,* the multiplex and singleplex real-time PCR assays had a higher sensitivity, but the difference was not significant (data not shown). When the stool samples were positive only for *Cryptosporidium* spp. or *G. intestinalis,* no such difference was seen.

DISCUSSION

E. histolytica, G. intestinalis, and *Cryptosporidium* spp. are common parasitic agents of diarrhea. The clinical presentation of these infections makes it difficult to differentiate them from other bacterial and viral causes of diarrhea. Diagnosis by microscopy is neither sensitive nor specific. Individual antigen detection tests and PCR tests are now available for these parasites, but multiplex antigen detection tests are still under development. The real-time multiplex PCR assay for the detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. developed in this study gives a useful alternative for diagnosis of these parasites.

There is one other report on real-time multiplex PCR assay for detection of these parasites in the literature.[21] Our multiplex PCR used different amplification primers and protocols for *E. histolytica* and *Cryptosporidium* spp. than this previous report but had comparable performance. Our work extended the application of multiplex PCR by showing the sensitivity of our multiplexed assay to detect individual parasites, and by

TABLE 4

Results of multiplex and singleplex real-time Taqman-based PCR assays for detection of *G. intestinalis* in stool specimens compared with "gold standard" (positive by antigen detection test and by another *G. intestinalis*–specific PCR)

	Multiplex real-time PCR assay		Singleplex real-time PCR assay	
"Gold standard diagnosis"	Positive	Negative	Positive	Negative
Positive	48	6	49	5
Negative	2	73	1	74
	50	79	50	79

TABLE 5

Results of multiplex and singleplex real-time Taqman-based PCR assays for detection of *Cryptosporidium* spp. in stool specimens compared with "gold standard" (positive by antigen detection test and by another *Cryptosporidium* spp.–specific PCR)

"Gold standard diagnosis"	Multiplex real-time PCR assay		Singleplex real-time PCR assay	
	Positive	Negative	Positive	Negative
Positive	28	3	29	2
Negative	0	98	0	98
	28	101	29	100

showing the use of the technique for a larger number of samples from a different geographic area, including importantly stool samples positive for more than one parasite.

In this study, the multiplex real-time PCR assay had a similar sensitivity and specificity compared with the singleplex PCR assay for detection of *E. histolytica, G. intestinalis,* and *Cryptosporidium* spp. The multiplex PCR assay developed in this study for three intestinal protozoan parasites can be easily used for two parasites (e.g. *G. intestinalis* and *Cryptosporidium* spp.) without any loss of sensitivity and specificity (data not shown). Detection of parasite-specific DNA by PCR is more sensitive than microscopy.[14,18,20,25,26] In the past, the testing of multiple stool samples had been recommended for parasites. Detection of parasite-specific DNA has the potential to reduce the number of stool samples requiring analysis for diagnosis of the infections, and multiplexing promises to make PCR more practical for routine laboratory use. We targeted oocyst wall protein gene instead of the rRNA gene for *Cryptosporidium* because we wanted to have similar annealing temperature of all the primers that we have used in this study. The primers that we used for *Cryptosporidium* are capable of detecting *C. parvum, C. hominis,* and *C. melagiardis* but not other species of *Cryptosporidium.*

The implementation of multiplex assays and the development of automated DNA isolation could have a tremendous impact on routine parasitology laboratory studies in the developed world. However, the cost of such real-time PCR assays in developing countries at present will likely prevent their application to routine diagnosis, although they could still have an important role in systematic surveillance efforts.

Received July 18, 2006. Accepted for publication December 9, 2006.

Financial support: The study was conducted at the ICDDR,B with the support of NIH Grant U19 AI056872 through the University of Virginia (UVA). ICDDR,B acknowledges with gratitude the commitment of NIH and UVA to the Center's research efforts.

Disclaimer: Dr. Petri received research support from TechLab and royalties from a patent license agreement with TechLab for a diagnostic test for amebiasis. These royalties accrue to the American Society of Tropical Medicine and Hygiene without benefit to Dr. Petri.

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