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Simultaneous Detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in Fecal Samples by Using Multiplex Real-Time PCR

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***Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium* are three of the most important diarrhea-causing parasitic protozoa. For many years, microscopic examination of stool samples has been considered to be the "gold standard" for diagnosis of *E. histolytica*, *G. lamblia*, *and C. parvum* infections. Recently, more specific and sensitive alternative methods (PCR, enzyme-linked immunosorbent assay, and direct fluorescent-antibody assay) have been introduced for all three of these parasitic infections. 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 costs of a stool examination. Therefore, a multiplex real-time PCR assay was developed for the simultaneous detection of *E. histolytica*, *G. lamblia*, and *C. parvum* in stool samples. The multiplex PCR also included an internal control to determine efficiency of the PCR and detect inhibition in the sample. The assay was performed on species-specific DNA controls and a range of well-defined stool samples, and it achieved 100 percent specificity and sensitivity. The use of this assay in a diagnostic laboratory would provide sensitive and specific diagnosis of the main parasitic diarrheal infections and could improve patient management and infection control.**

Diarrheal diseases are extremely common in the developed and developing worlds and are major causes of morbidity and mortality, affecting millions of individuals each year (6, 15, 20). The etiologies of diarrhea include viruses (e.g., Norwalk-like viruses, rotaviruses, and enteric adenoviruses), bacteria (e.g., *Campylobacter jejuni*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli*, and cytotoxigenic *Clostridium difficile*), and parasites (15), of which *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* are considered to be the most important (2, 5, 7, 25, 29).

E. histolytica is the cause of amoebic colitis, amoebic dysentery, and amoebic liver abscess, resulting in 100,000 deaths annually (34). In recent years the biochemical, immunological, and genetic differences between *E. histolytica* and *Entamoeba dispar*, which were previously known as pathogenic and nonpathogenic strains of *E. histolytica*, respectively, have resulted in their redescription as two separate species (8, 14). As the potentially invasive *E. histolytica* is morphologically indistinguishable from the noninvasive *E. dispar*, microscopy alone cannot provide a definite answer about the presence of *E. histolytica* cysts and/or throphozoits, and additional methods such as antigen detection or PCR have to be employed. Moreover, many of the infections will be missed because the sensitivity of microscopy has been shown to be low (16, 27, 31).

G. lamblia infections are very common throughout the world and are considered one of the main nonviral causes of diarrhea in industrialized countries (29). Classically, laboratory diagnosis of *G. lamblia* infections is performed by microscopic examination of stool samples. In recent years, direct fluorescent-antibody assay and antigen detection by using enzyme-linked immunosorbent assay (ELISA) have been accepted as cost-effective alternative diagnostic methods (12, 21). However, PCR-based methods have also showed excellent specificity and sensitivity compared with microscopy as well with antigen detection (13, 33).

C. parvum has been recognized as the cause of large waterborne and food-borne outbreaks of gastroenteritis (5, 25). *C. parvum*-associated diarrhea has become well known as a result of the severe manifestations in AIDS patients, and subsequently improved diagnostic methods were developed. Modified acid-fast staining techniques are commonly used for the detection of *C. parvum* oocysts in fecal smears. However, the sensitivity and specificity appear to be rather low, as identification depends on the experience and skills of the microscopist (36). Monoclonal antibodies against cryptosporidium antigens are successfully used for fluorescence microscopy and in antigen ELISAs (4, 12, 28). However, nonspecificity of antibody-based methods owing to cross-reactivity with other microorganisms and low sensitivity is reported to be problematic (3, 9, 10, 18, 36, 37). Alternatively, PCR has shown to be sensitive and specific for the detection of *C. parvum* in fecal samples (24, 37).

In summary, *E. histolytica*, *G. lamblia*, and *C. parvum*, are three diarrhea-causing intestinal protozoa with often similar clinical presentations (35). For all three infections, microscopy is not the method of choice any more, as sensitive and specific detection is achieved by other methods. However, the differential use of these methods is hampered by the nonspecific clinical presentation. Although PCR-based methods have been

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successfully used for all three infections, their application in routine diagnosis is still very limited. Introduction of PCR-based methods has been hindered by difficulties in DNA extraction from fecal samples (23). Moreover, the amplification and detection of DNA were prone to contamination as well as being time-consuming and expensive. In recent years, however, the isolation of parasitic DNA from fecal samples has been improved and simplified (32). The introduction of real-time PCR with fluorescent detection probes (19) can reduce the risk of contamination, labor time, and reagent costs through the possibility of combining assays for the detection of different targets into one assay.

Therefore, a multiplex real-time PCR was developed for the simultaneous detection of *E. histolytica*, *G. lamblia*, and *C. parvum* in fecal samples. Additionally, an internal control for the detection of possible inhibition of the amplification by fecal contaminants was included in the assay. The performance of the assay was evaluated with a range of control samples.

MATERIALS AND METHODS

Controls and samples. *E. histolytica* control DNA was obtained from an axenic culture of *E. histolytica* strain HM1. *G. lamblia* DNA was isolated from purified *G. lamblia* cysts, and *C. parvum* DNA was isolated from purified *C. parvum* oocysts (Waterborne Inc.).

Twenty stool samples in which *E. histolytica* was detected by microscopy and confirmed by specific PCR were selected (30, 31). Furthermore, 20 stool samples that were microscopically and antigen positive for *G. lamblia* were used. Twenty stool samples in which the microscopic examination of modified acid-fast stained fecal smears revealed *Cryptosporidium* oocysts were also tested. All samples used were from different patients. Additionally, eight fecal samples from an immunocompromised child taken over a 1-year period, in which microscopy revealed *Cryptosporidium* oocysts in four of eight samples, were tested. Also, 25 stool samples from patients with a negative result in microscopy, modified acid-fast staining, and *Giardia* antigen test were tested. For these negative samples, two subsequent stool samples from these patients tested negative by all conventional methods.

On arrival, unpreserved fecal samples were stored at 4°C. A fecal suspension (≈0.5 g/ml of phosphate-buffered saline containing 2% polyvinylpolypyrolidone [Sigma])was prepared within 1 week for DNA isolation. The fecal suspensions for DNA isolation were then stored at −20°C.

The specificity of the PCR was tested by using *E. dispar*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, or *Cyclospora cayetanensis* DNA as the template. Specificity of the assay was also tested on DNAs obtained from 12 different bacterial and yeast cultures: *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Yersinia enterocolitica*, and *Candida albicans.* Furthermore, 20 stool samples from different patients in which *E. dispar* was detected by microscopy and confirmed by specific PCR (31) were tested.

Microscopy and antigen detection. Microscopic examination for the presence of ova and cysts was performed routinely by examination of iodine-stained wet mounts after Formol-ether concentration with a magnification of ×400 (1). Modified acid-fast staining was performed on direct smears and Formol-ether concentrates for detection of coccidian parasites (17). *G. lamblia* antigen ELISA (Alexon-Trend Inc.) was performed on unconcentrated specimens according to the manufacturer's recommendations, with some minor adjustments as described previously (21).

DNA isolation. For DNA isolation, 200 μl of feces suspension (≈0.5 g/ml of phosphate-buffered saline containing 2% polyvinylpolypyrolidone [Sigma]), purified parasites, or suspension of bacterial colonies was heated for 10 min at 100°C. After sodium dodecyl sulfate-proteinase K treatment (2 h at 55°C), DNA was isolated with QIAamp tissue kit spin columns (Qiagen, Hilden, Germany) (32) In each sample, 10^3 PFU of phocin herpesvirus 1 (PhHV-1) per ml was added to the isolation lysis buffer to serve as an internal control (26).

PCR amplification and detection. *E. histolytica* PCR primers were designed by using Primer Express software (Applied Biosystems) with the small-subunit (SSU) rRNA gene sequences for *E. histolytica* and *E. dispar* (GenBank accession no. X64142 and Z49256, respectively) such that DNAs for *E. histolytica* and *E. dispar* should be amplified. The *E. histolytica* minor groove binding (MGB) TaqMan (Applied Biosystems) probe was designed with the same sequence such that amplified *E. histolytica* DNA should be detected specifically. The *E. histolytica*- and *E. dispar*-specific primers amplified a 172-bp fragment inside the SSU rRNA gene. The MGB TaqMan probe was used to detect *E. histolytica*-specific amplification.

G. lamblia-specific PCR primers and a detection probe were chosen by using Primer Express software (Applied Biosystems) on the basis of the known SSU RNA gene sequence for *G. lamblia* (GenBank accession no. M54878) such that a 62-bp fragment within the SSU RNA gene should be amplified and detected specifically for *G. lamblia* (33). The *G. lamblia*-specific primers and probe set consisted of forward primer Giardia-80F, reverse primer Giardia-127R, and the *G. lamblia*-specific double-labeled probe Giardia-105T (Biolegio, Malden, The Netherlands).

The *C. parvum*-specific primers and detection probe described by Fontaine and Guillot (11) were used. The *C. parvum*-specific primers consisted of CrF and CrR, which amplify a 138-bp fragment inside the *C. parvum*-specific 452-bp fragment. Specific DNA amplification was detected with the *C. parvum*-specific double-labeled probe Crypto (Biolegio).

The PhHV-1 specific primer and probe (26) set consisted of forward primer PhHV-267s, reverse primer PhHV-337as, and the specific double-labeled probe PhHV-305tq (Biolegio).

Serial 10-fold dilution series of DNA extracted from each pathogen were used to make a standard curve, and this was analyzed by means of the iCycler IQ real-time detection apparatus software (Bio-Rad) to determine assay efficiency and correlation coefficient. The dilution series were used to optimize all three of the PCRs as a monoassay and thereafter to assess the performance of each assay with and without other primers in the multiplex assay. The dilution series of each pathogen were also tested with and without the presence of the internal control DNA to determine the influence of the internal control. Each dilution series was also tested with and without the other targets to assess the ability to detect mixed infections.

Amplification reactions were performed in a volume of 50 μl with PCR buffer (HotstarTaq master mix; Qiagen), 5 mM $MgCl_2$, 6.25 pmol of each *E. histolytica*-specific primer, 6.25 pmol of each *G. lamblia*-specific primer, 25 pmol of each *C. parvum*-specific primer, 15 pmol of each PhHV-1-specific primer, 1.75 pmol of *E. histolytica*-specific MGB-TaqMan probe, 2.5 pmol of *G. lamblia*-specific double-labeled probe, 8.75 pmol of *C. parvum*-specific double-labeled probe, 2.5 pmol of PhHV-1-specific double-labeled probe, and 5 μl of the DNA sample. Amplification consisted of 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with the iCycler real-time detection system (Bio-Rad). Fluorescence was measured during the annealing step of each cycle. All primers and detection probes are described in Table 1.

RESULTS

The specificity of the real-time multiplex PCR was evaluated by using a range of controls: DNAs from *E. dispar*, *E. bieneusi*, *E. intestinalis*, or *C. cayetanensis* and from *B. cereus*, *E. faecalis*, *S. aureus*, coagulase-negative *Staphylococcus*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. enterica* serovar Typhimurium, *S. flexneri*, *Y. enterocolitica*, and *C. albicans*. In none of these samples was amplification detected with any of the four assays. Furthermore, 20 DNA extracts derived from feces positive for *E. dispar* and 25 DNA extracts derived from feces from individuals with no known history of parasitic infections were tested. No amplification of *E. histolytica*, *G. lamblia*, or *C. parvum* DNA was detected in any of these samples; only amplification of the internal control was detected, at the expected threshold cycle of approximately 33.

The cycle threshold (Ct) values obtained from testing the dilution series of each pathogen in the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. The individual performance of the assays was not influenced by the presence of other primers, the presence of DNA from the internal control, or the presence of DNA from the other targets.

TABLE 1. Oligonucleotide primers and detection probes for real-time PCR for the simultaneous detection of *E. histolytica, G. lamblia, C. parvum* and PhHV-1 (internal control)

Target organism and oligonucleotide	Oligonucleotide sequence	GenBank accession no. or reference
E. histolytica		
Ehd-239F	5′-ATTGTCGTGGCATCCTAACTCA-3′	X64142, Z49256
Ehd-88R	5′-GCGGACGGCTCATTATAACA-3′	X64142, Z49256
histolytica-96T	VIC-5′-TCATTGAATGAATTGGCCATTT-3′-nonfluorescent quencher	X64142
G. lamblia		
Giardia-80F	5′-GACGGCTCAGGACAACGGTT-3′	M54878
Giardia-127R	5′-TTGCCAGCGGTGTCCG-3′	M54878
Giardia-105T	FAM-5′-CCCGCGGCGGTCCCTGCTAG-3′-black hole quencher 1	M54878
C. parvum		
CrF	5′-CGCTTCTCTAGCCTTTCATGA-3′	11
CrR	5′-CTTCACGTGTGTTTGCCAAT-3′	11
Crypto	Texas Red-5′-CCAATCACAGAATCATCAGAATCGACTGGTATC-3′-black hole quencher 2	11
PhHV-1		
PhHV-267s	5′-GGGCGAATCACAGATTGAATC-3′	26
PhHV-337as	5′-GCGGTTCCAAACGTACCAA-3′	26
PhHV-305tq	Cy5-5′-TTTTTATGTGTCCGCCACCATCTGGATC-3′-black hole quencher 2	26

E. histolytica-specific amplification was detected in 20 of 20 DNA samples isolated from feces known to contain *E. histolytica* cysts. Ct values were found to be between 25.1 and 37.3, with a median threshold of 29.3 cycles. *G. lamblia* amplification was detected in all 20 samples in which *G. lamblia* was detected by microscopic examination and *Giardia* antigen test, with Ct values of between 24.2 and 37.7, with a median of 29.9 cycles. In 20 samples in which modified acid-fast staining revealed *Cryptosporidium* oocysts, *C. parvum* specific amplification was detected, with Ct values of between 24.0 and 36.7 and a median threshold of 29.7 cycles. Furthermore, in seven samples from an immunocompromised child with complaints of diarrhea, *C. parvum* DNA was detected with the multiplex real-time PCR. In only four of these samples were *Cryptosporidium* oocysts found by microscopic examination of modified acid-fast-stained fecal smears (Table 2).

TABLE 2. Comparison of microscopic examination of modified acid-fast-stained fecal smears and multiplex *E. histolytica-G. lamblia-C. parvum* real-time PCR for detection of *Cryptosporidium* infection in an immunocompromised child with complaints of diarrhea

Date of stool collection (day-mo-yr)	Microscopy result	Multiplex real-time PCR	
		Result	Ct value
31-5-2002	−	−	
12-9-2002	−	+	32.7
17-9-2002	−	+	28.0
10-2-2003	+	+	26.8
18-2-2003	+	+	27.9
10-4-2003	−	+	37.1
29-4-2003	+	+	31.2
13-5-2003	+	+	27.8

DISCUSSION

The most important parasitic causes of diarrhea are *E. histolytica*, *G. lamblia*, and *C. parvum.* The often nonspecific clinical presentation of the infections makes it difficult to choose more specific and sensitive methods for the detection of these pathogens. The multiplex real-time PCR for the detection of *E. histolytica*, *G. lamblia*, and *C. parvum* presented in this study gives a useful alternative for these individual additional methods.

With well-defined DNA and stool samples as controls, the multiplex real-time assay for the detection of *E. histolytica*, *G. lamblia*, and *C. parvum* achieved specificity of 100%. In all samples tested in which microscopy revealed the presence of *E. histolytica*, *G. lamblia*, or *C. parvum*, specific amplification was detected. There was no difference in the performance of the amplification of the specific targets in the individual assays compared with the multiplex PCR, so the multiplex PCR could be used with equal confidence as the individual assays.

PCR inhibition by fecal constituents is known to be a serious problem (23). However, in stool samples without a known history of parasitic infection and in stool samples with *E. dispar*, only the amplification of the internal control was detected. Hence, there was no evidence of inhibition of the amplification in any of these samples with this DNA isolation method.

Detection of parasite-specific DNA appears to be more sensitive than microscopy, as has been shown for *G. lamblia* infections with (real-time) *G. lamblia* PCR (13, 33), for *C. parvum* infections (24, 37), and for amoebic infection with *E. histolytica* and *E. dispar* specific (real-time) PCR (22, 31). In this study, *C. parvum* DNA was detected with the multiplex real-time PCR in the course of infection before microscopy became positive in an immunocompromised child with complaints of diarrhea (Table 1). Detection of parasite-specific

DNA has the potential to reduce the number of stool samples requiring analysis for the diagnosis of these infections.

In the future, other multiplex assays combining other parasitic targets could be developed, for example, for the detection of *E. histolytica*, *G. lamblia*, and *Cyclospora cayetanensis* in stool samples from travelers returning from the tropics and for the detection of *E. bieneusi*, *E. intestinalis*, and *C. parvum* in immunocompromised patients. The implementation of such multiplex assays and the development of automated DNA isolation procedures could have a tremendous impact on routine parasitology practice. Moreover, when combined with a comparable approach for the detection of viruses and bacteria that cause diarrhea, this would create a whole new dimension in the differential laboratory diagnosis of diarrheal diseases in general. The application of such an approach in a clinical or epidemiological setting, including the costs compared to those of a combination of other methods, has not been evaluated and needs further study.

In conclusion, the multiplex real-time PCR described here is a sensitive and specific method for the detection of *E. histolytica*, *G. lamblia*, and *C. parvum* and offers the possibility of introducing DNA detection as a feasible technique in the routine diagnosis of intestinal parasitic infections.

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