AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

PARASITOLOGY

A Real-Time PCR Assay for Simultaneous Detection and Differentiation of Four Common *Entamoeba* Species That Infect Humans

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ABSTRACT There are over 40 species within the genus *Entamoeba*, eight of which infect humans. Of these, four species (*Entamoeba histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*) are morphologically indistinguishable from each other, and yet differentiation is important for appropriate treatment decisions. Here, we developed a hydrolysis probe-based tetraplex real-time PCR assay that can simultaneously detect and differentiate these four species in clinical samples. In this assay, multicopy small-subunit (SSU) ribosomal DNA (rDNA) sequences were used as targets. We determined that the tetraplex real-time PCR can detect amebic DNA corresponding to as little as a 0.1 trophozoite equivalent of any of these species. We also determined that this assay can detect *E. histolytica* DNA in the presence of 10-fold more DNA from another *Entamoeba* species in mixed-infection scenarios. With a panel of more than 100 well-characterized clinical samples diagnosed and confirmed using a previously published duplex real-time PCR (capable of detecting *E. histolytica* and *E. dispar*), our tetraplex real-time PCR assay demonstrated levels of sensitivity and specificity comparable with those demonstrated by the duplex real-time PCR assay. The advantage of our assay over the duplex assay is that it can specifically detect two additional *Entamoeba* species and can be used in conventional PCR format. This newly developed assay will allow further characterization of the epidemiology and pathogenicity of the four morphologically identical *Entamoeba* species, especially in low-resource settings.

KEYWORDS *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba bangladeshi*, amebiasis, diagnosis, PCR, real-time PCR, pathogenicity

Amebiasis, a disease caused by the ameba *Entamoeba histolytica*, occurs in an estimated 500 million people worldwide and is responsible for 50,000 to 100,000 deaths annually, making it the third leading cause of death among parasitic diseases (1–3). It is transmitted through the fecal-oral route and is endemic in low-income regions that lack access to safe drinking water and sanitation, including Southeast Asia, Africa, Mexico, and South America (4–6). In developed countries, amebic infections are concentrated in certain high-risk groups, such as men who have sex with men, people who live in long-term-care facilities, and travelers returning from a country where the disease is endemic (6, 7). Nine of 10 *E. histolytica* infections remain asymptomatic; however, 10% of infections cause intestinal disease, such as amebic colitis (e.g., diarrhea or dysentery), or, less commonly, extraintestinal disease, such as amebic liver abscess (ALA), which can be fatal if not treated promptly (3, 8). In children, *E. histolytica* infections are linked to growth stunting (9) and delayed cognitive development (10). It

Citation Ali IKM, Roy S. 2021. A real-time PCR assay for simultaneous detection and differentiation of four common *Entamoeba* species that infect humans. J Clin Microbiol 59:e01986-20. https://doi.org/10.1128/JCM.01986-20.

Editor Bobbi S. Pritt, Mayo Clinic

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Received 29 July 2020
Returned for modification 23 August 2020
Accepted 21 October 2020

Accepted manuscript posted online 28 October 2020
Published 17 December 2020

remains unclear why only 10% of *E. histolytica* infections are symptomatic, but this is thought to be linked to parasite genotype (11, 12), host genetics (13, 14), and/or environmental factors (15, 16). The World Health Organization recommends treatment of all *E. histolytica* infections irrespective of associated symptoms (2). Treating asymptomatic carriers of *E. histolytica* not only eliminates future risk of disease but can prevent the spread of infectious ameba cysts excreted in stool.

While disease caused by *E. histolytica* is well documented, there are three additional species of *Entamoeba* also shown to infect humans: *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*. Traditionally, *E. dispar* has been almost universally accepted as a nonpathogenic species. Recent data suggest *E. moshkovskii* might be an emerging pathogen in children, causing diarrhea and dysentery in Bangladesh (17). *E. bangladeshi*, originally identified in two individuals in Bangladesh in 2012 (18) and recently detected in South Africa, occurs in both asymptomatic and diarrheal individuals (19) and is commonly observed in stool of those infected with other *Entamoeba* species; however, limited epidemiologic data prevent a definitive conclusion on pathogenicity (19).

Accurate diagnosis of amebiasis is challenging, especially in countries where the disease is endemic that still rely on microscopic detection of ameba cysts and trophozoites in stool samples (20). Morphologic *E. histolytica* resemblance to other nonpathogenic or "undetermined-pathogenic" species makes the majority of earlier epidemiological data on amebiasis questionable (21–23). In addition to the fact that microscopy is a nonspecific diagnostic tool due to the existence of other morphologically identical species, its use is also confounded by false-positive results due to the presence of host macrophages and other cells resembling *E. histolytica* (24, 25). Additionally, microscopy is highly insensitive as it relies on intact ameba structures and because the trophozoite form of *E. histolytica* begins to disintegrate within 1 to 2 h of stool production unless properly preserved prior to microscopic examination (20). In most ALA cases, stool microscopy is not helpful as concurrent intestinal infection is usually not seen in a majority of cases (26–29).

Several enzyme-linked immunosorbent assays (ELISAs) that detect amebic antigen in stool or liver aspirate pus samples show variable success rates in the detection of *E. histolytica* (reviewed in references 20, 30, and 31). Among these, the *E. histolytica* II ELISA (TechLab, Blacksburg, VA) is approved by the U.S. Food and Drug Administration. It performed well in most countries where the disease is endemic (32–34) but poorly in some of the countries where the disease is not endemic (35, 36). The assay performs poorly with frozen and preserved samples and with samples that are collected from patients already treated with antiamebic drugs (28). Similar assays for species-specific detection of *E. dispar*, *E. moshkovskii*, or *E. bangladeshi* are unavailable.

Molecular diagnosis performed with conventional and real-time PCR is more sensitive and specific than the ELISAs and enables species-specific detection. Species-specific detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii* is available in various formats, including conventional PCR (37, 38), nested PCR (39–41), and real-time PCR (42, 43). Several conventional and real-time PCR assays are also available for simultaneous detection and differentiation of *E. histolytica* and two other major diarrhea-causing parasites, *Giardia* and *Cryptosporidium* species (44–50). Real-time PCR assays have advantages over conventional PCR assays, as they are faster, can be monitored in real time, and are less prone to contamination since post-PCR processing steps to detect amplified products, such as running of gels, are not needed. However, real-time PCR assays require expensive equipment and supplies and technical expertise that are not readily available in resource-poor settings. Also, proper facilities (e.g., separate rooms for DNA purification and master mix preparation), knowledge (e.g., one-way direction of material and personnel, etc.), and participation in external quality assessment schemes may not be readily available in resource-poor settings. Recently, a hydrolysis probe-based real-time PCR for simultaneous detection and differentiation of four *Entamoeba* species (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*) was reported (19). While the probes in that assay are species specific, it utilizes primers that are common to all four *Entamoeba* species. As a result, the assay does not provide

species-specific information if used in the conventional PCR format in resource-poor settings, limiting its use to the real-time PCR format only.

We report a hydrolysis probe-based tetraplex real-time PCR assay developed to detect four *Entamoeba* species (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*) in clinically relevant stool and liver aspirate pus samples. Because it utilizes four sets of species-specific primers, it offers an advantage over the existing tetraplex real-time PCR assay by allowing species-specific detection in a conventional PCR format in the absence of a real-time PCR instrument and the associated supplies and technical expertise. The performance characteristics of this tetraplex real-time PCR were compared with those of an established duplex real-time PCR assay for *E. histolytica* and *E. dispar* using DNA extracted from more than 100 intestinal and ALA samples from clinical patients.

MATERIALS AND METHODS

Cultures of *Entamoeba*. *E. histolytica* HM-1:IMSS culture was maintained axenically in TYI-S-33 (Trypticase, yeast extract, and iron serum) medium at the U.S. Centers for Disease Control and Prevention (CDC) Free-Living and Intestinal Amebas (FLIA) laboratory as previously described (51). Extracted DNA from a known number of ameba trophozoites was used to determine the limit of detection (LOD) of the *E. histolytica* real-time PCR assay in singleplex and multiplexed real-time PCR assay formats. Axenic culture lysates with known numbers of trophozoites from *E. dispar* SAW760 and *E. moshkovskii* Laredo strains and xenic culture (52) lysate from known numbers of trophozoites of *E. bangladeshi* were kindly provided by Rashidul Haque in the Parasitology Laboratory of International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). These were used to determine LODs for the individual and multiplexed real-time PCR assays and were also used to determine the linear range of individual singleplex real-time PCR assays.

DNA from culture isolates and clinical samples. DNA from 27 xenic culture isolates (including 11 *E. histolytica* isolates, 9 *E. dispar* isolates, 3 *E. moshkovskii* isolates, 1 *E. bangladeshi* isolate, 2 *Blastocystis hominis* isolates, and 1 *Entamoeba coli* isolate), DNA from 46 clinical stool samples (including 12 samples positive for *E. histolytica*, 16 for *E. dispar*, and 1 for *E. moshkovskii* and 17 negative for *Entamoeba* spp.), and DNA from 41 aspirated liver abscess samples (including 38 samples positive for *E. histolytica* and 3 from patients with bacterial liver abscess) were utilized in this study. These DNAs were collected for other studies, but they were made available for Ph.D. research studies of I. K. M. Ali at the London School of Hygiene and Tropical Medicine, United Kingdom, during 2001 to 2005. The samples were deidentified prior to their use in this study. All samples had previously been characterized in ICDDR,B laboratories using one or more of the following methods (described elsewhere as indicated): isoenzyme analysis (24), nested PCR (24), antigen detection *E. histolytica* II ELISA (24) (TechLab, Blacksburg, VA), or a real-time PCR (53).

DNA purification. DNA was purified from 50 μl of culture pellet dispersed in 250 μl of lysis buffer (0.25% sodium dodecyl sulfate–0.1 M EDTA, pH 8.0) containing100 μg/ml of proteinase K using the cetyltrimethylammonium bromide (CTAB) method, as described previously (54). DNA was purified from 0.1 g of stool samples using a QIAamp DNA stool minikit (catalog no. 51604, Qiagen, USA) as described previously (55). DNA was purified from 200-μl volumes of aspirated liver abscess samples using a DNeasy blood and tissue kit (catalog no. 69506, Qiagen, USA) according to the manufacturer's instructions. A previously characterized *Entamoeba*-negative stool or liver abscess sample that had been spiked with an aliquot (~100 μl) of a known *Entamoeba* culture lysate served as a positive extraction control. The same previously characterized *Entamoeba*-negative stool or liver abscess sample served as a negative extraction control, which was not spiked with an *Entamoeba* culture lysate.

Primer and probe design. To design primers and probes, complete or partial 18S small-subunit rRNA gene sequences of *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* (only partial sequences were available for *E. bangladeshi*) were downloaded from PubMed (GenBank accession numbers: X75434.1 for *E. histolytica*, KP722600.1 for *E. dispar*, KP722602.1 for *E. moshkovskii*, and KR025411.1 for *E. bangladeshi*) and sequences were aligned using multiple-sequence-alignment software (http://multalin.toulouse.inra.fr/multalin/) (56). We chose the 18S small-subunit ribosomal rRNA genes as PCR targets because they occur in hundreds of copies per ameba genome (57). For each species, unique sequences were selected and sequence differences were chosen at the 3′ ends of primers to maximize the species specificity (Fig. 1). All probe sequences selected were species specific to increase the specificities of the individual and tetraplex real-time PCR assays (Table 1).

PCR assays. Real-time PCR assays were performed in tetraplex or singleplex format in duplicate in a total volume of 20 μl using commercially available Platinum Quantitative PCR SuperMix-UDG with ROX (catalog no. 11743-500, Fisher Scientific, USA). Each DNA template was diluted 1:2 and 1:20 with sterile distilled water to further minimize any adverse effect of the presence of PCR inhibitors that could have still been copurified during DNA extraction steps despite the use of an extraction kit that should have removed inhibitors. Next, 5 μl of each dilution was added to each PCR mixture in two separate wells. Positive controls (either a mixture of 4 ameba DNAs for tetraplex real-time PCR or a single relevant ameba DNA for the singleplex real-time PCR) and negative controls (sterile water) were included in each PCR run. One microliter each of forward and reverse primers from a 20 μM stock and 1 μl of each probe from a 2 μM stock were added to the final reaction mixture. The PCR assay described above was also run in a

(A) *E. histolytica*.

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      1
Eh:   CAGTAATAGTTTCTTTGGTTAGTAAAATACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Ed:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Em:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Eb:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG

                                                                      133
Eh:   ACGATCCAGTTTGTATTAGTACAAATGGCCA-ATTCATTCAATGAATTGAGAAATGACATTCTAAG
Ed:   ACGATCCAATTTGTATTAGTACAAAGTGGCCAATTTATGTAAGTAAATTGAGAAATGACATTCTAAG
Em:   ACGATCCGGTTTGTATTAGTACAAGTCGGCCACTCTCTTCACGGGGAGTGCGAATGCCATTCTGAAT
Eb:   ACGATCCGGTTTGTATTAGTACAAATTGGCCATACTCTGTAAGGGGTATGAAAAATGACATTCTAAG
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(B) *E. dispar*.

```
      1
Eh:   CAGTAATAGTTTCTTTGGTTAGTAAAATACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Ed:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Em:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG
Eb:   CAGTAATAGTTTCTTTGGTTAGTAAAGTACAAGGATAGCTTTGTGAATGATAAAGATAATACTTGAG

                                                                      134
Eh:   ACGATCCAGTTTGTATTAGTACAAAATGGCCA-ATTCATTCAATGAATTGAGAAATGACATTCTAAG
Ed:   ACGATCCAATTTGTATTAGTACAAAGTGGCCAATTTATGTAAGTAAATTGAGAAATGACATTCTAAG
Em:   ACGATCCGGTTTGTATTAGTACAAGTCGGCCACTCTCTTCACGGGGAGTGCGAATGCCATTCTGAAT
Eb:   ACGATCCGGTTTGTATTAGTACAAATTGGCCATACTCTGTAAGGGGTATGAAAAATGACATTCTAAG
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(C) *E. moshkovskii*.

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      1
Eh:   CAGATGGCTACCACTTCTAAGGAAGGCAGCAGGCSCGTAAATTACCCACTTTCGAATTGAAGAGGTAGTGACG
Ed:   CAGATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGTAAATTACCCACTTTCGAATTGAAGAGGTAGTGACG
Em:   CAGATGGCTACCACTTCTACGGAAGGCAGCAGGCGCGTAAATTACCCACTTTCGACGTGAAGAGGTAGTGACG
Eb:   CAGATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGTAAATTACCCACTTTCGAAGTGAAGAGGTAGTGACG

                                                                      145
Eh:   ACACATAACTCTAGAGTTGAGTAAAATCAATTCTTGAAGGAATGAGTAGGAGGTAAATTCTCCTACGAAATC
Ed:   ACACATAACTCTAGAGTTGAGTAAAATCAATTCTTGAAGGAATGAGTAGGAGGTAAATTCTCCTACGAAATC
Em:   ACAAATAACTCTCGAGGTGGTTAACTCCCACTTCTTGAAGGAATGAGTAAGAAGTAAATACTCTTACGAAATC
Eb:   ACACATAACTCTTGAGCTGAGTAAAATCAGTTCTTGAAGGAATGAGTAGGAGGTAAAGTCTCCTACGAAATC
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(D) *E. bangladeshi*.

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      1
Eh:   GTTTCTAGAAATGTTAAATTAAAATCAAAGAAGGAAACAATTCAAGTAATTGAGTTGTTATTACTT
Ed:   GTTTCTAGAAATGTTAAATTAAAATCAAAGAAGGAGACNNTTCAAGTAATTGAGTTGTTATTACTT
Em:   GTTTCTAGGAATGAGGTAATGAAACTAACGAAGGAGATGAAGTGAGTAATCACTTTATCATTACTT
Eb:   GTTTCTAGAGATGTGATAATGGTATTAAAGAAGGGGATGAGTCAAGTAATTGATTTGTCATTACTT

                                                                      132
Ed:   TGAATAAAATAAGGTGTTTAAAGCAAAACATTATGTTAATGAATATTCGAGCATGGGACAATGCTG
Eh:   TGAATAAAATAAGGTGTTTAAAGCAAAACATTATGTTAATGAATATTCAAGCATGGGACAATGCTG
Em:   TGAATAAAATAGAGTGTTTAAAGCAAAACATTAAGTTAATGAATATTCAAGCATGGGACAATGCTG
Eb:   TGAATAAAATAGGGTGTTTAAAGCAAAACATTAAGTTAATGGATATTCAAGCATGGGACAATATTG
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FIG 1 Locations of primers and probes in the 18S small-subunit rRNA genes. Alignment of corresponding 18S small-subunit rRNA genes of four *Entamoeba* species was performed to show locations of primer and probe sequences. Sequences of forward primers are underlined with solid lines, sequences of reverse primers are underlined with double solid lines, and the sequences of the probes are underlined with dashed lines and italicized. Primer and probe sequences that differ from those of at least one other *Entamoeba* species are shown by bold fonts. (A) *E. histolytica* (*Eh*). (B) *E. dispar* (*Ed*). (C) *E. moshkovskii* (*Em*). (D) *E. bangladeshi* (*Eb*). The GenBank accession numbers are as follows: X75434.1 (*E. histolytica*), KP722600.1 (*E. dispar*), KP722602.1 (*E. moshkovskii*), and KR025411.1 (*E. bangladeshi*).

conventional PCR format (i.e., without the use of probes) in singleplex PCR format. Results were considered valid when the results obtained with the replicates agreed. The duplex real-time PCR assay for *E. histolytica* and *E. dispar* was performed according to a method previously described by Qvarnstrom et al. (58). All PCR assays were carried out in an ABI7500 thermal cycler (Applied Biosystems, USA). The tetraplex or singleplex real-time PCR thermal cycles consisted of one cycle at 50°C for 2 min and 95°C for

TABLE 1 Primers and probes[a]

Primer or probe name	Sequence (5′ to 3′)[b]	Specificity	Product size (bp)
*Eh*F3	CAGTAATAGTTTCTTTGGTTAGTAAAA	*E. histolytica*	133
*Eh*R3	CTTAGAATGTCATTTCTCAATTCAT		
*Eh*P3	HEX-GTTTGTATTAGTACAAAATGGC-BHQ1		
*Ed*F3	CAGTAATAGTTTCTTTGGTTAGTAAAG	*E. dispar*	134
*Ed*R3	CTTAGAATGTCATTTCTCAATTTAC		
*Ed*P3n1	Cy5-GTATTAGTACAAAGTGGCCAA-BHQ3		
*Em*F4	CAGATGGCTACCACTTCTAC	*E. moshkovskii*	145
*Em*R4	GATTTCGTAAGAGTATTTACTTCT		
*Em*P4	FAM-CTCGAGGTGGTTAACTCCAC-BHQ1		
*Eb*F2	GTTTCTAGAGATGTGATAATGG	*E. bangladeshi*	132
*Eb*R2	CAATATTGTCCCATGCTTGAATATC		
*Eb*P2	TAMRA-GGGTGTTTAAAGCAAAACATTAA-BHQ2		

[a]18S small-subunit rRNA gene sequences of *Entamoeba* species were used to design primers and probes. In the primer and probe names, "F" indicates forward primer, "R" indicates reverse primer, "P" indicates probe, "*Eh*" indicates *E. histolytica*, "*Ed*" indicates *E. dispar*, "*Em*" indicates *E. moshkovskii*, and "*Eb*" indicates *E. bangladeshi*.

[b]5′ fluorescent dyes and the 3′ quencher molecules of the probes are shown at the respective ends. BHQ, black hole quencher; FAM, 6-carboxyfluorescein; HEX, 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein; TAMRA, 6-carboxytetramethylrhodamine.

2 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Real-time PCR data were analyzed using Applied Biosystems 7500 software for 7500 and 7500 Fast real-time PCR systems (v2.3). The conventional singleplex PCR was performed exactly the same as the singleplex real-time PCR except that no probes were added to the reaction mixture.

Gel electrophoresis. Amplified products (10 μl) from the conventional singleplex PCR assays were run on a 2% agarose gel (SeaKem LE Agarose, USA) containing a 1× final concentration of GelRed nucleic acid stain solution (Biotium, USA) for 2 h at a constant voltage (125 V) (59). A 100-bp DNA size marker (BioLabs, USA) was used to verify the sizes of amplicons.

Limit of detection (LOD) of singleplex and tetraplex real-time PCR assays. In order to detect the minimum quantity of organism DNA needed to obtain a positive amplification signal (i.e., LOD), *Entamoeba*-negative (verified by microscopy and PCR) stool or liver aspirate samples were spiked with known numbers of trophozoites of *E. histolytica*, *E. dispar*, *E. moshkovskii*, or *E. bangladeshi*. DNA was extracted by the use of Qiagen stool kits or Qiagen blood and tissue kits. Eluted DNA was then serially diluted 10-fold to produce 1.0-μl volumes containing DNA from 10,000, 1,000, 100, 10, 1, 0.1, or 0.01 trophozoite equivalents. DNA was assayed using the singleplex or tetraplex real-time PCR format with species-specific primers and probes.

LOD of *E. histolytica* real-time PCR in mixed infections. Mixed infections with more than one species of *Entamoeba* in the same individual are not uncommon. Detection of mixed infection of *E. histolytica* with another *Entamoeba* species is particularly important because only *E. histolytica* infection requires treatment. Here, we investigated the limit of the ability of *E. histolytica* real-time PCR and the tetraplex real-time PCR to detect *E. histolytica* DNA in the presence of various proportions of DNA from one or more of other *Entamoeba* species. We used *E. histolytica* DNA that had originated from 0.1, 1.0, 10, 100, 1,000, and 10,000 trophozoite equivalents in the presence of a base level of either 10 or 100 trophozoite DNA equivalents from other species in duplex or tetraplex PCR format.

Linear range of detection. In order to determine the linear range of detection for each individual singleplex real-time PCR, standard curves were generated using DNA originating from 0.1 to 10,000 trophozoite equivalents per reaction mixture. Each assay was repeated in triplicate.

Specificity. The specificity of each of the *Entamoeba* real-time PCRs was evaluated individually against the DNAs from other three *Entamoeba* species. For example, the *E. histolytica* primers and probe (Fig. 1) were allowed to amplify DNAs originating from 10,000 trophozoites of each of four *Entamoeba* species in separate wells. Additionally, the specificity of the primers and probes in this study was evaluated in singleplex and tetraplex formats against the DNAs from other intestinal organisms such as *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba polecki*, *Blastocystis hominis*, *Cryptosporidium*, *Giardia*, *Shigella*, *Campylobacter*, and *Escherichia coli*.

Data analysis and calculation of comparative sensitivity and specificity. In order to minimize the run-to-run variations, quantification cycle (C_q) values were manually adjusted with a known positive control in each run. If a clinical sample gave a C_q value of <37, it was considered positive. However, samples that gave late C_q values of between 37 and 40 were assayed at least twice, and if they still gave a similar or lower C_q value (i.e., <37), they were considered positive.

In comparison with the duplex real-time PCR for *E. histolytica* and *E. dispar*, the sensitivity and specificity of the tetraplex real-time PCR assay were calculated for the ability to detect *E. histolytica* and *E. dispar* with the following formulas: sensitivity = (no. of correct positive results)/(total no. of positive samples) × 100%; specificity = (no. of correct negative results)/(total no. of negative samples) × 100%.

TABLE 2 Limit of detection[a]

No. of trophozoite DNAs used	C_q LOD							
	[b]Singleplex real-time PCR				[c]Tetraplex real-time PCR			
	E. histolytica	*E. dispar*	*E. moshkovskii*	*E. bangladeshi*	*E. histolytica*	*E. dispar*	*E. moshkovskii*	*E. bangladeshi*
10,000	16.4	19.8	18.3	16.9	16.5	18.8	14.9	16.4
1,000	19.7	22.7	21.7	20.3	21	22.8	17.9	19.9
100	23.1	26.3	25.2	22.6	24.4	26	21.5	22.4
10	27.4	29.7	29	26.9	27.7	30.2	25.5	26.3
1	31.7	35.6	33.1	27.9	30.6	34.3	27.8	26.6
0.1	35.1	38.1	36	34.6	38.2	38.3	33.1	34.3
0.01	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
None	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q

[a]Limit of detection (LOD) values were calculated in the singleplex and tetraplex real-time PCR formats.
[b]For the singleplex PCRs, *Cq* values are shown with the respective primers and probe sets. All the signals were species specific. For example, *E. histolytica* primers and probe gave signals (i.e., *Cq* values) only with the *E. histolytica* DNA.
[c]DNA from all 4 species was mixed together to perform the tetraplex real-time PCR.

RESULTS

Limit of detection (LOD). Individually, *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* real-time PCR assays were sufficiently sensitive to detect the respective species DNAs originated from a 0.1 trophozoite equivalent (Table 2). The LODs remained the same when the real-time PCR was performed in the tetraplex PCR format (Table 2). Because the PCR target is multicopy small-subunit (SSU) ribosomal DNA (rDNA), which is maintained at about 200 copies per genome (57), a 0.1 trophozoite equivalent should contain about 20 copies of target DNA.

Linear range of detection. Standard curves for individual *Entamoeba* real-time PCR assays were calculated and found to be linear over a range from 0.1 to 10,000 trophozoite equivalents per reaction (Fig. 2) indicating that the individual real-time PCR assays could detect as little as 0.1 trophozoite equivalent per reaction, showing a high degree of correlation ($r^2 > 0.97$).

Specificity. Each of the individual *Entamoeba* real-time PCR assays was evaluated individually against the DNAs from other three *Entamoeba* species. *E. histolytica* real-time PCR gave positive signal only with the *E. histolytica* DNA and did not show any cross-reaction with any other *Entamoeba* DNAs. Similarly, the other three *Entamoeba* real-time PCRs gave amplification signals only with the respective species DNAs and did not show cross-reaction with any other *Entamoeba* species DNA. Additionally, each *Entamoeba* primer set was checked for cross-reaction with DNA from the other three *Entamoeba* species in separate reaction wells in conventional PCRs. Each primer set gave expected amplification only with the respective *Entamoeba* DNA and did not cross-react with other *Entamoeba* DNAs (Fig. 3).

The primers and probes in this study did not give nonspecific fluorescence signals in singleplex or tetraplex real-time PCRs against the DNAs from other intestinal organisms such as *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba polecki*, *Blastocystis hominis*, *Cryptosporidium*, *Giardia*, *Shigella*, *Campylobacter*, and *Escherichia coli*. Similarly, nonspecific amplifications were not detected in conventional PCRs either in singleplex or tetraplex formats (data not shown). We conclude that the *Entamoeba* primers and probes in this study were unique to the species level.

LOD of *E. histolytica* real-time PCR in mixed infections. In a mixed-infection scenario with more than one species of *Entamoeba* in the same individual, our results indicated that the *E. histolytica* real-time PCR assay was capable of detecting *E. histolytica* DNA in the presence of up to 100-fold more of another *Entamoeba* DNA in duplex real-time PCR or of up to 10-fold more of the remaining three *Entamoeba* DNAs in the tetraplex real-time PCR (Table 3). If the other *Entamoeba* DNAs were present at >100-fold compared to the *E. histolytica* DNA, then the *E. histolytica* real-time PCR failed to detect it. This is a limitation of this real-time PCR.

Comparison with the CDC diagnostic duplex real-time PCR for the detection of *E. histolytica* and *E. dispar* using clinical samples. DNA from cultured organisms and

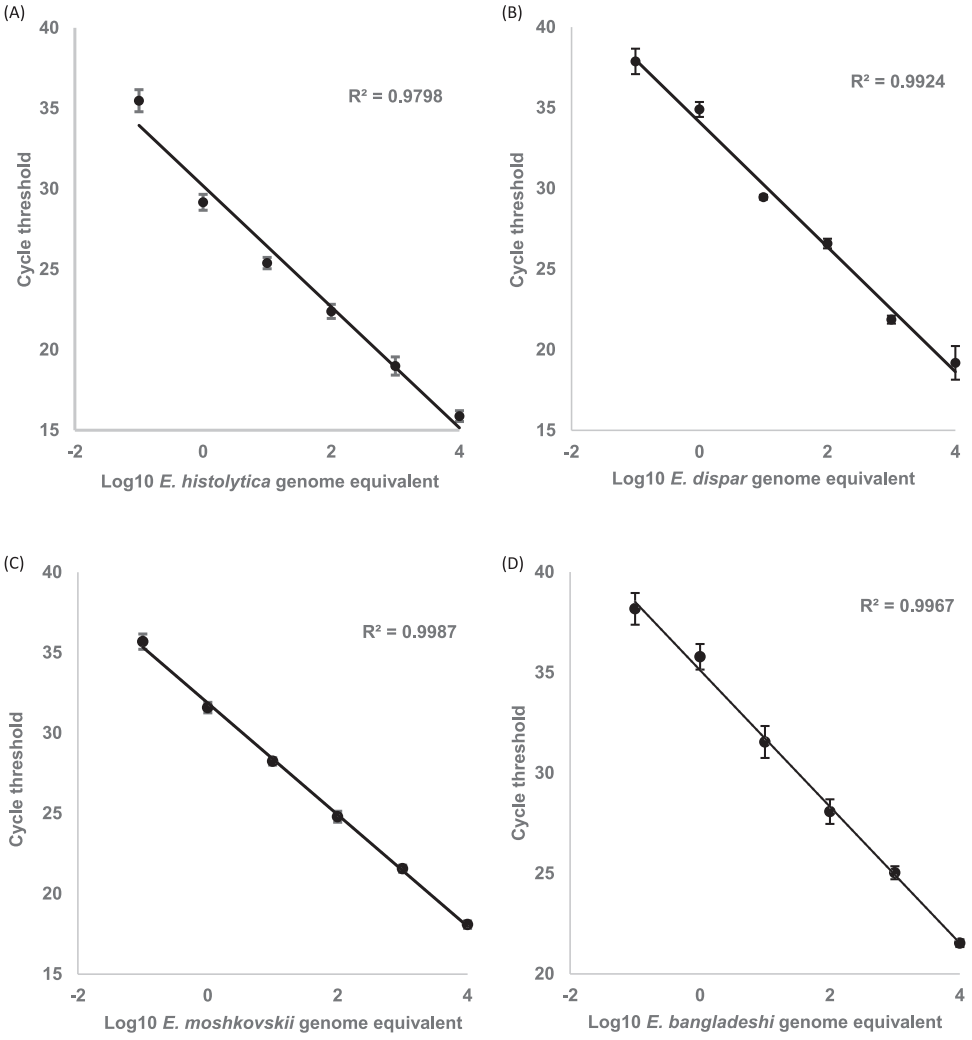

FIG 2 Linear dynamic range of the singleplex *Entamoeba* real-time PCR assays using the corresponding *Entamoeba* DNAs. (A) *E. histolytica*. (B) *E. dispar*. (C) *E. moshkovskii*. (D) *E. bangladeshi*.

stool and liver aspirate samples were available from 114 clinical patients from Bangladesh.

The duplex real-time PCR and tetraplex real-time PCR performed equally well with 11 *E. histolytica*-positive culture DNAs and 9 *E. dispar*-positive culture DNAs and detected all of these accurately (Table 4). Three *Entamoeba*-negative cultures (two of these were positive for *Blastocystis hominis*, and the other was positive for *Entamoeba coli*) were also accurately identified as negative for *E. histolytica* and *E. dispar* by both duplex and tetraplex real-time PCRs. Thus, the tetraplex real-time PCR showed 100% sensitivity and 100% specificity in detecting *E. histolytica* and *E. dispar* in culture DNAs compared to the duplex real-time PCR. Additionally, three *E. moshkovskii* culture DNAs and one *E. bangladeshi* culture DNA were also available. The tetraplex real-time PCR could positively identify the *E. moshkovskii* and the *E. bangladeshi* culture DNAs accurately; such identifications were outside the scope of the duplex real-time PCR, and it only identified these as negative for *E. histolytica* or *E. dispar*.

The tetraplex *Entamoeba* real-time PCR performed slightly better than the duplex *Entamoeba* real-time PCR in clinical stool samples ($n = 46$). It detected *E. histolytica* in 3 additional samples among 17 negatives that the duplex real-time PCR failed to detect. A nested genotyping PCR (in locus S^{TGA}-D followed by Sanger sequencing [54]) confirmed the presence of *E. histolytica* DNAs in these 3 "negative" samples (data not

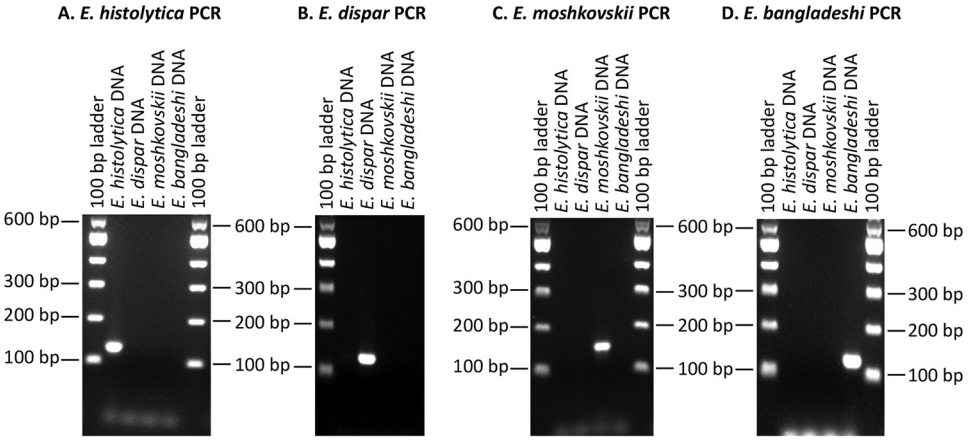

FIG 3 Specificity of individual *Entamoeba* PCRs in the conventional PCR format. The final sets of primers from four *Entamoeba* species were evaluated for their species specificity. Each conventional PCR (A, *E. histolytica* PCR; B, *E. dispar* PCR; C, *E. moshkovskii* PCR; D, *E. bangladeshi* PCR) used a single set of primers (for example, *E. histolytica* PCR used only an *E. histolytica*-specific primer set as described for panel A) in the presence of DNAs from four *Entamoeba* species that originated from 10,000 trophozoites each. Amplified products were run on a 2% agarose gel. In each of the PCRs, we observed species-specific amplification and no cross-amplifications with DNA from other *Entamoeba* species. The DNA size marker used was a 100-bp ladder (BioLabs, USA).

shown). These three samples were likely negative by the duplex real-time PCR due to lower number of target DNAs being below its detection threshold. The target amplicon size of the duplex real-time PCR is 231 bp (i.e., 42.4% larger than that of the tetraplex real-time PCR, 133 bp). PCR with a larger target is less sensitive than PCR a smaller target, and this may explain why the tetraplex real-time PCR was more sensitive than the duplex real-time PCR. The tetraplex *Entamoeba* real-time PCR could also detect mixed infections of *E. moshkovskii* with *E. dispar* in 3 additional samples that the duplex PCR identified as representing *E. dispar* only. The presence of *E. moshkovskii* DNA in these samples was verified using a nested PCR specific for *E. moshkovskii* (60). Overall, the tetraplex real-time PCR showed 100% sensitivity and 82.4% specificity in detecting *E. histolytica* and *E. dispar* compared to the duplex real-time PCR. Both real-time PCRs accurately detected 38 positive *E. histolytica* DNAs purified from the ALA samples, while they detected three liver aspirate DNAs from nonamebic sources as negative for *E. histolytica*. With liver abscess samples, the tetraplex real-time PCR showed 100% sensitivity and 100% specificity in detecting *E. histolytica* compared to the duplex real-time PCR.

DISCUSSION

Approximately half a billion people worldwide are thought to acquire *E. histolytica* infections annually. Because these data are based mostly on the microscopic identification of *E. histolytica*, there is uncertainty about their validity. We speculate that an unknown portion of these "*E. histolytica* infections" are actually due to other morphologically indistinguishable *Entamoeba* species such as *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*. In this study, our main objective was to develop a detection method that can specifically, and uniquely, identify the four *Entamoeba* species named above in clinical specimens and we successfully developed a highly sensitive and specific tetraplex real-time PCR assay. Not only can this PCR be performed in real-time PCR format, this novel PCR can be used as a conventional PCR if real-time PCR instrumentation, reagents, and expertise are unavailable, as is the case in most countries where amebiasis is endemic.

Performance characteristics of the tetraplex real-time PCR assay were compared with those of an existing duplex real-time PCR assay for *E. histolytica* and *E. dispar* (58) by the use of cultured ameba DNA and stool and liver abscess DNAs. Compared to the duplex real-time PCR assay, the tetraplex real-time PCR assay performed equally well

TABLE 3 Detection of mixed infections[a]

No. of *E. histolytica* trophozoite DNAs	No. of other *Entamoeba* trophozoite DNAs	Real-time PCR C_q value									
		Duplex-1		Duplex-2		Duplex-3		Tetraplex			
		E. histolytica	*E. dispar*	*E. histolytica*	*E. moshkovskii*	*E. histolytica*	*E. bangladeshi*	*E. histolytica*	*E. dispar*	*E. moshkovskii*	*E. bangladeshi*
0.1	10	31.2	28.8	35.5	26.2	33.1	28	No C_q	28.7	26.9	29.6
1	10	27.9	28.4	28.3	26.4	28.4	29.4	28.7	27.8	26.8	29.2
10	10	24.9	27.7	24.9	26	25.3	26.5	25.2	29.3	26.2	27.4
100	10	21.7	32.8	21.7	27.3	22.1	23.7	22.1	34.7	28.9	23.3
1,000	10	18.4	No C_q	18.5	No C_q	28.6	20.7	18.7	No C_q	No C_q	19.9
Blank	Blank	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
1	100	27.7	25.2	31.2	22.9	28.8	27.3	No C_q	25.1	23.4	27.8
10	100	24.7	24.8	25.1	22.7	25.6	25.9	25.2	24.9	23.3	26.2
100	100	21.7	24	21.6	22.6	22.1	23.6	21.8	24.7	23.2	23.7
1,000	100	18.5	28.6	18.5	23	18.7	20.3	18.7	29.4	24.9	20.5
10,000	100	15.4	No C_q	15.5	No C_q	15.6	17.5	15.8	No C_q	No C_q	17.6
Blank	Blank	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q

[a]Performance of *E. histolytica* real-time PCR in the presence of DNA of other *Entamoeba* species originating from 10 trophozoites or 100 trophozoites as indicated.

TABLE 4 Performance of tetraplex real-time PCR[a]

Source of DNA	No. of samples	Previous detection[b]	No. detected by:							
			Duplex real-time PCR[c]			Tetraplex real-time PCR				
			E. histolytica	*E. dispar*	Negative	*E. histolytica*	*E. dispar*	*E. moshkovskii*	*E. bangladeshi*	Negative
Culture ($n = 27$)	11	*E. histolytica*	11	0	0	11	0	0	0	0
	9	*E. dispar*	0	9	0	0	9	0	0	0
	3	*E. moshkovskii*	0	0	0	0	0	3	0	0
	1	*E. bangladeshi*	0	0	0	0	0	0	1	0
	3	Negative	0	0	3	0	0	0	0	3
Stool ($n = 46$)	12	*E. histolytica*	12	0	0	12	0	0	0	0
	16	*E. dispar*	0	16	0	0	16	3[d]	0	0
	1	*E. moshkovskii*	0	0	0	0	0	1	0	0
	17	Negative	0	0	17	3	0	0	0	14
LA ($n = 41$)	38	*E. histolytica*	38	0	0	38	0	0	0	0
	3	Negative	0	0	3	0	0	0	0	3

[a]The performance of the tetraplex real-time PCR was compared with that of an existing duplex real-time PCR for *E. histolytica* and *E. dispar* in clinical samples. LA, liver abscess (aspirate).

[b]Previous diagnosis was based on a combination of different tests such as culture (and zymodeme), conventional and nested PCR, antigen detection *E. histolytica* II ELISA (TechLab), and duplex real-time PCR. "0" indicates a negative test result. Negative, previously negative for *E. histolytica* and *E. dispar* species.

[c]Data were determined by the use of a previously described existing duplex real-time PCR (46).

[d]Tetraplex real-time PCR detected these as mixed infections of *E. dispar* and *E. moshkovskii*.

with the cultured and liver abscess DNAs and showed an increase in sensitivity with stool DNAs by detecting three additional *E. histolytica* DNAs that the duplex real-time PCR failed to detect.

A real-time PCR capable of simultaneous detection of the four *Entamoeba* species named above has recently been reported (19). It uses a common primer set for all four *Entamoeba* species but uses a unique species-specific probe set for each *Entamoeba* species. Because it uses a common primer pair for the four *Entamoeba* species, a major limitation of this assay is that it cannot be used in conventional PCR format in the absence of real-time PCR instruments, reagents, and expertise. The tetraplex real-time PCR developed in our study has the added advantage of being useful in a conventional PCR format, if needed, because it uses four pairs of unique primers specific for each *Entamoeba* species. Moreover, the sensitivity of the published tetraplex real-time PCR was not evaluated or reported, and the authors admitted that some of the true *Entamoeba*-positive samples (verified by Sanger sequencing) were missed by their real-time PCR, likely because of low ameba burdens. Additionally, it is not known how versatile their real-time PCR system is for the detection of mixed infections of two or more *Entamoeba* species. This is particularly important for the detection of mixed infections of *E. histolytica* with another *Entamoeba* species in order to make correct treatment decisions.

The tetraplex real-time PCR that we developed was highly sensitive, and it could detect *Entamoeba* DNA that had originated from the equivalent of 0.1 trophozoite per reaction. Detection of DNA originating from just 0.1 trophozoite equivalent is not surprising given the hundreds of copies of target 18S small-subunit ribosomal rRNA gene molecules per ameba genome (57). It could also detect the target pathogen, *E. histolytica* DNA, in the presence of up to 10-fold more DNA from one or more other *Entamoeba* species. Another advantage of this tetraplex real-time PCR is its smaller (132-to-145-bp) amplicon sizes in comparison with those used by Ngobeni et al. (19), which are 250 bp each, that is, approximately 42% to 47% larger than those used this study. A large (≥250-bp) amplicon is generally harder to amplify, and a PCR amplifying a larger amplicon is hence less sensitive than a PCR amplifying a smaller amplicon (61). In this study, during the screening and optimization of primers and probes, we noticed that the *E. dispar* real-time PCR was 10-fold less sensitive when a larger amplicon was used as the target (194 bp) than when a smaller amplicon was used as the target (134 bp, which was the target ultimately chosen) (data not shown). A comparison of the two tetraplex real-time PCRs is provided in Table 5.

TABLE 5 Comparison of tetraplex real-time PCRs developed previously by Ngobeni et al. and in this study

Feature	Result for tetraplex real-time PCR developed by:	
	Ngobeni et al. (19)	Ali and Roy (this study)
Simultaneous detection of *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*	Yes	Yes
Target DNA	18S SSU rDNA	18S SSU rDNA
Real-time PCR platform	Hydrolysis probes (TaqMan)	Hydrolysis probes (TaqMan)
Primers	One pair, common to all four *Entamoeba* species	Four pairs, each specific to an *Entamoeba* species
Probes	Four, each specific to an *Entamoeba* species	Four, each specific to an *Entamoeba* species
Sensitivity	Unknown	Well defined; capable of detecting ameba DNA originating from a single trophozoite
Amplicon size	Larger (250 bp)	Smaller (132–145 bp)
Use in conventional PCR format?	No	Yes
Detection limit for disproportionately infected mixed infections	Not tested	Can detect *E. histolytica* in the presence of up to 10-fold more DNA from one or more *Entamoeba* species
Compared with another established detection method?	No	Yes, compared with a diagnostic duplex real-time PCR for *E. histolytica* and *E. dispar* that is routinely used at U.S. CDC

Several real-time PCR-based assays are available for the detection of *E. histolytica* (and sometimes of *E. moshkovskii* as well) along with other enteric pathogens of bacterial or viral origin (50, 62–65). However, although they are sensitive and specific and allow detection of multiple pathogens in the same assay, a major disadvantage that limits their use in countries where *E. histolytica* infection is endemic is that they require sophisticated and expensive instruments and trained experts to run these assays. Also, they do not include *E. bangladeshi* detection and cannot be used in *Entamoeba* species-specific epidemiological surveys.

Our *Entamoeba* tetraplex real-time PCR has several limitations, however, and should be used with the following cautions. (i) We evaluated a limited number of DNAs from samples positive for *E. moshkovskii* and *E. bangladeshi*. (ii) This method may fail to detect an *Entamoeba* species in mixed-infection cases when the DNA from another *Entamoeba* is present at >100-fold. In such a scenario, only the predominant *Entamoeba* species is detected. (iii) For the majority of the intestinal samples that we tested, the clinical status of ameba-infected individuals was not provided for this study. (iv) Assay performance may be compromised in formalin-fixed specimens since formalin-induced DNA degradation can interfere with detection, leading to false-negative results. In this study, 9 of the 46 stool samples were sodium acetate-acetic acid-formalin (SAF)-fixed samples, and the remaining samples were fresh. Those nine SAF samples performed equally well in both duplex and tetraplex real-time PCRs. (v) This method is not suitable for determining the absolute burden of amebas in patients since amebas may not be excreted uniformly in each stool specimen. (vi) Since the stool samples were collected for other studies at the ICDDR,B, we were unable to investigate the effect of sample transportation time on the performance of real-time PCR. (vii) Experiments to detect LOD of *E. histolytica* DNA in mixed infections in the tetraplex real-time PCR were performed with purified trophozoites. As a consequence, we do not know if the results obtained with clinical samples would be equally accurate if the predominant form of *Entamoeba* were cyst. (viii) The *E. histolytica* real-time PCR would give positive amplification results with DNA from *Entamoeba nuttalli* (a species that infects nonhuman primates, especially macaques, but may occasionally infect humans) since the 18S SSU rDNA sequences used for the design of the primers and probe are identical in the species.

Concluding remarks. *E. histolytica* and *E. dispar* occupy opposite ends of the pathogenicity spectrum: *E. histolytica* is pathogenic, and *E. dispar* is nonpathogenic. However, the levels of pathogenicity of *E. moshkovskii* and *E. bangladeshi* likely lie somewhere between those extremes. We describe a sensitive and species-specific

tetraplex real-time PCR for the simultaneous detection and differentiation of *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* by the use of DNAs from clinical samples such as stool samples or liver aspirates. This novel real-time PCR will help elucidate the true epidemiology and pathogenicity of these four morphologically indistinguishable *Entamoeba* species. In resource-poor settings, where real-time PCR instruments and expertise are unavailable, this tetraplex real-time PCR can be used in a conventional PCR format.

ACKNOWLEDGMENTS

We acknowledge Rashidul Haque, International Centre for Diarrheal Disease Research, Bangladesh, for providing ameba culture lysates and/or DNA samples used in the real-time PCR LOD assay. We thank Jennifer Murphy, U.S. Centers for Disease Control and Prevention (CDC), for critical reading of the manuscript.

The findings and conclusions in this report are ours and do not necessarily represent the views of the Centers for Disease Control and Prevention.

I.K.M.A. conceived the idea for the study, designed the experiments, analyzed the data, and wrote the manuscript. S.R. designed the experiments, performed the experiments, and analyzed the data.

The efforts of I.K.M.A. and S.R. were funded by HHS and the Centers for Disease Control and Prevention (CDC).

We declare that we have no competing interests to disclose.

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