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PCR Detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in Stool Samples from Sydney, Australia▽

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This study investigated the presence of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in stool samples from a patient population in Sydney, Australia. Stool samples were tested by microscopy and PCR. Five patients were found with *E. histolytica* infections, while *E. dispar* and *E. moshkovskii* were observed in 63 (70.8%) and 55 (61.8%) patients, respectively, by PCR. This is the first study in Australia using molecular techniques to determine the presence of *E. histolytica*, *E. dispar*, and *E. moshkovskii*.

The genus *Entamoeba* comprises six species (*Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba poleki*, *Entamoeba coli*, and *Entamoeba hartmanni*) that live in the human intestinal lumen. *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically identical but are different biochemically and genetically (1, 2, 3, 5, 6). Although *E. histolytica* is recognized as a pathogen, the ability of the other two species to cause disease is unclear. *E. moshkovskii*, for example, is considered primarily a free-living ubiquitous amoeba found in anoxic sediments (2), and *E. dispar* is considered primarily a commensal of the human gut (3, 5).

Early studies of amebiasis in Australia have reported that the incidence of *Entamoeba* species varies from 1 to 4% in urban and rural communities, respectively (14). In another study, Law et al. (8) reported a 37% prevalence of *Entamoeba* in men who have sex with men. However, these studies did not differentiate *E. histolytica* from *E. dispar* or *E. moshkovskii*. The prevalence of *E. histolytica*, *E. dispar*, and *E. moshkovskii* (hereafter called the E complex) in the Australian population therefore remains unknown. The present study investigated the presence of the E complex in clinical samples by microscopy and PCR directly in stool samples collected from patients presenting with gastrointestinal symptoms.

All of the stool specimens (from a diverse patient population) submitted to the Department of Microbiology at St. Vincent's Hospital, Sydney, Australia, during January 2003 to June 2006 for investigation of diarrhea were included in this study. Specimens from outpatients were collected by the patient and submitted to the laboratory as fresh specimens along with a portion mixed with sodium acetate-acetic acid-formalin (SAF). Specimens from inpatients or received without a portion fixed in SAF were immediately preserved in SAF upon arrival at the laboratory. The SAF-fixed specimens underwent permanent staining with a modified iron-hematoxylin stain according to the manufacturer's recommendations (Fronine, Australia). Patients diagnosed with members of the E complex by microscopy of stained smears underwent further investigations.

DNA was extracted from fecal specimens (without fixatives) from patients either fresh or after storage at −20°C (immediately frozen upon arrival at the laboratory). Briefly, the stool sample (200 mg) was washed twice with 1 ml of sterile phosphate-buffered saline (pH 7.2), centrifuged for 5 min at 14,000 × *g*, and DNA extracted according to the manufacturers instructions with the QIAGEN kit (QIAGEN, Hilden, Germany).

Aspirated pus from a liver abscess was obtained from one patient. The DNA was extracted from the liver abscess with the QIAamp DNA tissue extraction kit.

PCR targeting of the 18S rRNA genes of *E. histolytica* and *E. dispar* was carried out by following the protocol previously described (15). For *E. moshkovskii*, a nested PCR was carried out with primers targeting the *E. moshkovskii*-specific 18S rRNA gene as previously described (1).

To exclude any inhibitory effect due to the presence of fecal material inhibition, control experiments were carried out and the DNA from the fecal specimens was spiked with an equal volume of genomic DNA (2 μl) from *E. histolytica* strain HTH-56:MUTM, *E. dispar* strain SAW 760, or the 18S rRNA gene cloned into plasmid pGEM-T. As a control for *E. moshkovskii*, genomic DNA of the Laredo strain was used in separate experiments.

A determination of the sensitivity of the PCR assay was performed with genomic DNAs from control samples of *E. histolytica*, *E. dispar*, and *E. moshkovskii*. Tenfold dilutions of the DNA were added to the stool samples negative for parasitic cysts and ova by microscopy and negative by PCR with *E. histolytica*-, *E. dispar*-, and *E. moshkovskii*-specific primers. DNA was extracted from spiked fecal samples by using the QIAamp DNA stool mini kit.

The PCR assay was also tested for specificity against a panel of DNAs extracted from 26 fecal samples, each containing one or a mixture of the following parasites: *Blastocystis hominis*, *Escherichia coli*, *E. hartmanni*, *Giardia intestinalis*, *Endolimax nana*, *Iodamoeba butschlii*, *Cryptosporidium* sp., *Cyclospora* sp., *Chilomastix mesnili*, and *Enteromonas hominis*.

For DNA sequencing, the PCR products were purified with

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TABLE 1. Clinical details of patients positive for *E. histolytica* by PCR

Patient age (yr) and sex	Microscopy result	TechLab ELISA[a] result	PCR (*E. histolytica*) result	Clinical history	Amebic serology	Overseas travel, risk factor
36, male	E complex, *B. hominis*	Negative	Positive	Amebic dysentery	Equivocal	Thailand
35, male	E complex, *E. hartmanni*, *B. hominis*	Negative	Positive	Gastroenteritis (2 wk)	Not done	None, MSM[d]
31, male	E complex, *E. coli*, *I. butschlii*, *B. hominis*	Negative	Positive[b]	Diarrhea, abdominal pain (1–2 wk)	Not done	None, MSM
53, male	E complex	Negative	Positive[c]	Gastroenteritis (>1 wk)	Not done	None, MSM
57, male	E complex, *I. butschlii*, *E. coli*, *E. hartmanni*	Negative	Positive	Amebic dysentery, extraintestinal disease, liver abscess[e]	Positive	Thailand

[a] ELISA, enzyme-linked immunosorbent assay.
[b] Positive for *E. dispar* and *E. histolytica* by PCR.
[c] Positive for *E. moshkovskii* and *E. histolytica* by PCR.
[d] MSM, men who have sex with men.
[e] Positive for *E. histolytica* by PCR and by TechLab ELISA.

the QIAquick PCR gel extraction kit (QIAGEN) in accordance with the manufacturer's instructions. The PCR products were then sequenced in both directions with an ABI Prism 3700. The 18S rRNA gene sequences obtained were from randomly selected PCR products derived from clinical specimens positive for each *Entamoeba* species. The sequences were compared to those available in the GenBank database with the BLASTN program run on the National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov/BLAST).

A total of 5,921 samples were tested over the period between January 2003 and June 2006, and 177 (2.9%) samples were microscopically positive for cysts or trophozoites of the E complex, either singly or in combination with other human protozoan parasites. Of the 177 microscopy-positive samples, only 110 were further studied as the rest of the samples were discarded because they could not be preserved. The E complex was found alone in 23 (21%) specimens, whereas the other 87 (79%) specimens contained the E complex with two or more different parasite species (data not shown). *B. hominis* (60.9%) was found to be the most common protozoan parasite, followed by *E. nana* (42.7%), *I. butschlii* (15.4%), *E. hartmanni* (11.8%), *E. coli* (11.8%), *E. hominis* (5.4%), *G. intestinalis* (2.7%), and *C. mesnili* (1.8%).

Of the 110 patients who provided specimens, 107 (97.3%) were male whereas only 3 (2.7%) were female. The majority of the patients (70/110 or 63.6%) were 31 to 50 years old.

Results of inhibition controls for all PCRs carried out to exclude the possibility that a negative PCR result was due to failure of amplification showed no inhibition for any of the samples.

The results of the PCR assays showed that they were able to detect as little as 10 pg of DNA of *E. histolytica*, 1 pg of DNA of *E. dispar*, and 0.506 fg of DNA of *E. moshkovskii*. All samples were tested twice. No cross amplification was observed when all of the sets of primer used in this study were tested against infected stool DNA containing different protozoal pathogens.

Microscopy-positive fecal samples containing the E complex ($n = 110$) were subject to PCR for confirmation of the diagnosis of E complex. PCR products were detected in 89 (81%) samples, whereas 21 (19%) samples were found to be negative by PCR assay. Of the 89 PCR-positive samples, 3 (3.4%) were shown to contain only *E. histolytica*, 30 (33.7%) contained *E. dispar*, and 22 (24.7%) contained only *E. moshkovskii*. Mixed infection with *E. dispar* and *E. moshkovskii* was found in 32 (36%) specimens. One sample contained both *E. histolytica* and *E. dispar*, while another sample contained both *E. histolytica* and *E. moshkovskii*. The clinical data from *E. histolytica*-positive patients are shown in Table 1. PCR of the DNA extracted from the liver abscess pus was positive for *E. histolytica*.

Representative PCR products were sequenced in both directions from all of the 5 specimens of *E. histolytica*, 15 specimens of *E. dispar*, and 15 specimens of *E. moshkovskii*. The sequences of all the 15 *E. dispar* amplicons showed 98.5% to 100% similarity to the *E. dispar* sequences in GenBank (e.g., accession no. Z49256), whereas all of the five *E. histolytica* sequences showed high similarity (99.7% to 100%) to the *E. histolytica* sequences in GenBank (e.g., accession no. X56991). All of the 15 *E. moshkovskii* amplicons showed 100% similarity to the *E. moshkovskii* sequences in GenBank (e.g., accession no. AF149906).

Of the 21 PCR-negative samples, 14 contained only trophozoites, 1 sample was positive for cysts only, and 6 contained trophozoites and cysts of the E complex. Every PCR-negative sample was retested by PCR, and each was again found to be PCR negative.

E. histolytica is the agent of human intestinal and extraintestinal amebiasis, a parasitic organism responsible for significant morbidity and mortality, mainly in developing countries and several communities of developed nations (10, 12). Accurate differentiation of *E. histolytica* from *E. dispar* and *E. moshkovskii* is crucial to the clinical management of patients. Current data indicate that *E. dispar* is perhaps 10 times more common than *E. histolytica* worldwide (12, 16), but the local prevalence may vary significantly, thereby necessitating the assessment of prevalence in different geographical regions. To date, several microscopy-based epidemiological surveys to study the prevalence of *E. histolytica* and *E. dispar* have been performed in different parts of Australia, but they were carried out without using molecular methods to accurately identify the presence of individual species. Hence, none of these studies have determined the true presence of *E. histolytica*, *E. dispar*, or *E. moshkovskii* (8, 14).

In this study, the overall prevalence of the E complex was found to be 2.9% by microscopy and permanent staining. By

molecular techniques, *E. dispar* and *E. moshkovskii* were found to be the most prevalent *Entamoeba* species. A recent study in Bangladesh has highlighted the prevalence of *E. moshkovskii* (21%), suggesting that this infection is common among children aged 2 to 5 years (1). A study in India linked *E. moshkovskii* infection with dysentery (11). In our study, all of the patients with *E. moshkovskii* infection were symptomatic. Therefore, further investigations are needed of control and other patient groups to determine the true pathogenic potential of *E. moshkovskii*.

Most (97.3%) of the patients with E complex were males and predominantly men who have sex with men. These results are in accordance with studies from other countries, which document a higher prevalence of *E. dispar* in men who have sex with men (4, 10). Of concern is the detection of *E. histolytica* in men who have sex with men, highlighting the need for continued monitoring of this particular population.

In this study, the PCR assay showed a sensitivity of 81% compared to microscopy, which is much higher than the sensitivity of 71.7% reported by Roy et al. (13), who used unfixed, frozen stool samples. However, higher sensitivity levels, ranging from 94 to 100%, obtained with microscopy-positive specimens have been reported for unpreserved frozen stool specimens (9, 15). In 21 (19%) samples positive for E complex by microscopy, we were not able to PCR amplify DNA from any member of the E complex with the primers used and no inhibition of the PCR was observed in control experiments. These results can potentially be explained by the presence of other *Entamoeba* species, which were detected by microscopy but not by PCR, or the presence of a low number of parasites in the sample, which fell below the detection limit of PCR. Another reason for this could be the fact that a majority of these samples (14/21) contained only trophozoites that could have degenerated with time. Similar to the findings of Kebede et al. (7), our PCR results show better concordance with the microscopic finding of E complex cysts than with the microscopic detection of E complex trophozoites. It therefore appears that the presence of cysts in the fecal samples, in contrast to trophozoites, can increase the chances of the PCR assay successfully detecting DNA from these three species.

This is the first study in Australia using molecular techniques to determine the true incidence of E complex and highlights the importance of molecular methods to differentiate among the three species. The discovery of *E. histolytica* within the homosexual community is of great public health concern. This study reports for the first time the identification of *E. moshkovskii* and *E. dispar* in clinical samples from Australia. Further studies are needed to determine the true prevalence and pathogenic potential of these two species.

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