J HEALTH POPUL NUTR 2005 Sep;23(3):292-295 © 2005 ICDDR,B: Centre for Health and Population Research
ISSN 1606-0997 $ 5.00+0.20

SHORT REPORT

Entamoeba moshkovskii and *Entamoeba dispar*-associated Infections in Pondicherry, India

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ABSTRACT

The prevalence of Laredo strain—*Entamoeba moshkovskii*—and non-pathogenic *E. dispar* in patients attending the Jawaharlal Institute of Postgraduate Medical Education and Research hospital, Pondicherry, India, is reported here. *E. moshkovskii* is reported for the first time in India. The species are morphologically indistinguishable from pathogenic *E. histolytica*. Of 746 stool samples screened, 68 showing cyst or trophozoite stage of *E. histolytica*, *E. dispar*, or *E. moshkovskii* were subjected to small subunit (SSU) rRNA gene-based polymerase chain reaction, which revealed a higher prevalence of *E. dispar* (8.8%) and *E. moshkovskii* (2.2%) compared to *E. histolytica* (1.7%) in patients. Only 19% of the 68 stool samples, resembling *E. histolytica* by microscopy, were actually *E. histolytica*, implying that 81% of suspected infections were misdiagnosed and would have been treated unnecessarily with anti-amoebic drugs.

Key words: *Entamoeba histolytica*; *Entamoeba moshkovskii*; *Entamoeba dispar*; Amoebiasis; Diagnosis, Laboratory; India

INTRODUCTION

Entamoeba moshkovskii is primarily a free-living amoeba. It is indistinguishable in its cyst and trophozoite forms from *E. histolytica*, the causative agent of amoebiasis. *E. moshkovskii* has so far rarely been shown to infect humans (1). *E. moshkovskii* in humans has been reported from North America, Italy, South Africa, and Bangladesh, but it has never been associated with disease (2). The prevalence of *E. moshkovskii* in India has not been reported earlier. The morphological similarity of *E. moshkovskii* and *E. dispar* to disease-causing *E. histolytica* makes it important to differentiate the three species by polymerase chain reaction (PCR). In the clinical setting, this may lead to a misdiagnosis and unnecessary treatment with anti-amoebic chemotherapy (3).

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MATERIALS AND METHODS

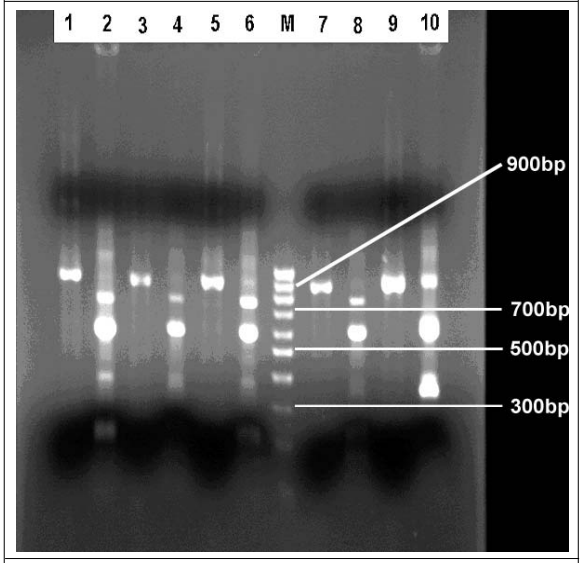
Stool specimens for this study were obtained from patients attending the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Pondicherry, India. In total, 746 stool samples from patients clinically suspected to have gastrointestinal infections were collected during July-December 2004 and were screened by microscopy, of which 68 showing trophozoite/cyst were subjected to *E. histolytica*, *E. dispar*, and *E. moshkovskii*-specific nested PCR.

The DNA was isolated using the cetyltrimethylammonium bromide (CTAB) extraction method (4). The extracted DNA was passed through DNA clean-up spin columns (Bangalore Genei, Bangalore) to remove PCR inhibitors. Based on the sequences of the small sub-unit

(SSU)-rDNA of *E. histolytica* and *E. dispar*, nested sets of primers (designated E-1/E-2, Eh-1/Eh-2, and Ed-1/Ed-2) were used for detecting *E. histolytica* and *E. dispar* in stool specimens. The PCR was given a hot start by pre-incubating the PCR mix at 96 °C for two minutes, followed by 30 cycles—each consisting of 92 °C for 60s, 43 °C for 60s, and 72 °C for 90s and 72 °C for five minutes—one cycle for the final extension (5). In the nested PCR, annealing temperature was raised to 62 °C, leaving the other parameters of the amplification cycles unchanged. *E. histolytica* and *E. dispar*-specific nested SSU-rDNA gene amplification products were double-digested with restriction endonuclease *Dra-I* and *Sau96-I* for two hours at 37 °C according to the instructions of the manufacturer (Bangalore Genei) to verify the identity of species. Products were visualized on a 1.3% agarose gel containing ethidium bromide (0.2 mg/mL).

The product of nested PCR from both *E. histolytica* and *E. dispar* showed 900-bp fragments which were further confirmed by restriction fragment length polymorphism (RFLP). The RFLP pattern for *E. histolytica* showed 550-bp and 350-bp fragments and undigested 900 bp, whereas for *E. dispar* it showed 700 bp, 550 bp, and confluent bands of 200 bp and 150 bp (Fig. 1).

Fig. 1. *Entamoeba histolytica* and *E. dispar*-specific nested SSU-rDNA PCR products. Odd- and even-numbered lanes represent undigested and *Dra-I* and *Sau96-I*-digested PCR products respectively



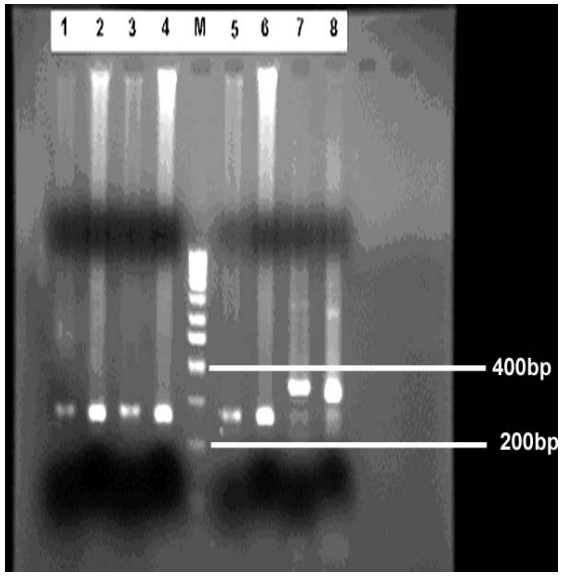
Lanes 1/2-7/8: DNA from stool samples showing *E. dispar*; lanes 9/10: DNA from stool samples showing *E. histolytica*. M: A 100-bp DNA ladder

Based on the sequence of the SSU-rDNA gene of *E. moshkovskii* Laredo (GenBank accession no. AF 149906), a nested set of primers (designated Em-1/Em-2 and nEm-1/nEm-2) was used (6) for detecting *E. moshkovskii* in stool DNA.

The PCR conditions were same as described above, except that the annealing temperature was 55 °C for the first PCR and 62 °C for the nested PCR. *E. moshkovskii*-specific nested SSU-rDNA gene amplification products were digested with restriction endonuclease *XhoI* for one hour at 37 °C according to the instructions of the manufacturer (Promega) to verify the identity of species. Products were visualized on a 1.8% agarose gel containing ethidium bromide (0.2 mg/mL).

The product of nested PCR from *E. moshkovskii* showed a 258-bp fragment which was further confirmed by RFLP. *XhoI* exclusively cuts the 258-bp product to produce 236-bp and 22-bp fragments (The 22-bp product is not visible in gel because it was too small to be resolved in 1.8% agarose gel). The standard strain of *E. moshkovskii* Laredo showed a higher molecular size (300 bp approximately) product compared to clinical isolates (Fig. 2).

Fig. 2. *Entamoeba moshkovskii*-specific nested SSU-rDNA PCR products. Odd- and even-numbered lanes represent undigested and *XhoI*-digested PCR products respectively



Lanes 1/2-5/6: DNA from stool samples showing *E. moshkovskii*; lanes 7/8: *E. moshkovskii* Laredo. M: A 100-bp DNA ladder

DNA from standard cultures of *E. histolytica* HM-1: IMSS, *E. dispar* SAW760, and *E. moshkovskii* Laredo was used as positive control, and stool samples showing no trophozoite/cyst were used as negative control.

RESULTS

The primer sequence for *E. moshkovskii*, *E. histolytica*, and *E. dispar*, blasted in the genome database of all organisms in the website (http://www.ncbi.nlm.nih.gov/blast/), was found to be specific for the study. Moreover, the amplified products of nested PCR were restriction-digested to rule out any non-specific amplification.

The reference strain—*E. moshkovskii* Laredo—gave a band at approximately 300 bp with the *E. moshkovskii*-specific SSU-rDNA-nested primers, whereas the control—*E. histolytica* HM-1: IMSS and *E. dispar* SAW760 DNAs—was negative.

The results of nested PCR-RFLP on the 68 stool DNA samples showing trophozoite/cyst resembling *E. histolytica* are shown in Table 1.

One sample, negative by stool PCR for both *E. histolytica* and *E. dispar*, was eventually positive for *E. moshkovskii*. Sixteen of 17 *E. moshkovskii*-positive stool samples were also positive for *E. histolytica*, *E. dispar*, or both by SSU-rDNA PCR.

Comparison of SSU-rDNA sequences from *E. moshkovskii*, *E. histolytica*, and *E. dispar* showed that the restriction endonuclease *XhoI* cut exclusively in the *E. moshkovskii*-specific, 258-bp–nested PCR product to produce 236-bp and 22-bp fragments. Products from all the 17 positive stool samples and the Laredo strain showed the presence of this site (Fig. 2).

The prevalence of *E. moshkovskii*, *E. dispar*, and *E. histolytica* among patients clinically suspected to have gastrointestinal infections, attending the JIPMER hospital, is shown in Table 2.

DISCUSSION

The study was conducted to identify the prevalence of *E. moshkovskii*, *E. dispar*, and *E. histolytica* in stool samples of patients attending the JIPMER hospital. The study, for the first time, reports the prevalence of *E. moshkovskii* in India.

The morphological similarity leads to confusion in diagnosis of amoebiasis in clinical settings. We have used nested PCR to detect infections due to *E. histolytica*, *E. dispar*, and *E. moshkovskii* because nested PCR increases the specificity and is more efficient in amplifying stool DNA.

Our study included patients from varied age-groups and from different geographical localities, which shows the wide distribution of *E. moshkovskii* and *E. dispar* in Pondicherry and its neighbouring areas.

The study has several interesting findings. Only one patient with dysentery showed *E. moshkovskii*-associated

Table 1. Summary of *Entamoeba dispar*, *E. histolytica*, and *E. moshkovskii*-specific nested PCR-RFLP results on stool specimens found microscopically positive for amoeba resembling *E. histolytica*

Type of infection	No. of samples positive by nested PCR-RFLP (n=68)	% of stools positive
E. dispar (mono-infection)	43	63.2
E. histolytica (mono-infection)	01	1.47
E. moshkovskii (mono-infection)	01	1.47
E. dispar + *E. moshkovskii* (mixed)	11	16.1
E. dispar + *E. histolytica* (mixed)	07	10.2
E. dispar + *E. histolytica* + *E. moshkovskii* (mixed)	05	7.3

PCR=Polymerase chain reaction; RFLP=Restriction fragment length polymorphism

Table 2. Prevalence of *Entamoeba dispar*, *E. histolytica*, and *E. moshkovskii* among patients attending the JIPMER hospital clinically suspected to have gastrointestinal infections

Type of *Entamoeba* species	No. of samples positive by nested PCR-RFLP	Prevalence (%)
E. moshkovskii	17	2.2
E. dispar	66	8.8
E. histolytica	13	1.7

Total no. of stool samples included in the study=746
JIPMER=Jawaharlal Institute of Postgraduate Medical Education and Research
PCR=Polymerase chain reaction
RFLP=Restriction fragment length polymorphism

mono-infection. The cause remained undetermined; bacterial aetiology by routine stool culture was negative. Other investigations, such as viral study, could not be done in the laboratory. The high prevalence of *E. moshkovskii* among the study population supports the view that humans are a true host for this free-living amoeba and are not just transiently infected (6). The study also answers the mystery of some microscopically-positive but antigenically- and PCR-negative results for *E. histolytica* and *E. dispar* (7), which, in our study, was due to *E. moshkovskii*. Eleven patients with mixed infections due to *E. dispar* and *E. moshkovskii* and five patients due to *E. dispar*, *E. histolytica*, and *E. moshkovskii* had no diarrhoea or dysentery, but had complaints of mild gastrointestinal discomfort.

The study has shown appreciably a high prevalence of *E. dispar* and *E. moshkovskii* in the patients compared to *E. histolytica* which reveals that only 19% of the 68 stool samples, resembling *E. histolytica* by microscopy, were actually *E. histolytica*, implying that 81% of suspected infections were misdiagnosed and would have been treated unnecessarily with anti-amoebic drugs when diagnosed based on microscopic findings alone.

Thus, epidemiologic studies and clinical diagnosis of *E. histolytica*-associated infection, which are based on morphological examination alone, are prone to error. Infections due to both *E. dispar* and *E. moshkovskii* are associated with asymptomatic carrier stage. The trophozoites of both *E. dispar* and *E. moshkovskii* lack the capability to invade the intestinal mucosa and do not have any ingested erythrocytes unlike that of *E. histolytica*. PCR is, therefore, essential to distinguish *E. histolytica* from *E. dispar* and *E. moshkovskii*.

ACKNOWLEDGEMENTS

We sincerely thank Dr. C. Graham Clark from London School of Hygiene & Tropical Medicine for providing us with lyophilized DNA of standard cultures of *E. histolytica* HM-1: IMSS, *E. dispar* SAW760, and *E. moshkovskii* Laredo.

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