11. ADDENDUM
Addendum

Based on this thesis work four papers were published in peer-reviewed international journals, one paper was presented in national conference, and one national newspaper has cited one of the published works.


- Khairnar K. Parija SC. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. BMC Microbiol. 2007 May 24;7:47.


_N.B: Reprints of the publications enclosed_
SHORT REPORT

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

Subhash Chandra Parija and Krishna Khairnar

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605 006, India

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).

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 Genie) 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.

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>No. of samples positive by nested PCR-RFLP (n=68)</th>
<th>% of stools positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. dispar</em> (mono-infection)</td>
<td>43</td>
<td>63.2</td>
</tr>
<tr>
<td><em>E. histolytica</em> (mono-infection)</td>
<td>01</td>
<td>1.47</td>
</tr>
<tr>
<td><em>E. moshkovskii</em> (mono-infection)</td>
<td>01</td>
<td>1.47</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. moshkovskii</em> (mixed)</td>
<td>11</td>
<td>16.1</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. histolytica</em> (mixed)</td>
<td>07</td>
<td>10.2</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. histolytica</em> + <em>E. moshkovskii</em> (mixed)</td>
<td>05</td>
<td>7.3</td>
</tr>
</tbody>
</table>

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

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
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.

**REFERENCES**

Detection of excretory Entamoeba histolytica DNA in the urine, and detection of E. histolytica DNA and lectin antigen in the liver abscess pus for the diagnosis of amoebic liver abscess

Subhash C Parija* and Krishna Khairnar†

Abstract

Background: Amoebic liver abscess (ALA) and pyogenic liver abscesses (PLA) appear identical by ultrasound and other imaging techniques. Collection of blood or liver abscess pus for diagnosis of liver abscesses is an invasive procedure, and the procedure requires technical expertise and disposable syringes. Collection of urine is a non-invasive procedure. Therefore, there has been much interest shown towards the use of urine as an alternative clinical specimen for the diagnosis of some parasitic infections. Here, we report for the first time the detection of E. histolytica DNA excreted in the urine for diagnosis of the cases of ALA.

Results: E. histolytica DNA was detected in liver abscess pus specimen of 80.4% of ALA patients by a nested multiplex polymerase chain reaction (PCR) targeting 16S-like rRNA gene. The nested PCR detected E. histolytica DNA in all 37 (100%) liver abscess pus specimens collected prior to metronidazole treatment, but were detected in only 53 of 75 (70.6%) pus specimens collected after therapy with metronidazole. Similarly, the PCR detected E. histolytica DNA in 21 of 53 (39.6%) urine specimens of ALA patients. The test detected E. histolytica DNA in only 4 of 23 (17.4%) urine specimens collected prior to metronidazole treatment, but were detected in 17 of 30 (56.7%) urine specimens collected after treatment with metronidazole. The enzyme-linked immunosorbent assay (ELISA) for the detection of lectin E. histolytica antigen in the liver abscess pus showed a sensitivity of 50% and the indirect haemagglutination (IHA) test for detection of amoebic antibodies in the serum showed a sensitivity of 76.8% for the diagnosis of the ALA.

Conclusions: The present study for the first time shows that the kidney barrier in ALA patients is permeable to E. histolytica DNA molecule resulting in excretion of E. histolytica DNA in urine which can be detected by PCR. The study also shows that the PCR for detection of E. histolytica DNA in urine of patients with ALA can also be used as a prognostic marker to assess the course of the diseases following therapy with metronidazole. The detection of E. histolytica DNA in urine specimen of ALA patients provides a new approach for the diagnosis of ALA.
Mortality from amoebiasis is mainly due to extra-intestinal pathology, of which amoebic liver abscess (ALA) is the most common. If left untreated, ALA can rupture into neighboring tissue and spread to the brain and other organs via hematological route producing serious morbidity and mortality. It is difficult to differentiate clinically the ALA from pyogenic liver abscess (PLA) as well as from other space occupying lesions of liver such as hydatid cyst and liver hepatoma [2,3].

Imaging techniques like ultrasound, computed tomography, and magnetic resonance although are highly sensitive to detect abscesses in the liver of varied aetiology, however fail to distinguish specifically ALA from that of PLA. Less than one third of patients with ALA have active diarrhea [3]. Hence, stool microscopy and stool antigen detection is not very helpful for diagnosis of ALA. In fact less than only 10% of ALA patients have identifiable E. histolytica in stool specimens [4].

Laboratory diagnosis of ALA is usually established by conventional antibody-based serological tests. Nevertheless, the main disadvantage with antibody detection is that serum antibody levels in individuals living in endemic areas, continues to remain positive even for years after infection with E. histolytica [5-7]. The demonstration of amoebic antibodies in the serum, therefore, fails to demonstrate the amoebic infection whether it is recent or old. Furthermore, serum amoebic antibodies are not demonstrated in up to 10% of the patients with acute ALA [3].

The demonstration of E. histolytica trophozoite in liver abscess pus aspirates by microscopy confirms the diagnosis of ALA, but in best of the laboratories, the amoebic trophozoites can be demonstrated in only 15% of the liver pus [8]. Since the trophozoites of E. histolytica are found mainly in the periphery of the abscess diagnosis of ALA by culture of liver pus for E. histolytica is also unsatisfactory

Demonstration of amoebic antigen in the liver pus is a recent approach for specific diagnosis of the ALA. A monoclonal antibody-based second generation TechLab enzyme-linked immunosorbent assay (ELISA) kit (Blackburg, Va.) has been reported to be 78% sensitive for the detection of E. histolytica lectin antigen in the liver pus for the diagnosis of ALA in Dhaka, Bangladesh [10]. Studies conducted in various laboratories worldwide including ours have shown that polymerase chain reaction (PCR) is sensitive and specific method for detecting Entamoeba DNA in stool samples and for differentiating the morphologically similar E. histolytica from Entamoeba dispar and Entamoeba moshkovskii [11-19]. However, only few studies using the PCR have been reported for the detection of Entamoeba DNA in liver abscess pus for the diagnosis of the ALA [20,21,9,10].

Collection of blood or liver abscess pus is an invasive procedure, and the procedures require technical expertise and disposable syringes [22]. The method if not carried out under stringent conditions is associated with the risk of acquiring needle-borne infections such as hepatitis B virus and human immunodeficiency virus (HIV). Therefore, of late much interest has been shown towards the use of urine as a specimen alternate to the blood for the diagnosis of some parasitic infections including malaria, schistosomiasis, kala-azar, cystic echinococcosis and neurocysticercosis [22]. Urinary antigen for cystic echinococcosis (CE) and neurocysticercosis has been reported for the first time from our laboratory at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India [23,24]. Our laboratory has developed for the first time a counter-current immunoelectrophoresis (CIIEP) and co-agglutination (Co-A) to detect the hydatid antigen excreted in the urine for the diagnosis of CE [23,25], and Co-A to detect cestode antigen in the urine for the diagnosis of neurocysticercosis [24].

Detection of DNA in urine by PCR has been employed for the diagnosis of Toxoplasma gondii, Neisseria gonorrhoeae, Borrelia burgdorferi, Mycobacterium tuberculosis, Mycobacterium leprae and Chlamydia trachomatis infections [26-30]. Some studies have also shown that the kidney barrier in rodents and humans is permeable to DNA molecules large enough to be analyzed by standard genetic methodologies [31,32]. To the best of our knowledge till now there is no report available on detection of Entamoeba DNA in the urine for the diagnosis of ALA. In the present study, therefore for the first time, we have evaluated a nested multiplex PCR for detection of Entamoeba DNA excreted in the urine for the diagnosis of ALA.

Results

The quantification of DNA in the liver abscess pus and urine specimen by Spectrophotometric analysis showed the DNA yield to be approximately 85 and 3 μg/ml respectively. The purity of DNA extract from liver abscess pus and urine specimens was found to be satisfactory as the value of ratio of readings at 260 nm and 280 nm (OD260/OD280) was approximately 1.8-1.85.

The sequencing result of PCR product of E. histolytica from liver abscess pus and urine specimen showed 99% identities to the sequence deposited in GenBank [accession number: X56921]. The result of assessment of competition of other non target DNA present in liver abscess pus (PLA pus negative for E. histolytica) and urine (negative control group) specimen with target DNA showed...
expected amplification and no nonspecific amplification in nested multiplex PCR.

Estimation of minimum number of Entamoeba cells detectable by nested multiplex PCR showed that the detection limit of PCR was found to be approximately 15 E. histolytica cells as even 1.5 μl of template DNA from 1000 E. histolytica cells/100 μl of Tris- ethylenediamine tetraacetic acid (EDTA) (TE) buffer produced a positive signal.

The IHA test was positive for serum antibodies in the serum of 86 (61.9%) of 139 patients provisionally diagnosed as ALA. The test was positive in a higher number of serum (71 of 102 [69.6%]) samples of patients who had received prior treatment with metronidazole than those who had not received any prior treatment with metronidazole (15 of 37 [40.5%]) and this difference was statistically significant (χ² = 8.53, P = 0.003). Metronidazole treatment was initiated from a few days to several weeks before collection of the blood samples in these patients. Two (4.6%) out of 43 sera from control cases were positive for antiamebic antibody by IHA.

The TechLab E. histolytica II test was positive for E. histolytica Gal/GalNAc lectin antigen in the liver abscess pus of 98 (40.3%) of 243 provisionally diagnosed ALA patients. The TechLab E. histolytica II test detected lectin antigen in 80 (81%) of 37 liver abscess pus of patients which were collected prior to treatment with metronidazole. In contrast, the TechLab E. histolytica test detected the lectin antigen in only 26 (25.5%) of 102 liver pus (χ² = 32.61, P < 0001), collected after initiation of therapy with metronidazole. The probability of E. histolytica antigen detection in liver abscess pus by ELISA was found to be 12 times more in patients who had not received prior treatment with metronidazole (Odds Ratio (OR) = 12.53, 95% Confidence Interval (CI) = 4.55 to 35.86) than in the patients who received prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1.

Microscopy of the liver pus demonstrated E. histolytica trophozoites in 10 of 139 (7.2%) liver abscess specimens, but only 2 (1.4%) pus specimens were positive by culture for E. histolytica. All 10 patients whose liver pus were positive for E. histolytica by microscopy and/or culture were also positive for E. histolytica Gal/GalNAc lectin antigen in the liver pus by the TechLab E. histolytica II test and serum amoebic antibodies by the IHA test.

A total of 102 out of 139 (73.4%) liver abscess pus were negative for aerobic bacteria by Gram’s staining and bacterial culture. Twenty seven liver abscess pus specimens were positive for aerobic bacteria by Gram’s staining and bacterial culture. These included Klebsiella pneumoniae (n = 9), Proteus species (n = 5), Enterobacter (n = 5), Escherichia coli (n = 3) and Pseudomonas (n = 5). Ten liver abscess pus specimens showed secondary infection of ALA with aerobic bacteria by Gram’s staining and bacterial culture. These included K. pneumoniae (n = 3), Enterobacter (n = 2), E. coli (n = 1), group B Salmonella species (n = 1), Enterococcus (n = 1) and Coagulase negative Staphylococci (n = 2). Such secondary infection of ALA with bacteria has been reported previously in the literature [33,34].

In the present study, a total of 112 out of 139 (80.6%) provisionally diagnosed ALA patients were diagnosed as ALA and remaining 27 patients were diagnosed as PLA, by following the criteria mentioned in this study elsewhere.

The result of nested multiplex PCR performed on the liver abscess pus is depicted in Figure 1. The nested multiplex PCR test was positive for E. histolytica DNA in 90 (80.4%) of 112 liver abscess pus specimens (Table 1). The nested multiplex PCR could detect E. histolytica DNA in the liver abscess pus of all 37 ALA patients (100%), who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly decreased the ability of the PCR to detect E. histolytica DNA in the liver abscess pus, with only 53 (70.6%) of 75 liver pus samples positive (Fisher’s Exact test, P = 0.0006). The probability of E. histolytica DNA detection in liver abscess pus by nested multiplex PCR was 31 times more in patients who had not received prior metronidazole therapy (OR = 31.54, 95% CI = 1.879 to 624.2) than in the patients who received prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1.

The nested multiplex PCR did not detect E. histolytica DNA in a total of 49 liver abscess pus specimens, which included 27 PLA and 22 ALA pus specimens. The probability of negative nested multiplex PCR results, in these 49 liver abscess pus specimens due to PCR inhibitors was ruled out by the inclusion of an internal amplification control (IAC) in the nested PCR reaction.

The comparison of results of nested multiplex PCR and TechLab E. histolytica II ELISA test on liver abscess pus from ALA patients is summarised in the table 1.

The nested multiplex PCR result on urine specimen is shown in Figure 1. The nested multiplex PCR was performed on urine specimen collected from 68 patients (including 53 ALA and 15 PLA) and 43 controls. The nested multiplex PCR test detected E. histolytica DNA in 21 (39.6%) of 53 urine samples collected from patients with ALA (Table 2). The test did not detect E. histolytica DNA in urine samples collected from all 15 PLA patients and 43 controls.
The nested multiplex PCR test detected *E. histolytica* DNA in the urine specimens of 4 (17.4%) of 23 ALA patients who were tested prior to treatment with metronidazole, and in 17 (56.7%) of 30 ALA patients who were tested after treatment with metronidazole ($\chi^2 = 6.83$, P = 0.009). All of the 4 ALA patients, who did not receive prior treatment with metronidazole and whose urine specimens were positive for *E. histolytica* DNA, were available for follow-up study. Urine specimens were collected from these patients every week for 4 weeks after starting of the therapy with metronidazole, and were tested for *E. histolytica* DNA by PCR. It was observed that 2 weeks after treatment with metronidazole, 3 (75%) out of 4 urine specimens became negative for *E. histolytica* DNA. One urine specimen became negative for *E. histolytica* DNA after 4 weeks of treatment with metronidazole.

### Discussion

In this study, we have made an attempt to detect excretory *E. histolytica* DNA in urine by applying nested multiplex PCR and to assess the diagnostic potential of the test for detection of *E. histolytica* DNA in urine for the diagnosis of ALA. Also we have studied the kinetics of the excretion of *E. histolytica* DNA in urine during the course of therapy with metronidazole.

In our study, 76.8% (86 of 112) of ALA patients were positive for anti amoebic antibody in serum by the IHA. This result was similar to that reported from Bangladesh where serum anti amoebic antibodies were found in 78% of ALA patients [10], but differed from that of the study reported from South Africa, where serum anti amoebic antibodies were found in a higher 99% of ALA patients [35].

Only two out of 10 ALA pus samples which were positive for *E. histolytica* trophozoite by microscopy were positive for the amoebae by culture and the rest were negative, this may be due to the inhibition of growth by culture itself. In majority of patients, *K. pneumoniae* was the major bacterial pathogen responsible for PLA and as secondary bacterial infection of ALA. One of 10 ALA pus specimens was positive for Group B *Salmonella* species, this patient had liver abscess with perihilar collection, with severe sepsis and disseminated intravascular coagulation, finally the patient died. In this study, the anaerobic culture of liver abscess pus aspirate was not done. Therefore, the possible etiology of liver abscess due to anaerobic organisms such as *Bacteroides* remained undetermined.

In the present study, 50% (56 of 112) of liver abscess pus were positive for *E. histolytica* lectin antigen. The sensitivity of the test in our study was observed to be slightly higher than that of the study using the same TechLab *E. histolytica* II kit (40.7% sensitivity) on liver pus reported from Bangladesh [10]. However, results of other studies using polyclonal antibody based ELISA showed a very high sensitivity for the detection of amoebic antigen in the liver pus. Amoebic antigen was detected in liver abscess pus in 97.6% (41/42) of ALA cases by ELISA as reported from China [21] and in 92% and 96% of liver pus by
using immunoelectrophoresis and ELISA respectively, from India [36].

In developing countries like India where amoebiasis is endemic, antiamoebic drugs and antibiotics are used indiscriminately, making it difficult to obtain an accurate treatment history. Most of the patients in the present study had already been treated with metronidazole at the time of collection of clinical specimens. The serum amoebic antibodies were detected in higher percentage (94.7%) of ALA patients treated earlier with metronidazole, but were detected in only 40.5% of patients who did not receive any prior treatment with metronidazole. This might be due to the late antibody response during the course of the disease.

Unlike serum amoebic antibody detection, E. histolytica lectin antigen was detected in liver pus by TechLab ELISA in a higher percentage (81%) of ALA patients, who were tested prior to treatment with metronidazole, but was detected in only 34.6% of ALA patients, who were tested after the initiation of therapy with metronidazole. This might be due to the rapid clearing of amoebic antigen from the liver pus due to killing of E. histolytica trophozoites on treatment with metronidazole.

The PCR for the detection of E. histolytica DNA in liver abscess pus had a much higher sensitivity (100%) when tested prior to treatment with metronidazole, but had a lower sensitivity (70.6%) when tested after the initiation of treatment with metronidazole. This might be attributed to the clearing of E. histolytica DNA from the liver abscess due to the death and lysis of E. histolytica trophozoites on treatment with metronidazole. The percentage of agreement between E. histolytica DNA detection and ELISA for the histolytica antigen detection in liver abscess pus was found to be 67.9% in the present study (Table 1). The kappa statistic was found to be 0.36 which indicates a fair agreement between the two tests. PCR for detection of E. histolytica DNA and ELISA for detection of E. histolytica lectin antigen in liver abscess pus were evaluated for the diagnosis of ALA (McNemar's $\chi^2 = 30.25, p < 0.0001$). The sensitivity of PCR was 80.4%. This was found to be significantly higher than that of ELISA (50 %) using McNemar's $\chi^2$ test ($p < 0.0001$). All 27 liver abscess pus aspirates diagnosed as PLA were negative for E. histolytica DNA by PCR and for E. histolytica lectin antigen by TechLab ELISA, which represents a specificity of 100%. ELISA for detection of liver abscess pus E. histolytica lectin antigen demonstrated a 100% positive predictive value and a 32.5% negative predictive value. PCR for the detection of liver abscess pus E. histolytica DNA demonstrated a 100% positive predictive value and a 55.1% negative predictive value.

In the present study E. histolytica DNA was detected in the urine specimen of 4 (17.4%) of 23 ALA patients, who were tested prior to treatment with metronidazole and in 17 (56.7%) of 30 ALA patients, who were tested after treatment with metronidazole by PCR. The probability of E. histolytica DNA detection in urine by PCR was 6 times more in ALA patients who had received prior metronidazole therapy (OR = 6.2, 95% CI = 1.47 to 28.37) than in the ALA patients who did not receive prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1. This might be due to release of increased E. histolytica DNA from the dying E. histolytica trophozoites when metronidazole therapy was initiated, leading to excretion of E. histolytica DNA in the urine. One study has demonstrated that the DNA from dying cells can cross the kidney barrier in rodents and humans and can get excreted with urine, which can be used for genetic analysis [31].

PCR for detection of E. histolytica DNA in liver abscess pus and urine specimen were evaluated for the diagnosis of ALA (McNemar's $\chi^2 = 26.28, p < 0.0001$). The sensitivity of PCR for urine was 39.6%. This was found to be signifi-
cantly lower than that of PCR for liver abscess pus (80.4%) using McNemar’s χ² test (p < 0.0001). All urine specimens from 15 PLA patients and 43 control group individuals were negative for *E. histolytica* DNA by PCR. This represents a specificity of 100% (Table 2). PCR for the detection of urinary *E. histolytica* DNA demonstrated a 100% positive predictive value and a 31.9% negative predictive value.

As per the PCR kinetics the likelihood of amplifying smaller PCR product is more than amplifying larger PCR product. We feel that if the PCR product smaller than 400 bp would have been amplified, the PCR might show higher sensitivity.

*E. histolytica* DNA in urine did not persist longer in ALA patients after treatment with metronidazole as observed in the present study. Three of 4 (75%) urine specimens positive for *E. histolytica* DNA became negative for *E. histolytica* DNA within 2 weeks of treatment with metronidazole. This might be attributed to the reduced excretion of *E. histolytica* DNA in the urine as a result of reduction of *E. histolytica* infection load following treatment with metronidazole. The effect of metronidazole in killing of *E. histolytica* and clearing of angenaemia in hamsters suffering from hepatic amoebiasis has been well documented in a study reported by Thammapalerd et al. Results of the present study therefore indicate that the PCR can be used to monitor excretion of *E. histolytica* DNA in urine as a prognostic marker during therapy of ALA with specific antiamoebic drugs.

In the present study, none of the liver abscess pus and urine PCR results were positive for either *E. dispar* or *E. moshkovskii* specific PCR products, which confirm the non-invasive nature of these species (Table 2).

The detection of *E. histolytica* DNA and *E. histolytica* specific lectin antigen in the serum specimen of ALA patients was not carried out in this study. A controlled prospective study to evaluate the detection of *E. histolytica* DNA and *E. histolytica* specific lectin antigen in the serum specimen of ALA patients has been intended to be carried out in future in our laboratory.

**Conclusion**

The present study for the first time shows that the kidney barrier in ALA patients is permeable to *E. histolytica* DNA molecule resulting in excretion of *E. histolytica* DNA in urine which can be detected by PCR. The study also shows that the PCR for detection of *E. histolytica* DNA in urine of patients with ALA can also be used as a prognostic marker to assess the course of the diseases following therapy by metronidazole. The detection of *E. histolytica* DNA in urine specimen of ALA patients provides a new approach for the diagnosis of ALA.

**Methods**

**Sample details**

The subjects in the present study included 139 patients provisionally diagnosed as ALA, who were admitted to JIPMER hospital, Puducherry, as well as 43 controls during a period from September 2004 to March 2006. The provisional diagnosis of ALA was made by the physicians on the basis of patient's history and clinical features, unfortunately these features are often nonspecific to confirm the diagnosis of ALA. Of the 139 provisionally diagnosed ALA patients, 102 had received prior treatment and 37 did not receive prior treatment with metronidazole. The patients and controls were residing in neighboring area of Puducherry. Informed consent was obtained from the patients. This study has been performed with the approval of Institute Research Council of JIPMER, Puducherry.

The control group included 43 individuals who had no history of recent dysentery or diarrhea and whose stool samples were negative for *E. histolytica* infection by microscopy and culture. Thirty five of the controls were healthy asymptomatic volunteers, and the other 8 patients included, hydatid cyst in liver (n = 2), liver hematoma (n = 1), liver cirrhosis (n = 3), and viral hepatitis (n = 2).

The diagnosis of ALA was established on the basis of radiological, symptomatological and laboratory criteria as follows [3,10]: (i) a space-occupying lesion in the liver diagnosed by ultra sonography and suggestive of abscess, (ii) clinical symptoms (fever, pain in the right hypochondrium (often referred to the epigastrum), lower chest, back, or tip of the right shoulder), (iii) enlarged and/or tender liver, usually without jaundice, (iv) raised right dome of the diaphragm on chest radiograph, (v) improvement after treatment with antiamoebic drugs (e.g., metronidazole) (vi) positive IHA of serum antibody showing a titer (≥ 1:128) against *E. histolytica*, (vii) liver aspirate appeared like anchovy sauce but was bacteriologically sterile.

**Sample collection**

Liver abscess pus: The aspiration of liver abscess pus was indicated only under the following conditions [3], (i) to rule out a pyogenic abscess; (ii) the failure to respond clinically in 3 to 5 days; (iii) the threat of imminent rupture; and (iv) the prevention of rupture of left-lobe abscess into the pericardium. The liver abscess pus aspirations were performed, only for clinical purposes as judged by the clinicians for the patient care and not for the purpose of this study. Liver abscess pus was obtained under ultrasound guidance from all 139 provisionally diagnosed ALA
patients and was stored at -20°C in a sterile container until used.

Urine: Urine specimen could be collected from 68 out of 139 provisionally diagnosed ALA patients and all 43 control group individuals included in the study. 10 ml of urine specimen was collected in a sterile container using aseptic techniques; urine sample was stored at -20°C until use.

Blood: Blood specimen was collected from all 139 provisionally diagnosed ALA patients and 43 control group individuals included in the study. Venous blood (5 ml) was collected in a sterile container; sera were separated and stored at -20°C until used.

Microscopy for Entamoeba
Liver abscess pus: The specimens were examined immediately after the aspiration of abscess. The liver abscess pus was first centrifuged at 2,500 g for 5 mins and a loopful (usually inoculating needle loop) of sediment was mixed with a drop of warm saline on a microscope slide. Microscopic examination of an amoebic abscess aspirate from liver may reveal haematothrophic trophozoites.

Culture for Entamoeba
Liver abscess pus: Liver abscess pus specimens were cultured for Entamoeba species in Locke-egg (LE) medium (pH modification of Boeck and Drbohlav’s medium) as previously described [38]. The liver abscess pus was first centrifuged at 2,500 g for 5 mins and a loopful of sediment was inoculated into the LE medium. It is to be noted that in case of culturing Entamoeba from liver abscess aspirates, since the abscess is sterile, bacterial flora (ATCC E. coli) was added before inoculation of amoebae into xenic culture.

Gram stain and bacterial culture for liver abscess pus aspirates
Direct smear Gram staining and bacterial culture was done for all liver abscess pus aspirates. The liver abscess pus specimens were inoculated on to sheep blood agar, MacConkey agar and chocolate agar plates. The MacConkey agar plates were incubated at 37°C for 18-24 hours whereas the blood agar and chocolate agar plates were incubated in a candle jar at 37°C for 48 hours. Based on the colony morphology and result of culture smears, necessary biochemical tests were done to identify bacteria to the species level.

TechLab E. histolytica II ELISA test
The TechLab E. histolytica II test was performed on liver abscess pus specimens to detect E. histolytica as per the method described earlier [10].

Antiamoebic antibody detection by rapid-IHA test
The Rapid-IHA was performed on serum specimen as per the method described earlier [39]. Briefly, double aldehyde stabilized chick red blood cell (RBC) sensitized with the amoebic antigen was used in the test. The haemagglutination test was performed on test serum samples including known positive and negative control sera in each batch. The chick RBC settled quickly and their haemagglutination pattern could be determined within 30 to 45 min of incubation at room temperature with test sera [39]. A titer of ≥ 1:128 was considered positive for ALA [40].

Extraction of Entamoeba genomic DNA
Liver abscess pus: Briefly, 1 ml of liver abscess pus was taken in 1.5 ml centrifuge tube and centrifuged at 12,000 g for 8 minutes. The supernatant was discarded and 50 μl of pellet was dispersed in 250 μl of lysis buffer (0.25% sodium dodecyl sulfate (SDS) in 0.1 M EDTA, pH 8.0), followed by addition of 100 μg/ml of proteinase K. The lysate was incubated at 55°C for 2 hours. Then 75 μl of 3.5 M sodium chloride (NaCl) followed by 42 μl of 10% cetyltrimethylammonium bromide (CTAB)/0.7 M NaCl (heated to 55°C) was added. After the components were mixed, the sample was incubated at 65°C for 30 min. This was followed by extractions with equal volumes of chloroform and then phenol-chloroform-isoamyl alcohol, and the DNA was precipitated with ice cold ethanol. The dried DNA pellet was dissolved in 50 μl of sterile distilled water.

Urine: Briefly, 10 ml of the urine sample was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was discarded and the pellet was suspended in 500 μl of sterile distilled water. The suspension was boiled for 10 minutes followed by sudden cooling. Next, 5 μl of proteinase-K (10 mg/ml) and 60 μl of 10% SDS were added to the suspension and incubated at 65°C for three hours. Then 80 μl of 5 M NaCl and 20 μl of 10% CTAB were added to the mixture and incubated at 65°C for 45 min. This was followed by extractions with equal volumes of chloroform and then phenol-chloroform-isoamyl alcohol. The DNA was precipitated with ice cold ethanol. The dried DNA pellet was dissolved in 50 μl of sterile distilled water.

The protocol for extraction of DNA from liver abscess pus and urine specimen has been modified in our laboratory from CTAB DNA extraction protocol originally described for DNA extraction from amoebic culture [41]. The extracted DNA from liver abscess pus and urine sample was passed through DNA clean-up spin columns (Bangalore Genei KT-62, Bangalore). The DNA was stored at -20°C until used.
Quantification of DNA in liver abscess pus and urine specimen

DNA quantification in spin column purified DNA extract from liver abscess pus and urine specimen was determined by UV absorbance using a Cintra 5 double beam spectrophotometer. DNA yields were calculated on the basis of UV absorbance at 260 nm. The purity of the nucleic acid in the samples was estimated by the ratio of readings at 260 nm and 280 nm (OD260/OD280).

Primers design

The genus specific primers were designed using nucleotide sequences of 16S-like rRNA gene of E. dispar, E. histolytica and E. moskowskii Laredo deposited in GenBank [accession number: Z42556], [accession number: X56991] and [accession number: AF149906] respectively.

The comparison of all the three 16S-like rRNA gene sequences of E. dispar, E. histolytica and E. moskowskii Laredo revealed significant differences enough to design species specific primers. In this study, we have used an IAC targeting human 18S ribosomal RNA gene to rule out false negative results in clinical specimens. All the primers were designed using Prime3 online software [42]. The primers used in PCR are shown in Table 3.

Standard strains

Three standard strains used in this study were E. histolytica HM-1 1IMSS, E. dispar SAW760, and E. moskowskii Laredo these were used as positive control in the present study. The lyophilized DNA of these strains was generously gifted by Dr. C. Graham Clark from London School of Hygiene & Tropical Medicine, London, UK.

Table 3: Primer sequence used in PCR.

<table>
<thead>
<tr>
<th>Genus specific primers (First PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entamoeba genus</td>
</tr>
<tr>
<td>E-1 5' TAAGATGCGACGAGCGAAAA 3' (forward primer)</td>
</tr>
<tr>
<td>E-2 5' GTACAAAGGGGCGGGACGTA 3' (reverse primer)</td>
</tr>
<tr>
<td>Species specific primers (Second nested multiplex PCR)</td>
</tr>
<tr>
<td>E. histolytica species</td>
</tr>
<tr>
<td>EH-1 5' AAGCGATTCTTAGAGCTGAG 3' (forward primer)</td>
</tr>
<tr>
<td>EH-2 5' AAGAGGCTTCAACCCGAAATTAG 3' (reverse primer)</td>
</tr>
<tr>
<td>E. moskowskii species</td>
</tr>
<tr>
<td>M-1 5' GAAACCCAGGTTTCCAAC 3' (forward primer)</td>
</tr>
<tr>
<td>M-2 5' CAATATAAGGCGCTTGATGAT 3' (reverse primer)</td>
</tr>
<tr>
<td>E. dispar species</td>
</tr>
<tr>
<td>ED-1 5' TCTTTTCTTAGAAGCTCT 3' (forward primer)</td>
</tr>
<tr>
<td>ED-2 5' TCCCTACCTATTAGACATGC 3' (reverse primer)</td>
</tr>
</tbody>
</table>

Internal amplification control (IAC) primer for PCR

<table>
<thead>
<tr>
<th>Human 18S ribosomal RNA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAC-1 5' GGCTTTGGGTGACTCTAGATA 3' (forward primer)</td>
</tr>
<tr>
<td>IAC-2 5' CGTTAAGGGATTAAAGTGTT 3' (reverse primer)</td>
</tr>
</tbody>
</table>

Nested multiplex PCR protocol

Liver abscess pus PCR: For a reaction volume of 25 µl, comprising 2.5 µl of 10X PCR buffer (Biogene). 2.0 µl of 25 mM MgCl2 (Bangalore genei), 0.75 µl of deoxynucleoside triphosphate mix (10 mM each, each dNTP, Biogene). 0.3 µl (5 IU/µl) of Taq polymerase (Biogene), 10 picomoles of target DNA primers (IDT) and 5 picomoles of IAC primers (IDT) were added in genus and species specific PCR. The template DNA volume was 2 µl for both genus and species specific PCR. The PCR tubes were finally placed in an Eppendorf Thermal cycler [Master cycler gradient].

Urine PCR: The PCR mix composition was the same as described earlier for liver abscess pus PCR, except that 1.0 µl of 25 mM MgCl2 and 2.5 µl of template DNA was added.

The conditions for genus specific PCR were as follows; the PCR mix was subjected to an initial denaturation at 96 °C for 2 minutes, followed by 30 cycles – each consisting of 92 °C for 60 seconds (Denaturation), 56 °C for 60 seconds (Annealing), and 72 °C for 90 seconds (Extension). Finally one cycle of extension at 72 °C for seven minutes was performed. In the species specific nested multiplex PCR (which had multiple primer sets in the same tube), only the annealing temperature was changed to 48 °C, leaving the other parameters of the amplification cycles unchanged.

3.5 µl of the amplification product was separated by electrophoresis through 1.8% agarose gel (Agarose Low EEO, Bangalore genei products, Bangalore, India) containing ethidium bromide in 0.5 x Tris-borate-EDTA at 120 V for 45 min and was visualized under UV light for bands of DNA of appropriate sizes (Figure 1). A negative control...
reaction was included with each batch of samples analyzed by PCR.

**Primer validation**

The primer sequences designed for *E. moshkovskii*, *E. histolytica*, *E. dispar* and Human IAC were subjected to Basic Local Alignment Search Tool (BLAST) in the genome database of all organisms [43] and were found to be specific for the study. The amplified PCR products of *E. histolytica* species in liver abscess pus and urine samples was confirmed by getting both the strands of DNA sequenced on ABI3730XL sequencer (Macrogen, Seoul, South Korea). The sequencing was done using species specific primers i.e. EH-1/EH-2 for *E. histolytica*. All sequences were analyzed for homology by using the nucleotide-nucleotide BLAST search feature [43].

The identity between the sequencing results of PCR product of *E. histolytica* from liver abscess pus and urine with the sequence deposited in GenBank (accession number: X56922) were analyzed by using the 'Align two sequences (bl2seq)' feature [43].

**Assessment of competition of non target DNA**

During the standardization to assess the competition of other non target DNA present in liver abscess pus and urine specimens with target DNA, the nested multiplex PCR was checked with reference DNA (DNA from standard culture of *E. histolytica*, *E. dispar* & *E. moshkovskii*) spiked with DNA from liver abscess pus (PLA pus negative for *E. histolytica*) and urine (negative control group) followed by nested multiplex PCR amplification.

**Estimation of minimum number of Entamoeba cells detectable by nested multiplex PCR**

This was performed for *E. histolytica* with Locke-egg (LE) medium (NHI modification of Boeck and Drbohlav's medium) liver abscess pus cultures; the amoebae were counted using a standard haemocytometer. A cell pellet containing 10⁶ cells was preferred for determining the detection limit of nested multiplex PCR for *E. histolytica*. The cell pellet containing 10⁶ cells of *E. histolytica* was diluted 10 folds in phosphate buffer saline (PBS) to obtain different concentration of cells, such as 10⁵, 10⁴, 10³, 10² and 10 cells/ml. The different dilutions of cells ranging from 10⁴ to 10⁶ cells/ml were centrifuged and the remaining pellet of each dilution was added to 0.05 μl of liver abscess pus (PLA pus negative for *E. histolytica*) followed by DNA extraction and PCR as per the aforementioned protocol.

**Statistical data analysis**

Sensitivity was calculated as follows: number of patients with positive test results/total number of patients × 100. Specificity was calculated as follows: number of controls with negative test results/total number of controls × 100. The positive predictive value was calculated as follows: number of true positives/(number of true positives + number of false positives) × 100. The negative predictive value was calculated as follows: number of true negatives/(number of true negatives + number of false negatives) × 100. The agreement between the tests was calculated using the Kappa statistics. To determine the statistical significance of differences between the proportions, Chi-square (x²) test and Fisher's exact test were used. The x² test and odds ratio were found using "Epi Info Version 6". To calculate the significance of the difference in sensitivities, McNemar's Chi-square test was applied. The McNemar's test was performed using "Graph Pad Software".

**Authors' contributions**

SCP supervised and coordinated the study, and helped to draft the manuscript. KK carried out the experimental works, and drafted the manuscript.

**Acknowledgements**

We sincerely thank Dr. K.V. Karubasankar and Dr. Gowri sankar from Department of Preventive and Social Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry for doing the statistical data analysis for this work.

**References**


42. Primer3 Input [http://www.rockefeller.edu/~nlstr/]

43. BLAST: Basic Local Alignment and Search Tool [http://www.ncbi.nlm.nih.gov/blast/]

Publish with BioMed Central and every scientist can read your work for free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:
- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here
http://www.biomedcentral.com/info/publishing_adv.asp

Page 10 of 10 (page number not for citation purposes)
Diagnosis of intestinal amoebiasis by using nested polymerase chain reaction-restriction fragment length polymorphism assay

KRISHNA KAIRNAR, SUBHASH CHANDRA PARIJA, and RAVISANKAR PALANIAPPAN

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605006, India

Background. Microscopy is unreliable to distinguish the pathogenic Entamoeba histolytica from the nonpathogenic Entamoeba dispar or Entamoeba moshkovskii in stool specimens. Methods. Nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was carried out to detect E. histolytica, E. dispar, and E. moshkovskii DNA in stool samples of 202 patients positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture and in 35 controls. The lab performed E. histolytica II enzyme-linked immunosorbent assay (ELISA) to detect Gal/NAc lectin in 45 stool samples positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture. Rapid-indirect hemagglutination assay (IHA) was performed to detect serum anti-amoebic antibodies in the 85 patients positive for E. histolytica, E. dispar, or E. moshkovskii in their stool specimens and in the 35 controls. Results. Nested PCR-RFLP was positive in 75 of 202 (36.6%) patient stool samples and was negative in all 35 negative control stool samples. ELISA was positive in 29 of 45 (64.4%) patient stool samples. The IHA test was positive in 19 of 85 (22.4%) patient serum samples and in one (2.8%) of the 35 control serum samples. Nested PCR-RFLP detected E. histolytica DNA in stool specimens of 12 (63.2%) of 19 seropositive patients, and in 31 (47%) of 66 seronegative patients. ELISA detected E. histolytica antigen in stool specimens of six (54.5%) of 11 seropositive patients, and in 23 (67.6%) of 34 seronegative patients. Conclusions. Nested PCR-RFLP was useful for the specific detection of E. histolytica, E. dispar, and E. moshkovskii in stool samples.

Key words: PCR, RFLP, E. moshkovskii, Pondicherry

Introduction

Entamoeba histolytica causes amoebiasis, including both intestinal and extraintestinal amoebiasis. Entamoeba histolytica results in 34 million to 50 million symptomatic cases of amoebiasis worldwide every year, causing 40,000 to 100,000 deaths annually. Entamoeba histolytica, the pathogenic species of amoeba, is indistinguishable in its cyst and trophozoite stages from those of E. moshkovskii, a free-living amoeba, or E. dispar, a noninvasive amoeba, by microscopy, except in cases of invasive disease, when the E. histolytica trophozoite may contain ingested red blood cells, but such a finding is rarely seen. This leads to a confusing scenario for the definite identification and differentiation of E. histolytica from E. moshkovskii and E. dispar in the diagnosis of intestinal amoebiasis.

The sensitivity of light microscopy is limited to only 60% at best. Isoenzyme analysis of cultured amoebas allows the differentiation of E. histolytica from E. dispar. An isoenzyme analysis takes usually one to several weeks before the results are reported, and also requires special laboratory facilities, making it impractical for use in the routine diagnosis of intestinal amoebiasis. Moreover, factors such as delays in the stool specimen processing and initiation of amoebic treatment prior to sample collection can lead to E. histolytica-negative culture results even in those patient stool samples showing cysts/trophozoites by light microscopy.

At present, amoebic coproantigen detection and polymerase chain reaction (PCR) analysis of stool samples are reported to be specific and sensitive methods for detection of E. histolytica in stool specimens. The sensitivity and specificity of monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the detection of E. histolytica-specific antigens in the stool specimen of intestinal amoebiasis patients have been reported to be 85% and 90%, respectively. ELISA for
antigen detection and nested PCR targeting the 16S-like rRNA gene of *E. histolytica* have shown comparable sensitivities of 85% and 87%, respectively, when performed directly on fresh stool specimens. The main limitation of ELISA is that it can specifically detect *E. histolytica* and *E. dispar* but not *E. moshkovskii*. Until now, *E. moshkovskii* in humans has been reported from North America, Italy, South Africa, Bangladesh, and India. Our report of *E. moshkovskii* was the first such report from India. The presence of morphologically similar forms such as *E. dispar* and *E. moshkovskii* has made the differentiation of the three species by PCR necessary, especially in those countries, including India, where all three species, *E. histolytica*, *E. dispar*, and *E. moshkovskii*, have been reported to be prevalent.

The differential diagnosis of pathogenic *E. histolytica* from nonpathogenic *E. dispar* and free-living *E. moshkovskii* will enable clinicians to avoid unnecessary treatment with antiamoebic drugs, as colonization with *E. dispar* and *E. moshkovskii* does not warrant antiamoebic therapy.

**Methods**

**Sample details**

A total of 1720 stool samples were collected and examined from patients presenting at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Puducherry, India, with general complaints of gastrointestinal discomfort during July 2004 to July 2006. As negative controls in the present study, we used 35 stool samples collected from healthy persons as well as patients with other intestinal infections who showed no trophozoites or cysts by either microscopy or culture.

The 35 control stool samples included 15 from healthy persons negative for common enteric pathogens, six positive for enteric bacteria by bacterial culture, ten positive for *Giardia intestinalis* cysts, and four for eggs of *Ascaris lumbricoides*. All of these 35 stool samples were negative for *E. histolytica*, *E. dispar*, and *E. moshkovskii* complex cysts and trophozoites by both microscopy and culture.

Fresh unpreserved stool samples were collected in sterile capped containers for examination by microscopy and culture for *Entamoeba* species. Aliquots of fresh unpreserved stool samples were stored at −20°C until used for PCR and ELISA tests.

**Stool microscopy**

Both saline and iodine wet mounts of fresh stool samples were examined microscopically for *E. histolytica*, *E. dispar*, or *E. moshkovskii* cysts and trophozoites as previously described.

**Stool culture**

Stool samples were cultured for *Entamoeba* species in Locke-egg medium (NIH modification of Boeck and Drbohlav's medium) within 6 h of collection as previously described.

**Nested PCR-restriction fragment length polymorphism**

**Extraction of Entamoeba genomic DNA.** DNA was isolated from stool specimens by the cetyltrimethylammonium bromide (CTAB) extraction method, modified from a previously described method for DNA isolation from in vitro cultures of *Entamoeba*. The extracted DNA was passed through DNA clean-up spin columns (Bangalore Genei, Bangalore, India) to minimize PCR inhibitors and improve the amplification performance. The DNA was stored at −20°C.

**Quantification of DNA in stool specimens.** DNA in the spin column purified DNA extract from stool specimen was quantified by UV absorbance using a Cintra 5 double beam spectrophotometer (Bristol, UK). DNA yields were calculated as UV absorbance × dilution. The purity of the nucleic acid in the samples was estimated by the ratio of readings at 260 and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>).

**Primers used.** Based on the sequences of the 16S-like rRNA gene of *E. histolytica* and *E. dispar*, nested set of primers (designated E-1/E-2, Eh-1/Eh-2, and Ed-1/Ed-2) were used, as described in 1998 by Haque et al. for detecting *E. histolytica* and *E. dispar* in stool specimens. In addition, based on the sequence of the 16S-like rRNA gene of *E. moshkovskii* Laredo, a nested set of primers (designated Em-1/Em-2 and nEm-1/nEm-2) was used, as described in 2003 by Ali et al. for detecting *E. moshkovskii* in stool specimens. The primer sequences used for nested PCR are shown in Table 1.

**Standard strains used.** The three standard strains *E. histolytica* HM-1: IMSS; *E. dispar* SAW760; and *E. moshkovskii* Laredo were used as positive controls in this study. The lyophilized DNA of these strains was generously provided by Dr. C. Graham Clark of the London School of Hygiene and Tropical Medicine.

Nest ed PCR-restriction fragment length polymorphism for *E. histolytica* and *E. dispar*. For a reaction volume of 25 μL, comprising 2.5 μL of 10× PCR buffer (Bangalore Genei), 1.5 μL of 25 mM MgCl<sub>2</sub> (Bangalore Genei), 1.4 μL of deoxynucleoside triphosphate mix (5 mM of each dNTP, ABgene, Epsom, UK)
<table>
<thead>
<tr>
<th>Primer sequences used for nested polymerase chain reaction (PCR)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>First PCR primer for <em>Entamoeba histolytica</em> and <em>E. dispar</em></td>
<td>E-1: 5’ TTTGTATTAGTACAAA 3’ (forward primer)</td>
</tr>
<tr>
<td>Second nested PCR primer for <em>E. histolytica</em> species</td>
<td>E-2: 5’ GTA[AG]TTTGTATTACT 3’ (reverse primer)</td>
</tr>
<tr>
<td>Second nested PCR primer for <em>E. dispar</em> species</td>
<td>Eh-1: 5’ AATGGCCAATTCATTCAAGT 3’ (forward primer)</td>
</tr>
<tr>
<td>First PCR primer for <em>E. moshkovskii</em></td>
<td>Eh-2: 5’ TTTAGAAACAATGCTTCT 3’ (reverse primer)</td>
</tr>
<tr>
<td>Second nested PCR primer for <em>E. moshkovskii</em> species</td>
<td>Ed-1: 5’ AGTTGCCAATTTGATAGC 3’ (forward primer)</td>
</tr>
<tr>
<td></td>
<td>Ed-2: 5’ TTTAGAAACAATGCTTCTC 3’ (reverse primer)</td>
</tr>
<tr>
<td></td>
<td>Em-1: 5’ CTCTTCAGGGGGAGTGGTGC 3’ (forward primer)</td>
</tr>
<tr>
<td></td>
<td>Em-2: 5’ TCGTATTTTCTCACCAGT 3’ (reverse primer)</td>
</tr>
<tr>
<td></td>
<td>nEM-1: 5’ GAATAAGGGTATGTGGC 3’ (forward primer)</td>
</tr>
<tr>
<td></td>
<td>nEM-2: 5’ AAGTGGAGTTAACCACCT 3’ (reverse primer)</td>
</tr>
</tbody>
</table>

For 0.75 μl of deoxynucleoside triphosphate mix (10 mM each dNTP, Biogene, Kimbolton, UK), 0.3 μl (51 U/μl) Taq polymerase (Bangalore Genei), 0.3 μM of each primer (Bangalore Genei), and 2.5 μl of template DNA were added in the first PCR and the second (nested) PCR. The PCR was performed in an Eppendorf thermal cycler (master cycler gradient; Westbury, NY, USA).

The conditions for genus-specific PCR were as follows: The PCR mix was subjected to an initial denaturation at 96°C for 2 min, followed by 30 cycles—each consisting of 92°C for 60s (denaturation), 43°C for 60s (annealing), and 72°C for 30s (extension). Finally, one cycle of extension at 72°C for 5 min was performed. In one species-specific nested PCR (i.e., the second PCR), the annealing temperature only was raised to 62°C, leaving the other parameters of the amplification cycles unchanged.

A volume of 3.5 μl of the amplification products was separated by electrophoresis on a 1.0% agarose gel (Agarose Low EE-0, Bangalore Genei) containing ethidium bromide in 0.5 x TBE; ethidium bromide at 120 V for 45 min and visualized under UV light for bands of DNA of appropriate sizes (Fig. 1). Positive and negative control reactions were included with each batch of samples analyzed by nested PCR.

The nested PCR product of both *E. histolytica* and *E. dispar* showed approximately 900-bp fragments, which were further confirmed by restriction fragment length polymorphism (RFLP) analysis.

To verify the identity of the species, *E. histolytica* and *E. dispar*-specific nested smaller subunit (SSU)-rDNA gene amplification products were double-digested with the restriction endonuclease *Dra*I and *Sau*96I for 2 h at 37°C according to the manufacturer's instructions (Bangalore Genei). RFLP-digested product was visualized by loading 10 μl of RFLP digest on a 3% agarose gel containing ethidium bromide. The RFLP pattern for *E. histolytica* showed 550-bp and 350-bp fragments and an undigested 900-bp fragment, whereas that for *E. dispar* showed 700-bp and 550-bp fragments, and consistent bands of 200 bp and 150 bp (Fig. 1).

![Fig. 1. *Entamoeba histolytica*- and *E. dispar*-specific nested polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Odd and even numbered lanes represent undigested and *Dra*I and *Sau*96I-digested PCR products respectively. Lane 1/2, DNA from stool specimen showing *E. dispar*, lane 3/4, DNA from stool specimen showing *E. histolytica*; lane M, a 100-bp DNA ladder](image)
Xho-I for 1 h at 37°C according to the manufacturer's instructions (Promega). RFLP-digested product was visualized by loading 10 μl of RFLP digest on a 1.8% agarose gel containing ethidium bromide. Xho-I exclusively cuts the 258-bp nested PCR product to produce 236-bp and 22-bp fragments. The 22-bp product is not visible on the gel because it was too small to be resolved on a 1.8% agarose gel (Fig. 2).

Primer validation

The sequences of the primers E-1/E-2, Eh-1/Eh-2, Edl/Edl-2, Em-1/Em-2 and nEm-1/nEm-2 to be used for identification of E. histolytica, E. dispar, and E. moshkovskii were first subjected to a BLAST search of the genome database of all organisms (http://www.ncbi.nlm.nih.gov/blast/) and were found to be specific for the study. The amplified PCR products of E. histolytica, E. dispar, and E. moshkovskii species in stool samples were confirmed by sequencing both strands of DNA with an ABI 377 sequencer (Indian Institute of Science, Bangalore, India). All sequences were analyzed for homology by using the nucleotide–nucleotide BLAST search feature (http://www.ncbi.nlm.nih.gov/blast/BLAST).

The identities between the sequencing results of the PCR products of E. dispar, E. histolytica, and E. moshkovskii with the sequences deposited in GenBank accession numbers Z49256, X56991, and AF 149906, respectively) were analyzed by using the BLAST sequence alignment (bl2seq) feature (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

**TechLab E. histolytica II ELISA**

The TechLab E. histolytica II test, designed to detect specifically the E. histolytica lectin antigen, was performed on 45 stool specimens randomly selected from 202 patients with stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture. The TechLab E. histolytica II test was performed on stool specimens as per the manufacturer's instructions.

**Antiamoebic antibody detection by rapid indirect hemagglutination test**

Blood samples were available from only 85 of 202 patients with stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture. Blood specimens were also collected from all 35 control group individuals included in the study. Venous blood (5 ml) was collected in a sterile container, and the serum was separated and stored at −20°C until used. A rapid indirect hemagglutination (IHA) test was performed on serum specimens of all 85 patients (positive for E. histolytica, E. dispar, or E. moshkovskii in stool specimens by microscopy or culture) and of the 35 control group individuals included in the study with the method described earlier. A titer of ≥1:128 was considered positive for amoebiasis.

**Statistical data analysis**

Sensitivity was calculated as the number of patients with positive test results/total number of patients × 100. Specificity was calculated as the number of controls with negative test results/total number of controls × 100. The agreement between the tests was calculated using kappa statistics. The kappa statistic was obtained with Microsoft Excel 2003. To determine the statistical significance of differences between the proportions, χ-squared and Fisher's exact tests were used. The χ-squared test was performed and the odds ratio determined with Epi Info Version 6 software.

**Results**

**Microscopy and culture of stool**

Of the 1720 stool samples screened, 202 were positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture (Table 2). The male to female ratio among these 202 patients was 1:1. All 35 negative
Table 2. Comparison of results of nested PCR-restriction fragment length polymorphism (RFLP) and microscopy and culture of stool specimens

<table>
<thead>
<tr>
<th>Nested PCR-RFLP result</th>
<th>Microscopy and culture result*</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive by microscopy and culture</td>
<td>Positive by microscopy only</td>
</tr>
<tr>
<td>E. dispar (monoinfection)</td>
<td>77</td>
<td>15</td>
</tr>
<tr>
<td>E. histolytica (monoinfection)</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>E. moshkovskii (monoinfection)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. dispar + E. moshkovskii (mixed)</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>E. histolytica + E. moshkovskii (mixed)</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>E. histolytica + E. moshkovskii (mixed)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. dispar + E. histolytica + E. moshkovskii (mixed)</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>total</td>
<td>164</td>
<td>22</td>
</tr>
</tbody>
</table>

*Microscopy and culture could not distinguish among cysts and trophozoites of E. histolytica, E. dispar, or E. moshkovskii in stool specimens.

Control stool samples were negative for Entamoeba species by microscopy and culture.

Nested PCR-RFLP

The results of the quantification of DNA in the stool specimens showed the DNA yield to be approximately 45 μg/ml. The purity of the DNA extract from stool specimens was satisfactory, as the OD260/OD280 ratio was approximately 1.8. The sequencing of the PCR products of E. dispar, E. histolytica, and E. moshkovskii from stool samples showed reasonable identity with the GenBank sequences of accession number Z49256, accession number X56991, and accession number M149906, respectively.

The nested PCR-RFLP was performed on a total of 45 stool specimens, including 202 stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy or culture and 35 negative control stool specimens. The nested PCR-RFLP was positive in 175 of the 202 stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii trophozoites or cysts by microscopy or culture (Table 2), for a sensitivity of 86%. Nested PCR-RFLP results for representative stool specimens are shown in Figs. 1 and 2.

All 35 negative control stool samples that were negative by both microscopy and culture for E. histolytica, E. dispar, or E. moshkovskii trophozoites or cysts were also negative by nested PCR (Table 2), for a specificity of 100%.

The probability of negative nested PCR results in the negative control stool samples being caused by PCR inhibitors was ruled out by spiking the DNA of the negative stool specimens with standard Entamoeba DNA, followed by nested PCR amplification.

The nested PCR-RFLP detected monoinfection with E. histolytica in 5.4% (11/202 stool samples), E. dispar in 47.5% (96/202), and E. moshkovskii in 1.0% (2/202) of stool samples. The nested PCR-RFLP detected mixed infections of E. dispar with E. moshkovskii in 7.9% (16/202), E. dispar with E. histolytica in 17.8% (36/202), E. histolytica with E. moshkovskii in 1.0% (2/202), and with all the three species in 5.9% (12/202) of stool samples (Table 2).

Two stool samples were positive for E. moshkovskii exclusively by nested PCR-RFLP. This finding may explain the previous finding of some of microscopically positive but antigenically and PCR negative results for E. histolytica and E. dispar,* suggesting that E. moshkovskii may be present in such stool samples.

The prevalence of E. moshkovskii, E. dispar, and E. histolytica among patients at JIPMER hospital clinically suspected to have gastrointestinal infections is shown in Table 3.

TechLab E. histolytica II ELISA

The TechLab E. histolytica II ELISA was positive for E. histolytica-specific lectin antigen in 29 of 45 stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii by culture (Table 4). Nested PCR identified E. histolytica DNA in 32 of these 45 culture-positive stool specimens.

Comparison of results of the TechLab E. histolytica II ELISA and the nested PCR-RFLP on stool specimens showed that of the 29 specimens positive for E. histolytica by the antigen detection test, nested PCR detected E. histolytica DNA in 27 specimens (17 positive for both E. histolytica and E. dispar; one positive for both E. histolytica and E. moshkovskii; six positive...
Table 3. Prevalence of *Entamoeba dispar*, *E. histolytica*, and *E. moshkovskii* among patients presenting at the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital clinically suspected to have gastrointestinal infections

<table>
<thead>
<tr>
<th>Type of Entamoeba species</th>
<th>No. of samples positive by nested PCR-RFLP</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. moshkovskii</em></td>
<td>32</td>
<td>1.9</td>
</tr>
<tr>
<td><em>E. dispar</em></td>
<td>160</td>
<td>9.3</td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td>61</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Total no. of stool samples included in the study = 1720

Table 4. Comparison of results of culture and the TechLab *E. histolytica* II enzyme-linked immunosorbent assay (ELISA) performed on stool specimens

<table>
<thead>
<tr>
<th>No. of patient specimens with reaction in culture for Entamoeba</th>
<th>TechLab <em>E. histolytica</em> II stool specimen result*</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

* *E. histolytica* II ELISA is designed to detect only *E. histolytica* specific lectin antigen in stool specimens and therefore can confirm only the presence of *E. histolytica*.
* *E. histolytica* was detected by PCR in 5 of these 16 antigen negative stool specimens.
* *E. histolytica* was detected by PCR in 27 of these 29 antigen positive stool specimens.

Table 5. Comparison of results of nested PCR-RFLP and TechLab *E. histolytica* II ELISA on stool specimens

<table>
<thead>
<tr>
<th>Nested PCR-RFLP result</th>
<th>TechLab <em>E. histolytica</em> II stool specimen test result</th>
<th>Total no. of specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><em>E. histolytica</em> + <em>E. moshkovskii</em> (mixed)</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. histolytica</em> (mixed)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>E. histolytica</em> + <em>E. moshkovskii</em> (mixed)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><em>E. dispar</em> (monoinfection)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>E. moshkovskii</em> (monoinfection)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. histolytica</em> (monoinfection)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>E. moshkovskii</em> (dual)</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

for *E. histolytica*, *E. moshkovskii*, and *E. dispar*; and three positive for *E. histolytica*), for a correlation of 81% (Table 5).

Antiamoebic antibody detection by rapid IHA test

The rapid IHA test was positive for antiamoebic antibody in serum specimens of only 19 of 85 (22.4%) patients positive for *E. histolytica*, *E. dispar*, or *E. moshkovskii* in stool specimens by microscopy or culture. Of these 19 seropositive patients, 12 (63.2%) had stool specimens positive for *E. histolytica* (as mono- or mixed infections) by nested PCR-RFLP, whereas 31 (47%) of 66 seronegative patients had stool specimens positive for *E. histolytica* (as mono- and mixed infections) (Table 6). One (2.8%) of 35 control subjects was positive for antiamoebic antibody by IHA of serum.

Comparison of the results of stool lectin antigen detection with serum antiamoebic antibody detection in 45 patients showed that of the 11 seropositive patients, six (54.5%) had stool specimens positive for *E. histolytica* by the antigen detection test, whereas 23 (67.6%) of 34 seronegative patients had stool specimens positive for *E. histolytica* infection (Table 7).
Table 6. Comparison of the nested PCR-RFLP results performed on stool specimens and antiamoebic antibody test result in serum of patients presenting at JIPMER hospital with gastrointestinal discomfort

<table>
<thead>
<tr>
<th>Nested PCR-RFLP result</th>
<th>Positive</th>
<th>Negative</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. dispar (monoinfection)</td>
<td>6</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>I. histolytica (monoinfection)</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>I. moshkovskii (monoinfection)</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>I. dispar + E. moshkovskii (mixed)</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>I. dispar + E. histolytica (mixed)</td>
<td>6</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>I. histolytica + E. moshkovskii (mixed)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>I. dispar + E. histolytica + E. moshkovskii (mixed)</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>66</td>
<td>85</td>
</tr>
</tbody>
</table>

*Serum indirect hemagglutination titer ≥ 1:128 was considered positive.

Table 7. Comparison of the TechLab E. histolytica II ELISA results performed on stool specimens and the antiamoebic antibody test result in serum of patients presenting at JIPMER hospital with gastrointestinal discomfort

<table>
<thead>
<tr>
<th>No. of specimens with reaction for antiamoebic antibody in serum</th>
<th>TechLab E. histolytica II stool specimen test result</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

*Includes two stool specimens positive for E. histolytica by nested PCR-RFLP.

Discussion

It is now possible to detect and distinguish E. histolytica, E. dispar, and E. moshkovskii in stool samples by exploiting the genetic differences between E. histolytica and E. dispar, several research groups have developed PCR-based assays for the detection of these two species. Most of these PCR analyses are based on the use of sequences of the extrachromosomal circular plasmid DNA gene present in about 200 copies in each Entamoeba cell.

A few reports have considered E. moshkovskii along with E. histolytica and E. dispar for detection in stool specimens by PCR. In the present study, nested PCR was negative in 27 of 202 stool specimens positive in Entamoeba species trophozoites or cysts by microscopy or culture (Table 2). The negative PCR result in these 27 stool samples may be due to the presence of other Entamoeba species or may be imputed to a limitation of the sensitivity of the nested PCR technique. The nested PCR-RFLP results reported here showed a sensitivity of 86.6% and a specificity of 100%, indicating that it is a dependable method for specific detection of E. histolytica in stool specimens.

The overall correlation between the nested PCR-RFLP results for stool specimens and those of antigen detection tests for detecting E. histolytica infection was greater than 90%. This agreement between techniques provides confidence that any one of the techniques may be used alone to accurately assess the presence of E. histolytica in a stool specimen. Nested PCR-RFLP has the advantage of identifying and differentiating all three morphologically similar forms of amoeba, E. histolytica, E. dispar, and E. moshkovskii, which is not possible with ELISA or culture. Our results clearly indicate the superiority of PCR over ELISA for detecting and characterizing the species of amoeba at the DNA level.

The percentage agreement between ELISA for E. histolytica lectin antigen detection in stool specimens and IHA seropositivity for antiamoebic antibody was
37.78%. The kappa statistic was -0.084, which indicates poor agreement between the two tests.

We also found no significant association between seropositivity for amoebic antibodies and stool positivity for *E. histolytica* by ELISA. In the present study, 20.7% of the patients that were infected with *E. histolytica* were seropositive, and 31.2% of the patients whose stools were *E. histolytica*-negative were seropositive (Fisher’s exact test, *P* = 0.483) (Table 7). *Entamoeba histolytica* lectin antigen was detected in stool specimens from 23 of 34 seronegative patients (Table 7). We also found no significant association between seropositivity and positivity for stool infection with *E. histolytica* by PCR-RFLP. In the present study, 27.9% of the patients infected with *E. histolytica* were seropositive, and only 16.7% of patients whose stools were *E. histolytica*-negative were seropositive (χ² = 0.27, *P* = 0.603) (Table 1).

These results contrast with those from Bangladesh, where it was found that 52% of children colonized with *E. histolytica* were seropositive. Our data also differ from those of a study conducted in India that found 12.8% of seropositive individuals to be colonized with an *E. histolytica*-*E. dispar* complex (as defined by microscopic examination of stool), whereas 20.3% of seronegative individuals were colonized. Results similar to ours have been reported from Brazil, where no correlation was observed between seropositivity and stool colonization with *E. dispar* or *E. histolytica*. In the Brazilian study, 19.5% of the individuals colonized with *E. histolytica* were seropositive; this result is similar to our findings in India.

Previous reports suggest that differentiation by culture/isoenzyme analysis or antigen detection kits might overlook mixed infections with *E. histolytica* and *E. dispar*. Since such infections were detected only after PCR was used directly on the same stool specimens previously assayed. Regarding antigen-detection methods, a small number of *E. dispar* organisms present among a large population of *E. histolytica* would be difficult to detect using an *E. histolytica*-specific monoclonal antibody or a monoclonal antibody cross-reactive with *E. histolytica* and *E. dispar*. In such cases, only the use of PCR or an *E. histolytica*- and *E. dispar*-specific monoclonal antibody would enable differentiation. These observations may explain why mixed infections with *E. histolytica* and *E. dispar* were not reported prior to the development of more specific and sensitive molecular techniques.

In the present study, occult infection (PCR-positive but ELISA-negative stool specimens for *E. histolytica*) was observed. Occult infection may possibly be caused by degradation of lectin antigen in stool specimens after prolonged storage at -20°C before testing by ELISA, which might result in ELISA negativity in PCR-positive stool specimens. The percentage agreement between ELISA for *E. histolytica* antigen detection and PCR for *E. histolytica* DNA detection in stool specimens was 82.22%. The kappa statistic was 0.60, which indicates moderate agreement between the two tests.

This study included patients from various age groups and from different geographical localities, which shows the wide distribution of *E. moshkovskii* and *E. dispar* in Puducherry and its environs. The study has several interesting findings.

First, of two patients with *E. moshkovskii* mono-infection, one suffered from dysentery, although the associated bacterial etiology by routine stool culture was negative. Other investigations, such as viral studies, could not be done in our 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 merely transiently infected.

Second, this study highlights the risk of relying on microscopy for the diagnosis of amoebiasis. Of all patients with gastrointestinal discomfort diagnosed with amoebiasis by microscopy or culture, only 30.2% were proven to have *E. histolytica* infection when nested PCR-RFLP was used. The study showed an appreciably high prevalence of *E. dispar* in the patients compared with *E. histolytica*, indicating that only 61 of 202 stool samples with organisms resembling *E. histolytica* by microscopy actually contained *E. histolytica*, implying that the majority of suspected infections are misdiagnosed and might be treated unnecessarily with anti-amoebic drugs when diagnosed on the basis of microscopic findings alone. Other investigators have also found that infection with *E. dispar* is more common than infection with *E. histolytica*. One report, however, claims that infection with *E. histolytica* is predominant in a population.

Third, the present study showed that the rate of mono-infection with *E. dispar* was highest (47.5%; 96/202 stool samples) among patients presenting at JIPMER hospital. The study also shows that the rate of con-infection of *E. histolytica* with *E. dispar* is highest (17.8%; 36/202 stool samples) compared with *E. dispar* with *E. moshkovskii* (7.9%; 16/202 stool samples) or *E. histolytica* with *E. moshkovskii* (1.0%; 2/202 stool samples). Coinfection with both *E. dispar* and *E. histolytica* in the same stool specimen has been reported earlier.

In conclusion, epidemiologic studies and clinical diagnosis of *E. histolytica*-associated infection based on morphological examination alone are prone to error. Infections due to both *E. dispar* and *E. moshkovskii* are associated with an asymptomatic carrier stage. PCR is, therefore, essential for distinguishing *E. histolytica* from *E. dispar* and *E. moshkovskii*. 
Acknowledgments. We thank DSTE (Department of Science, Technology and Environment), Puducherry and ICMR (Indian Council of Medical Research), New Delhi, for funding the project. We sincerely thank TechLab, Inc. (Blacksburg, VA) for generously providing the TechLab E. histolytica II ELISA test kit free of cost for the purpose of research. We also thank Dr. K. V. Kirubasankar and Dr. Gowrisankar from Department of Preventive and Social Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, for doing the statistical data analysis of this work.

References

33. Pillai DR, Keystone JS, Sheppard DC, MacLean JD, MacPherson DW, Kain KC. Entamoeba histolytica and Entamoeba dispers epi-


K. Khairnar et al.: Nested PCR-RFLP for Entamoeba


A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of Entamoeba histolytica, E. moshkovskii and E. dispar DNA in stool samples

Krishna Khairnar and Subhash C Parija*

Address: Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India

Email: Krishna Khairnar - kk.khairnar@yahoo.co.in; Subhash C Parija* - parijasc@vsnl.com

* Corresponding author

Abstract

Background: E. histolytica, a pathogenic amoeba, is indistinguishable in its cyst and trophozoite stages from those of non-pathogenic E. moshkovskii and E. dispar by light microscopy. We have developed a nested multiplex PCR targeting a 16S-like rRNA gene for differential detection of all the three morphologically similar forms of E. histolytica, E. moshkovskii and E. dispar simultaneously in stool samples.

Results: The species specific product size for E. histolytica, E. moshkovskii and E. dispar was 439, 553 and 174 bp respectively, which was clearly different for all the three Entamoeba species. The nested multiplex PCR showed a sensitivity of 94% and specificity of 100% for the demonstration of E. histolytica, E. moshkovskii and E. dispar DNA in stool samples. The PCR was positive for E. histolytica, E. moshkovskii and E. dispar in a total of 190 out of 202 stool specimens (94% sensitive) that were positive for E. histolytica/E. dispar/E. moshkovskii by examination of stool by microscopy and/or culture. All the 35 negative control stool samples that were negative for E. histolytica/E. dispar/E. moshkovskii by microscopy and culture were also found negative by the nested multiplex PCR (100% specific). The result from the study shows that only 34.6% of the patient stool samples that were positive for E. histolytica/E. dispar/E. moshkovskii by examination of stool by microscopy and/or culture, were actually positive for pathogenic E. histolytica and the remaining majority of the stool samples were positive for non-pathogenic E. dispar or E. moshkovskii as demonstrated by the use of nested multiplex PCR.

Conclusion: The present study reports a new nested multiplex PCR strategy for species specific detection and differentiation of E. histolytica, E. dispar and E. moshkovskii DNA in stool specimens. The test is highly specific, sensitive and also rapid, providing the results within 12 hours of receiving stool specimens.

Background

Infection with Entamoeba histolytica results in 34 million to 50 million symptomatic cases of amoebiasis worldwide each year, causing 40 thousand to 100 thousand deaths annually [1]. E. histolytica, the pathogenic amoeba, is indistinguishable in its cyst and trophozoite stages from those of non-pathogenic E. dispar [1] and E. moshkovskii [2], except in rare cases of invasive disease when E. histo-
**Results**

**Microscopy and culture of stool**

A total of 202 out of 1,720 stool samples screened were positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* by microscopy and/or culture. These included 164 specimens positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* by both microscopy and culture, 22 positive by microscopy and 16 positive by culture (Table 1). All the 35 negative control stool samples were negative for *E. histolytica*/*E. dispar*/*E. moshkovskii* by microscopy and culture.

**Nested multiplex PCR**

Quantification of *DNA* in stool specimen

The DNA yield was found to be approximately 49 μg/ml. The purity of DNA extract from stool specimen was found to be satisfactory as the value of ratio of readings at 260 nm and 280 nm (OD 260/OD 280) was approximately 1.8.

---

Table 1: Comparison of results of nested multiplex PCR (Polymerase Chain Reaction), microscopy and culture on stool specimens.

<table>
<thead>
<tr>
<th>Nested multiplex PCR result</th>
<th>Microscopy and culture result</th>
<th>Total no. of specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive by microscopy and culture</td>
<td>Positive by microscopy only</td>
</tr>
<tr>
<td>E. dispar (mono infection)</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>E. histolytica (mono infection)</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>E. moshkovskii (mono infection)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. dispar + E. moshkovskii (mixed)</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>E. dispar + E. histolytica (mixed)</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>E. histolytica + E. moshkovskii (mixed)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. dispar + E. histolytica + E. moshkovskii (mixed)</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>164</strong></td>
<td><strong>22</strong></td>
</tr>
</tbody>
</table>

*Microscopy and culture was unable to distinguish between cyst and trophozoite of *E. histolytica*, *E. dispar* and *E. moshkovskii* in stool specimens.*
Primer validation
The sequencing result of PCR product of E. dispar, E. histolytica and E. moshkovskii Laredo showed 99% to 100% identity to the sequences of E. dispar, E. histolytica and E. moshkovskii Laredo, deposited in GenBank with accession number [GenBank:Z49256], [GenBank:K56592] and [GenBank:AF149908] respectively.

Assessment of competition for non-target DNA
The assessment of competition of other non-target DNA present in stool DNA extract with a target DNA in nested multiplex PCR showed expected amplification without any non-specific amplification.

Estimation of minimum number of Entamoeba cells detectable by nested multiplex PCR
The nested multiplex PCR detected E. histolytica, E. dispar and E. moshkovskii DNA, even at the minimum parasite concentration tested (1000 parasites/0.05 grams of feces). The detection limit of nested multiplex PCR for E. histolytica, E. dispar and E. moshkovskii was found to be approximately 25 Entamoeba protozoa cells, since 2.5 μl of template DNA (1000 parasite/100 μl of TE buffer) gave positive signal (Figure 1).

Estimation of nested multiplex PCR to detect mixed infections with E. histolytica, E. dispar and E. moshkovskii species
1000 cells of E. dispar and E. moshkovskii species as the background allowed for the detection of a 0.01 cell of E. histolytica; 1000 cells of E. histolytica and E. moshkovskii species as the background allowed for the detection of a 0.001 cell of E. dispar; and 1000 cells of E. histolytica and E. dispar species as the background allowed for the detection of a 0.1 cell of E. moshkovskii (Figure 2).

Cross checking the results of nested multiplex PCR
The cross checking of the results of nested multiplex PCR was satisfactory as the same results were reproduced in randomly selected samples showing a mixed infection when subjected to individual species specific PCR in separate tubes.

Nested multiplex PCR
The nested multiplex PCR developed and evaluated in the present study showed that the size of diagnostic fragments of PCR products was clearly different for all the three Entamoeba species, the species-specific product size for E. histolytica, E. moshkovskii and E. dispar was 439, 553 and 174 bp respectively (Figure 3).

The nested multiplex PCR was performed on a total of 237 stool specimens including 202 stool specimens positive for E. histolytica, E. dispar, or E. moshkovskii by microscopy and/or culture, and 35 amoebae-negative control stool specimens. The nested multiplex PCR was positive in 190 out of 202 stool specimens that were positive for E. histolytica/E. dispar/E. moshkovskii complex trophozoites/cysts by microscopy and/or culture, thus showing a sensitivity of 94%. All the 35 negative control stool samples were negative by nested multiplex PCR thus showing a specificity of 100%.

The probability of negative nested multiplex PCR results in 35 control stool samples due to PCR inhibitors was ruled out by spiking the DNA of negative stool specimens with standard DNA of Entamoeba followed by nested multiplex PCR amplification.

The nested multiplex PCR detected mono-infection with E. histolytica in 7.4% (15 of 202), E. dispar in 49.5% (100 of 202) and E. moshkovskii in 1.0% (2 of 202) of stool samples. The PCR also detected mixed infections by both E. dispar and E. moshkovskii in 8.9% (18 of 202), E. dispar and E. histolytica in 18.8% (38 of 202), and E. histolytica and E. moshkovskii in 1.0% (2 of 202) of stool samples. The test also detected mixed infections by all the three species (E. histolytica, E. dispar and E. moshkovskii) in 7.4% (15 of 202) of stool samples (Table 1). The result of the nested multiplex PCR as compared with microscopy and culture is also summarized in Table 1.
Differential detection of *E. histolytica*, *E. moshkovskii* and *E. dispar* by nested multiplex PCR on stool samples. The *E. moshkovskii* (EM), *E. histolytica* (EH) and *E. dispar* (ED) bands are 553, 439 and 174 bp, respectively. Lane-1, *E. moshkovskii* (mono-infection); Lane-2, *E. moshkovskii*, *E. histolytica* and *E. dispar* (mixed infection); Lane-3, *E. histolytica* and *E. dispar* (mixed infection); Lane-4 *E. moshkovskii* and *E. histolytica* (mixed infection); Lane-5, *E. moshkovskii* and *E. dispar* (mixed infection); Lane-6 *E. histolytica* (mono-infection), Lane-7 *E. dispar* (mono-infection); Lane-8, 100 bp DNA ladder (Bangalore genei, Bangalore).

PCR positivity of *E. dispar*, *E. histolytica* and *E. moshkovskii* among the stool samples that were positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* by microscopy and/or culture is presented in Table 2.

**TechLab E. histolytica II ELISA**

The TechLab *E. histolytica* II ELISA test was performed to detect *E. histolytica* coproantigen in 45 randomly selected stool samples that were positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* complex by microscopy and/or culture.

The TechLab *E. histolytica* II ELISA detected coproantigen in 29 out of 45 (64.4%) stool specimens positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* complex by microscopy and/or culture. These 29 amoebic antigen positive stool specimens included 4 specimens positive for *E. histolytica* as mono-infection, 17 specimens positive for *E. histolytica* and *E. dispar* as mixed-infection, 1 specimen positive for *E. histolytica* and *E. moshkovskii* as mixed-infection, and 7 specimens positive for all the three species namely *E. histolytica*, *E. moshkovskii* and *E. dispar* as mixed-infection by the nested multiplex PCR (Table 3).

Comparison of results of nested multiplex PCR and TechLab *E. histolytica* II ELISA test performed on 45 stool specimens is summarised in the table 3.

**Discussion**

To be able to detect and distinguish *E. histolytica*, *E. dispar* and *E. moshkovskii* in stool samples is extremely important
for accurate diagnosis of intestinal amoebiasis and for knowing the true prevalence of pathogenic *E. histolytica* in the community. Various DNA-based molecular methods have been evaluated for accurate detection of *E. histolytica*, *E. dispar* or *E. moshkovskii* [2,6,9-16].

In the present study, describes a new nested multiplex PCR strategy for species-specific detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* DNA directly in the stool samples of patients.

Recently a single round PCR to detect *E. histolytica*, *E. dispar* and *E. moshkovskii* in stool samples has been reported by Hamezah et al [17], the study reported that out of 27 stool samples positive for *Entamoeba* spp. by microscopy only 7 were successfully identified at species level by PCR, which included 1 positive for *E. histolytica* and 6 for *E. dispar*, but no amplification of *E. moshkovskii* was observed in a Thai population [17]. In contrast, our study has shown the presence of *E. moshkovskii* in the Indian population. The negative result for *E. moshkovskii* in Thai population may be attributed to the small sample size used in the study. A detailed comparative study between these two newly described PCR techniques may yield useful information especially in the field of molecular-based diagnosis of intestinal amoebiasis.

Nested PCR was used in the present study because it increases sensitivity [18]. Clinical specimens such as stool often contain PCR inhibitors even after purification steps. The two rounds of PCR might have assisted in compensating the effects of inhibitors present in clinical specimens. The first PCR product may be in too low concentration for detection with ethidium bromide-stained gels using a UV transilluminator. The detection limit of agarose gel electrophoresis with ethidium bromide stain using an UV transilluminator is approximately 10 ng of DNA [19]. The product of first PCR may be just enough to provide adequate templates for the synthesis of second PCR product in the nested reaction to be detected by ethidium bromide staining.

The nested multiplex PCR was negative in 12 out of 202 stool specimens that were positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* complex trophozoites/cysts by microscopy and/or culture. The negative result due to inhibition of PCR in all these 12 stool samples was ruled out by spiking with standard DNA of *Entamoeba* followed by nested multiplex PCR amplification.

The negative PCR result in these 12 stool samples may be due to the presence of other *Entamoeba* species. However, we feel that this supposition needs to be proven by further development of molecular tools to confirm the presence of other *Entamoeba* species commonly found in humans such as *E. coli*, *E. hartmanni* or other similar looking *Entamoeba* species. Till then these negative results may be imputed to the sensitivity limitation of the nested multiplex PCR technique.
In the present study by using nested multiplex PCR it was shown that the rate of mono infection with *E. dispar* was the highest. *E. dispar* was demonstrated in 100 out of 202 stool samples (49.5%) amongst patients attending JIPMER hospital. The study also shows that the rate of co-infection with *E. histolytica* and *E. dispar* was the highest (18.8%) as compared to both, *E. dispar* and *E. moshkovskii* (8.9%), and *E. histolytica* and *E. moshkovskii* (1.0%). The occurrence of co-infection with *E. dispar* and *E. histolytica* in the stool specimens has been documented by several studies reported earlier [9,10,12,20,21].

Nested multiplex PCR was positive for *E. histolytica* DNA in 6 stool specimens which were negative for coproantigen by TechLab ELISA. The possible reason for such occult infection (PCR-positive, ELISA-negative stool specimens for *E. histolytica*) may be due to degradation of lectin antigen in stool specimen due to prolonged storage (approximately 30–60 days) at -20°C prior to testing, thus resulting in a negative test for amoebic coproantigen by the ELISA.

In the present study the overall correlation between the results of nested multiplex PCR and that of TechLab *E. histolytica* II ELISA to detect *E. histolytica* in stool specimen was greater than 90%. This agreement between these two techniques shows clearly that the techniques may be used alone to yield an accurate assessment of the presence of *E. histolytica* in a stool specimen, but not for the detection of either *E. dispar* or *E. moshkovskii* in stool specimens by TechLab *E. histolytica* II ELISA.

The inability of TechLab *E. histolytica* II ELISA to detect either *E. dispar* or *E. moshkovskii* in stool specimens is the noted disadvantage of the test. The nested multiplex PCR, on the other hand appears to be more useful for simultaneous detection of all the three species *E. histolytica, E. moshkovskii* and *E. dispar* when performed directly on the stool specimens. This is the main advantage of this test, and the importance of the fact that there is an increasing documentation of both *E. moshkovskii* and *E. dispar* from different parts of the world [2,6,9,11-17], including from Puducherry, the southern union territory of India [10]. The coexistence of non-pathogenic *E. dispar* and *E. moshkovskii* as mixed infection or solely as mono-infection amongst the patients showed an increased possibility of false diagnosis when the identification of *E. histolytica* was based primarily on morphology by microscopic examination of stool. The high PCR positivity of *E. moshkovskii* among the study population supports the view that humans are a true host for this free-living amoeba [2].

The inability to distinguish *E. histolytica* from those of morphologically similar *E. dispar* or *E. moshkovskii* in the stool samples is the main limitation of microscopy or culture. As shown in the present study only 34.6% of 202 stool specimens positive for *E. histolytica*, *E. dispar* or *E. moshkovskii* complex trophozoites/cysts by either microscopy or culture were actually *E. histolytica* and the remaining majority of stool specimens were positive for *E. dispar* and/or *E. moshkovskii*. In the absence of tests such as the nested multiplex PCR or ELISA for specific detection of *E. histolytica*, the majority of suspected infections would have been wrongly diagnosed as *E. histolytica* infection and treated unnecessarily with anti-amoebic drugs. We therefore, recommend the use of the nested multiplex PCR for simultaneous detection and accurate identification of all the three *Entamoeba* species in stool specimens.

**Conclusion**

The present study reports a new nested multiplex PCR strategy for species specific detection and differentiation of *E. histolytica, E. dispar* and *E. moshkovskii* DNA in stool specimens. The test is highly specific, sensitive and also rapid; results of the test are available within 12 hours of receipt of stool specimens.

**Methods**

**Sample details**

A total of 1,755 stool samples were collected in the present study during a study period of two years from July 2004 to July 2006. This included 1,720 stool specimens collected from patients attending JIPMER hospital, Puducherry, India, with complaints of gastrointestinal discomfort. It also included 35 stool samples, as control samples, collected from healthy persons as well as patients with other intestinal infections. The 35 control stool samples included 15 stool samples negative for common enteric pathogens from healthy persons, 6 samples positive for enteric bacteria by bacterial culture, 10 positive for *Giardia intestinalis* cysts and 4 for eggs of *Ascaris lumbricoides*. All these 35 stool samples were negative for *E. histolytica/*E. dispar/*E. moshkovskii* complex cysts and trophozoites by microscopy and culture.

Fresh unpreserved stool samples were collected in sterile capped containers for examination by microscopy and culture for *E. histolytica/*E. dispar/*E. moshkovskii* complex. Aliquots of fresh unpreserved stool samples were stored at -20°C until used for PCR and ELISA tests.

**Microscopic examination of stool**

Both saline and iodine wet mounts of fresh unpreserved stool samples were examined microscopically for demonstrating *E. histolytica/*E. dispar/*E. moshkovskii* complex cysts and trophozoites as previously described [22]. Briefly, saline wet mounts were made by mixing approximately 2 mg of stool with a drop of physiological saline on a glass microscope slide and placing a cover slip over the stool...
by adding approximately 2 mg of stool to a drop of lugol’s iodine (diluted 1:5 with distilled water) on a glass microscope slide and placing a cover slip on the stool suspension. Similarly, iodine wet mounts were prepared initially by using a low-power (10×) objective and then using a high-power (40×) objective of a compound light microscope. The wet mount was read in approximately 5 min to view at least 100 fields per slide. Each stool sample was screened by at least three well-trained microscopists before reporting negative results in our laboratory.

Stool culture

Stool samples were cultured for Entamoeba species in Locke-egg (LE) medium (NIH modification of boeck and ortbohlav’s medium) within 6 h of collection as previously described [23, 24].

Nested multiplex-polymerase chain reaction (PCR)

Quantification of DNA in stool specimen

Quantification of DNA in spin column purified DNA extract from stool specimens was determined by UV absorbance using a Cintra 5 double beam spectrophotometer. DNA yields were calculated on the basis of UV absorbance × dilution. The purity of the nucleic acid in the samples was estimated by the ratio of readings at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>). The quantification of DNA was done only for representative stool specimens to know the DNA yielding capacity of the CTAB-DNA extraction method and also to estimate the purity of extracted DNA for its suitability to be used in PCR.

Primer design

The genus specific primers were designed using nucleotide sequences of 16S-like rRNA gene of E. dispar, E. histolytica and E. moshkovskii Laredo deposited in GenBank with accession number [GenBank:Z49256], [GenBank:X56929] and [GenBank:AE149806] respectively. The comparison of all the three 16S-like rRNA gene sequences of E. dispar, E. histolytica and E. moshkovskii Laredo revealed significant differences enough to design species specific primers. The primers were designed using Primer 3 on-line software [26]. The primer sequences used for nested multiplex PCR are shown in table 4.

Primer validation

The primer sequences designed for E. moshkovskii, E. histolytica, and E. dispar were subjected to a Basic Local Alignment Search Tool (BLAST) in the genome database of all organisms [27] and were found to be specific for the study.

The amplified PCR products of all the three species in stool samples were confirmed by getting both the strands of DNA sequenced on ABI PRISM 377 sequencer (Indian Institute of Science, Bangalore, India). Briefly, the ABI PRISM 377 DNA sequencer automatically analyzes DNA molecules labeled with multiple fluorescent dyes. After samples are loaded onto the system’s vertical gel, they

### Table 4: Primer sequences used for nested multiplex PCR

<table>
<thead>
<tr>
<th>Genus specific primers (First PCR)</th>
<th>Species specific primers (Second nested multiplex PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entamoeba genus</td>
<td></td>
</tr>
<tr>
<td>E. histolytica species</td>
<td></td>
</tr>
<tr>
<td>E. moshkovskii species</td>
<td></td>
</tr>
<tr>
<td>E. dispar species</td>
<td></td>
</tr>
</tbody>
</table>

E. histolytica species primer sequences used in the PCR reaction:

- **E. histolytica species**: EH-1 5' AAGCATTTGTCTTATGCTGAG 3' (forward primer)
- **E. moshkovskii species**: Mos-1 5' GAAACCAAAGTTTCCACAC 3' (forward primer)
- **E. dispar species**: Dis-1 5' TCAATTGCGTACATCCTGAG 3' (forward primer)
undergo electrophoresis, laser detection, and computer analysis. Electrophoretic separations are viewed on-screen in real-time. Software enables this system to support sequencing and fragment analysis applications. ABI PRISM 377 DNA sequencer can generate readings up to 900 bases per sample with 98.5% accuracy. The sequencing was done using species specific primers of each species. For example, the PCR product of E. histolytica DNA was sequenced using the species specific primer EH-1/EH-2, E. dispar using ED-1/ED-2 and E. moshkovskii using Mos1/Mos-2.

The PCR product from stool samples showing mixed infection by multiplex PCR were sent for sequencing which included stool samples with mixed infection containing all the three species (E. histolytica + E. dispar + E. moshkovskii). Each species DNA was amplified separately with respective species specific primers before sending for sequencing. All sequences were analyzed for homology by using the nucleotide-nucleotide "BLAST" search feature [27]. The identities between the sequencing result of PCR product of E. dispar, E. histolytica and E. moshkovskii with the sequence deposited in GenBank, with accession number [GenBank:Z249256], [GenBank:K535921] and [GenBank:AF110900] respectively, were analyzed by using the "Align two sequences (bl2seq)" feature [27].

Standard strains

Three standard strains used in this study were E. histolytica HM.1.18555, E. dispar SAW760, and E. moshkovskii Laredo. These were used as a positive control in the present study. The lyophilized DNA of these strains was generously gifted by Dr. C. Graham Clark from London School of Hygiene & Tropical Medicine, London, UK.

Nested multiplex PCR protocol

For a reaction volume of 25 μl comprising 2.5 μl of 10X PCR buffer (Biogene), 1.5 μl of 25 mM MgCl₂ (Bangalore genie), 1.4 μl of deoxyribonucleotide triphosphate mix (5 mM each dNTP, Biogene), 0.3 μl (5 μl/μl) of Taq polymerase (Biogene), 0.3 μM of each primer (IDT) and 2.5 μl of template DNA was added in genus specific and species specific PCR. The PCR tubes were finally placed in an eppendorf Thermal cycler [Master cycler gradient].

The conditions for genus specific PCR were as follows

The PCR mix was subjected to an initial denaturation at 94°C for 2 minutes, followed by 30 cycles - each consisting of 92°C for 60 seconds (Denaturation), 56°C for 60 seconds (Annealing), and 72°C for 90 seconds (Extension). Finally one cycle of extension at 72°C for seven minutes was performed. In the species specific nested multiplex PCR (which had multiple primer sets in the same tube), only the annealing temperature was changed to 48°C, leaving the other parameters of the amplification cycles unchanged.

Three micro litres of the amplification products were separated by electrophoresis through 1.8% agarose gel (Agarose Low EEO, Bangalore genie products, Bangalore, India) in 0.5 Tris-borate-EDTA at 120 V for 45 min and were visualized by ethidum bromide staining under UV light for bands of DNA of appropriate sizes (Figure 3). Positive and negative control reactions were included with each batch of samples analyzed by nested multiplex PCR.

Assessment of competition for non-target DNA

To assess the competition of other non-target DNA present in stool samples with target DNA, the nested multiplex PCR was checked with reference DNA (DNA from standard strain of E. histolytica, E. dispar and E. moshkovskii) spiked with DNA from negative control stool samples (negative by microscopy and culture) followed by nested multiplex PCR amplification.

Estimation of minimum number of Entamoeba cells detectable by nested multiplex PCR

To determine the minimum number of Entamoeba cells detectable by nested multiplex PCR, all the three species were studied by Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav's medium) cultures and the amoebae were counted using a standard haemocytometer. A cell pellet containing 10⁶ cells was preferred for determining the detection limit of nested multiplex PCR for each Entamoeba species. The cell pellet containing 10⁵ cells of E. histolytica, E. dispar and E. moshkovskii was diluted ten folds in Phosphate buffer saline (PBS) to obtain different concentrations of cells, such as 10⁵, 10⁴, 10³, 10² and 10 cells. The different quantities of cells ranging from 10⁵ to 10⁰ cells were added to 0.05 gm of faeces (negative control stool samples) followed by DNA extraction and PCR as per the aforementioned protocol.

Estimation of nested multiplex PCR to detect mixed infections with E. histolytica, E. dispar and E. moshkovskii species

A variable number of lysed E. histolytica cells (ranging from 1000 cells to 0.001 cell) were mixed with a constant number (that is 1000 cells) of each of the lysed E. dispar and E. moshkovskii cells: a variable number of lysed E. dispar cells (ranging from 1000 cells to 0.001 cell) were mixed with a constant number (that is 1000 cells) of each of the lysed E. histolytica and E. moshkovskii cells; a variable number of lysed E. moshkovskii cells (ranging from 1000 cells to 0.001 cell) were mixed with a constant number (that is 1000 cells) of each of the lysed E. histolytica and E. dispar cells, followed by DNA extraction and PCR as per the aforementioned protocol.
Cross checking the results of nested multiplex PCR
Some of the representative stool samples showing mixed infection by nested multiplex PCR were selected randomly and subjected to species specific individual nested PCR, with species specific primers for each species, in separate PCR tubes.

**TechLab E. histolytica II ELISA test**
The TechLab E. histolytica II ELISA test was performed to detect E. histolytica coproantigen in 45 randomly selected stool samples, positive for E. histolytica/E. dispar/E. moshkovskii complex, by microscopy and/or culture, and also positive for E. histolytica, E. dispar and E. moshkovskii DNA by nested multiplex PCR. The TechLab E. histolytica II ELISA test was performed as per the instructions of the manufacturer. The kit was generously given for the purpose of research by TechLab, Inc. (Blacksburg, Va.).

**Authors' contributions**
AK carried out the experimental works, and drafted the manuscript. SC supervised and coordinated the study, and helped in the completion of the manuscript.

**Acknowledgements**
We sincerely thank Dr. C. Graham Clark from London School of Hygiene & Tropical Medicine for providing us with hypoxilized DNA of standard cultures of E. histolytica HM-1:IMSS, E. dispar SAW760, and E. moshkovskii "A" strain. We are also grateful to TechLab, Inc. (Blacksburg, Va, USA) for providing the TechLab E. histolytica II ELISA kit free of cost for the purpose of research.

**References**

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:
- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright
Common disease, simple test

Reliable diagnosis of amoebic liver abscess (ALA) — a common parasitic infection — requires extracting blood or pus from the abscess, an invasive procedure. Now, microbiologist Subhash Parija at the Jawaharlal Institute of Postgraduate Medical Education and Research (Jipmer), Pondicherry, has shown for the first time that *Entamoeba hystolytica* — the parasite that causes the disease — may also be detected through a simple urine test. Parija and his colleague, Krishna Khairnar, have demonstrated that the genetic material of this parasite passes through the human kidney and is excreted in urine. Doctors estimate that amoebiasis annually causes illness in some 34 to 50 million people worldwide and leads to 40,000 to 100,000 deaths, mainly from ALA. The Jipmer study, published in the journal *BMC Microbiology*, may pave the way for a new way to diagnose ALA. Monitoring the excretion of the parasite's DNA in urine may also provide a way to track the response of a patient who has been given metronidazole, the standard treatment for the infection.