
Downloaded from: http://researchonline.lshtm.ac.uk/17191/

DOI:
Entamoeba moshkovskii Infections in Children in Bangladesh

Ibne Karim M. Ali,*†1 Mohammad Bakhtiar Hossain,†1 Shantanu Roy,† Patrick F. Ayeh-Kumi,‡ William A. Petri, Jr.,§ Rashidul Haque,† and C. Graham Clark*

Entamoeba moshkovskii cysts are morphologically indistinguishable from those of the disease-causing species E. histolytica and the nonpathogenic E. dispar. Although sporadic cases of human infection with E. moshkovskii have been reported, the organism is considered primarily a free-living amoeba. No simple molecular detection tool is available for diagnosing E. moshkovskii infections. We used polymerase chain reaction (PCR) to detect E. moshkovskii directly in stool. We tested 109 stool specimens from preschool children in Bangladesh by PCR; 17 were positive for E. histolytica (15.6%) and 39 were positive for E. dispar (35.8%). In addition, we found that 23 (21.1%) were positive for E. moshkovskii infection, and 17 (73.9%) of these also carried E. histolytica or E. dispar. The high association of E. moshkovskii with E. histolytica and E. dispar may have obscured its identification in previous studies. The high prevalence found in this study suggests that humans may be a true host for this amoeba.

Entamoeba moshkovskii, considered to be primarily a free-living amoeba, is indistinguishable in its cyst and trophozoite forms from E. histolytica (the cause of invasive amebiasis) and E. dispar (a common noninvasive parasite), except in cases of invasive disease when E. histolytica trophozoites may contain ingested red blood cells. E. moshkovskii has so far rarely been shown to infect humans; however, the organism appears to be ubiquitous in anoxic sediments. Although the early isolations of this species were from sewage, E. moshkovskii can also be found in environments ranging from clean riverine sediments to brackish coastal pools (1). E. moshkovskii is osmotolerant, can be cultured at room temperature, and is resistant to emetine, all characteristics that distinguish it from E. histolytica and E. dispar (2–5). Human isolates of E. moshkovskii to date have come from North America, Italy, South Africa, and Bangladesh, and they have never been associated with disease (5,6). However, few studies have actually set out to identify such infections (7).

The structural resemblance of the apparently innocuous E. moshkovskii to the disease-causing E. histolytica makes differentiating the two species important. In the clinical setting, for example, an E. moshkovskii–infected patient could be diagnosed as infected with E. histolytica and be treated unnecessarily with antiamebic chemotherapy. Most studies that have investigated the prevalence of E. histolytica and E. dispar have not considered the possible presence of E. moshkovskii, partly because of a lack of tools to detect E. moshkovskii other than cultivation, which is labor-intensive, not always successful, and problematic in the case of mixed infections. We report for the first time the application of tools to detect the species directly in stool and investigate the prevalence of E. moshkovskii in humans, a group of children in an E. histolytica– and E. dispar–endemic area where the first human infection with E. moshkovskii from Bangladesh was detected (6).

Materials and Methods

Stool Specimens

Fecal specimens included in this study were from 109 preschool children ages 2–5 years from Mirpur, an urban slum in Dhaka, Bangladesh. Based on results of polymerase chain reaction (PCR) on stool DNA samples, 39 were E. dispar–positive, 17 were E. histolytica–positive, and 1 was positive for both E. histolytica and E. dispar. Of the 52 samples negative by stool PCR, 18 were eventually found positive for E. histolytica, E. dispar, or both, either by PCR from culture DNA or by antigen detection tests performed on stool specimens, and the remaining 34 samples were negative by all methods. Only four of the samples were from children with diarrhea.

Cell Culture and Isoenzyme Analysis

All stool samples were cultured for Entamoeba species in Robinson’s medium (8) within 6 hours of collection, and hexokinase isoenzyme analysis was performed when possible as previously described (9). E. moshkovskii strains Laredo and FIC were maintained axenically in LYI-S-2
medium (10) with 10% adult bovine serum. Laredo (ATCC 30042) is a human isolate, and FIC (ATCC 30041) is an environmental isolate. *E. histolytica* HM-1:IMSS clone 9 (ATCC 50528) and *E. dispar* SAW760 (ATCC 50484) were used as controls.

**Antigen Detection Tests for *E. histolytica* and *E. dispar***

The TECHLAB, Inc. (Blacksburg, VA) *Entamoeba* test (designed to detect but not differentiate *E. histolytica* and *E. dispar* antigen in stool specimens) and *E. histolytica* test (designed to detect specifically *E. histolytica* in stool specimens) were performed on stool specimens according to the manufacturer’s instructions (9).

**Preparation of DNA**

Stool DNA was isolated by using a modified version of the silica-DNA binding method of Katzwinkel-Wladarsch et al. as previously described (11,12). Culture DNA was isolated by a cetyltrimethylammonium bromide (CTAB) extraction method as previously described (13), dissolved in 10 mM Tris-Cl (pH 8.5), and passed over a Microspin S-200 HR column (Amersham Biosciences UK Ltd, Chalfont St. Giles, England). RNA was removed by the addition of RNase A (Promega UK, Ltd, Southampton, England) to 0.05 µg mL⁻¹.

**Small Subunit rRNA Gene Amplification**

Based on the sequences of the small subunit rRNA genes (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, as described (11), to detect *E. histolytica* and *E. dispar* in stool specimens (Table 1). 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 designed (unpub. data) and used to detect *E. moshkovskii* in stool DNA (Table 1). In the initial PCR (total vol. 25 µL), 1.0 µL of stool or culture DNA was used. Thermal cycler conditions included 30 cycles, each consisting of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension of 5 min at 72°C. The ArgTCT amplification products from *E. moshkovskii* Laredo, *E. moshkovskii* MS15-3646 (one of the infections detected above), and *E. dispar* SAW760 were cloned into the pGEM-T Easy vector (Promega) and sequenced (MWG Biotech Ltd, Milton Keynes, England). From the sequence results, an *E. moshkovskii*–specific primer pair, EmR-1 and EmR-2, was designed to amplify the *E. moshkovskii* ArgTCT gene fragment specifically (Table 1). PCR amplification was performed at an annealing temperature of 58°C as described for ArgTCT gene amplification.

**Results**

**Culture and Isoenzyme Analysis**

All 109 stool specimens were added to Robinson’s medium for growth of *Entamoeba* species. Incubation led to growth of *E. histolytica*/*E. dispar*/*E. moshkovskii* in 33 cultures and *E. coli* in 8 cultures (no growth of *E. hartmanni* or *Endolimax nana* was observed). Hexokinase isoenzyme analysis was possible for 10 cultures; 4 of them showed the band pattern of *Entamoeba histolytica*, 5 showed *E. dispar*, and 1 showed the band pattern of *E. dispers* with an extra band just behind the faster moving band, perhaps indicating a mixed culture with *E. moshkovskii*.

**Detection of *E. moshkovskii* by Nested PCR**

The reference strain *E. moshkovskii* Laredo gave the expected band at approximately 260 bp with the *E. moshkovskii*–specific SSU-rDNA nested primers, whereas
control *E. histolytica* HM-1:IMSS and *E. dispar* SAW760 DNAs were negative. Twenty-three of 109 (21%) stool DNA samples were positive by nested PCR for *E. moshkovskii* (Table 2). Of these, seven were positive for amoebae by culture; one DNA sample extracted from these cultures was positive for *E. moshkovskii*. Seventeen of the 23 *E. moshkovskii*–positive samples were also positive for *E. histolytica, E. dispar*, or both, by either PCR of stool SSU rDNA (13/17) or by TECHLAB Entamoeba or *E. histolytica* tests (15/17) (Figure 1). One of the four children with diarrhea was positive for *E. moshkovskii* and coinfect-ed with *E. dispar*. The cause of his diarrhea remained undetermined.

A comparison of SSU-rDNA sequences from *E. moshkovskii, E. histolytica*, and *E. dispar*, showed that the restriction endonuclease XhoI cuts exclusively in the *E. moshkovskii*–specific, 258-bp–nested PCR product to produce 236-bp and 22-bp fragments. Products from all 23 positive stool samples and the Laredo strain showed the presence of this site (Figure 1).

### Table 2. Nested SSU rDNA polymerase chain reaction (PCR) (for *Entamoeba histolytica, E. dispers*, or both) and stool antigen-detection test results of the 17 *E. moshkovskii*–positive samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Stool antigen-detection test results</th>
<th>SSU rRNA gene PCR for <em>E. histolytica/E. dispar</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;3&lt;/sup&gt;</td>
<td><em>E. histolytica</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>2</td>
<td><em>E. dispar</em></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>4</td>
<td><em>E. histolytica</em></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>6&lt;sup&gt;3&lt;/sup&gt;</td>
<td><em>E. dispar</em></td>
<td>Mixed</td>
</tr>
<tr>
<td>7</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>8</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>9</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>11</td>
<td><em>E. dispar</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>12</td>
<td><em>E. dispar</em></td>
<td>0</td>
</tr>
<tr>
<td>13&lt;sup&gt;3&lt;/sup&gt;</td>
<td><em>E. dispar</em></td>
<td><em>E. histolytica</em></td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td><em>E. dispar</em></td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td><em>E. histolytica</em></td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>3</sup>NC, no culture; 0, negative. All stool antigen tests that are positive for *E. histolytica* can also be used because no specific *E. dispar* antigen test exists.

<sup>3</sup>Patients 1 and 6 likely had mixed infections with *E. histolytica* and *E. dispar*, in which *E. histolytica* was much lower in number than *E. dispar* in the stool specimen. For patient 1, SSU rDNA PCR failed to detect *E. histolytica*, though both species grew in the culture. For patient 6, although SSU rDNA PCR could detect *E. histolytica* in stool DNA, the *E. histolytica* antigen-detection test failed to detect *E. histolytica*, and only *E. dispar* survived in the culture.

<sup>3</sup>The stool specimen of patient 13 was marginally negative by the *E. histolytica* antigen-detection test (optical density value was 0.13 where the cut-off value for a positive result was 0.15).

![Figure 1. *Entamoeba moshkovskii*–specific nested SSU rDNA polymerase chain reaction (PCR) products. Odd- and even-numbered lanes represent undigested and XhoI-digested PCR products, respectively. Lanes 1/2, *E. moshkovskii* Laredo; lanes 3/4–5/6, DNA from stool samples. M, a 50-bp DNA ladder (Invitrogen Corp.).](image)

**Figure 1.** *Entamoeba moshkovskii*–specific nested SSU rDNA polymerase chain reaction (PCR) products. Odd- and even-numbered lanes represent undigested and XhoI-digested PCR products, respectively. Lanes 1/2, *E. moshkovskii* Laredo; lanes 3/4–5/6, DNA from stool samples. M, a 50-bp DNA ladder (Invitrogen Corp.).
contains no short tandem repeat sequences (Figure 2C).

The EmR primers amplified the expected 265-bp fragment from E. moshkovskii Laredo DNA and did not amplify E. histolytica HM-1:IMSS or E. dispar SAW760 DNA. However, they successfully amplified 10 of a possible 23 E. moshkovskii–positive stool DNA samples. The most likely reason why these primers did not amplify the other 13 E. moshkovskii DNA samples is that they differed in sequence in the primer-binding regions. Although the PCR product size of the 10 positive samples was slightly different from that of Laredo, they were very similar in size to each other (Figure 3). The DNA of the previously reported E. moshkovskii ICDDRB:717, isolated from humans in the same geographic location (6), also gave a product of the same size (Figure 3, lane 2). The EmR primers successfully amplified DNA from environmental E. moshkovskii isolate FIC, but its product size was quite different from that of the human isolates of E. moshkovskii (Figure 3, lane 7).

Discussion

The main objectives of this study were to develop molecular tools to identify E. moshkovskii and to investigate its prevalence and diversity in humans. We were successful in developing a simple diagnostic technique: a nested SSU-rDNA PCR followed by restriction endonuclease digestion. We chose to use nested PCR to detect E. moshkovskii infections because our previous experience in this area showed that nested PCR was much more efficient in amplifying stool DNA (14). Our attempt to produce a species-specific polymorphic marker was not completely successful. The EmR primers failed to amplify 13 of 23 E. moshkovskii–containing samples, probably because of sequence differences in primer-binding sites. However, the ArgTCT primers, originally designed to amplify E. histolytica and E. dispar DNA, did amplify most of the E. moshkovskii samples, producing a product distinct in size from those of E. histolytica and E. dispar.

Our study has some limitations. The subjects were children 2–5 years of age, so we do not know whether these subjects are representative of all age groups. All previous human isolates of E. moshkovskii have belonged to ribodeme 2 (5). Our attempts to perform riboprinting on these infections were unsuccessful, likely because of the size of the amplification target (approximately 1.95 kb). Even if PCR had been successful, the presence of mixed infections with other eukaryotes would have prevented successful typing.

This study has several important findings. The overall E. moshkovskii prevalence (21%) suggests that this infection is common among these children. E. dispar–infected children were almost twice as likely to have a mixed infection with E. moshkovskii (35%) compared to those with (18%) or without E. histolytica (18%) infections. None of

Figure 2. ArgTCT locus. ArgTCT sequences from Entamoeba histolytica HM-1:IMSS (GenBank accession no. A2535059), E. dispar SAW760 (GenBank accession no. AF525284), E. moshkovskii Laredo (GenBank accession no. AF 525285), and MS15-3646 (GenBank accession no. AF525286) were aligned at the 5´(A) and 3´ (B) ends to design E. moshkovskii–specific primers. The EmR primer sequences are shown in italic and bold with E. moshkovskii–specific positions underlined. C. Schematic representation of ArgTCT loci from E. histolytica HM-1:IMSS, E. dispar SAW760, and E. moshkovskii. Locations of the primers used in polymerase chain reaction amplification are indicated by small arrows, the RNA genes are indicated by large arrows, and the short tandem repeats by shaded boxes (not to scale.)
the six children with *E. moshkovskii* monoinfections had diarrhea or dysentery, which suggests that *E. moshkovskii* is a noninvasive parasite. The high prevalence of *E. moshkovskii* infection may have been unnoticed over the years because most such infections (74%) were mixed infections with *E. histolytica*, *E. dispar*, or both. Previous attempts to identify human *E. moshkovskii* infections (7) may have failed because the human intestinal flora was unsuitable for cultivation at room temperature.

The high prevalence of *E. moshkovskii* shown in this study population indicates that perhaps humans are a true host for this putatively free-living amoeba and are not just transiently infected. This prevalence may also explain some of the microscopy-positive/antigen-negative results obtained when using the *Entamoeba* test kit (15). Epidemiologic studies of *E. histolytica* infection should include tools to diagnose all three of these species individually, simultaneously, and accurately, and the prevalence of *E. moshkovskii* infection in other regions of the world should be investigated.

### Acknowledgments

The International Centre for Diarrheal Disease Research, Dhaka, Bangladesh, acknowledges with gratitude the commitment of University of Virginia to the Centre’s research efforts. Mr. Ali is funded by the Commonwealth Scholarship Commission.

This research was supported in part by a grant received by International Centre for Diarrheal Disease Research, Dhaka, Bangladesh, Centre for Health and Population Research, with the support of University of Virginia (NIH grant AI-43596) and by grant 067314 from the Wellcome Trust awarded to C.G. Clark.

Mr. Ali is a senior research officer at the International Centre for Diarrheal Disease Research, Dhaka, Bangladesh, on leave and studying for his doctorate in the Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine. His current research focus includes genetic diversity in *Entamoeba* species.

### References


Address for correspondence: C. Graham Clark, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, England; fax: +44-207-636-8739; email: graham.clark@lshtm.ac.uk