HIGH PREVALENCE RATE OF ENTAMOEBA HISTOLYTICA ASYMPTOMATIC INFECTION IN A RURAL MEXICAN COMMUNITY

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Abstract. The frequency of Entamoeba histolytica and Entamoeba dispar infection was analyzed in a rural community in the state of Morelos, Mexico, using polymerase chain reaction (PCR). Sociodemographic variables as risk factors for the infection were assessed. Results highlighted the number of individuals with intestinal parasites (43.1%) in the community, indicating extensive fecalism. A high frequency of E. histolytica asymptomatic infection, higher than E. dispar infection (13.8% versus 9.6%), was detected by PCR. Anti-amebic antibody levels (IgG) in serum and saliva (IgA) samples were not associated with E. histolytica intestinal infection. These findings suggest a predominant distribution of E. histolytica strains of low invasive potential in this community.

INTRODUCTION

Mexico is considered an endemic country for amebic infections. Recent seroepidemiological surveys showed a seroprevalence rate of 8.4%. After defining two important Entamoeba species, Entamoeba histolytica and Entamoeba dispar, it is important to reassess the epidemiologic data on intestinal infection and invasive disease in Mexico. Both species behave differently in infected hosts, even though they share biological cycles, ecological niches, and transmission mechanisms in human hosts. E. histolytica is pathogenic and often the cause of intestinal or extraintestinal invasive disease; E. dispar is a nonpathogenic species. To date, there is no evidence of tissue lesions attributed to E. dispar in human hosts. However, E. histolytica can also infect humans more frequently than expected, causing no symptoms suggestive of tissue damage. We therefore conducted a field study in the state of Morelos, 200 km south of Mexico City, to determine the frequency of E. histolytica and E. dispar in Coahuixtla, a rural community with a population of 903. We also studied the sociodemographic conditions associated with infection and the frequency of association of anti-amebic antibody response.

MATERIALS AND METHODS

Study protocol. A descriptive transversal study was conducted from September to October 2000. The rural community of Coahuixtla is located at the southern border of the state of Morelos. The geographic characteristics and the weather correspond to a tropical valley, with 28–32°C temperature during the spring and summer and 20–26°C during the months of autumn and winter. The rainy season begins in late May and usually ends in late September. Sociodemographic conditions were assessed in each family with emphasis placed on housing characteristics, access to drinking water, waste disposal, and hygienic habits. A history of diarrheal episodes, associated with E. histolytica infection or amebic liver abscess 6 months prior to the study, was also assessed.

Individuals. Every family was interviewed by a member of the research team and were explained the purpose of the study, the number and types of samples required (blood, saliva, and feces), and the procedure for taking and transporting the samples to the health center in town. Informed consent was granted by 290 individuals that accepted to participate in the study. The protocol was previously approved by the Ethics Committee for Human Studies of the Faculty of Medicine at the National Autonomous University of Mexico, and by the Ethics Committee at the Hospital 1° de Octubre, ISSSTE, in Mexico City.

Samples. Three stool samples from consecutive days were obtained from each individual and kept refrigerated (4°C) and untreated until used. Saliva was stored in 5.0 mL vials with screw caps (Costar, Cambridge, MA) without induction and kept frozen at −20°C until used for anti-E. histolytica antibody detection. Ten-milliliter venous blood samples were drawn using a Vacutainer device (Becton Dickinson de México, Mexico D.F.), and after storing at room temperature (RT), the samples were centrifuged (200 × g, 10 minutes, RT). The serum was separated and placed in other vials and kept frozen at −20°C until used.

Microscopic examination of stool samples. The presence of parasites was detected by microscopic examination of fresh stool samples stained with iodine solution (4%) and thereafter using a flotation technique in a zinc-sulfate gradient (d = 1.192). Briefly, stool specimens were homogenized in 0.15 M NaCl solution and passed through gauze to discard larger detritus; the homogenate was centrifuged at 600 × g for 1 minute at RT. The pellet was suspended in the zinc-sulfate gradient solution and centrifuged again at 600 × g. The flotation disk was separated using a Pasteur pipette and washed with 0.15 M NaCl solutions at 600 × g for 1 minute at RT until the supernatant was clear. A sample of the obtained pellet was used for microscopic observation in 4% iodine solution.

Molecular characterization of E. histolytica and E. dispar species. DNA was extracted from cysts found in stool samples submitted to the zinc-sulfate gradient flotation tech-
Cysts were transferred to a 2 mL Eppendorf tube, washed 4 times with 0.15 M NaCl, and resuspended in 300 μL of lysis buffer (100 mM EDTA pH 8, 0.25% SDS). The tubes were subjected to three freezing cycles in ethanol–dry ice and thawed in a 37°C water bath. Afterwards, 3 μL of 20 mg/mL of proteinase K were added. The sample was incubated for 1 hour at 55°C. After digestion with proteinase K, lysates were brought to 0.7 M NaCl and 1% CTAB (Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 65°C for 30 minutes, and the samples were then extracted with chloroform, phenol/chloroform, and chloroform followed by the precipitation of DNA with ethanol. DNA was suspended in 50 μL water and passed over a Sephadex G-25 spin column (Pharmacia Biotech, Uppsala, Sweden).

The DNA obtained was used for amplification by polymerase chain reaction (PCR) in a 20 μL reaction mixture with the following primers: RD (sense: ATC TGG TTG ATC CTG CCA GT; antisense: ATC CTT CCG CAG GTT CAC CT); PsP (sense: GGC CAA TTC ATT CAA TGA ATT GAG; antisense: CTC AGA TCT AGA AAC AAT GCT TCT C); NPsp (sense: GGC CAA TTC ATT AGT TAA GTA AAT TGA G; antisense: CCT GGA TTT ATG TAA GTA AAT GAG; antisense: AAC AAT GAA TGA TCT AGT TGA G); nSRPEh (sense: TGA AGA TAA TGA AGA TGA AGA TG; antisense: TAT TAT TAT CGT TAT CTG AAC TAC TCT CGT); and SRPEd (sense: GTA GTT CAT CAA ACA CAG GTG A; antisense: CAA TAG CCA TAA TGA AGA CAA).

RD primers are unspecific and amplify a segment of the small rRNA gene subunit consisting of 1950 base pairs (bp) in E. histolytica and E. dispar.10,11 PsP primers are specific for E. histolytica, and NPsp primers are specific for E. dispar.10,11 In both cases, it generated an 876-bp fragment that is part of the RD amplicon. In these three cases, the conditions of the PCR were 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2 mM MgCl₂, 0.2 mM of each nucleotide, 0.025U (Ampli Taq Gold, Applied Biosystems, Foster City, CA) and 1 μM of each primer. Thirty-five cycles at 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 2 minutes followed an initial 10-minute step at 95°C. A final step of 8 minutes at 72°C was included. For amplification with RD primers, 2 μL of DNA sample was used as a template and 1 μL of the product obtained diluted 1:10 was used as a template for amplifications with the PsP and NPsp primers.

The SRPEd5′/3′ primers (designed by the authors) are specific for E. histolytica and generate a fragment of 553 bp in the HM1:IMSS strain. From this amplification, the nSRPEd5′/3′ primers generated a fragment of 452 bp.12 In both cases, the PCR conditions were 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2.5 mM MgCl₂, 0.2 mM each nucleotide, 0.025U (Ampli Taq Gold, Applied Biosystems), and 1 μM of each primer. Thirty-five cycles at 94°C for 1 minute, 67°C for 1.5 minutes, and 72°C for 2 minutes followed an initial step of 10 minutes at 95°C. A final step at 72°C for 8 minutes was included. For the amplification with SRPEd primers, 2 μL of the DNA sample was used as a template, and 1 μL of the obtained product diluted at 1:10 was used as a template for the amplifications with the nSRPEd primers.

The SRPEh5′/3′ primers (designed by the authors) are specific for E. dispar and generate a fragment of 567 bp in the SAW760 RR strain clone A. The conditions of the PCR were the same as for SRPEd, with the exception of the annealing temperature, which in this case was 64°C. When the results were unclear, a reamplification was done using 1 μL of the amplified product, the same primers, and under the same conditions.

The PCR products were visualized using electrophoresis in 1.5–2% agarose gels in a TBE buffer stained with ethidium bromide and photographed for its later analysis.

**Anti-amebic antibodies in serum and saliva samples.** Anti-E. histolytica antibody detection was performed using the enzyme-linked immunosorbent assay (ELISA) technique and the E. histolytica antigen from a 72-hour axenic culture as previously reported.13 Serum samples were diluted 1:1000 in PBS-BSA-Tw. Fifty microliters per well were incubated for 12 hours at 4°C and submitted to mild agitation. Due to IgA concentration variations related to the circadian cycle,14 total IgA was titered in each saliva sample and then adjusted to 50 μg/mL. Fifty microliters per well of the saliva dilution was incubated for 12 hours at 4°C.

**Statistical analysis.** Data collected during the study were computed, coded, and analyzed using SPSS 10.0 version software (SPSS, Chicago, IL). The χ² test and Fisher’s exact test were used for categorical variables for comparing proportions. In all instances, P < 0.05 was considered to be statistically significant. Marginal odds ratio (ORs) and 95% confidence intervals (95% CIs) were used to measure the strength of association between the risk factors and the intestinal parasitic infection, as well as the presence of high levels of IgA or IgG anti-amebic antibodies and E. histolytica infection.15

**RESULTS**

**Population characteristics.** Coahuixtla is a rural community with a population of 903. The age and gender distributions are shown in Figure 1. Individuals under 25 years of age compose the highest density population. The male to female ratio was 1:1.08. Drinking water is available, however sewage services are not. Roads are unpaved and housing is very heterogeneous, ranging from scarce well-constructed homes to wood and grass built houses. Only a small number of houses have running water, and latrines are often used. A significant number of houses do not have fecal disposal sewage.

**Sociodemographic variables associated with intestinal parasite infection.** The sociodemographic variable analysis of the 290 individuals participating in this study highlighted statistically significant differences between parasitized and nonparasitized individuals. These variables are shown in Table 1. In
intestinal parasitic infection was observed preferentially in individuals between 1 to 14 years old.

The evaluation of parasite infections in the community demonstrated that the population is highly infected (Table 2, A). The parasites more frequently observed were protozoa, some of them pathogenic for humans, and others related to fecalism.

The prevalence of *E. histolytica/E. dispar* detected by microscopic examination was 12.8%. A high number of multiple infections were observed, and the most commonly *E. histolytica/E. dispar* associated parasites were *Entamoeba coli* (75.7%) and *Endolimax nana* (56.8%) (Table 2, B).

**Prevalence of *E. histolytica* and *E. dispar* species as detected by PCR.** The *E. histolytica* species was detected by PCR analysis in 2 of 37 *E. histolytica/E. dispar* microscopically positive stool samples (5.4%). *E. dispar* species was observed in 18 samples (48.6%). Both species of *Entamoeba* were detected in 6 stool samples (16.2%). However, 7 of these 37 *E. histolytica/E. dispar* microscopically positive samples were infected with intestinal protozoa different from *E. histolytica* or *E. dispar*. Also, 4 microscopically *E. histolytica/E. dispar* positive samples were negative in the PCR analysis (Table 3).

Eighty-eight samples microscopically infected with intestinal protozoa different from the *E. histolytica/E. dispar* complex were detected as shown in the PCR analysis as follows:

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Positive samples</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Entamoeba coli</em></td>
<td>78</td>
<td>26.9</td>
</tr>
<tr>
<td><em>Endolimax nana</em></td>
<td>67</td>
<td>23.1</td>
</tr>
<tr>
<td><em>Entamoeba histolytica/Entamoeba dispar</em></td>
<td>37</td>
<td>12.8</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>29</td>
<td>10.0</td>
</tr>
<tr>
<td><em>Hymenolepis sp.</em></td>
<td>14</td>
<td>4.8</td>
</tr>
<tr>
<td><em>Iodamoeba bütschlii</em></td>
<td>9</td>
<td>3.1</td>
</tr>
<tr>
<td><em>Chilomastix mesnili</em></td>
<td>4</td>
<td>1.4</td>
</tr>
<tr>
<td><em>Ascaris sp.</em></td>
<td>2</td>
<td>0.7</td>
</tr>
<tr>
<td><em>Enteromonas hominis</em></td>
<td>1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**TABLE 2**
Prevalence rate of intestinal parasitic infection (A) and prevalence rate of intestinal parasites associated with *Entamoeba histolytica/ Entamoeba dispar* infection (B)

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Associated parasite</th>
<th>Positive samples</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Entamoeba coli</em></td>
<td></td>
<td>28</td>
<td>75.7</td>
</tr>
<tr>
<td><em>Endolimax nana</em></td>
<td></td>
<td>21</td>
<td>56.8</td>
</tr>
<tr>
<td><em>Iodamoeba bütschlii</em></td>
<td></td>
<td>7</td>
<td>18.9</td>
</tr>
<tr>
<td><em>Hymenolepis sp.</em></td>
<td></td>
<td>4</td>
<td>10.8</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td></td>
<td>4</td>
<td>10.8</td>
</tr>
</tbody>
</table>

* Values reflect the frequency of association between anti-*Entamoeba* antibody levels over a cutoff point and *E. histolytica* intestinal colonization. The cutoff point was defined with the mean of OD values measured at 490 nm plus 2 standard deviations obtained by enzyme-linked immunosorbent assay (ELISA) of saliva (IgA) and serum (IgG) samples of non-parasitized individuals. The cutoff point for IgG was 0.435 and for IgA 0.474. Differences between groups were not statistically significant (P = 0.28 for IgA and P = 0.52 for IgG) (χ² test).

**Associated with levels of anti-amebic antibody and *Entamoeba* infection**

<table>
<thead>
<tr>
<th>Parasite</th>
<th>ELISA positive samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgA (N = 212)</td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td>0.0% (0/27)</td>
</tr>
<tr>
<td><em>E. dispar</em></td>
<td>11.2% (2/18)</td>
</tr>
<tr>
<td>Non-<em>E. histolytica</em> or <em>E. dispers</em></td>
<td>4.0% (4/101)</td>
</tr>
<tr>
<td>Nonparasitized</td>
<td>7.6% (5/66)</td>
</tr>
</tbody>
</table>

* Values reflect the frequency of association between anti-*Entamoeba* antibody levels over a cutoff point and *E. histolytica* intestinal colonization. The cutoff point was defined with the mean of OD values measured at 490 nm plus 2 standard deviations obtained by enzyme-linked immunosorbent assay (ELISA) of saliva (IgA) and serum (IgG) samples of non-parasitized individuals. The cutoff point for IgG was 0.435 and for IgA 0.474. Differences between groups were not statistically significant (P = 0.28 for IgA and P = 0.52 for IgG) (χ² test).
point, while 5% (4/80) were positive for anti-ameba IgG antibodies in serum samples.

In nonparasitized individuals, 7.5% (5/66) of the saliva samples had anti-amebic IgAs antibody levels over the cutoff point, and 4.1% (2/49) of the serum samples were positive for anti-amebic IgG antibodies.

The analysis of association between anti-amebic antibody response and the *E. histolytica* intestinal infection in this community demonstrated that anti-amebic IgA antibody levels or serum anti-amebic IgG levels do not correlate with the *E. histolytica* infectious condition \((P = 0.28\) for IgAs and \(P = 0.52\) for IgG) \((\chi^2\) test\) (Table 4).

**DISCUSSION**

*E. histolytica* infection is still a serious public health problem in Mexico. The incidence rate for intestinal amebiasis and amebic liver abscesses during 2000 were 1,353.43 and 6.37 per 100,000 inhabitants, respectively.16 Data from the state of Morelos during the same period showed 2,210.81 cases of intestinal amebiasis and 3.02 cases of amebic liver abscesses per 100,000 inhabitants.16 The sociodemographic conditions and the hygienic behavior of the general population clearly favor the transmission of intestinal parasites. The lack of drinking water and the crowded population index were responsible for the multiple parasitism observed, including *E. histolytica* and *E. dispar* intestinal infection (Table 1).

Even though in our design we did not consider the follow-up of included individuals, the absence of intestinal amebiasis cases in the studied population 6 months prior to and during the 3 months of our stay in town was unexpected, considering the high prevalence of parasitism in the community, as well as the predominance of *E. histolytica* and mixed (Eh + Ed) infections over *E. dispar* infections (Table 3).

This is in agreement with the epidemiologic assertion before the characterization of *E. histolytica* and *E. dispar* species in the 1990s, that 90% of *E. histolytica*-infected individuals are asymptomatic cyst passers.17

The pathogenic characteristic of several human *E. histolytica* isolates studied at different laboratories and tested in vitro and in vivo for their pathogenetic capacity showed differences that can be associated with variations in the virulence potential between strains.18,19 The advancements in molecular biology and genetics, now applied to amebic research, allow for a more precise search of intraspecies diversity markers associated with intestinal or extraintestinal invasive capacity of this protozoan in humans. Our group and others are making efforts in that direction.

The second important point is the study of anti-amebic secretory and systemic antibody response to the invasive mechanisms displayed by the *E. histolytica* species.

In our study, the anti-ameba antibody response in a population of *E. histolytica* asymptomatic cyst passers, without any clinical sign of sickness while we were there, compared with noninfected and infected individuals with other parasites different to *E. histolytica* species, demonstrated that there is no association between the presence of high levels of secretory or circulating anti-amebic antibodies and the presence of *E. histolytica* (sensu stricto) intestinal infection (Table 4). These results agree with our previous results in a HIV+/AIDS cohort study conducted in Mexico (Morán P and others, unpublished data), and with results obtained in a Brazilian population.20 However, there are previous reports where the presence of high levels of circulating IgG anti-amebic antibodies are clearly coincident with the *E. histolytica* species intestinal infection.21,22 In reference to saliva anti-amebic IgAs antibodies, there are reports where the presence of high levels of secretory anti-amebic IgA seems to correlate with the presence of *E. histolytica/E. dispar* complex detection in microscopically analyzed stool samples.23,24

The important question to be solved is if the host secretory and/or systemic antibody response is related to resistance against colonization or to invasive mechanisms displayed by the *E. histolytica* species. Haque and others observed that the presence of anti-galNac inhibitable lectin antibodies seems to protect Bangladeshi children from *E. histolytica* infection and reinfection.25 Differences between results previously reported and our data may be a consequence of the type of population studied or the *E. histolytica* antigen used in the antibody detection. Without discarding differences in the pathogenic behavior of prevalent strains of *E. histolytica* in specific geographic areas. In the studied Mexican population, the pathogenic capability of prevalent strains seems to be low. However, further studies on human *E. histolytica* infection and disease in different geographic endemic areas are required, particularly to obtain relevant genetic markers that may be used for the detection of particular invasive strains of *E. histolytica*.

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