

Brief Original Article

Identification of *Entamoeba histolytica* and *E. dispar* infection in Maceió, Alagoas State, northeast Brazil

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Abstract

Introduction: A cross-sectional study was carried out to assess the prevalence of *E. histolytica* and *E. dispar* by examining stool samples obtained from 1,003 students of public schools in Maceió, Alagoas, Brazil.

Methodology: All stool samples were processed using the spontaneous sedimentation technique and examined microscopically for the presence of *Entamoeba* species. In order to distinguish infections caused by *E. histolytica*, fecal samples presenting cysts of *Entamoeba* were subjected to specific enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

Results: The analysis of the fecal specimens by microscopy identified 6.4% (64/1,003) students positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* cysts. The prevalence of *E. histolytica* detected by ELISA was 3.0% (30/1,003) and by PCR 2.8% (28/1,003), but the difference is not statistically significant ($p > 0.05$). The prevalence of *E. dispar* in schoolchildren was 5.0% (50/1,003). Mixed infections with *E. histolytica* and *E. dispar* were also detected by PCR. Even though immunological and molecular methods have shown similar results for identification of *E. histolytica*, ELISA is advantageous over the PCR since it is relatively cheaper and easier to perform.

Conclusions: Our study demonstrated the occurrence of *E. histolytica* in Maceió and highlights the need to introduce a specific diagnostic test to detect amoebiasis cases in public laboratories.

Key words: *Entamoeba histolytica*; *Entamoeba dispar*; northeast Brazil

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Introduction

Amoebiasis is a severe infection caused by *Entamoeba histolytica*, and it is considered an important cause of morbidity and mortality in the world [1,2]. Higher prevalences are found in tropical areas of South and Central America, Africa, the Asian subcontinent, and Pacific countries, mainly because of unsanitary living conditions. The similarities between these regions include low socioeconomic development and poor environmental sanitation, both of which favor parasite transmission [2-4]. *E. dispar* and *E. moshkovskii* are considered commensal non-pathogenic species of amoeba, morphologically indistinguishable from *E. histolytica*, the causative agent of invasive amoebiasis. A differential diagnosis between these species is critical because, according to the World Health Organization, treatment should only be administered to patients who have been diagnosed as having *E. histolytica* [1]. The most commonly used diagnostic methods to specifically separate these species are enzyme-linked immunosorbent assay

(ELISA) for the detection of *E. histolytica* antigen, and polymerase chain reaction (PCR) to amplify amoebic DNA [1].

In Brazil, the frequency of *Entamoeba* infection displays regional variations. The highest levels of amoebiasis prevalence rates are observed in the northern region of the country. In Belém and Manaus municipalities, both in the Amazon region, the prevalence of *E. histolytica* infections recorded were 29% and 6.8%, respectively [5-7]. In the northeast region, the prevalence for amoebiasis in Fortaleza (Ceará State) is 14.9% [3], whereas in Pernambuco and Bahia States, no case of infection due to *E. histolytica* has been reported in the literature [8-11].

The present study aimed to assess the specific occurrence of infection by *E. histolytica* and *E. dispar* in Maceió, Alagoas State in northeast Brazil.

Methodology

Design of the study and sample calculation

A cross-sectional epidemiological study was conducted in the urban area of Maceió, state of Alagoas, Brazil (Figure 1), from March 2008 to December 2010. The reference population comprises 67,738 students 4–15 years of age selected from the four education coordination units (CES) representing all geographical areas of the city. This age group is considered to be at increased risk for *Entamoeba* infection [2]. In each CES, public schools were stratified according to the overall average of students (645 students/school), resulting in two groups of schools, one with more and other with fewer than this average number of students. This criterion was adopted because of the possibility that students from larger schools have different characteristics from those who attend smaller schools. Sample size calculation using an expected frequency of 11% of *Entamoeba* infection [12] with an acceptable error of 20% and a confidence level of 95% returned a minimum sample size of 768 subjects. The study protocol was approved by the ethics committee of the Universidade Federal de Alagoas (reference number 011577/2005-19).

Microscopic examination

Fresh unpreserved stool samples were collected and processed using the spontaneous sedimentation technique [13]. Four slides of each sample stained with Lugol's iodine solution were prepared and examined microscopically by four different technicians. Samples positive for *E. histolytica*/*E. dispar*/*E. moshkoviskii* cysts were further characterized for distinction of the species using immunological and molecular techniques.

Antigen detection

Microscopically positive fecal specimens for *Entamoeba* were tested by ELISA for *E. histolytica* lectin-specific antigen. The commercially available kit *E. histolytica* II (Techlab, Blacksburg, USA) was used according to the manufacturer's instructions. This kit uses monoclonal antibodies against the Gal/GalNAc-specific lectin (adhesin molecule) of *E. histolytica*. A sample was considered positive if the optical density (OD), measured at 450 nm, was ≥ 0.05 after subtracting the negative control.

DNA amplification

The same fecal specimen sediments used for antigen identification were used for DNA extraction. DNA was extracted from 200–400 mg of each stool sample with the PSP Spin Stool DNA Kit (Invitex,

Figure 1. Map showing the survey location.



Hayward, USA), following the manufacturer's instructions.

Genomic DNA was subjected to PCR using the species-specific primers p11plus/p12plus for *E. histolytica* (p11plus: 5'GGAGGAGTAGGAAAGTTGAC3', p12plus: 5'TTCTTGCAATTCCTGCTTCGA3') and p13plus/p14plus for *E. dispar* (p13plus: 5'AGGAGGAGTAGGAAAATTAGG3', p14plus: 5'TTCTTGAACTCCTGTTTCTAC3') [14].

The amplification was done as described Rivera *et al.* [15], with minor modifications. The PCR reagents comprised 20 mM Tris-HCl, 50 mM KCl, 200 μ M dNTP, 1.5mM MgCl₂, 20 pM of the specific pair of primers (*E. histolytica* or *E. dispar*), 2U Taq Polymerase Platinum (Invitrogen, Carlsbad, USA), 400 ng of bovine serum albumin, 2 μ L of DNA and ultra-pure H₂O to a final volume of 25 μ L. Amplification conditions consisted of 35 cycles of 60 seconds at 94°C, 60 seconds at 59°C, and 60 seconds at 72°C (PCR Express Thermo Hybaid, Ashford UK). PCR products were subjected to electrophoresis in 2% agarose, staining with ethidium bromide (10 mg/mL) and visualization in a UV transilluminator (Vilber Lourmat TFX-20.M, Eberhardzell, Germany). The species-specific product size for *E. histolytica* and *E. dispar* was 100 and 101 bp, respectively.

DNA extracted from *E. histolytica* and *E. dispar* cultures were used as positive controls in each PCR run. DNA extraction of cultured parasites was done as described by Sambrook *et al.* [16].

Statistical analysis

The data was analyzed in Epi-Info version 3.5.1 computer software (Centers for Disease Control and Prevention). Chi-Square (χ^2) and student t test were used to compare proportions. Statistically significant

difference was defined as $p < 0.05$. Risks were estimated by odds ratio (OR).

Results

Stool samples were collected from 1,003 schoolchildren (54% males and 46% females) with a mean age of 9.7 ± 2.6 years, attending any of the 16 chosen public schools located in different districts of the city. Students from 40 out of the 50 districts of Maceió were selected.

A total of 64 (6.4%) of all 1,003 examined fecal specimens were identified as having *Entamoeba* cysts, either singly or in combination with other intestinal parasites. *E. histolytica* coproantigen by ELISA was detected in 30 (3.0%) of these microscopically positive samples. The average age of infected children was 10 ± 6.8 years, and no difference was observed in relation to the infection by age group ($p > 0.05$) (Table 1).

The PCR detected mono-infection with *E. histolytica* in 21.9% (14/64) and with *E. dispar* in 56.3% (36/64) of the stool specimens microscopically found with *Entamoeba* cysts. Mixed infection with both *E. histolytica* and *E. dispar* species were detected in 21.9% (14/64) of stool samples identified as positive for *Entamoeba* spp.

Table 2 summarizes the ELISA and PCR results of the 64 fecal specimens microscopically positive for *Entamoeba* infection. Though two samples positive by ELISA showed negative results by PCR (Table 2), there was no difference in the positivity between the two techniques ($p > 0.05$). When ELISA was used as a reference standard, the PCR sensitivity and specificity were 93.3% and 100%, respectively, with a Kappa correlation index of 0.96.

Stool samples from 48 students presenting no parasite cysts by microscopy were tested for *E. histolytica* and *E. dispar* by PCR, with negative results. Considering that microscopy screened all negative samples for *E. histolytica*/*E. dispar* cysts, and regarding mono- and co-infection cases, the overall prevalence for *E. histolytica* was 2.8% (28/1,003), and 5.0% (50/1,003) for *E. dispar*. The difference between the proportions of each species was statistically significant ($p < 0.05$). Thus, in the studied area, the non-pathogenic parasite *E. dispar* was 1.8 times more common than *E. histolytica* infection (OR: 1.8 [95% CI: 1.14–20.93], $p < 0.05$; χ^2 : 6.46).

Discussion

The present results are consistent with epidemiological studies carried out in different regions of Brazil and the world, in which the two species of *Entamoeba* were found infecting the population [1,5,6,17]. In Brazil, there are only few locations where no cases of amoebiasis been found, such as Pernambuco and Bahia states [8-11].

In general, there is a higher number of *E. dispar* cases in relation to the *E. histolytica* infections, as reported in other studies, including in Brazil, as well as the occurrence of mixed infections with *E. histolytica* and *E. dispar* [18-22].

Antigen detection by ELISA and DNA amplification through PCR showed similar results and good correlation for specific amoebiasis diagnosis, as observed by other authors [23]. This agreement between ELISA and PCR indicates that either one is suitable to the specific detection of *E. histolytica* in a stool specimen. The choice of the method will depend on the budget and the objective of the study [23-25].

Table 1. Distribution based on age group of microscopy-positive *Entamoeba histolytica*/*E. dispar* stool samples among school children from Maceió, Alagoas State, northeast Brazil.

Age groups (years)	n	Microscopy ^a
4–7	239	13 (5.4%)
8–11	508	35 (6.9%)
12–15	256	16 (6.3%)
Total	1,003	64 (6.4%)

^aDetection by microscopy of *E. histolytica*/*E. dispar*/*E. moshkovskii* complex cysts.

Table 2. Comparison of results obtained by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) performed on 64 stool specimens microscopically positive for *Entamoeba histolytica*/*E. dispar* complex.

PCR ^b	ELISA ^a		Total
	Positive	Negative	
Positive	28	0	28
Negative	2	34	36
Total	30	34	64

^aELISA test detected *E. histolytica*-specific antigen in stool specimen; ^bPCR amplified specific DNA fragment of *E. histolytica*.

The two samples found to be ELISA positive but PCR negative may be due the presence of DNA amplification inhibitors in the fecal samples. Bacterial proteases, nucleases, cell debris, and other factors may interfere in the PCR, preventing DNA amplification [26].

Although no specific test was performed to detect *E. moshkovskii*, most infections caused by this amoeba worldwide are commonly reported as a mixed infection [27,28]. In Brazil, there is a lack of data concerning the prevalence of this *Entamoeba* species.

Conclusions

In Brazil, particularly in the northeast, there are few surveys indicating the occurrence of amoebiasis cases. The present study contributes to the knowledge of the epidemiological situation of this morbidity in the country. The results indicate the need for specific diagnostic techniques to detect *E. histolytica* in the evaluated population.

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