

Original Article

Use of real-time polymerase chain reaction to identify *Entamoeba histolytica* in schoolchildren from northwest Mexico

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Abstract

Introduction: *Entamoeba histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically identical, but intestinal amebiasis is caused only by *E. histolytica*. Mexico is among the countries with high amebae infection rates, although the contribution of pathogenic amoeba to the total detected cases remains unknown, especially in the northwestern dry region. Therefore, the aim of this study was to identify the actual prevalence of *E. histolytica* using real-time polymerase chain reaction (PCR) in schoolchildren of northwestern Mexico.

Methodology: Participants were children from five public elementary schools in the low-socioeconomic-level suburban areas of Hermosillo, Sonora, Mexico. One stool sample was collected from each child and analyzed by the Faust technique for *Entamoeba* spp. and by real-time PCR for *E. histolytica*.

Results: Analysis of stool samples from 273 children (9.0 ± 1.5 years of age) resulted in 25 (9.2%) positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* by the Faust technique; of these, 3 were positive for *E. histolytica* by real-time PCR. In addition, 2 samples that were negative for *E. histolytica*/*E. dispar*/*E. moshkovskii* by the Faust technique were positive by real-time PCR.

Conclusions: The actual prevalence of *E. histolytica* in our study population was 1.8%, which is lower than those reported in previous studies in other Mexican regions.

Key words: *Entamoeba histolytica*; real-time PCR; schoolchildren; identification; Mexico.

J Infect Dev Ctries 2017; 11(10):800-805. doi:10.3855/jidc.9350

(Received 25 August 2016 – Accepted 28 February 2017)

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Introduction

In 2010, the World Health Organization (WHO) estimated that *E. histolytica* infection caused more than 103 million cases of diarrhea, the complications of which led to around 5,450 deaths worldwide [1]. *E. histolytica* infection is more common in tropical climate areas and in developing countries [2] such as Mexico, where amebiasis displays endemic behavior, affecting children under 15 years of age [3]. According to the most recent report of the Mexican Health Ministry (2014), intestinal amebiasis is one of the 20 principal causes of disease, with 299,242 cases occurring per year [4]. However, the contribution of *E. histolytica* to the prevalence of infection in Mexico remains unclear, because it shows similar morphology to other nonpathogenic *Entamoeba* species, such as *E. dispar* and *E. moshkovskii* [3], and there are no studies that properly differentiate between these species.

The worldwide prevalence of *E. histolytica*, based on microscopic techniques, has been estimated to be 10%; however, it is probably an overestimation of the actual prevalence [5]. The distribution of *E. moshkovskii* has been scarcely studied, but *E. dispar* has been found to be ten times more prevalent than *E.*

histolytica [6-7]. Therefore, serology or molecular techniques must be employed to properly identify *E. histolytica*.

In Mexico, a national survey found that 8.4% of the population had antibodies against *E. histolytica* [8]. In addition, a molecular approach found a prevalence of 13.8% for *E. histolytica* in a rural community of central Mexico, with a high number of asymptomatic cases [9]. In the desert northwest Mexican region, the true prevalence of *E. histolytica* remains unknown, and a prevalence of 10% for *Entamoeba* spp. (*E. histolytica*/*E. dispar*/*E. moshkovskii*) has been described [10]. Based on this information, the aim of this study was to identify and to determine the prevalence of *E. histolytica* by real-time polymerase chain reaction (PCR) and to compare this result with those by microscopy in schoolchildren from a suburban area of the municipality of Hermosillo in northwest Mexico.

Methodology

Study population and sample collection

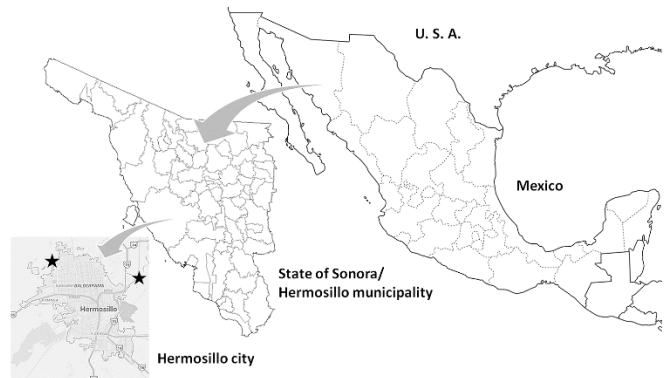
The study was approved by the ethical committee of the Centro de Investigación en Alimentación y

Desarrollo A. C. (CE/016/2014). It was a cross-sectional study with convenience non-probability sampling, conducted between October 2014 and December 2015, in Hermosillo, Sonora (884,273 inhabitants, 81,619 school-age children) [11] in northwest Mexico (Figure 1). This is a desert area, with a semidry to very dry climate. In 2014, Hermosillo had maximum mean temperatures from June to August (33.0–35.4°C) and minimum mean temperatures (19.0–20.7°C) from January to February, with precipitation from July to September (80.5–104.0 mm³) [12].

This study focused on children in second to sixth grades in five public elementary schools located in suburban communities of Hermosillo, located in areas of low socioeconomic status [13]. Families living around the selected schools were characterized by a high percentage of parents with only elementary education and a high number of homes lacking drainage (using septic tank or latrine), potable water, and proper sanitary conditions.

Apparently healthy children, for whose parents or guardians provided informed consent to participate in the study, were enrolled in the study. A stool sample was collected from each child and transported, properly refrigerated, to the Parasitology Lab at the Centro de Investigación en Alimentación y Desarrollo for analysis. Two 1.5 mL aliquots of each sample were separated and stored at -70°C until DNA extraction for real-time PCR analysis. The remaining fecal material was analyzed using the Faust technique for identification of intestinal parasites. The feces sample was diluted in distilled water (1:10). The suspension was filtered, centrifuged at 945 g for 5 minutes, and the precipitate was resuspended in distilled water (1:10). Centrifugation and resuspension were repeated three times. In the last centrifugation cycle, distilled water was replaced by zinc sulfate (33% w/v, density 1.180). After 20 minutes resting at room temperature, three drops of the supernatant were placed on a slide with one drop of lugol for microscopic examination (10× and 40×) [14]. Results were given to parents so children received the proper medical treatment when required.

Figure 1. Geographical location of the study site. Stars mark the two suburban zones where the elementary schools are located.



DNA extraction and real-time PCR

DNA extraction from stools was performed with the QIAmp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer’s protocol. DNA concentration and 260/280 ratio were measured in a Nanodrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, USA).

Identification of *E. histolytica* by real-time PCR was done using specific primers for a 134 bp fragment in the 16S-like small-subunit rRNA gene (Ehf: 5-AAC AGT AAT AGT TTC TTT GGT TAG TAA AA-3; and Ehr: 5-CTT AGA ATG TCA TTT CTC AAT TCA T-3) [15]. Reactions were performed using 10 µL of SYBR Green Supermix (Bio-Rad, Jurong, Singapore), 5 µM of each primer, 100 ng of template DNA, and sterile milli-Q water to a final volume of 20 µL. Cycling conditions began with an initial hold at 95°C for 10 minutes, followed by 40 cycles consisting of 95°C for 15 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Reactions were run in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, USA) enabling a melting curve to obtain the melting temperature (T_m) of the amplicon. Amplicon length was confirmed with electrophoresis in 2% agarose gel stained with GelRed (Biotium, Fremont, USA).

Table 1. Parasites detected in the analyzed samples (n = 273) using the Faust technique.

| Parasite | Positive samples | Prevalence (%) | 95% CI (%) |
|---|------------------|----------------|------------|
| <i>Giardia lamblia</i> | 61 | 22.3 | 17.4–27.3 |
| <i>Endolimax nana</i> | 57 | 20.9 | 16.1–25.7 |
| <i>Entamoeba coli</i> | 39 | 14.3 | 10.1–18.4 |
| <i>Entamoeba histolytica/E. dispar/E. moshkovskii</i> | 25 | 9.2 | 5.7–12.6 |
| <i>Hymenolepis nana</i> | 12 | 4.4 | 2.0–6.8 |
| <i>Iodamoeba bütschlii</i> | 5 | 1.8 | 0.2–3.4 |

Statistical analysis

Descriptive statistics were used to calculate the proportions of schoolchildren with intestinal parasitic infections and to estimate the prevalence of *E. histolytica*. The 95% confidence intervals for proportions were calculated, all using the statistical software NCSS 7.0 (NCSS, LLC., Kaysville, USA).

Results

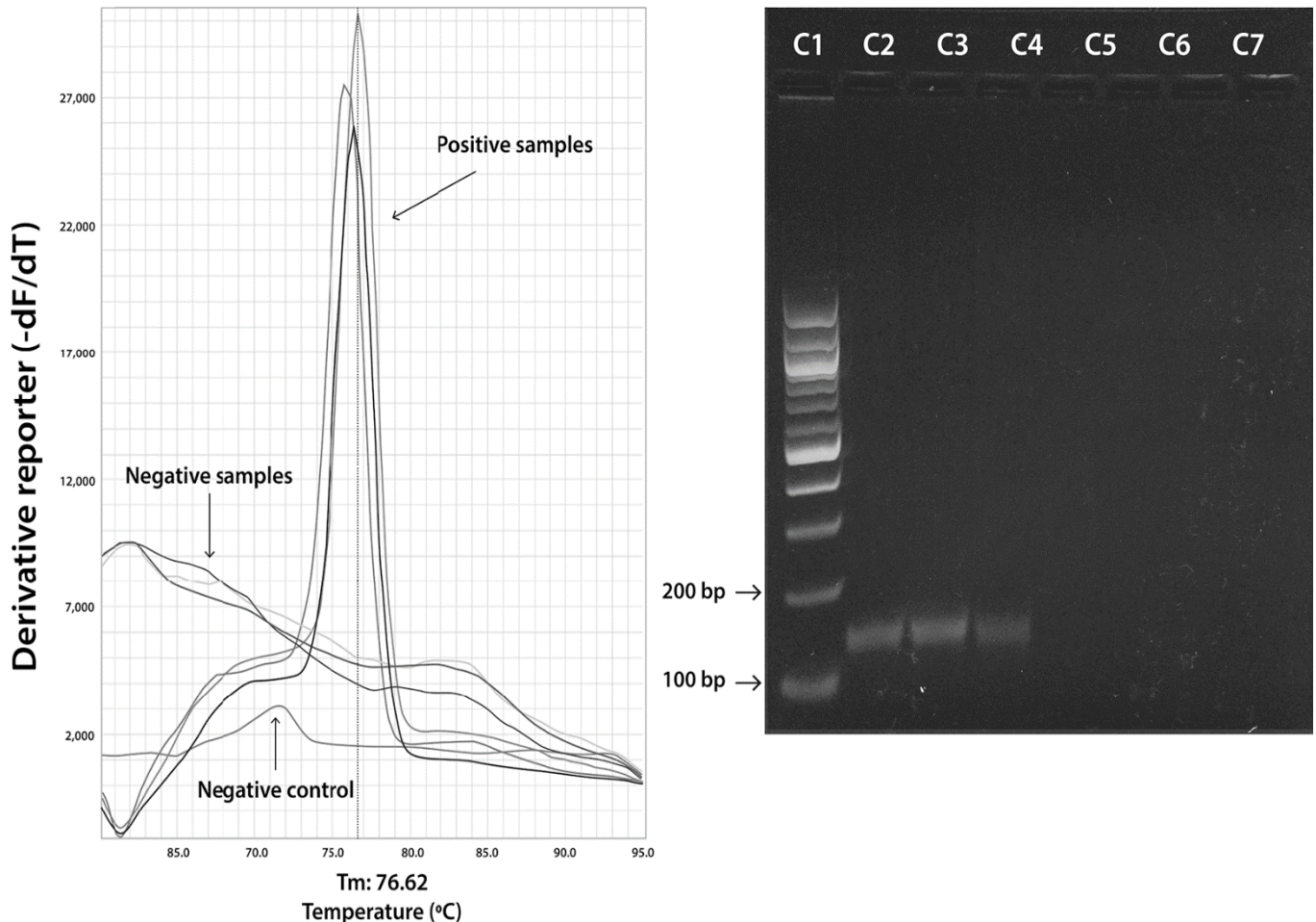
A total of 745 children were invited to this study; ultimately, 273 (36.6%) participated. The children’s mean age was 9.1 ± 1.5 years, and 51.3% (n = 140) were girls. Stool sample analysis using the Faust technique exhibited intestinal parasitic infection in 54.9% (n = 150) of the children and polyparasitism (more than one parasite species) in 29.7% (n = 81). The complex *Entamoeba* spp. (*E. histolytica*/*E. dispar*/*E. moshkovskii*) was found in 9.2% (n = 25) of the analyzed samples (Table 1).

All fecal samples were tested by real-time PCR to identify *E. histolytica*. Three of the 25 *Entamoeba* spp. detected by the Faust technique were positive for *E. histolytica*. A T_m of 76.62°C was obtained in the melt curve (Figure 2A), and the expected amplicon size (134 bp) was obtained in the electrophoresis analysis (Figure 2B). Among the rest (n = 248) of the negative samples for *Entamoeba* spp. by microscopy, two samples resulted positive for *E. histolytica* by real-time PCR. Thus, the overall prevalence of *E. histolytica* infection in the study population was 1.8% (95% confidence interval: 0.2%–3.4%).

Discussion

In developing countries and resource-limited areas, microscopy is the routine test for parasite infection diagnosis due to its low cost [16]. Compared to immunoassay, microscopy has a specificity of 97% and sensitivity of only 20% for *E. histolytica*/*E. dispar*

Figure 2. A) Melt curve of positive and negative samples for *E. histolytica* obtained in real-time PCR. B) Agarose gel electrophoresis of positive (C2, C3, and C4) and negative (C5, C6, and C7) samples for *E. histolytica*.



identification [5]. When compared to real-time PCR, the immunoassay test has 79% and 96% sensitivity and specificity, respectively [15].

Nowadays, the highest sensitivity and specificity (nearly 100%) for *E. histolytica* infection diagnosis is achieved with real-time PCR [17]. Based on its high specificity, we were able to delimit the 25 samples (9.2%) positive for *Entamoeba* spp. obtained by the Faust technique, to 3 (1.1%) positive for *E. histolytica*. In addition, real-time PCR sensitivity enabled us to identify two more positive samples for *E. histolytica* among those negatives for *Entamoeba* spp. by the Faust technique. Using real-time PCR allowed us to estimate an overall prevalence for *E. histolytica* of 1.8% (n = 5) in our study population.

Several authors have reported that Mexico is one of the countries with the highest rate of *E. histolytica* infection [3,7,18-19]. This was mainly based on a national survey carried out in 1994 in a representative sample of the Mexican population, which found a seroprevalence of 8.41% for *E. histolytica* using indirect hemagglutination [8]. However, a further analysis by immunoenzyme assay in solid phase (ELISA) found antibodies against *E. histolytica* only in 4.49% of the same serum samples [20]. Therefore, although not by completely comparable techniques, our results were much lower, perhaps attributable to the geographical conditions discussed below.

A PCR-based study performed in a rural community of central Mexico that included 290 children and adults found a prevalence of *E. histolytica* of 13.8% by conventional PCR [9]. This is higher than that found in our study (1.8%), though *E. histolytica* infection is more common in children in Mexico [3]. Regardless, the socioeconomic and hygienic conditions were similar for both populations. The schoolchildren included in our study lived in poor neighborhoods with no paved roads, in houses constructed with low-quality materials and with no drainage, comparable to the conditions of the population studied in the central Mexican community [9].

The main difference between our study and that performed in central Mexico is the kind of weather in the study area; they have a tropical valley climate [9], while a desert climate prevails in our region. *E. histolytica* infection is more common in tropical climates and poor-hygiene areas [2]. For example, in Brazil, prevalence of 36.6% and 19.4% of *E. histolytica* were found by ELISA in two urban populations of western Amazonia [21], but in another rural region with water scarcity, the prevalence, identified by PCR, was 10.3% [6]. In India and Malaysia, prevalence values

were 13.7% [22] and 9.2% [23], respectively, using PCR. In contrast, in drier and hotter climates, lower prevalence of *E. histolytica* has been detected by PCR, such as in northwest Ethiopia (1.7%) [7] and northern Ghana (0.4%) [24], where children and adults have been studied.

An absence of *E. histolytica* infection has been reported in schoolchildren in some African countries that are believed to have high infection rates. For example, in southwestern Nigeria, 199 stool samples of children (6–14 years of age) from a semirural community were analyzed by real-time PCR, and none was positive for *E. histolytica* [25]. Similarly, not a single positive sample was detected among 363 stool samples of primary schoolchildren from various regions of Ethiopia, also analyzed by real-time PCR. The authors discussed that the absence of *E. histolytica* was probably due to the extended use of metronidazole [26]. In Mexico, albendazole is given to schoolchildren biannually in the national deworming campaign, and it has been reported that a single dose of albendazole (400 mg) reduces the *E. histolytica*/*E. dispar* infection rate in more than 50% of children 7–15 years of age [27]. This, in combination with the prevailing climate conditions, may contribute to the low prevalence of *E. histolytica* found in our study.

Conclusions

Although the sample size was not representative of the entire northwest region, the low prevalence found for *E. histolytica* in our study population is likely an indicator of the actual *E. histolytica* infection status in the schoolchildren of the region. Additional studies using highly sensitive techniques for *E. histolytica* identification in Mexico are required. Estimation of the *E. histolytica* prevalence must consider the climate, urbanization, hygienic conditions, and access to health services, as these account for differences in infection rates. The identification of *E. histolytica* among the *E. histolytica*/*E. dispar*/*E. moshkovskii* complex will allow the correct diagnosis of amebiasis, the implementation of strategies to reduce the morbidity of *E. histolytica*-associated diarrhea, and to reduce the unnecessary treatment of patients with nonpathogenic *Entamoeba* spp., particularly in regions with a low prevalence of *E. histolytica*. Thus, the actual distribution of *E. histolytica* in our country should be reanalyzed, and the high prevalence found in central Mexico must not be generalized to all Mexican regions.

Acknowledgements

The authors want to thank to M.Sc. Gabriel Arvayo and M.Sc. Manuel Castro for their technical support. Sandra Aguayo-Patrón thanks CONACYT the Ph.D. scholarship (377998). CIAD A.C. is a research center with federal government resources from CONACYT.

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Conflict of interests: No conflict of interests is declared.