Validation and utilization of PCR for differential diagnosis and prevalence determination of *Entamoeba histolytica/Entamoeba dispar* in Salvador City, Brazil

ABSTRACT

Amoebiasis is an infection caused by *Entamoeba histolytica* and is a potential health risk in countries in which health barriers are inappropriate. Since the discovery of *Entamoeba dispar*, the prevalence of amoebiasis has been modified. **Objective:** This study has standardized the PCR technique applied for the diagnosis of different species of the *E. histolytica/E. dispar* complex and has evaluated the prevalence of infection among patients attending private and public clinical laboratories in Salvador City, Bahia State, Brazil. **Results:** Analysis of 52,704 stool samples by microscopic examination demonstrated that 1,788 (3.4%) were positive for the *E. histolytica/E. dispar* complex and infection occurred more often in samples originated from public clinical laboratories (5.0%) than those that came from private laboratories (3.2%). PCR performed in approximately 15% (262) *E. histolytica/E. dispar* complex positive samples, randomly chosen, amplified 227 samples (86.6%), all of them positive for *E. dispar*. The non-amplified 35 samples (13.4%) were also negative for *E. histolytica*-specific galactose adhesin. Moreover, to exclude a probable infection caused by *E. hartmanni*, morphometric analysis demonstrated that non-amplified samples had cyst sizes comparable to *E. histolytica/E. dispar* (>10 μm). **Conclusion:** The absence of amplification of these samples indicates the presence of PCR inhibitors in the stool samples or the presence of DNA from *Entamoeba* species other than *E. dispar, E. histolytica* or *E. hartmanni.*

Keywords: amebiasis; *Entamoeba histolytica*; diagnosis; prevalence.

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Authors
Fred Luciano Neves Santos[1]
Marilda de Souza Gonçalves[2]
Neci Matos Soares[3]

[1]MSc, PhD Student, Health and Investigative Medicine Biotechnology (CPqGM-FIOCRUZ), Bahia, Brazil
[2]PhD; Professor, Clinical Hematology, Faculdade de Farmácia, Universidade Federal da Bahia; Researcher, Laboratory of Pathology and Molecular Biology, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Bahia, Brazil
[3]PhD; Professor, Clinical Parasitology,Faculdade de Farmácia, Universidade Federal da Bahia, Bahia, Brazil

INTRODUCTION

Parasitic infections are endemic and represent a major public health problem in developing countries.[1-6] In particular, *Entamoeba histolytica*, the etiologic agent of amoebic colitis and liver abscess, causes human infections on a global scale, resulting in significant human suffering and death. Approximately 50 million people have this invasive disease annually, resulting in 100,000 deaths per year and making this the second most common cause of parasitic death in humans.[7] The high prevalence of infection is due to fecal contamination of food and water supply; thus, the disease is predominantly seen in developing countries.[8]

E. histolytica cysts measure 10-15 μm in diameter, possess a rigid cyst wall and can contain up to four nuclei. They are morphologically indistinguishable from cysts of the commensal *Entamoeba dispar* and share characteristics with cysts of *Entamoeba hartmanni*, which are smaller than 10 μm in diameter. Optical microscopy is a desirable tool for the diagnosis of amoebiasis:[9] it is simple and cheap to execute and does not require sophisticated technology. However, microscopy is unable to differentiate between species belonging to the *E. histolytica/E. dispar* complex. Moreover, intermittent releasing of cysts decreases the sensitivity of this method. Therefore, the positivity rate is enhanced by the use of concentration procedures, one of which is formalin-ether sedimentation.[10] Concentrated and purified cysts of *E. histolytica/E. dispar* can improve diagnostic sensitivity to differentiate *E. histolytica* from *E. dispar* by polymerase chain reaction (PCR), especially in feces that contain low numbers of cysts. The coproantigen ELISA technique has been suggested for use in routine diagnostic procedures and epidemiological studies. However, PCR is used for final confirmatory identification of intestinal amoebiasis.[11-14]

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Correspondence to:
Fred Luciano Neves Santos
Departamento de Análises Clínicas e Toxicológicas, UFBA
Rua Barão de Geremoabo S/N. Ondina,
40170115, Salvador, Bahia, Brazil
Phone: 55 71 3283 6950
flucianon@hotmail.com

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The prevalence of the *E. histolytica/E. dispar* complex differs among the five regions of Brazil with 2.5-11% in the South and Southeast, 19% in the North and the Amazon region and approximately 10% in the Northeast and Midwest.[4,15] This variation in prevalence is associated with regional differences in sanitation and socio-economic conditions, mainly related to housing, sewage facilities, water quality, and other as yet unknown factors.[16]

Given the medical importance of differentiating species that belong to the *E. histolytica/E. dispar* complex and the fact that the prevalence of each species is unknown in Salvador City, the purpose of this study was to perform a survey to determine the prevalence of *E. histolytica* and *E. dispar* using a nested and multiplex PCR technique with genomic DNA extracted from stool specimens of individuals residents in Salvador City.

MATERIALS AND METHODS

Sample details

A total of 52,704 stool samples were collected from patients attending Datalab and NKB-Bahia groups (private clinical laboratories, n = 47,080) and the Clinical Laboratory of Pharmacy Faculty and University Hospital, *Universidade Federal da Bahia* (public clinical laboratories, n = 5,624), from February to August 2006. A single fresh stool specimen was collected from each patient, and diagnosis of the *E. histolytica/E. dispar* complex was performed by the spontaneous sedimentation technique.[17] Daily, around 8-15 samples were positive for *E. histolytica/E. dispar* complex. From these, 262 samples were randomly selected for cysts concentration and extraction of genomic DNA. Approximately 800 mg of each positive sample was preserved without fixative and stored at -20° C for immunologic diagnosis.

This study was carried out in Salvador City (Bahia State, Northeast Brazil), which has a population of 2,892,625 inhabitants,[18] and was approved by Committee of Ethics in Research of the Gonçalo Moniz Institute number 100/2006. Informed consent for participation was obtained from patients (or legal guardians in the case of minors) during collection of clinical specimens. A form for personal (age, sex) and epidemiologic data (e.g., race, signs and symptoms, drugs) was completed for all patients positive for *E. histolytica/ E. dispar* complex.

The sample was estimated for different scenarios based on the following parameters: error $\alpha = 0.05$ and power of test $(1-\beta) = 0.90$, minimum detectable odds ratio (OR) = 2.0 and frequency of exposure 3.2%, according to Santos *et al.*[19]

Cyst concentration and morphometric analysis

To concentrate the cysts of these parasites, 25 *E. histolytica/ E. dispar*-positive samples were used to test the efficiency of spontaneous sedimentation, formalin-ether and flotation by zinc sulfate or sucrose.[20] The cysts were concentrated from 5 g of fresh stools as described by Troll *et al.*[21] Approximately 25 µL of the concentrated sample and 50 µL of iodine were mixed, spotted on glass slides and covered by a coverslip (24 x 24 mm). The slides were analyzed by 40 x magnification, and the average of cysts was quantified in 20 microscopic fields.

Morphometric analysis of cysts was performed using reticule calibration in a millimeter scale (mm). Each 0.01 mm corresponded to 10 µm in cyst size.

Extraction of genomic DNA

Cysts purified from 5 g of fresh stools by the formalin-ether technique were used for DNA extraction. A 50 µL pellet was washed four times with distilled water at 2,000 x *g* for 30s in an Eppendorf microfuge. The pellet was resuspended in a small volume (50 - 100 µL) of a solution containing 100 mM Tris, pH 8.0, and 2.5 mM EDTA. The tubes were immersed in a mixture of dry ice and ethanol for 4 min and incubated at 50°C for 3 min. This process was repeated six times to rupture the cysts. The samples were then sonicated three times by being immersed in picked ice in an ultrasonic cleaner (model 250, Branson Sonifier, USA) for 30s at an amplitude of 35 without pulses. Then, 200 µL of buffer containing 100 mM Tris (pH 8.0), 1% sodium dodecyl sulfate, 25 mM EDTA and 200 µg of proteinase K (Boehringer, Mannheim, Germany) was added to each tube, mixed, incubated at 50°C for 24h, boiled for 10 min and centrifuged at 12,000 x g for 5 min. The DNA in the supernatant was precipitated with 360 µL ice-cold isopropanol, resuspended with 20 µL of 10 mM Tris and 1 mM EDTA and frozen for analysis by PCR.

Multiplex PCR

Nested and multiplex PCR was carried out according to the protocol described by Evangelopoulos *et al.*,[12] with some modifications. A set of oligonucleotide primers based on small subunit rDNA (SSU-rDNA) sequences of *E. histolytica* and *E. dispar* were prepared. The outer primer set, E1 (5'-TGC TGT GAT TAA AAC GCT-3') and E2 (5'-TTA ACT ATT TCA ATC TCG G-3'), which specifies a 1,076-bp fragment, is common to and specifically designed for both species. The inner primer pair for pathogenic sequences, Eh-L (5'-ACA TTT TGA AGA CTT TAT GTA AGT A-3') and Eh-R (5'-CAG ATC TAG AAA CAA TGC TTC TCT-3'), brackets a 427-bp region, whereas the inner primer pair Ed-L (5'-GTT AGT TAT CTA ATT TCG ATT AGA A-3') and Ed-R (5'-ACA CCA CTT ACT ATA CCT ACC-3') is specific for *E. dispar*, resulting in a 195-bp fragment.

Amplification was performed in a total volume of 50 µL containing 0.5 µM of each primer, 5 µL

10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.75U Taq DNA polymerase (Gibco BRL, Rockville, MD) and 3 µL of DNA sample. An initial DNA amplification was performed using the E1, E2 primer set in a GenAmp PCR system 2400 (AB Applied Biosystems) thermal cycler. The first cycle of 5 min at 94°C was followed by 45 cycles of denaturation for 1 min at 94°C. Primers were annealed for 1.5 min at 47° C and elongated for 2.5 min at 72°C. As positive controls, 5 µL of DNA from cultured *E. histolytica* strain HM-1:IMSS, grew in TYI-S-33 medium axenically, and 5 µL of DNA from cultured *E. dispar* strain MCR, grew in Pavlova medium polyxenically (kindly provided by Dr. Maria Aparecida Gomes, Amoebiasis Laboratory, *Universidade Federal de Minas Gerais*, Brazil) were used. For subsequent amplifications, 5 µL of DNA from the first reaction and primer sets Eh-L, Eh-R and Ed-L, Ed-R were used under the conditions described above, except that the annealing temperature was 58°C. The analysis of PCR products was performed using gel electrophoresis. DNA fragments were separated on a 1.5% (w/v) agarose gel (Invitrogen Life Technologies, USA) containing 0.5 µg ethidium bromide/mL. Gels were photographed under ultraviolet illumination (Sigma Chem. Co., USA, model T1201).

Immunoenzymatic assay

The presence of *E. histolytica*-specific galactose adhesin was determined in 35 stool samples, without preservatives, that were negative by PCR and 60 randomly selected stool samples that were positive for *E. dispar.* The ELISA test was performed according to the manufacturer's instructions (TechLab *E. histolytica* II ELISA, USA).

Statistical analysis

The statistics tests used in this study were performed using the SPSS program 15.0 for Windows. The Wilcoxon test was used to evaluate the statistical significance between analyzed variables; a two-tail p-value less than 0.05 was considered significant.

RESULTS

Cyst concentration

The effects of spontaneous sedimentation, formalin-ether and flotation by sulfate zinc or sucrose on the concentration of cysts of the *E. histolytica/E. dispar* complex were evaluated. The formalin-ether technique yielded more cysts than any other method analyzed with a mean of 5.88 cysts under 40 x magnification, varying from 0.1 to 8 cysts (Table 1). The mean number of *E. histolytica/E. dispar* complex cysts by formalin-ether was 3.42 ($p = 0.02$), 8.17 ($p < 0.001$) and 9.19 ($p < 0.001$) times more than that of the spontaneous sedimentation, zinc sulfate and sucrose flotation procedures, respectively. Likewise, the formalin-ether technique concentrated 7.73 ($p = 0.008$), 3.25 ($p = 0.021$) and 4.5 ($p = 0.01$) times more cysts of *Entamoeba coli* than the above mentioned techniques. Statistically significant differences between other concentration methods were not observed ($p > 0.05$).

Amplification of DNA extracted from stool samples

The standardization of nested and multiplex PCR was performed using DNA from cultured trophozoites of *E. histolytica* strain HM-1 and *E. dispar* strain MCR. The detection limit was determined by the addition of 100 µL of stools free

Table 1. Comparison of four techniques for concentration of amoeba cysts

Cysts	Methodology	Mean number of cysts in 40X (Microscopic field)	p*
E. histolytica/E. dispar complex	Spontaneous sedimentation	1.72	0.02
	Formalin-ether	5.88	-
	Flotation by sulfate zinc	0.72	< 0.001
	Flotation by sucrose	0.64	< 0.001
E. coli	Spontaneous sedimentation	5.71	0.008
	Formalin-ether	44.18	-
	Flotation by sulfate zinc	13.59	0.021
	Flotation by sucrose	9.82	0.01

The cysts were concentrated from 5 g of fresh stools with a positive diagnosis for *E. histolytica/E. dispar* or *E. coli.* The analysis of statistical significance between differences in numbers of cysts concentrated by formalin-ether or other methodologies was performed using the Wilcoxon Test.

of parasites to either different concentrations of *E. histolytica* DNA (12 ng/mL, 6 ng/mL, 3 ng/mL, 1.5 ng/mL and 0.75 ng/mL; data not shown) or different concentrations of *E. dispar* DNA (18 ng/mL, 9 ng/mL, 4.5 ng/mL, 2.25 ng/mL and 1.12 ng/mL; Figure 1). Multiplex PCR was capable of detecting the specific target sequence when a minimum of 1.5 ng/mL of DNA template was used for *E. histolytica* and when 2.25 ng/mL was used for *E. dispar.*

A positive stool sample for the *E. histolytica/E. dispar* complex was fractionated into six aliquots and stored at -20°C. At monthly intervals, one aliquot was thawed, and its DNA was used to observe the inter-testing reproducibility of the PCR (Figure 2). Compatible profile bands are shown with the species of *amoeba* tested. Similar results were obtained when the samples were amplified twice in the same reaction to demonstrate the reproducibility of intra-testing of PCR, as shown in Figure 3.

Figure 1. Limit detection of Multiplex-PCR in detection of DNA from E. dispar. Amplified PCR products using Eh-R/Eh-L and Ed-L/Ed-R primers and a 1.5% electrophoresis gel stained by ethidium bromide. M, molecular weight ladder (100-bp ladder); Lane 1, negative control (presence of primer dimer); Lane 2, positive control (PCR products from 12 ng/mL DNA from *E. histolytica* – 427 bp); Lane 3, positive control (PCR products from 18 ng/mL DNA from *E. dispar* – 195 bp); Lane 4, PCR products from 9 ng/mL DNA from *E. dispar*; Lane 5, PCR products from 4.5 ng/mL DNA from *E. dispar*; Lane 6, PCR products from 2.25 ng/mL DNA from *E. dispar* ; Lane 7, PCR products from 1.12 ng/mL DNA from *E. dispar*.

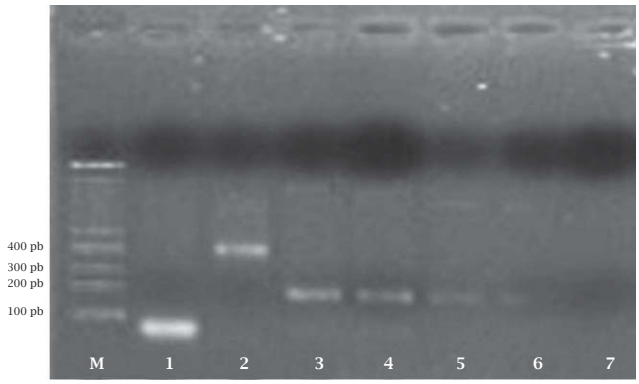


Figure 2. Evaluation of inter-testing reproducibility. Amplified products in a 1.5% electrophoresis gel stained by ethidium bromide. PM, molecular weight ladder (100-bp ladder); Lane 1, negative control; Lane 2, positive control (DNA of *E. histolytica* – 427 bp); Lane 3, positive control (DNA of *E. dispar* – 195 bp); Lanes 4 to 9, clinical sample positive for *E. dispar* amplified monthly during a period of six months; Lane 10, clinical sample negative for the *E. histolytica/E. dispar* complex (presence of primer dimer).

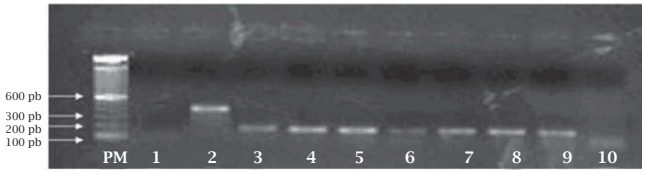
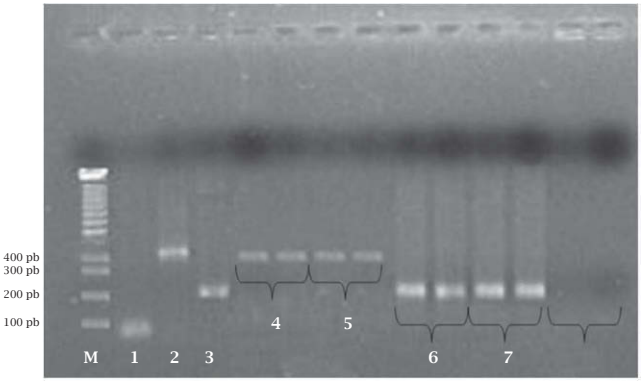


Figure 3. Evaluation of intra-testing reproducibility. Amplified products in a 1.5% electrophoresis gel stained by ethidium bromide. M, molecular weight ladder (100-bp ladder); Lane 1, negative control (presence of primer dimer); Lane 2, positive control (DNA of *E. histolytica* – 427 bp); Lane 3, positive control (DNA of *E. dispar* – 195 bp); Lanes 4 and 5, clinical samples spiked with 3.0 ng/mL of DNA from the HM-1 strain of *E. histolytica*; Lanes 6 and 7, clinical samples positive for *E. dispar*; Lane 8, clinical sample negative for the *E. histolytica/E. dispar* complex.



Analyses of stool samples

Analyses of 52,704 stool samples by microscopic examination demonstrated that 3.4% (1,788) of the samples were positive for the presence of four-nucleus amoebae. Of these stool samples, 47,080 were from private clinical laboratories, and 5,624 were from public clinical laboratories. According to the source of samples, the prevalence of the *E. histolytica/E. dispar* complex was 3.2% (1,507) and 5% (281) in the private and public laboratories, respectively (Table 2).

Table 2. Prevalence of* E. histolytica/E. dispar *in laboratories from Salvador-BA

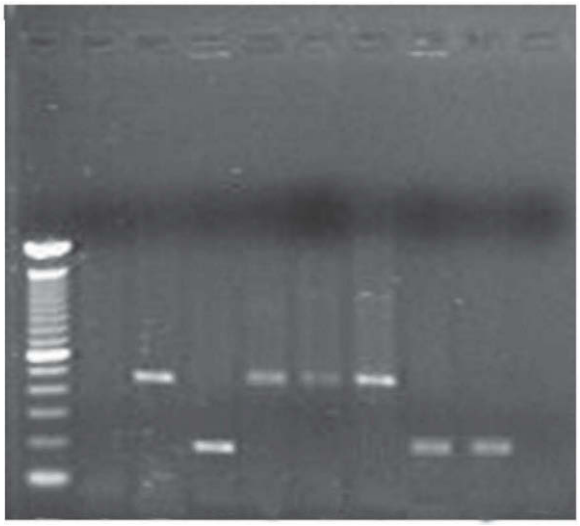
Laboratories	Samples	
	Total (n)	Positives (%)
Not public	47,080	1,507 (3.2%)
Datalab	25,996	984 (3.8%)
Dirceu Ferreira	18,501	473 (2.6%)
Qualitech	2,583	50 (1.9%)
Public	5,624	281 (5.0%)
HUPES[1]	2,078	86 (4.1%)
Pharmacy School (UFBA)[2]	3,546	195 (5.5%)
Not public + public	52,704	1,788 (3.4%)

[1]Professor Edgard Santos University Hospital.
[2]*Universidade Federal da Bahia.*

Extraction of DNA from 262 samples positive for the *E. histolytica/E. dispar* complex followed by PCR showed that 227 samples (86.6%) were positive for *E. dispar*, as demonstrated by amplification of the species-specific fragment (195 bp). No amplification was observed for pathogenic *E. histolytica*. To evaluate the efficiency of detecting *E. histolytica* fragments, we spiked negative samples with DNA template from *E. histolytica* strain HM-1:IMSS. Thereafter, the *E. histolytica* DNA fragment (495 bp) was amplified from all samples. This demonstrates that nested and multiplex PCR can be used for both *E. histolytica* and *E. dispar* species (Figure 4).

The mean number of cysts found in amplified samples was 116 cysts per gram of stool, while the mean number of cysts in the 35 non-amplified samples was 173 cysts. After successive dilution (1:20 to 1:160), these 35 samples remained negative. Even after having carried out the spike technique the samples did not amplify. In order to resolve this problem, non-amplified samples and 60 randomly chosen samples positive for *E. dispar* by PCR were submitted to an *E. histolytica*-specific galactose adhesin immunoenzymatic assay. This technique demonstrated that all 35 non-amplified samples were negative for *E. histolytica*-specific galactose adhesin, suggesting a high PCR specificity in *E. histolytica* diagnosis. Furthermore, to distinguish a probable infection caused by *E. hartmanni,* morphometric analysis demonstrated that non-amplified samples had cyst sizes comparable to *E. dispar* (> 10 μm).

Figure 4. Analysis of 262 *E. histolytica/E. dispar* positive samples by Multiplex-PCR. Amplified PCR products using Eh-R/Eh-L and Ed-L/Ed-R primers and a 1.5% electrophoresis gel stained by ethidium bromide. M, molecular weight ladder (100-bp ladder); Lane 1, negative control (presence of primer dimer); Lane 2, positive control (DNA of *E. histolytica* – 427 bp); Lane 3, positive control (DNA of *E. dispar* – 195 bp); Lanes 4 to 6, clinical samples spiked with 3 ng/mL of DNA from the HM-1 strain of *E. histolytica*; Lanes 7 and 8, clinical samples positive for *E. dispar*; Lane 9, negative sample for the *E. histolytica/E. dispar* complex.



Characteristics of *E. dispar* infection

The mean age of the 262 *E. histolytica/E. dispar* complex-positive individuals submitted to amplification reactions was 35.7 years (with a range of 2 to 79 years), and the median was 35 (25 and 75% quartiles were 22.5 and 50, respectively). Sixty-four percent were male, and 65.5% were of African descent ($p < 0.005$), with a monthly income of less than 5 minimum wages ($p < 0.005$) and an educational level at the 2 grade ($p < 0.005$).

The study population was asymptomatic or presented non-specific symptoms that could be attributed to amoebiasis. The primary reason for seeking medical attention was routine medical check-up, as reported by 67.2% of participants. Among the cases, 21.3% reported having gastrointestinal disturbances, flatulence and diarrhea, 11.5% reported using metronidazole, and no confirmation of *E. histolytica* infection. We identified 31 individuals with liquid stool samples (12% samples) and none with mucus or blood in their stools. A total of 45 individuals (17%) had loose stool samples, and 186 (71%) had formed stool samples.

DISCUSSION AND CONCLUSION

In this report, we described a PCR-based technique for selective identification of *E. histolytica* and *E. dispar* in stool samples. The technique consists of an initial amplification of a 1,076-bp fragment of the SSU-rDNA sequence of both species, followed by an additional amplification for the two species differentiation. This multiplex PCR permits specific identification in a single reaction mixture and is therefore more cost-effective and less laborious than other PCR-based methods.[12] Some studies have shown methods used to isolate DNA from in vitro amoeba cultures.[5,27,28] However, the cultivation of amoebae before concentrating cysts is more expensive and time-consuming.[29] Here, we isolated DNA for PCR amplification of amoeba DNA directly from the stools, reducing time and the possibility of false negative results.

However, PCR presents some disadvantages. It can be difficult to purify DNA from stool samples due to hardness of the cyst wall and the presence of inhibiting substances, which can inhibit the Taq polymerase.[13,30-32] According to Campos-Górgora *et al.*,[33] the major component of the *Entamoeba* cyst wall is chitin, a homopolymer of beta-(1,4)-linked N-acetyl-D-glucosamine that confers great rigidity and resistance. Therefore, the utilization of both chemistry and physical conditions was sufficient to yield a great quantity of genetic material to proceed with the PCR.

Until now, there had not been an ideal method for extracting *Entamoeba* DNA from feces because various substances could act as inhibitors.[34,35] In fact, inhibition

occurred in 13.3% of samples. It is likely that non-specific substances inhibited amplification, since the number of cysts found in these samples did not influence the reaction and the presence of non-specific substances in feces, indirectly evaluated by spike, showed that added DNA did not suffer similarly in amplification of successive dilutions. Specific column chromatography could minimize the influence of enzymatic inhibitors present in feces. According to Verweij *et al.*,[13] it is fast and simple to use. The authors observed that only 1.7% of 657 samples could not be amplified by PCR. We observed an inhibition in 13.3% of our samples, and the utilization of the columns might have lowered this figure. However, specific column chromatography use increases the cost of PCR, making this technique impracticable in laboratory diagnosis.

In 1997, it was formally accepted that the species once called *E. histolytica* comprises two distinct species: the potentially invasive *E. histolytica* and the non-invasive, commensal *E. dispar*.[7] The cysts and trophozoites of these species cannot be distinguished microscopically. As no inexpensive or practical diagnostic procedures are currently available for the identification of *E. histolytica* at health centers in countries with limited resources, we are left with the common practice of identifying the *E. histolytica/E. dispar* complex as "*E. histolytica.*" The likely consequence of this is over-diagnosis and over-treatment, which could be the cause of anti-amoebic drug resistance.[22] Therefore, a method capable of differentiating both species is essential for appropriate treatment and follow-up of infected individuals.

As a result, there has been a lot of progress in the search for molecular methods to distinguish *E. histolytica/E. dispar* complex species in the last few decades. Coproantigen searching, for example, has innumerable advantages relative to other methodologies. However, antigens can denature during the preservation process, yielding false negative results. Another alternative to differentiating the species is PCR. This technique has been used frequently in epidemiological investigations worldwide.[5,23-26]

In this report, the prevalence of *E. histolytica/E. dispar* based on fecal examination by optical microscopy was 3.4%. This finding corroborates the results in the literature that state that the prevalence of infection varies a great deal throughout Brazil, reaching 19% in the Amazon and varying from 2 to 29.5% in other regions.[4,15,16,36] In the present study, the analysis of 262 samples with *E. histolytica/E. dispar* using specific set of primers showed that 227 (86.6%) were positive for *E. dispar*. No amplification was observed in 35 samples (13.4%) that were negative for *E. histolytica*-specific galactose adhesin. Moreover, to exclude a probable infection caused by *E. hartmanni*, morphometric analysis demonstrated that non-amplified samples had cyst sizes comparable to *E. histolytica/E. dispar* (> 10 μm). The absence of amplification of these samples indicates the presence of PCR inhibitors in the stool samples or the presence of DNA from Entamoeba species other than *E. dispar*, *E. histolytica* or *E. hartmanni.*

In fact, Oliveira-Costa *et al.*[37] in Belo Horizonte City, Southeast Brazil, and Dourado *et al.*[38] in Pernambuco State, Northeast Brazil, described only the presence of *E. dispar* in their studies. Santos *et al.*[5] found a prevalence of 21% for four-nucleus amoebae in two slums in Rio de Janeiro State, Southeast Brazil, but only two samples were positive for *E. histolytica*. It appears that *E. histolytica* is more common in North and extreme Northeast Brazil and is rare in other regions.[4,39]

Our described protocol provides a method to sensitively and selectively detect and diferentiate *E. histolytica* and *E. dispar* DNA directly from stool specimens without the need for prior cultivation. Apart from often unsuccessful and time-consuming cultivation attempts, possible misdiagnoses by one strain displacing the other in mixed infections can be avoided. We believe that difficulties in the differential diagnosis of the *E. histolytica/E. dispar* complex need to be overcome for the adequate treatment of *E. histolytica*. PCR is expensive, and the majority of laboratories do not have adequate infrastructure for its use. The utilization of this method in Brazil is in its initial phase and restricted to research centers located in big cities. However, once the method is established and standardized, its cost will decrease. The main limitations to this technique are the difficulty in obtaining conserved DNA from cysts, and the presence of unspecific inhibitors. More inquiries about simpler, faster and cheaper methodologies should be encouraged and, after approval, should be established in laboratories that compose the public and private health network.

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