

Nested PCR reveals elevated over-diagnosis of *E. histolytica* in Barcelona, Venezuela.

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Keywords: *Entamoeba*, diagnosis, PCR, *E. histolytica*.

Abstract. The aim of this study was to identify the presence of *Entamoeba histolytica* and *E. dispar* by nested PCR in children attending the “Dr. Luis Razetti” Hospital, Barcelona, Anzoátegui State. Of the 1,141 fecal samples coproparasitologically evaluated by conventional microscopy, 150 were diagnosed positive for *E. histolytica* in 0-10 year-old-children, of both sexes. The signs, symptoms and a full coproparasitological report were obtained from all of these and nested PCR was performed to identify *E. histolytica* and *E. dispar*. The conventional microscopy results showed a diagnostic frequency of *E. histolytica* in 13.2% of the cases, of which 79.3% were positive only for this pathogen. However, nested PCR showed that of these, only 28% (42/150) were actually infected by *Entamoeba* spp., revealing a high over-diagnosis of *E. histolytica*. We also identified 9.3% *E. histolytica*, 4% *E. dispar* and 4.7% mixed infections. Diarrhea was the most common symptom, followed by abdominal pain and fever. Bloody stools were statistically associated with *E. histolytica*, but were also found for *E. dispar* infections. This study demonstrates that molecular techniques complementary to conventional methods enable the correct identification of *Entamoeba* spp., thus contributing to an improved epidemiological assessment of these parasites and implementation of the appropriate treatment.

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Palabras clave: Entamoeba, diagnóstico, PCR, *E. histolytica*.

Resumen. Esta investigación planteó detectar por nested PCR *Entamoeba histolytica* y *E. dispar* en niños del Hospital "Dr. Luis Razetti" de Barcelona, estado Anzoátegui y su asociación con síntomas clínicos. De 1.141 muestras fecales evaluadas parasitológicamente por microscopía convencional, 150 fueron positivas a *E. histolytica* en niños de 0-10 años y de ambos sexos. Se obtuvo información de signos, síntomas y reporte parasitológico completo de cada uno de los pacientes y se realizó nested PCR para identificar *E. histolytica* y *E. dispar*. Los resultados de la microscopía convencional demostraron una frecuencia de diagnóstico de *E. histolytica* del 13,2%. En el 79,3% de estas positivas se reportó esta especie como único patógeno. Sin embargo, la nested PCR evidenció que sólo 28,0% (42/150) de las mismas presentaron infecciones por *Entamoeba*, evidenciándose un elevado sobrediagnóstico de *E. histolytica*. Además se identificaron 9,3% infecciones por *E. histolytica*, 4,0% *E. dispar*, y 4,7% infecciones mixtas. La diarrea fue el síntoma más común, seguido de dolor abdominal y fiebre. La presencia de sangre demostró asociación estadísticamente significativa con *E. histolytica*, pero también se reportó en infecciones por *E. dispar*. Este estudio demuestra que las técnicas moleculares complementarias a los métodos convencionales, permiten la identificación correcta de especies de *Entamoeba*, contribuyendo con una mejor evaluación epidemiológica de estos parásitos y la aplicación adecuada del tratamiento.

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INTRODUCTION

Entamoeba histolytica is the causal agent of intestinal amoebiasis, one of the principal causes of mortality in humans worldwide (1-4). Other *Entamoeba* species, such as *E. dispar* and *E. moshkovskii* have also been found in patients with gastrointestinal symptoms (5-9). However, there is as yet no definitive evidence demonstrating that these two species are pathogenic to humans (10-12).

Clinical features of amoebiasis range from asymptomatic colonization to amoebic dysentery and invasive extra intestinal

amoebiasis, the latter in some cases in the form of liver abscesses (13). An estimated 50 million people suffer from this invasive disease worldwide, producing an annual death toll of between 40,000 and 100,000 (14, 15). Although this parasite is distributed throughout the world, prevalences exceeding 10% have only been reported from some developing countries (16). Despite the availability of effective treatments against *E. histolytica*, morbidity and mortality rates have persisted, suggesting that measures to eliminate or limit the disease are at present largely ineffective. Nevertheless, as humans are apparently the only

hosts, an appropriate control program should be able to eradicate the infection (17, 18).

E. histolytica infections are diagnosed based on the study of the clinical symptoms, as well as the microscopic examination of serial stool samples. The latter is a faster and easier procedure, but its sensitivity is limited and requires an experienced observer to accurately distinguish between pathogenic and non-pathogenic species; a serious cause of error for the correct diagnosis of the disease. For this reason, direct diagnosis by itself is considered to be insufficient for the identification of *E. histolytica* and should be complemented by fixation techniques such as permanent trichrome staining that permit the intracellular elements to be visualized more easily (19,20). Haque *et al.* (1), however, argue that trichrome and iron hematoxylin staining are not good methods for the detection of *E. histolytica*, because they do not differentiate between this and other non-pathogenic species which are morphologically identical.

The diagnosis of false negatives of *E. histolytica* is thus at least partly due to a delay in sample processing, short analysis time, analyses performed by technicians without adequate theoretical and practical training and a lack of complementary methods that help to improve the visualization of hematophagous trophozoites (21). False positives, on the other hand, occur due to the incorrect identification of non-pathogenic species of amoeba and host cells such as macrophages (22-24). The difficulties of differentiating *E. histolytica* from other *Entamoeba* spp. is, in many cases, the reason why the prevalence of this infection vary so greatly from one region to another (5).

In order to provide a solution to the problems of the diagnosis of *Entamoeba* related infections, rapid and highly sensitive Polymerase Chain Reaction (PCR)-based

protocols have been developed, and although several studies have compared the different methods of species-specific diagnoses (25-27), molecular techniques have been effective for the successful detection and differentiation of *E. histolytica* and *E. dispar* in clinical samples. Parija and Khaimar (7) analyzed 746 stool samples with cysts and trophozoites of *E. histolytica*, *E. dispar* and *E. moshkovskii*, using PCR to amplify the small rRNA subunit. These authors found a greater prevalence of *E. dispar* (8.8%) compared to *E. histolytica* (1.7%). *E. histolytica* was, in fact, only actually present (as diagnosed by PCR) in 19% of the 68 stool samples registered as containing *E. histolytica* by microscopic examination, implying that 81% of suspected infections were wrongly diagnosed and patients were thus unnecessarily treated with antiamoebic medication. This over-diagnosis of *E. histolytica*, has led to a re-evaluation of the epidemiology of amoebiasis in terms of prevalence and morbidity, particularly in geographical areas with high endemicity (19,28).

In Venezuela, 6,872,282 cases of diarrhea, mainly in children, were reported between 2007 and 2010. Of these, 574,225 were the result of amoebiasis, with Zulia State having the largest number of cases and Anzoátegui and Sucre being the most affected states in the eastern region of the country (29). In addition, changes in the frequencies of the *E. histolytica*/*E. dispar* complex have been reported, with high prevalences in different regions (30-33). The application of PCR by Rivero *et al.* (34) in Maracaibo (Western Venezuela), and Mora *et al.* (35), in Cumaná (Eastern Venezuela), have shown significant differences in the frequencies of infections by species of *Entamoeba* between the microscopic and molecular detection.

The fact that an important number of infections by *Entamoeba* spp. has been re-

ported in Venezuela, means that more appropriate, sensitive and specific methods that allow for the correct detection and differentiation of species within this genus should be implemented. We thus undertook this investigation in order to assess the real frequencies of *E. histolytica* and *E. dispar* in stool samples diagnosed as positive for *E. histolytica* and/or *E. dispar* by conventional methods in patients from Barcelona, Venezuela.

METHODOLOGY

Study population

A total of 150 stool samples collected from children aged 0-10 of both sexes, between January and August 2009, who attended the Pediatric Department "Dr. Rafael Tobías Guevara" at the Dr. "Luis Razetti" Hospital, Barcelona, Anzoátegui State, were diagnosed as microscopically positive for *Entamoeba histolytica*, when they were observed using wet preparations with 0.85% physiological saline solution and Lugol's solution. The physical characteristics of the samples such as: appearance, consistency, color, odor, pH, presence or absence of mucus, blood and/or adult worms, were noted as well as the signs and symptoms of the children at the time of the collection of the sample (fever, diarrhea, abdominal pain, among others). In addition, intestinal worms and protozoa were detected and identified microscopically in each of the samples.

The consent of the legal representatives of the children who participated in the study was sought in each case after informing them about the aims of the research. All of the methods used were previously approved by the Bioethics and Biosafety Committee at the Instituto de Investigaciones en Biomedicina y Ciencias Aplicadas, Universidad de Oriente (IIBCAUDO), Cumaná, Venezuela. Once the representative had au-

thorized the participation of their child or children and the stools had been processed by microscopy, 1 g of fecal matter was removed from each sample and placed in a 1.5 mL sterile Eppendorf tube with 500 μ L phosphate buffered saline (PBS) 1X. The tubes were then transported on ice to the Molecular Genetics Laboratory, IIBCAUDO, where they were stored at -20°C until analysis by PCR.

DNA extraction

Genomic DNA was extracted from the 150 stool samples using the Promega Wizard® Genomic DNA purification Kit, employing the protocol suggested by the manufacturer with the following modifications: 200 μ L of each of the fecal samples preserved in PBS were placed in a 1.5 mL Eppendorf tube, centrifuged at 14 000 g for 3 min at 18°C and the supernatant discarded. Then 400 μ L nuclei lysis solution was added and the mixture homogenized by pipetting the sediment. Cyst walls were fractured by sonication, using a high intensity sonicator (Autotone Ultrasonic) set at the minimum speed for 2 sec and repeated three times, with the samples held on ice. 10 μ L proteinase K was then added. Samples were incubated for 2 hours at 65°C and then left to cool for 5 min at room temperature. The extraction procedure was then continued using the protocol recommended by the kit and the DNA obtained stored at -20°C , until its subsequent amplification by PCR.

(PCR) polymerase chain reaction

The Nested PCR procedure has been proposed by the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh (ICDDR, B) (36), as well as the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India (7) as the optimal method for the detection and differentia-

tion of *E. histolytica* and *E. dispar*, using the 16S rRNA-like gene sequence as the genomic target.

Nested PCR was performed by first amplifying a fragment specific to the *Entamoeba* genus (E1-E2 primers 898 bp) and afterwards the fragments specific to *E. histolytica* and *E. dispar* (439 bp, and 174 bp, respectively) following the protocol described by Parija and Khairnar (7). A cultivated strain of *E. histolytica*; IULA-0593:2 (NER), donated by the Immunology Institute, Universidad de los Andes (ULA), Mérida, Venezuela; and a positive sample for *E. dispar*, UDO402 strain (35), were used as positive controls in each PCR run. The amplified products were separated by electrophoresis on a 2% agarose gel (EC330 Primo Minicell Gel Electrophoresis System) in Tris-boric acid-EDTA (TBE) buffer 1X at 80 V for 45 min, stained with ethidium bromide (0.5 µg/mL) and visualized in a ultraviolet transilluminator. The molecular marker used was the Axygen100 pb ladder.

Statistical analysis

The results obtained are shown in the tables and figures. Associations between the *Entamoeba* species assessed in this study with the age, sex and symptoms of the patients and the macroscopic characteristics of the fecal material, were tested

with the Chi-square (χ^2) test (37, 38), using version 11.5 of the SPSS statistical package.

RESULTS

E. histolytica was found in 13.2% (150) of the 1,141 stool samples analyzed microscopically by bioanalysts in the Pediatric Department Laboratory at the "Dr. Luis Razetti" Hospital, Barcelona, Anzoátegui State, of which 79.3% contained cystic forms and trophozoites of *E. histolytica* (Table I). In addition, in 19.3% of the samples, contained forms of other intestinal protozoa such as *Giardia duodenalis*, *Blastocystis hominis*, *E. coli* and *Chilomastix mesnili*; and in 1.4%, co-infections with the eggs of intestinal helminthes, such as *Trichuris trichiura* and *Hymenolepis nana*, were found (Table I), demonstrating a high prevalence of infections caused by intestinal protozoa in the samples analyzed. However, it must be emphasized that these samples were not concentrated as part of the clinical diagnosis, thus the frequency of helminthes and other intestinal protozoa observed may have been underestimated.

A total of 98.7% of the children showed symptoms related to the infection, with acute diarrhea being the most prevalent symptom (68%), followed by fever

TABLE I
FREQUENCY OF CHILDREN INFECTED WITH *E. histolytica* ONLY AND *E. histolytica* ASSOCIATED WITH OTHER INTESTINAL PROTOZOA AND HELMINTHES FOR EACH SYMPTOM OBSERVED

Symptom	<i>E. histolytica</i>	<i>E. histolytica</i> , <i>G. duodenalis</i> <i>B. hominis</i>	<i>E. histolytica</i> , <i>E. coli</i> <i>C. mesnili</i>	<i>E. histolytica</i> , <i>T. trichiura</i> <i>H. nana</i>	Total
Acute diarrhea	80	12	8	2	102
Fever	19	3	2	0	24
Abdominal pain	16	1	2	0	19
Dysentery	2	0	1	0	3
Asymptomatic	2	0	0	0	2
Total	119	16	13	2	150

(16%) and abdominal pain (12.7%) (Table I). When conventional microscopy was used as the diagnostic method, *E. histolytica* was identified as single infection in 79.3% of symptomatic and asymptomatic children and in only 12% of samples they were accompanied by other protozoa and pathogenic intestinal helminths. In 8.7% of samples, *E. histolytica* was found causing infections together with commensal protozoa such as *E. coli* and *C. mesnili* (Table I).

The first round of amplification of the nested PCR, corresponding to the 898 bp fragment specific for *Entamoeba* spp., allowed us to detect a 28% (42/150) infection rate by any of the *Entamoeba* species. The second round of amplification produced species specific fragments in all of these samples, of which 29 (19.3%) were identified as *E. histolytica*, 6 (4%) as *E. dispar*, and 7 (4.7%) as mixed infections by *E. histolytica/E. dispar* (*Eh/Ed*) (Figs. 1 and 2).



Fig. 1. Representative gels for the second round of the nested PCR showing the presence of *E. histolytica* as diagnosed from stool samples collected from the studied children. M: 100 bp molecular weight marker (Axygen). C+: *E. histolytica* NER strain positive control: 439 bp. Evaluated stool samples: wells 1-74. C-: negative control.

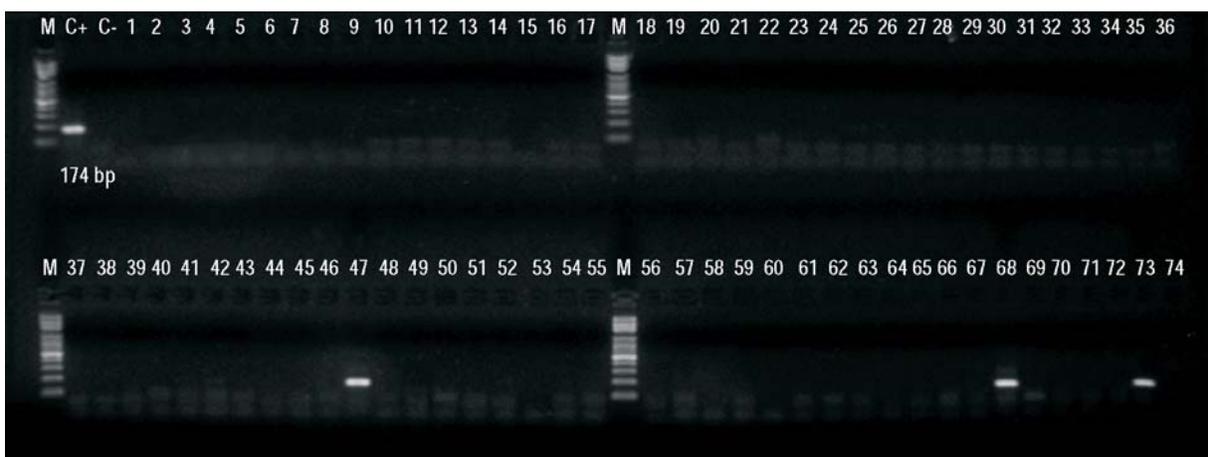


Fig. 2. Representative gels for the second round of the nested PCR showing the presence of *E. dispar* as diagnosed from stool samples collected from the studied children. M: 100 bp molecular weight marker (Axygen). C+: *E. dispar* UDO402 strain positive control: 174bp. Evaluated stool samples: wells 1-74. C-: negative control.

The PCR procedure applied to the 150 stool samples microscopically diagnosed as positive for *E. histolytica* showed that microscopic analysis over-diagnosed the incidence of amebiasis by 72% (108/150). As already mentioned above, PCR showed that only 42 of the 150 samples were, in fact, positive for the *Entamoeba* species diagnosed in this investigation.

Furthermore, PCR revealed that in 89.7% (26/29) of infections, *E. histolytica* was the only parasite involved, whereas in 10.3% (3) of the cases there was a co-infection with *E. coli* and *C. mesnili*. In those infections caused by *E. dispar* and mixed *Eh/Ed* infections no other parasites were found, except for a mixed infection with *E. coli* and *C. mesnili*. It should be noted that *E. histolytica* was not detected in samples where it had been microscopically diagnosed as co-infecting with other protozoa and pathogenic intestinal helminthes. This could be due to the difficulty of differentiating *Entamoeba* spp. from host cells when using SSF and Lugol's solution as the staining agents during parasitological diagnosis.

Regarding the symptoms associated with the infections (Table II), diarrhea was also the most common symptom of infection by *E. histolytica* (62.1%), followed by abdominal pain (20.7%) and fever (13.8%). Diarrhea was also present in 66.6% of all *E.*

TABLE II
FREQUENCY OF THE SYMPTOMS SHOWN BY THE CHILDREN UNDER STUDY FOR EACH OF THE INFECTING AGENTS AS DIAGNOSED BY PCR

Symptoms	Eh	Ed	M
Acute diarrhea	18	4	5
Fever	4	2	1
Abdominal pain	6	0	1
Dysentery	0	0	0
Asymptomatics	1	0	0
Total	29	6	7

Eh: *Entamoeba histolytica*. Ed: *Entamoeba dispar*. M: mixed Eh/Ed infection.

dispar and mixed *Eh/Ed* infections, and 33.3% of children infected by *E. dispar* also suffered fever. These observations thus show that both *E. histolytica* and *E. dispar* may be found in the same group of individuals with intestinal symptoms.

We could not find any species specific pattern as regards stool consistency when we macroscopically examined the PCR positive stool samples (Table III), as both soft and liquid stools were infected by either species. Mixed infections did, however, predominate in liquid samples (71.4%). Similarly, the presence of blood was not necessarily indicative of pathogenic species, although it

TABLE III
MACROSCOPIC CHARACTERISTICS OF THE PCR-POSITIVE AND NEGATIVE STOOL SAMPLES COLLECTED FROM THE CHILDREN UNDER STUDY

PCR Results	N	Consistency		Mucus	Blood
		Liquid	Soft		
<i>E. histolytica</i>	29	10	19	28	17
<i>E. dispar</i>	6	3	3	6	3
<i>E. histolytica/E. dispar</i>	7	5	2	6	4
Negative	108	47	61	0	0
Total	150	65	85	40	24

was statistically associated with *E. histolytica* ($\chi^2 = 4.753$; $P < 0.05$) (Table IV). Lastly, mucus was found in the fecal samples of most of the *E. histolytica*, *E. dispar* and mixed *Eh/Ed* infections (Table III).

DISCUSSION

In general, infections caused by intestinal protozoa predominated in the children examined. In this regard, authors such as Ouattara *et al.* (39) indicate that poly-parasitism by intestinal protozoa is fre-

quent (80.2%) in the 6-16 year-old age-group, with the most common species being, *Endolimax nana*, *E. coli*, *G. duodenalis* and the *Eh/Ed* complex; this last with a prevalence of 11.3%. This confirms the presence and range of these micro-organisms and indicates that an understanding of their distribution in areas of transmission of pathogenic protozoa could aid the development of disease control programs that combine treatment with prevention.

With respect to the prevalence of *E. histolytica* in Venezuela, large variations in

TABLE IV
SUMMARY OF THE RESULTS OF THE χ^2 ANALYSIS OF THE ASSOCIATION OF THE MACROSCOPIC CHARACTERISTICS OF THE STOOL SAMPLES, AGE, SEX AND GASTROINTESTINAL SYMPTOMS OF THE CHILDREN WITH THE PRESENCE OF *E. histolytica* AND *E. dispar* AS IDENTIFIED BY PCR

Species	Variable	χ^2	p
<i>E. histolytica</i>	Sex	0.068	0.794
	Age	1.071	0.586
	Diarrhea	1.033	0.309
	Fever	0.000	1.000
	Consistency	0.054	0.973
	Mucus	0.220	0.639
	Blood	4.753	0.029*
	pH	1.397	0.237
	Leucocytes	0.070	0.792
	Red bloodcells	0.130	0.718
<i>E. dispar</i>	Sex	0.004	0.950
	Age	0.274	0.872
	Diarrhea	0.273	0.601
	Fever	0.421	0.515
	Consistency	2.410	0.300
	Mucus	1.358	0.244
	Blood	0.727	0.394
	pH	0.007	0.935
	Leucocytes	1.125	0.289
	Red bloodcells	0.000	0.997

*Significantly associated variable, $P < 0.05$.

different regions of the country have been reported. In the West, a prevalence of 4% for the *E. histolytica*/*E. dispar* recomplex was detected by parasitological analysis (33). A study of the Yukpa ethnicity in rural areas of Zulia state, showed a prevalence of 21.9% *E. histolytica* infections in children aged 0-14 (32). In addition, Rivero *et al.* (34), obtained a prevalence of 10.8% *E. histolytica* infections in Maracaibo, Zulia state using PCR, compared with that of 20.6% mixed *E. histolytica*/*E. dispar* infections diagnosed by parasitological analysis. These researchers found no significant associations between infection by these species and age and/or sex.

In Bolívar state (southeast Venezuela), Devera (40) reported the absence of this intestinal protozoa after microscopic analysis. However, Mora *et al.* (35) found a prevalence of 5.4% for *E. histolytica* and 3.5% for *E. dispar* infections using molecular detection by PCR in individuals with gastrointestinal symptoms in Cumaná, Sucre state. According to recent reports there is a higher prevalence of *E. histolytica* in Sucre state, compared to other states. This coincides with our results for Barcelona, Anzoátegui state, showing higher frequencies in North-eastern Venezuela, compared to the South-eastern area.

Our study agrees with that of Mora *et al.* (35), in that both indicate an important over-diagnosis of pathogenic species by parasitological methods. They reported 51.2% false positives from conventional microscopic analysis. On the contrary, Rivero *et al.* (34) found that under-diagnosed by 10.6% by microscopic examination: 42 positive samples were detected by this method compared to 47 positives by PCR, however only 22 (10.78%) of these were identified as *E. histolytica*.

The tendency of microscopic analysis to produce false results due to confusion between macrophages and trophozoites, and

polymorphonuclears (PMN) and cysts, leading to the incorrect identification of amoebas, is well known (19, 22, 23). Thus, the current recommendation when species specific diagnosis is not possible is to report: "*E. histolytica*/*E. dispar*/*E. moshkovskii*" in order to describe the presence of species with identical morphologies in stool samples. Researchers and technicians are also encouraged to use new technologies wherever possible in order to elucidate the true epidemiology and pathogenesis of *Entamoeba* spp., including the least studied *E. moshkovskii* (41).

The fact that 108 samples were diagnosed by conventional microscopy as positive for *E. histolytica*, but were found to be negative for this species by PCR, may be explained by the presence of intestinal amoebas morphologically similar to *E. histolytica* such as *E. hartmanni* and *E. polecki*. This last, although it is a parasite of monkeys and pigs, and is uncommon in humans, has been reported from the latter on eight occasions in Venezuela (42-45). *E. hartmanni*, on the other hand, has often been reported from western regions of Venezuela since 1976, using staining and concentration techniques (31, 45, 46-50). All these studies have pointed out that *E. hartmanni* may be less frequently detected in fresh stools or concentrated samples due to its small size, or because its morphological features make it indistinguishable from other *Entamoeba* species, thus hindering a specific diagnosis.

The macroscopic characteristics of the stool are important to indicate the correct diagnostic methodology to use in the search for intestinal pathogens. In this study, however, the characteristics of PCR-positive samples (consistency and presence of mucus) were not reliable indicators of the presence of the different *Entamoeba* spp. Nevertheless, the presence of blood, although not exclusive to one pathogenic species, did show a statistically significant association

with *E. histolytica*. Different research papers have pointed out that, while *E. dispar* is considered to be a commensal amoeba, it has also been identified in patients with gastrointestinal symptoms (5, 9, 38, 51). We now know that hematophagism is not exclusive to pathogenic amoebae, since it has been demonstrated that *E. dispar* cells from stools samples can contain red blood cells inside, which has been corroborated by *in vitro* studies, showing the ability of *E. dispar* to ingest red blood cells (5, 53).

The difficulties of correctly identifying *Entamoeba* spp. has led to the use of PCR-based procedures as the method of choice for clinical and epidemiological studies in developed countries (54-57), a practice strongly endorsed by the World Health Organization. The use of molecular biology techniques has enabled the identification of *E. histolytica* in a variety of clinical specimens, including feces, tissue, liver abscesses and aspirates (19).

Lastly, according to our experience at the Molecular Genetics Laboratory (LGM) at the IIBCAUDO, the application of PCR for the diagnosis of intestinal amoebiasis, as well as more sensitive and rapid molecular techniques (58,59) that are less expensive and can be applied in the field (60), provides a complementary method to the conventional protocols that enables the correct identification of *Entamoeba* spp. Thus, PCR provides us with an extremely useful tool for a better understanding of the biology, diagnosis and epidemiology of these species in different regions.

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