ARTÍCULO ORIGINAL

Entamoeba histolytica and Entamoeba dispar: differentiation by Enzyme-Linked Immunosorbent Assay (ELISA) and its clinical correlation in pediatric patients

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ABSTRACT

Studies were carried out at a mexican pediatric hospital to determine the ratio between the pathogenic species *Entamoeba histolytica* and non-pathogenic species *E. dispar* using an enzyme-linked immunosorbent assay (ELISA) to detect the lectin (1 galactose N-acetyl D-galactosamine) of *E. histolytica* in feces. A close correlation was noted between the presence of the *E. histolytica* lectin and clinical symptoms. In the study, amebas were detected by microscopy in 120 children (either *E. histolytica* or *E. dispar*). But while almost all (13/14) of the children with *E. histolytica* had clinical symptoms, dysentery-feces with mucus and blood, diarrhea, cramping abdominal pain, tenesmus rectal, flatulence, vomiting and headache, almost none (1/106) of the children infected with the non-pathogenic ameba *E. dispar* had signs and symptoms. This suggests that much of the amebiasis diagnoses made in Mexico are, in fact, due to non-pathogenic *E. dispar*.

Key words: *Entamoeba histolytica/E. dispar*, Entamoebosis intestinal, Clinical laboratory techniques.

INTRODUCTION

Studies on the prevalence of intestinal amebiasis in Mexico have been carried out under microscopic detection of amebas (either *Entamoeba histolytica* or *E. dispar*). Published reports show significant differences in the findings, from 79%¹, 38%², 23%³, 19 %⁴ to 10%⁵. In 2004, the Mexican Ministry of Health reported 5,467,208 cases of intestinal infectious diseases, it is including ameba infections, the majority in tropical areas⁶. Worldwide intestinal amebiasis is frequent, with approximately 500,000,000 persons infected every year^{7,8}.

Of the vast number infected, the great majority are infected with *E. dispar*, a non-pathogenic parasite, which explains, in part, the low percentage of disease in infected persons^{4,8}. It is now estimated that only 10% of reported cases are due to *E. histolytica*^{9,10}.

Since 1925, has been proposed the existence of two species of ameba, one pathogenic, the other non-pathogenic¹¹. In the pathogenesis of the disease, *E. histolytica* cysts invade the terminal ileum, each quadrinucleate cyst giving rise to eight unnnucleated trophozoites. The trophozoites adhere primarily to the cecum, sigmoid colon and rectum, and begin to divide

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and bind to exposed target cell glycoproteins through N-acetyl-D-galactosamine (galactose) adhesin⁸.

The galactose adhesins of *E. histolytica* can be differentiated from those of *E. dispar* by the use monoclonal antibodies, resulting in a diagnostic ELISA test¹². Unfortunately, most laboratories still diagnose amebic infection solely by microscopic examination of fecal specimens¹³. Recent data have led to a re-description of the two amebic species. *E. histolytica* has been shown to be the causative agent of amebiasis disease, thus separating it from *E. dispar*, a non-pathogenic species^{14,15}. Because of this, the World Health Organization now suggests that amebic diagnosis based only on microscopy is inadequate since it fails to differentiate *E. histolytica* from *E. dispar*; such “double” diagnoses must then be joined in to as *E. histolytica/E. dispar*¹⁶. In our country, few studies have been carried out for differentiate the two ameba species^{4,8}. We report here a study of 120 children with ameba infection (positive by microscopy) in which *E. histolytica* was differentiated from *E. dispar* by ELISA test; in addition, the infection could be correlated with the presence of clinical symptoms.

MATERIALS AND METHODS

This investigation was carried out at a third-level pediatric hospital in Mexico City. The children enrolled for this study were examined for the presence of common intestinal parasites by microscopy and produced stools which were positive for *E. histolytica/E. dispar* trophozoites or cysts. Three stool samples were obtained on three-day intervals from each of the children. The parents of all of the children signed a form of consent which gave full explanation about the purpose and the techniques of the study. The study protocol were reviewed and approved by the Committees of Investigation, Ethics and Biosecurity of the HIMFG.

Laboratory Techniques Used

Microscopic examination for amebas in feces: Three fecal samples were taken from each child and processed the same day: direct smears (saline and iodine), and examinations by concentration (flotation) with zinc sulfate were prepared from each sample and examined

microscopically at low (20x) and high (40x) magnifications. These examinations were carried out in accordance with NCCLS recommendations¹⁷.

Identification of parasites in feces: The identification of *E. histolytica/E. dispar* trophozoites was made by the characteristic movement of the protozoan and the presence of phagocytized red blood cells. The identification of amebic cysts (*E. histolytica/E. dispar*) was based on morphologic characteristics, (10-15 µm, spherical form, mature tetranucleated cysts having a central endosome). The identification of other commensal and pathogenic parasites was made from morphological characteristics¹⁸.

Enzyme-linked immunosorbent assay (ELISA) for detection of galactose/N-acetyl D-galactosamine lectin for *E. histolytica* in stools; (*E. histolytica* Test TechLab, Blacksburg, VA, USA): Only fresh samples were used, obtained no more than 48 hours prior to testing; no specimens treated with formalin or other preservative (SAF or PVA) are allowed.

Overall, assays were done in duplicate, with one well per sample. In the test, one drop of conjugate (mouse monoclonal antibodies specific for adhesin from *E. histolytica*; coupled to horseradish peroxidase) were added to a well (one per each sample) in a 96-well plastic plate previously coated with polyclonal antibodies binding adhesin. Then, 0.2 ml of a diluted fecal specimen were added at room temperature to the well. A positive and negative controls were included in each test. After two hours incubation, the liquid in the well was decanted and washed with phosphate buffered saline. After washing, the residual fluid was removed by striking the inverted plate on a paper towel. One drop of substrate (tetra methyl benzidine TMB) was added and the wells were incubated for 10 minutes; finally, a drop of stop solution (sulfuric acid 1.0 M) was added to prepare the liquid for measuring on a ELISA reader at 450 nm. The instrument should be blanked against air. A positive result was defined as an optical density reading of >0.05 (after subtraction of the negative control optic density). The TechLab *E. histolytica* test was used according to manufacturer's instructions¹².

Clinical Charts: The clinical charts of all of the 120 children were revised under the supervision of the principal investigator and a

pediatrician resident and were examined for evidence of intestinal amebic infection on the date of stool sampling. This included dysentery (three to five muco-sanguineous evacuations per day with moderate colic pain), diarrhea (more than two soft stools within the past 24 hours), cramping abdominal pain (stomach ache similar to cramping), rectal tenesmus, flatulence, vomiting and headache. Asymptomatic when the patients haven't had any gastrointestinal symptoms.

Statistical analysis. The percentage of results was calculated. The patient was considered to be true positive if the children had clinical symptoms and *E. histolytica* adhesin test positive, and considered true negative if the children was asymptomatic with *E. histolytica* adhesin test negative (*E. dispar* positive). The data were calculated in the contingency table 2x2. The correlation percentage between ELISA *E. histolytica* adhesin test and the clinical symptoms was taken to be the total number of true positive plus the total number of true negative result among the total number of patients studied per 100.

RESULTS

During the 2000-2002 study period, one hundred and twenty children with amebic *E. histolytica*/*E. dispar* trophozoites or cysts in feces were detected by microscopy. Their ages varied from 9 months to 17 years of age: 7 (6%) were under 2 years of age, 19 (16%) were 2 to 5 years old, 63 (52%) were 5 to 12 years old, and 31 (26%) were 12 to 17 years old. There were 52 females and 68 males. Cysts forms morphologically attributable to *E. histolytica*/*E. dispar* were identified by microscopy in 95% (114/120), and trophozoites forms in 5% (6/120) of the total. Of the 120 patients with *E. histolytica*/*E. dispar*, 34 (28%) had a single parasite infection, 49 (41%) had additional non-pathogenic parasites (*Entamoeba coli*, *Endolimax nana*, *Iodamoeba bütschlii* and *Chilomastix mesnili*), 22 (18%) had other pathogenic parasites (*Giardia lamblia*, *Blastocystis hominis*, *Hymenolepis nana*, *Trichuris trichiura*, *Ascaris lumbricoides* and *Strongyloides stercoralis*), 15 (13%) had both non-pathogenic and pathogenic parasites. Eighty six children (72%) with *E. histolytica*/*E. dispar*, had more that one parasitic species. Ninety two

percent (110/120) had only protozoa, 2% (3/120) had helminths, and 6% (7/120) of subjects were infected by both. Differentiation into *E. histolytica* and *E. dispar* by ELISA tests revealed 12% (14/120) of patients to be positive for *E. histolytica* versus 88% (106/120) of patients to be positive for *E. dispar*. ($p < 0.01$) (Figure 1). When the clinical symptoms were correlated to the ELISA test, it was noted that 13 of the 14 children positive for *E. histolytica* had gastrointestinal symptoms, while only one child infected with *E. dispar* had symptoms (Figure 2). The correlation between the presence of *E. histolytica* in feces and gastrointestinal symptoms was 98% (118/120) (Table 1).

Figure 1. Enzyme-linked immunosorbent assay (ELISA) for detection of galactose / N-acetyl D-galactosamine lectin for *E. histolytica* in stool. Distribution of results from ELISAs of stool specimens from 120 children with *Entamoeba histolytica* and *Entamoeba dispar*. Optical density (O.D.) determined by TechLab *E. histolytica* test were plotted for each stool sample. Optical density > 0.05 after subtracting negative control were considered positive for each test, (cut off).

Table 1. Correlation of *Entamoeba histolytica* and *Entamoeba dispar* with clinical symptoms from 120 patients

ELISA test	Symptomatic	Asymptomatic	Total
<i>E. histolytica</i>	13*	1	14
<i>E. dispar</i>	1	105**	106
Total	14	106	120***

* The total number of the true positive children (*E. histolytica* plus symptomatic patients).

** The total number of the true negative children (*E. dispar* plus asymptomatic patients).

*** The total number of patients studied.

Figure 2. Clinical symptoms in 120 children and their correlation to the presence of either *E.histolytica* or *E.dispar* in feces. *E.histolytica* with symptoms was found in 13 cases, without symptoms in one case. *E.dispar* with symptoms in one case and *E.dispar* without symptoms in 105 cases.

DISCUSSION

In Mexico, the morbidity and mortality of amebiasis has declined in recent years. Nevertheless, it is known that prevalence in rural communities is higher than in urban zones; amebiasis often affects people of low socioeconomic status, with practices that favor fecal-oral transmission.

The classical method for diagnosis has been microscopy, although that test does not discriminate between *E. histolytica* and *E. dispar*^{12,13}. It is remarkable that, whenever an alternative method to differentiate these two types of ameba was used, the majority (88%) were *E. dispar*. When the clinical histories were compared, 13 of the 14 children (93%) infected with *E. histolytica* were found to have symptoms, while in the group infected with *E. dispar* only one of 106 (1%) was found symptomatic, a 98% correlation between *E. histolytica* and the presence of symptoms. Similar results were previously reported in a small village of Ecuador, where the majority (88.8%) of the population studied had *E. dispar* and did not show any symptoms, and only the 11.2% with symptoms were infected with *E. histolytica*¹⁹. *E. dispar* was more prevalent in another group studied in the Philippines²⁰, and colonization with *E. dispar* was 3-7 times more frequent than with *E.*

histolytica in non-symptomatic children in Bangladesh²¹. A study in Kilimanjaro indicated that *E. dispar* infection was 14.4 time more prevalent than *E. histolytica* infection²². Furthermore, our results demonstrate that *E. dispar* was seven times more frequent than *E. histolytica* in children.

Those children found infected with amebas in hospitals in Mexico are treated for amebic infection, in spite of the fact that the majority are most likely infected with a type of ameba that does not cause symptoms. The anti-parasitic treatment of those persons can be considered unnecessary, as suggested by recommendations published by the World Health Organization¹⁶. In our study the presence of symptoms in the majority of patients with *E. histolytica* (13/14) and the high correlation (98%) between symptoms and the presence of *E. histolytica* suggest the diagnosis of invasive ameba and indicate that treatment should be administered only in those cases. In a previous report of children in Puebla, Mexico, the authors found no relation between *E. histolytica* and the classical clinical manifestations, but they failed to differentiate *E. histolytica* from *E. dispar*, and the majority of children were probably infected with *E. dispar*¹. In our results, we observed six fecal specimens with trophozoites with ingested red blood cells in the 13 symptomatic children (of the 14 positive for *E. histolytica*); other authors suggested that erythrophagocytic ameba is specific and predictive of infection with invasive *E. histolytica* and their results correlated with zymodeme analysis¹³. We found that microscopic observation of erythrophagocytic *E. histolytica* trophozoites correlated with the ELISA test. The discrepancy between the number of infected persons and the occurrence of amebiasis disease could be explained by the high frequency of non-pathogenic *E. dispar*. We found two children in the which the relation between *E. histolytica* or *E. dispar* infection and clinical symptoms was unexpected. The patient with *E. histolytica* but without symptoms was a girl with diagnosis of glomerulopathy who frequently took anti-parasitic treatment. The child infected with *E. dispar* who had symptoms had a concomitant infection with *G. lamblia*, suggesting symptomatic giardiasis.

Several sophisticated techniques are available for differentiation of these two related species

such as PCR, real-time PCR and isoenzyme characterization^{23,24}. Few mexican laboratories discriminate between the two species since they require well-trained personnel and more time⁴, but the ELISA for *E. histolytica* fecal adhesins permits rapid detection and can be used for specimens submitted for routine clinical testing from adults or children. In addition ELISA test is highly sensitive, as little as 0.2-0.4 ng of parasitic antigen can be detected from stool samples. Indeed, it has been shown to be as sensitive and specific as culture with isoenzyme analysis^{25,26} and the amebic liver abscess can be diagnosed by detection of circulating antigen²⁷.

In conclusion, our investigation shows a low incidence of amebiasis infection in our hospital and demonstrated that *E. dispar* is the predominant species found among children. The routine use of a practical (ELISA) test to differentiate *E. histolytica* from *E. dispar* would help to determine the true prevalence of the two species in our country for future decision about the treatment. Moreover, we might suggest that in patients with gastrointestinal symptoms the microscopic examination of *E. histolytica*/*E. dispar* could be attributed to pathogenic *E. histolytica*.

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Acknowledgments. We are very grateful to Dr. Beatriz Zepeda Gutiérrez (pediatrician resident) for her participation in the revision of clinical charts.

FE DE ERRATA:

En el trabajo de los autores Calderón-Argueda O. et al., publicado en nuestro fascículo Vol 60 (Nº 3-4) 2005, se cometieron algunos problemas de edición, y uno de ellos el cometido en la Tabla 1, hace que el trabajo sea ininteligible. Por tanto, hacemos la corrección en esta fe de errata, editando la tabla 1 en forma correcta.

Tabla 1. Promedio de larvas de *Synthesiomyia nudiseta* colectadas por día durante los cuatro ciclos de observación

DPE	Ciclos de Muestreo							
	I		II		III		IV	
	Media	DE*	Media	DE*	Media	DE*	Media	DE*
9			6,0	5,6			16,3	27,4
11			3,0	5,2			35,7	14,3
14			2,7	4,6	16,0	17,7	42,3	6,1
16			5,0	8,7	37,0	41,2	28,7	2,1
18	5,3	9,2	11,3	15,5	38,0	26,1	46,7	25,8
21	5,0	8,6	23,7	37,5	40,3	29,6	3,3	3,0
23	1,6	2,9	12,7	21,9	24,3	28,0	1,7	2,9
25	0,6	1,1	13,3	23,0	35,7	36,0	1,7	2,9
28	0,3	0,5	12,7	21,9	29,3	25,6		
30	3,6	6,3	9,3	16,2	31,7	27,8		
32	1,3	2,3	8,3	14,4	27,3	30,3		
35	0,3	0,5			7,0	12,1		
37	0,0	0,0						
39	0,0	0,0						
42	0,0	0,0						
44	0,0	0,0						
46	2,3	4,0						
49	1,0	1,7						
51	0,7	0,5						
53	0,0	0,0						
55	2,0	3,5						
57	5,0	8,7						
59	2,3	4,0						
62	0,3	0,5						
64	0,0	0,0						
66	0,3	0,5						
69	0,0	0,0						
71	0,0	0,0						
73	0,0	0,0						
76	0,3	0,5						
78	1,3	2,3						
80	2,7	4,6						

* Desviación Estándar.