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Research Article

Detection and differentiation of E.histolytica and E.dispar by PCR in Al-Dewaniyah city

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ABSTRACT
Differential distinguishing proof of E.histolytica and E.dispar is fundamental for both fitting patient treatment and plague intelligent purposes. To decide the predominance of these single adaptable cell diseases in Al-Dewaniyah city, a PCR examine utilizing explicit introductions for every species was normalized and applied. 204 feces tests were investigated through direct minuscule assessment with SSF (0.85%) and lugol, formol-ether focus, and PCR. Under direct micros-duplicate, 42 people (20.58%) introduced the E. histolytica/E. dispar complex. In the interim PCR indicated 47 positive cases for these single adaptable cells: 22 E. histolytica (10.78%), 16 E. dispar (7.84%), and 9 (4.41%) blended diseases. There was not cant distinction within the sight of E. histolytica and/or E. dispar as indicated by either sex or age. There were no instances of these one-celled critters in youngsters under 2 years old. Watched recurrence of E. histolytica (31/204) shows the endemic idea of single adaptable cell disease in this network. Entamoeba ; Parasites; Defecation; Microscopy; Poly-simple Chain reaction.

Keywords:E.histolytica,E.dispar

INTRODUCTION

The term amebiasis defines all cases of human infection with Entamoeba histolytica, regardless of presence or absence of clinical manifestations in the individual. To what Over time, multiple unknowns accumulated nitas related to case variations symptomatic and asymptomatic in patients infected with this amoeba. This generated the the existence of pathogenic strains and not pathogens of E. histolytica [1,2,3] as well as the existence of two different species, but with identical morphological characteristics [4] . Multiple biochemical, immunological and genomic studies allowed that finally in 1997, the experts recognized the existence of two different species of Entamoeba: E. histolytica as the causative species of invasive and extra-intestinal disease and E. dispar as the non pathogenic species [5] . Acceptance of the existence of E. dispar changes dramatically the epidemiology of amebiasis and leads to estimates of the actual prevalence of E. histolytica worldwide are reinterpreted. Despite having clarified this situation, a number of problems related to the diagnosis of amebiasis. On the one hand, ignorance of the new classification by many profes- professionals in the health area, and on the other, laboratory methodology now required for the differentiation of E. histolytica and E. dispar. microscopic examination is extremely subjective, depending to a high degree on the observer's ability to differentiate the morphology of the protozoan morphology of other species of commensal amoebae such as: E. coli , E. hartmanni , Iodamoeba butschlii or Endolimax nana , as well as other elements such as leukocytes and macrophages and not lead to over-diagnoses amebiasis. For all the above it is imperative to recognize that special tests, such as screening techniques antigens specific to E. histolytica or polymerase chain reaction techniques (PCR) in order to discriminate the presence of E. histolytica and / or E. dispar in the feces of a determined , the deficient infrastructure structures and low budgets existing in the regional public health centers, limit the application of these techniques in laboratories. This difficulty has prevented establishing the truth prevalence of amebiasis in the state and in the country. This research aims to standardize a PCR technique that allows the identification and di-differentiate E. histolytica and E. dispar in individuals from a community in the Maracaibo municipality, Zulia State, Venezuela, as a pioneering study in determining the exact prevalence of these amoebas in our region.

MATERIALS AND METHODS

Population

A descriptive study was carried out, not experimental mental in individuals in the community of **Al-Dewaniyah city**, in the period from January to July 2006, this means that some houses are built on land firm and others on the water. The facts that influenced the choice of this community were, the size of it, its precarious hygienic-sanitary conditions and proximity to the University of Zulia. As a criterion for inclusion sion was required not to have ingested medications antiparasitics at least six months before of taking the sample. Consent was obtained written consent of the parents, representatives or heads of family in the community, which allows had 204 individuals agree to participate in the study and met the requirements of the same. Samples and parasitological procedure One fecal specimen was collected per individual, in a large, new, clean plastic container wide mouth and screw cap. These samples are kept refrigerated (4 ° C) and without treatment any chemical, until its processing in the Laboratory of Parasitology of the School of Bio-analysis of the University of Zulia. Each sample This was divided into two parts, one of them being gelled at -20 ° C, for subsequent molecular analysis (PCR) and the other portion was kept unfrozen for parasitological analysis. This consisted of the fresco montage with SSF and lugol, as well as the formaldehyde-ether concentration method [8] , for identify the presence of some evolutionary form of Entamoeba .

Molecular characterization of E. histolytica and E. dispar

The extraction of genomic DNA from Entamoeba sp. from stool samples, approximately 0.5 to 0.7g into a 2mL Eppendorf tube, re-suspended in 1mL of PBS and filtered through double gauze mesh. It is sterile. The homogenate was centrifuged at 10,000rpm and the resulting sediment was washed three times with 1.5mL of PBS to remove con- soluble taminants. The resulting sediment is washed with 1mL 0.15M NaCl three times or until that the supernatant was clear. Sediment was resuspended in 600mL of lysis buffer and entered 5 freeze-thaw cycles, incubating the tube in dry ice-isopropylic for 5 minutes and thawing at 37 ° C for three minutes. After the last treatment, 10mL of 20mg / mL proteinase k was added and it was incubated at 55 ° C overnight. Up to date next, 60mL of CTAB-NaCl (0.7M NaCl, 1% CTAB) and incubated at 65 ° C for one hour. An extraction was made with 500mL of chloroform, then with phenol-chloroform and chloroform. I will be- collected the aqueous phase into another Eppendorf tube and the DNA was precipitated with 600mL of isopropanol, incubating it at room temperature for 45 minutes and then centrifuging for 30 minutes cough at 14,000rpm. The sediment was re-suspended in 50mL of TE buffer. 10mL of the sample was used for amplification assays. The same treat- procedure was followed for DNA extraction genomic from E. histolytica cultures IULA: 1092: 1. In these cases, 2.5mL of DNA for PCR amplification assays controls. A reaction mixture was prepared for a final volume of 50mL, consisting of 10mL of 10X Go taq DNA polymerase buffer (Promega), oligo-nucleotide (primers) 50mM (1mM each oligonu-cleotid). 0.5mL of Taq DNA polymerase was used 5U / mL for each reaction. This reaction mixture was used for PCR amplification of se- SRPEh 5 \ 'gene sequences -> CTTGAAAAG CTTGAAGAAGCT G 3 '; 3 \ 'AAC AAT GAA TGG ACTTGA TGC A - <5 '; and SRPEd 5 \ '-> GTA GTT CAT CAAACA CAG GTG A 3 '; 3 \ 'CAA TAG CCA TAA TGAAAG CAA - <5 \ ´; including oligonucleotides specific for each reaction. For amplification of E. histolytica genome sequences and E. dispar , two pairs of oligonucleotides targeting the SREHP gene sequence, whose specificity has been previously reported you 10. The oligonucleotides SRPEd5 / 3 are specimens of E. dispar and generate a fragment of 567bp, while the oligonucleotides SRPEh5 / 3 specific to E. histolytica generate a 553bp fragment. The preparation of the PCR mixes was carried out carried out in a sterile work area and for reactions amplification times, a thermos-cycling was used. MJ Research PTC-100 (GMI Inc.). The PCR products were separated on gels agarose in horizontal chambers (Bio-Rad Laboratoires). The concentration of agarose used day was 1%. As run buffer, we used TBE (89mM Tris-Borate, 2mM EDTA pH 8). The Running was carried out at 40v / cm for 2-3 hours. The gels were stained with ethidium bromide, visualized in ultraviolet transilluminator and photo to graphed with photodocumentation system DigiDoc UVP. Molecular weight marker was included. cular 100bp DNA Ladder from Promega.

Statistical analysis

The statistical package was used for the analysis. co Statgraphics Plus version 5.1 for Windows (Statistical Graphics Corp., Herndon, United United). using the Z test. by the Pearson's Chi-square statistic. A value of $p < 0.05$ was considered as the critical level of significance [16].

RESULTS AND DISCUSSION

Evaluation of parasitic infections in community, showed that the population is high- mind infected by enteroparasites: (177/204) 86.77% general prevalence (Table 1). The most frequently observed parasites were protozoa, some human pathogens and others related to fecalism.

The prevalence of E. histolytic a / E. dispar detected by microscopic examination was 20.58% (42/204). Other studies carried out in the indigenous communities and educational institutions Zulia State reflect similar values, which cilan between 7.3% and 27% [17,18,19] . Were not observed hematophagous trophozoites, so In this case, the presence of E. histolytica using this procedure. The recognition of E. histolytica as is-pathogenic species and E. dispar as a non-toxicity, and their classification as separate species Rare, but microscopically indistinguishable, have induced the World Organization for Health (WHO) to recommend the development and application of specific methods for diagnosis of E. histolytica 20 . Various publications have reviewed by PCR as the most sensitive method and specific for the diagnosis of amebiasis [15,21-24] and have proposed it as the "test of gold "to determine this infection. The PCR technique described here demonstrated safe detection and differentiation of the two species that make up the E. histolytica / E. dispar , by DNA extraction directly from fecal samples without effect take previous cultures (Figure 1). The sensibility and specificity of PCR for the diagnosis of E. histolytica was 87% and 91% respectively; while for E. dispar it was 92% and 89%. The results of the PCR test applied to the studied samples are shown in Table 2. The species E. histolytica was detected in 22 of 204 fecal samples (10.78%), E. dispar was observed in 16 samples (7.84%) and both species of Entamoeba were detected in 9 samples (4.41%). The overall prevalence of infection by E. histolytica [(Eh + (Eh + Ed)] in the community studied, 31 cases (15.19%) was greater than the infection by E. dispar [(Ed + (Eh + Ed)], 25 cases (12.25%). The occurrence of mixed infections between E. histolytica and E. dispar has been reported previously given 9 . The results of the Z test ($p < 0.01$) showed that the frequency of E. histolytica is significant for this population. Although many authors [9-11,21,25-30] point out a higher prevalence of E. dispar with relation to E. histolytica , the finding of a higher number of cases of the latter (15.19%) no surprising for this community, in under the deplorable hygienic conditions

Table 1: Prevalence of parasitic species identified in stool samples by light microscopy *.

Parasitic species	n	%
Protozoa		
Blastocystis hominis	110	53,92
Entamoeba coli	43	21,08
Complex E. histolytica/E. dispar	42	20,58
Giardia lamblia	40	19,61
Endolimax nana	26	12,75
Chilomastix mesnili	7	3,43
Iodamoeba butschlii	4	1,96
Pentatrichomonas hominis	3	1,47
Helminths		
Trichuris trichiura	88	43,14
Ascaris lumbricoides	72	35,29
Hymenolepis nana	10	4,90
Strongyloides stercoralis **	5	2,45
Enterobius vermicularis **	4	1,96
Ancylostomideos	1	0,49

* Including parasitic associations; ** Values obtained without the use of specific techniques for the diagnosis of these parasites.

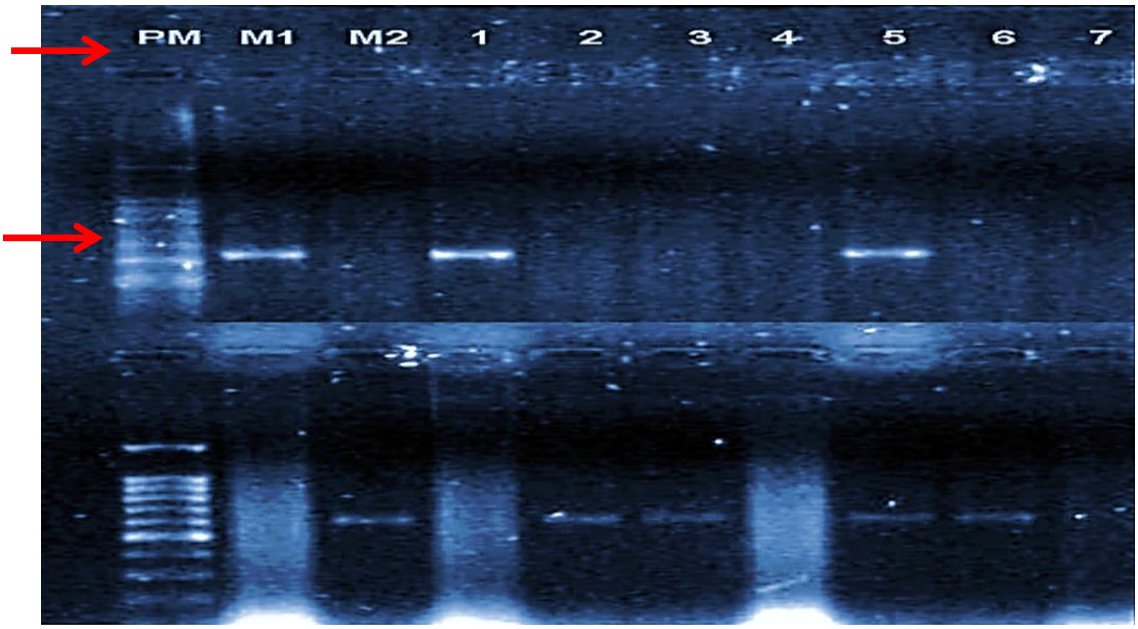

Fig 1: Identi fi cation by PCR of E. histolytica and E. dispar. MW: molecular weight marker; M1: reference control of E. histolytica; M2: reference control of E. dispar; lanes 1 to 7: DNA extracted from patient stool samples, you can note that sample 5 had both species. Top: PCR amplification, using SRPEh oligonucleotides 5/3, 553bp (specific to E. histolytica). Bottom: amplification products, obtained using oligonucleotides SRPEd 5/3, 567bp (specific for E. dispar).

observed health and safety, and alerts on the potential Tencial health problem of amebiasis, such as morbidity and mortality factor in this pa - rroquia. Most of the individuals living in stilt houses, they usually throw their excreta to the water, bathe in them and then reuse these same waters in their homes. In Venezuela, there is only one previous report that refers to the use of discriminatory techniques between the two amoebas. Mora et al. [31] studied using nested-multiplex PCR, 428 patients with symptoms gastrointestinal, finding a prevalence of E. histolytica of 6.31%, 4.44% of E. dispar and 4 cases of mixed infection. Although other authors have reported the presence blood in samples with E. histolytica [32,33].

Table 2: Results of the PCR * technique applied to stool samples.

Parasitic species	n	%
E. histolytica	22	10.78
E. dispar	16	7.84
Infection mixed of E. histolytica y E. dispar	9	4.41
Negative for both amoebae	157	76.96
Total	204	100.00

*** PCR with primers: SRPEh and SRPEd; ** Signi fi cantly different from negative samples ($p<0.01$) when applying the Z test.**

In the present investigation, only one patient fected with E. histolytica (1/31) , presented blood in the fecal sample at the time of the physical croscopic, so it was not possible to relationship between these variables. It is possible to justify the absence of blood in the stool of patients included in this study, if for the moment in which the individuals submitted their sample, the parasite has not yet invaded the mucosa

intestinal. In general, a high number of multiple infections (polyparasitism) between individuals from the community (66.17%). When do the cases that were positive are analyzed by PCR, protozoa most frequently associated with E. histolytica were Blastocystis hominis (74.19%), E. coli (41.93%) and Giardia lamblia (22.58%). In individuals with E. dispar , established association with the same parasitic species and also with E. nana . Some publications refer to E. coli as one of the main organizations associated with E. histolytica infections and have suggested that there must be a common transmission mechanism or a specific susceptibility to these parasitosis [9] . Rivera et al. [11] studied the distribution distribution of E. histolytica and E. dispar in northern Philippines and detected a strong association between E. coli and infection with E. histolytica / E. dispar . No significant difference was observed between the global percentages of the amoebae studied and the age group to which the individuals belonged
duos ($p > 0.05$). Similar results were obtained nests by Povoa et al. [34], when studying the prevalence of E. histolytica in Brazil, by detecting of coproantigens (ELISA). Despite not existing di-important differences by age group, it is observed the absence of cases of amebiasis in infants minor and major tees, which is related to previous reports. Silva et al. 35 found a higher prevalence of E. histolytica in the group of individuals over 14 years of age, when studying a population in Brazil, through various techniques. It is important to highlight the absence of cases amebiasis in children under two years of age, since none were parasitized with E. histolytica , E. dispar or both (Table 3). It is possible that in this group there is indeed a low valence of infection, but the small number of individuals studied does not allow obtaining results conclusive data for this age group. By On the other hand, this situation can be explained by the maternal care that generally receives children from newborn to about damente 20 months of age. Later, children come to have more contact with the contaminated environment and for this reason
increases the probability of acquiring the infection. This point is important, because in our midst it is appreciated with concern, a high report of amoebiasis cases in the children under two years of age. The Yearbook Mortality of the year 2005 36 indicates 114 deaths
due to amoebiasis in the country, of which 23 occurred rum in children under two years. The realization of more sensitive and specific techniques for the diagnosis The diagnosis of amebiasis will contribute significantly to mind in clarifying this situation. He greater number of individuals parasitized with E. histolytica and E. dispar occurred in the group of 7-12 years. Rivera et al. 11 got results similar, since they did not observe different between age groups, but a higher prevalence of these amoebae in individuals from 5-14 years old. Of the 204 individuals studied, 94 belong to They were male and 110 were female. Of the 47 samples that were positive for amoeba by PCR (34.04%), 31 (65.96%) specimens belonged to individuals of the sex female and 16 male. In female sex child, the prevalence of amoebae.

Table 3: Frequency of amoeba species by age group. Age group *

Age group	Individuals studied	Microscopy Complex E. histolytica / E. dispar n (%)	E. histolytica n (%)	PCR E. dispar n (%)	Association of E. histolytica / E. dispar
Younger infant (1-11 months)	7	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)
Older infant (12-23 months)	6	0 (0,00)	0 (0,00)	0 (0,00)	0 (0,00)
Pre-school (2 -6 years)	48	8 (19,04)	4 (18,18)	3 (18,75)	2 (22,22)
School (7-12 years)	46	11 (26,19)	8 (36,36)	5 (31,25)	2 (22,22)
Adolescents (13-19 years)	16	5 (11,90)	3 (13,63)	1 (16,25)	1 (11,11)
Young adult (20-39 years)	48	11 (26,19)	3 (13,63)	5 (31,25)	3 (33,33)
Middle adult (40-64 years)	29	6 (14,28)	4 (18,18)	2 (12,5)	0 (0,00)
Older adult (≥ 65 years)	4	1 (2,38)	0 (0,00)	0 (0,00)	1 (11,11)
Total	204	42 (100,00)	22 (100,00)	16 (100,00)	9 (100,00)

Chi square ($\chi 2$) = 8.5155; p = 0.9015 (not significant); confidence interval: 0.3-1.3.
* Classi fi cation according to Masalán & Gonzalez (37).

Statistical analysis

showed that no there was a significant difference between the frequency of amoebas and sex. Like other studies that report a prevalence of infections by E. histolytica equivalent between males and females Women 11 . The differentiation of species by PCR is a necessary and valuable tool for the diagnosis of amebiasis, as it allows you the clinician to discriminate true infections by E. histolytica and avoid unnecessary treatments when E. dispar is present.

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