Comparison of Multiplex-PCR and Antigen Detection for Differential Diagnosis of *Entamoeba histolytica*

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Amebiasis is an infection caused by *Entamoeba histolytica*. However, differentiation between *E. histolytica* and *Entamoeba dispar*, which are morphologically identical species, is essential for treatment decision, precaution of the invasive disease and public health. The purpose of the present study was to evaluate a Multiplex -PCR for detection and differentiation of *E. histolytica* from *E. dispar* from fresh stool samples in comparison with the coproantigen commercial ELISA. Microscopic examination of stools using the Coprotest method, detection of stool antigen by enzyme-linked immunosorbent assay kit and a home made Multiplex-PCR, were used for the diagnosis of amoebiasis infection. Analysis of the 127 stools samples by microscopy examination demonstrated that only 27 (21%) samples were positive for *E. histolytica/E. dispar* complex. Among these stool samples, 11 were positive by Multiplex-PCR, with nine presenting the diagnostic fragment characteristic of *E. dispar* (96 bp) and two presenting diagnostic fragment of *E. histolytica* (132 bp). Among negative samples detected by microscopic examination, three positive samples for *E. dispar* and one positive for *E. histolytica* by Multiplex-PCR was observed. This denotes a low sensibility of microscopic examination when a single stool sample is analyzed. Assay for detection of *E. histolytica* antigen was concordant with multiplex-PCR in relation to *E. histolytica*. Statistical analysis comparing the sensibility tests was not done because of the low number of *E. histolytica* cases. The results demonstrate the importance of the specific techniques use for the differentiation between *E. histolytica* and *E. dispar*.

Key-Words: **Multiplex-PCR, *Entamoeba histolytica*, amebiasis.**

Amoebiasis is an infection caused by *Entamoeba histolytica* with or without clinical manifestations [1]. *E. histolytica* infect approximately 10% of the world's population, with a higher incidence in tropical and subtropical countries, due to poor sanitary and socioeconomic conditions and non-hygienic practices [2,3].

Individuals infected with *E. histolytica* may show a wide range of clinical manifestations, from asymptomatic colonization to amoebic dysentery and invasive extraintestinal amoebiasis. It has been related that majority of individuals infected are asymptomatic [4-8]. These individuals are reservoir of infection and represent the most neglected category of infected subjects, what might interfere in an epidemiological study and in control of this infectious. Moreover, they should progress to invasive disease [5, 7, 9,10].

The WHO/Pan American Health Organization/UNESCO Expert Consultation on amoebiasis recognized *E. dispar* as a new specie morphologically indistinguishable from *E. histolytica* and recommended the development of improved methods, using appropriate technologies for specific diagnosis of *E. histolytica* infection in developing countries. The correct identification of this parasite is very important since *E. histolytica* is the only specie within the genus *Entamoeba* associated with intestinal disease [11]. Identification of other species of *Entamoeba* is important because they show morphologic similarities between cysts and trophozoites, when diagnostic investigation is done by microscopic examination. In addition, differential diagnosis between *E. dispar* and *E. histolytica* has critical significance for treatment decision, prevention of the invasive disease and health public. Previous studies have related that *E. dispar* can be capable of producing variable focal lesions by erosion of mucosa intestinal in animals [12,13] and of destroying epithelial cells monolayer "in vitro" [14]. Is *E. dispar* non-pathogenic as its former designation would indicate? *E. dispar* would be a non-invasive pathogenic. Although, there is no evidence of tissue lesions caused by *E. dispar* in human hosts.

Diagnosis of *E. histolytica* is usually based on microscopic examination of protozoan morphology. However, it reaches about 60% sensitivity and can give false-positives due to misidentification of non-pathogenic *Entamoeba* species [15-18]. The examination of fecal samples by optic microscope is not able to identify or differentiate *E. histolytica* from *E. dispar* unless erythrophagocytosis (the presence of ingested RBCs in trophozoites) is seen during microscopic examination [11,19]. This feature has been observed among patients with dysentery. Some investigators suggest that this classical feature has long been considered the definitive diagnostic criterion for *E. histolytica* [1,6,11,14]. However, it is rarely observed in chronic amoebic infections.

Methods for antigen detection in stool and polymerase chain reaction (PCR) have been evaluated as diagnostic tools. Antigen detection may be useful as an additional assay to the microscopic diagnosis since this assay detects the galactose-inhibitable adherence protein specifically for *E. histoltyica* in

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stool. The coproantigen ELISA technique has been suggested to be used in routine diagnosis procedure and epidemiologic studies. However, a comparative study on the use of the ELISA and PCR for detection of *E. histolytica* indicated that PCR was more sensitive [6,20]. In reference laboratories, PCR is the method of choice for differentiation between the pathogenic specie (*E. histolytica*) from the non-pathogenic (*E. dispar*). Many investigations have reported successful application of PCR to the diagnosis of amoebiasis as a tool for final confirmatory identification of intestinal amoebiasis [5,6,20-26].

The main purpose of the present study was to evaluate PCR designed for differential detection of *E. histolytica* and *E. dispar* from fresh stools samples.

Material and Methods

Stool Samples

A total of 127 stool specimens were evaluated using Multiplex-PCR. Specimens from asymptomatic individuals living in two villages in state of Rio de Janeiro (Sumidouro and São Gonçalo) Brazil were obtained. Housing is inadequate in these areas for settlement expansion aside from poor economic condition. This study was reviewed and approved by the Human Investigation Committee of Universidade Federal Fluminense and Fundação Oswaldo Cruz (local ethic committee). Stool sample was taken from individuals who had given their informed consent prior to the collection.

Stool samples from 115 individuals of a rural area in Sumidouro were divided in two aliquots. One aliquot was preserved in formalin, for later microscopic examination, and another was immediately frozen at –20°C for the antigen detection and DNA extraction for PCR analyses. Two stool samples were colleted in different days from 12 individuals living in urban area São Gonçalo and the same procedure as described before was done.

Microscopic Examination of Parasites

A single fresh stool specimen from each individual was collected in special containers with formalin. Microscopic examination for the presence of parasite (*E. histolytca*/*E. dispar* complex cysts and trophozoites) was performed by examination of iodine-stained wet mount after formalin ethyl acetate concentration technique. This test was performed according to the manufacturer's instructions, using a commercial kit, Coprotest [27].

Immunoenzymatic Assay

Antigen detection was performed on the stool specimens without preservative, using *E. histolytica* II test kit (Techlab, Inc., Blacksburg, VA), recommended to detect specifically *E. histolytica*, according to the manufacturer's instructions.

Cultured *E. histolytica* and *E. dispar* Trophozoites

E. histolytica strain HM1:IMSS was grown in TYI-S-33 medium axenically and the *E. dispar* strain was grown in Pavlova medium polyxenically. After 48 hours of growth, the culture tubes were placed in ice-cold bath for 5 min and trophozoites were centrifuged, resuspended in phosphate–buffered saline pH 7.2 and the parasite number was determined. Analytical sensibility of Multiplex-PCR was estimated using cultured trophozoites. Variable amount of trophozoites (200, 100, 50, 25, 15 and 5) were used to spike a volume of 100 uL stool free of parasite. DNA from the HM1-IMSS strain (*E. histolytica*) and from *E. dispar* isolated from stool sample, characterized by isoenzyme analysis, were used as control for all PCR analyses.

Multiplex-PCR

Extraction of Nucleic Acids

DNA from *Entamoeba* trophozoites and cysts were obtained according to the protocol previously described (Picher et al. 1989) [28] with slight modifications. Approximately 1 g of unpreserved stool (stored at –20°C) was homogenized in distilled water and passed through gauze to discard larger detritus. The homogenates were centrifuged at 500g per 5 min. The pelllet was resuspended in distilled water and washed three times by centrifugation (500 g for 5 min). The sediment was resuspended in 3 mL of distilled water. The fecal suspension was stored at – 20°C. For extraction, aliquots (100µL) of fecal suspension was placed in a 1.5 mL Eppendorff tube, and parasites were lysed with 0.5 mL of 5 M guanidine isothiocyanate (Promega corporation, USA). The tubes were agitated and incubated at room temperature for 10 min. Lysate materials were cooled on ice for 10 min. After that, 0.25 mL of cold 7.5 M ammonium acetate was added. The mixture was kept on ice for 10 min and then, 0.5 mL of chloroform/isoamyl alcohol (24:1 v/v) was added. Phases were mixed thoroughly, transferred to a 1.5 mL Eppendorf tube, centrifuged at 13,800 g for 10 min and the sediment suspended with 0.54 mL of 2-propanol. After centrifugation at 3,500g for 20 s, the sediment was washed five times with 70% ethanol by centrifugation (4,000g for 20 min) and dried at 37°C for 24 hr. After that, the dried sample was suspended with 100 uL of TE buffer at 37°C for 1 h. This material corresponds to the DNA.

Amplification Reactions

The Multiplex-PCR was carried out according to a protocol described by Nuñez et al. 2001 [21], with some modifications. Based on the sequences tandemly repeated in the respected extrachromosomal circular DNAs of *E. histolytica* and *E. dispar*, a set of oligonucleotide primers specific for *E. dispar*, (EDP1 – 5'ATGGTGAGGTTGTAGCAGAGA3' and EDP2 - 5'CGATATTG AC CTAGTACT3') and *E. histolytica* (EHP1- 5'CGATTTTCCCAGTAGAAATTA3' and EHP2- 5'CAAAATGGTCGTCTAGGC3') were prepared. Each primer set was used to specifically amplify a 132 bp fragment from *E. histolytica* (EHP1/EHP2) and a 96 bp fragment from *E. dispar* (EDP1/ EDP2). Multiplex-PCR reaction was performed in a volume of 50 µL reaction containing 20 mM of Tris-HCl pH 8.4; 50 mM of KCl; 1.5 mM of $MgCl_2$; 40 pmoles of each

oligonucleotide primer; 250 µM of each deoxynucleoside triphosphate (dNTPs) and 1.25 U of Taq DNA polymerase (Invitrogen Life technologies, USA), 0.1% of bovine serum albumin (BSA Sigma Chem. Co., USA) e 2 µL of DNA sample. PCR was carried out using an GenAmp PCR system 2400 (AB Applied biosystems) thermal cycler and amplification condition were: 3 min at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; 5 min at 72°C. Amplified products were analyzed by electrophoresis using 2.0% of agarose gel containing 0.5 µg of ethidium bromide/ mL.

Results

The standardization of Multiplex-PCR was done using DNA from *E. histolytica* and *E. dispar* cultured trophozoites. The detection limit was determined by contamination of 100 uL of stool free of parasite with 200, 100, 50, 25, 15 and five trophozoites or with different concentration of DNA (32 ng to one fg). Multiplex-PCR was capable to detect the specific target DNA sequence when a minimum of five trophozoites or 40 fg of DNA template were used (Figures 1 and 2). Different concentrations of *E. histolytica* DNA (16 ng/mL, 8 ng/mL, 1.6 ng/mL and 0.8 ng/mL) were assayed at the same tubes with one fix DNA concentration (9.2 ng/mL) from *E. dispar*. In this condition, Multiplex-PCR detected both species in all thesamples (Figure 3).

Analysis of the 127 stool samples by microscopy examination demonstrated that 27 (21%) samples were positive for *E. histolytica*/*E. dispar* complex. Amongst these stool samples, only 11 were positive by Multiplex-PCR, of which nine presenting the diagnostic fragment characteristic of *E. dispar* (96 bp) and two presenting diagnostic fragment of *E. histolytica* (132 bp) (Table 1). No mixed infection was detected. Among the stool samples in which no *E. histolytica*/ *E. dispar* cysts and trophozoites complex were detected by microscopic examination, three were identified as *E. dispar* and one as *E. histolytica* when analyzed by Multiplex-PCR. The result obtained with coproantigen ELISA test was in agreement with those obtained by the Multiplex-PCR (Table 2).

Multiplex-PCR was negative for 15 stool samples that were positive for *E. histolytica*/*E. dispar* complex by microscopy examination. In order to clear up this problem, we spiked these samples with 800 pg of DNA template from *E. histolytica* strains HM1:IMSS. All samples had the *E. histolytica* DNA fragment amplified showing that the previous negative results are not due to the presence of inhibitors in the sample.

Discussion

Diagnosis of amoebiasis has been done by traditional microscopic examination of protozoan morphology since amoeba trophozoites description in stool by a physician, Fedor Lösh in 1875. The recent recognition of *E. dispar* as a new non-pathogenic specie, which is morphologically indistinguishable from *E. histolytica*, has indicated the need

Figure 1. Agarose gel of Multiplex-PCR products amplified by *E. histolytca* primers EHP1/EHP2 and *E. dispar* primers EDP1/EDP2. DNA was extracted from stool containing a predetermined number of trophozoites stain HM1-IMSS (see Materials and Methods). *E. dispar* positive control (lane 1), *E. histolytica* positive control (lane 2), negative control (lane 3), *E. histolytica* and *E. dispar* control (lane 4), stool containing 50 trophozoites (lane 5), stool containing 25 trophozoites (lane 6), stool containing 15 trophozoites (lane 7), stool containing 5 trophozoites (lane 8). 100-pb, molecular size ladder marker (M). The arrow head indicates the position and size of marker.

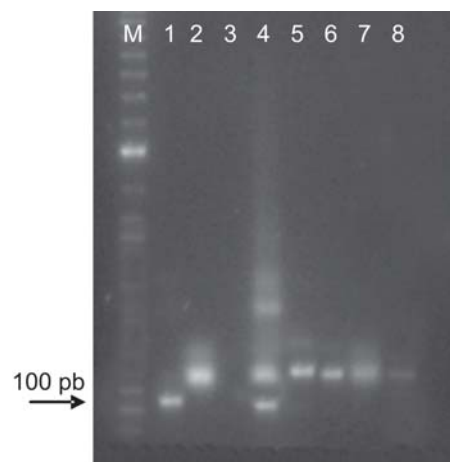


Figure 2. Limit detection of Multiplex-PCR in detection of DNA sample from reference stain (see Materials and Methods). *E.histolytica* positive control (lane 1), *E. dispar* positive control (lane 2), negative control (lane 3), *E. histoltyica* and *E. dispar* control (lane 4), 2 ng DNA (lane 5), 400 fg DNA (lane 6), 80 fg DNA (lane 7), 40 fg DNA (lane 8), 4 fg DNA (lane 9), 4 fg DNA (lane10), *E. histoltyica* and *E. dispar* control (lane 11). 100-pb, molecular size ladder marker (M). The arrow head indicates the position and size of marker.

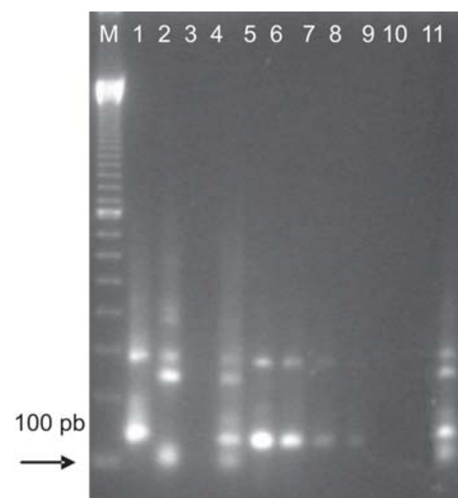
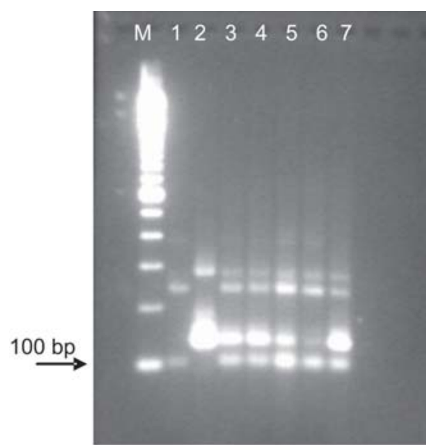


Figure 3. Agarose gel of Multiplex-PCR products amplified by *E. histolytca* primers (EHP1/EHP2) and *E. dispar* primers (EDP1/EDP2). Multiplex PCR mixtures containing different amounts of DNA from *E.histolytica* and *E. dispar* (lane 3-7). 100-pb, molecular size ladder marker (M),*E. dispar* positive control (lane 1), *E.histolytica* positive control (lane 2), PCR products from a mixture of 8.0 ng/mL DNA from *E.histolytca* and 9.2 ng/mL *E. dispar* (lane 3) 1.6 ng/mL DNA from *E.histolytca* and 9.2 ng/mL from *E. dispar* (lane 4), 0.8 ng/mL DNA from *E.histolytca* and 9.2 ng/mL from *E. dispar* (lane 5), 16 ng/mL DNA from *E.histolytca* and 9.2 ng/mL from *E. dispar*(lane 6).



of alternative methods able to differentiate these two species, as recommended by WHO [1]. Petri et al. (2000) reported that microscopic examination of stool should not be used to diagnosis amoebiasis since it is a method with low sensibility, specificity and present false positive results [18]. Dysentery due to entities such as bacteria, virus and other agents should be considered [3,5,7,10,29] and probably may be misdiagnosed as amoebic colitis if microscopy is the sole method used.

Culture of stool samples following isoenzyme analysis has been considered as the gold standard for *E. histolytica* definitive diagnosis, although it is known to be far from 100% sensitive [16,31]. This method takes one or more weeks to carry out, is laborious and not practical for routine diagnosis laboratories. Antibodies detection is useful as additional test to confirm the diagnosis of invasive extraintestinal amoebiasis, but not for intestinal form of the disease. It is unable to differentiate acute infection from past infection since antibodies can persist for years after clinical cure [11,16,28].

At the present time, only one commercial test (Techlab *E. histolytica*) can be used to identify *E. histolytica* protein in the stool sample. Several PCR assays designed to differentiate *E. histolytica* for *E. dispar* have been described [5,6,21,22-25,32,33]. Most of them targeted either the small subunit ribosomal RNA gene or specific episomal repeats species. The sensibility and specificity of PCR methods for diagnosis of *E. histolytica* are very similar to stool culture followed by isoenzyme analysis. However, PCR amplification for detection of small subunit ribosomal RNA genes is almost 100 times more sensitive than currently available ELISA kit for detection of *E. histolytica* antigens, when parasite forms isolated from cultured stool were used [20,24,34]. Recently studies showed that PCR with culture and antigen detection methods from stool samples have the same performance [35].

The primary advantage of using PCR is the possibility of differentiation between *E. histolytica* and *E. dispar* in area where the presence of other *Entamoeba* species is common. PCR is more accurate to understand the epidemiology of *E. histolytica* and *E. dispar* infection, contrary to the TechLab *E. histoltyica* II test, because it is allowed to distinguish the two *Entamoeba* species. Besides, coproantigen kit detection contains specific antibodies for *E. histolytica* that recognize antigens on the surface of the trophozoites only, which are generally identified in diarrhea, and not in the cystic stage of the parasite.

In the present study, the data with cultured trophozoites of *E. histolytica* clearly indicate that PCR technique is sensible and reliable for species differentiation and can be applied for diagnosis in clinical samples. Then, when PCR was used in stool samples from individuals living in two villages of Rio de Janeiro state, Brazil, discrepancy between microscopy and Multiplex-PCR was found. The probability of false negative results by PCR inhibition by fecal constituents is known to be a serious problem. In all PCR negative samples, inhibition factors were checked by spiking these samples with 800 ng of DNA obtained from *E. histolytica* culture forms. No evidence of inhibition was found in any of the Multiplex-PCR negative samples. This result suggested that other species of *Entamoeba* are presentin this area.

Similar discrepancy has been reported by Pinheiro et al., 2004, [22] when they analyzed 59 cultured stool samples, where 31 samples had *Entamoeba* trophozoites, but only 23 samples were identified as *E. dispar* and eight samples were negative for both species. Other study, conducted in Ethiopia with 108 stool samples, demonstrated that only one sample was *E. histolytica* and 77 *E. dispar* when PCR was used. The remaining 30 samples were negative for both species [19].

High prevalence of *E. dispar* has been described in different countries [19,22,25,32,36]. Several studies that have investigated the prevalence of *E. histolytica* and *E. dispar* have not considered the presence of other species such as *E. hartmanni* and *E. moshkovskii* [19,22,23,36]. These species were reported in areas of Ghana, Pondicherry and Bangladesh [37-39]. These results suggest that species of *Entamoeba* that not belong to *E. histolytica/E. dispar* complex may not be identified. A differential characterization of *E. histolytica* from other intestinal protozoa is essential because only *E. histolytica* infection requires a specific drug treatment. The discriminate used of such drug can induce development of resistance.

Multiplex-PCR is a robust procedure and easily adapted to routine use in the context of well equipped laboratories and can serve as a tool for the confirmation of microscopy results.

Table 1. Results of Multiplex-PCR and microscopic examination analysis using stool specimens

Microscopy Examination	Multiplex-PCR			
	E. histolytica	*E. dispar*	Negative	Total
E. histolytica/E.dispar complex	02	09	16	27
Other parasites	01	03	34	38
Negative	00	02	60	62
Total	03	14	110	127

Table 2. Results of Multiplex-PCR and antigen detection analysis using stool specimen

	Microscopy Examination	ELISA *E. histolytica*	Multiplex-PCR	
			E. histolytica	*E. dispar*
Positive	27	03*	03*	09
Negative	100	83	124	118
Total n° of specimens	127	86	127	127

*Same positive specimens.

However, PCR techniques do not substitute the direct microscopy stool examination, which widely screen for virtually intestinal parasite, but could be a useful tool for diagnosis and epidemiological studies in areas where *E. histolytica* is endemic.

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References

1. World Health Organization. Amoebiasis. Report on the WHO/ Pan American Health Organization/ UNESCO Expert Consultation, Mexico City. Geneva- WHO. W Epidemiol Rec **1997**;72:97-100.
2. Rivera W., Tachibana H., Kanbara H. Field study on the distribution of *Entamoeba histolytica* and *Entamoeba dispar* in the Northern Philippines as detected by the polimerase chain reaction. Am J Trop Med Hyg **1998**;59:916 -21.
3. Ramos F., Valdez E., Morán P., et al. Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in a Highly Endemic Rural Population. Arch Med Res **2000**;31:S34-5.
4. Walsh J.A. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. Rev infect Dis **1986**;8:228-38.
5. Evangelopoulos A., Spanakos G., Patsoula E., Vakalis N. A nested multiplex PCR assay for the simultaneous detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in faeces. Ann Trop Med Parasitol **2000**;94:233-40.
6. Gonin P., Trudel L. Detection and Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR and Enzyme-Linked Immunosorbent Assay. J Clin Microbiol **2003**;41:237-41.
7. Stanley S. L. Amoebiasis. Lancet **2003**;361:1025-34.
8. Huston C. D. Parasite and host contributions to the pathogenesis of amebic colitis. Trends Parasitol **2004**;20:23-6.
9. Haque R., Huston C. D., Hughes M., et al. Current concepts: Amebiasis. N Engl J Med **2003**;348:1565-73.
10. Blessmann J., Van L. P., Nu P.A.T., et al. Epidemiology of amebiais in a region of high incidence of amebic liver abscess in Central Vietnam. Am J Trop Med Hyg **2002**;66:578-83.
11. Tanyuksel M., Petri W.A.P. Laboratory Diagnosis of Amebiasis. Clin Microbiol Rev **2003**;16:713-29.
12. Chadee K., Smith J.M., Meerovitch E. *Entamoeba histolytica*: electrophoretic isoenzyme parttners of stains and their virulence in the cecum of gerbilis (Meriones unguiculatus). Am J. Trop Med Hyg **1985**;34:870-8.
13. Vohra H., Bhatti N.K., Ganguly N.K., Marajan R.C. Virulence of pathogenic and non-pathogenic zymodemes of *Entamoeba histolytica* (Indian strains) in guinea pigs. Trans R Soc trop Med Hyg **1989**;83:648-50.
14. Espinosa-Cantellano M. ,Gonzalez-Robles A., Chavez B., et al. *Entamoeba dispar*: ultrastructure, surface properties and cytopathic effect. J Euk Microbiol **1998**;45:265-72.
15. Gonzalez-Ruiz A., Haque R., Aquire A., et al. Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. J Clin Pathol **1994**;47:236-9.
16. Haque R., Petri W.A. Diagnosis of Amebiasis in Bangladesh. Arch Med Res **2006**;37:273-6.
17. Urdaneta H., Rangel A., Martins M.S., et al. *Entamoeba histolytica*: Fecal antigen capture immunoassay for the diagnosis of enteric amebiasis by a monoclonal antibody. Rev Inst Med Trop São Paulo **1996**;38:39-44.
18. Petri W.A., Haque R., Lyerly D., Vines R.R. Estimating the Impact of Amebiasis on Health. Parasitol Today **2000**;16:320-1.
19. Kebede A., Verweij J.J. , Petros B., Polderman A.M. Misleading Microscopy in Amoebiasis. Trop Med Int Health **2004**;9:651-2.
20. Mirelman D., Nuchamowitz Y., Stolarsky T. Comparison of use Enzyme-Linked Immunosorbent Assay-Based Kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E. dispar*. J Clin Microbiol **1997**;35(9):2405-7.
21. Nuñez Y.O., Fernández M.A., Torres-Nuñez D., et al. Multiplex polymerase chain reaction amplification and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* DNA from stool samples. Am J Trop Med Hyg **2001**;64:293-7.
22. Pinheiro S.M., Carneiro R.M., Aca I.S., et al. Determination of the prevalence of Entamoeba histolytica and E. dispar in the Pernambuco state of northeastern Brazil by a polymerase chain reaction. Am J Trop Med Hyg **2004**;70:221-4.
23. Verweij J.J., Blotkamp J., Brienen E.A.T., et al. Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* cysts using polimerase chain reaction on DNA isolated from faeces with spin columns. Eur J Microbiol Infect Dis **2000**;19:358-61.
24. Freitas M.A.R., Vianna E.N., Martins A.S., et al. A Single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba díspar*. Parasitology **2004**;128:625-8.

25. Rivera W.L., Tachibana H., Kanbara H. Application of the Polymerase Chain Reaction (PCR) in the epidemiology of *Entamoeba histolytica* and *Entamoeba dispar*. Tokai J Exp Clin Med **1999**;23:413-5.
26. Qvarnstrom Y., James C., Xayavong M., et al. Comparison of Real-Time PCR Protocolos for Differential diagnosis laboratory diagnosis of Amebiasis. J Clin Microbiol **2005**;43:5491-7.
27. Serqueira F.L. Coprotest: metodologia confiável para o exame parasitológico de fezes. Laes & Laes **1988**;9:5-2.
28. Pitcher D.G., Saunders N.A., Owen R.J. Rapid extraction of bacterial genomic DNA with guanidine thiocyanate. Lett Appl Microbiol **1989**;4:1513-21.
29. Petri A.W., Singh U. Diagnosis and Management of Amebiasis. Clin Infec Dis **1999**;29:1117-25.
30. Sehgall R.M., Abd-Alla A.H., Moody P.L., Ackers J. Comparison of two media for the isolation and short-term culture of *Entamoeba histolytica.* Trans R Soc Trop Med Hyg **1995**;89:394.
31. Ackers J.P. The diagnostic implications of the separation of *Entamoeba histolytica* and *Entamoeba dispar*. J. Biosci **2002**;27:573-8.
32. Acuna-Soto R.J., Samuelson P.D., Girolami L., et al. Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. Am J Trop Med Hyg **1993**;48:58-70.
33. Novati S., Sironi S.M., Granata S., et al. Direct sequencing of the PCR amplified SSU rRNA gene of *Entamoeba dispar* and the design pf primers for rapid differentiation from *Entamoeba histolytica*. Parasitology **1996**;112:363-9.
34. Troll H., Marti H., Weiss N. Simple differential detection of *Entamoeba histolytica* and *Entamoeba dispar* in fresh stool specimens by sodium acetate-acetic acid-formalin concentration and PCR. J Clin Microbiol **1997**;35:1701-5.
35. Haque R., Ali I.K., Akther S., Petri W.A. Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of Entamoeba histolytica infection. J Clin Microbiol **1998**;36:449-52.
36. Heckendorn F., Goran E.K.N., Ferger I., et al. Species-specific field testing of *Entamoeba spp.* in area of high endemicity. Trans R Soc Trop Med Hyg **2002**;96:521-8.
37. Ali I.B., Hossain M.B., Roy S., et al. *Entamoeba moshkovskii* Infections in Children in Bangladesh. Emerg Infec Dis **2003**;9:580-4.
38. Parija S.C., Khairnar K. *Entamoeba moshkovskii* and *E. dispar* associated Infections in Pondicherry, India. J Health Popul Nutr **2005**;23:292-5.
39. Verweij J.J., Oostvogel F., Brienen E.A., et al. Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in Northern Ghana. Trop Med Int Health **2003**;8:1153-6.