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Original Article

Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in Gonbad City, 2006, Iran

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Abstract

Background: Differential diagnosis of two protozoan parasites *Entamoeba histolytica* and *E. dispar* is of great clinical and epidemiological importance, but except in the case of haematophagous trophozoites in acute dysentery, it is not possible to differentiate them by microscopy. The present study was carried out from February 2005 to July 2006 in order to determine the prevalence of *E. histolytica* and *E. dispar* in Gonbad City, by using a PCR method.

Methods: Five hundred and sixty four fecal samples were collected from primary health care centers of Gonbad both urban and rural areas. The stool specimens were examined by light microscopy (Direct slide smear, Iodine wet mount, Formal-ether concentration and Trichrome staining) to distinguish *E. histolytica/E. dispar* complex and differentiate them from other non-pathogenic intestinal amoebae.

Results: The microscopy results of stool exams showed a frequency rate of 23 positive samples (4.07%) for cyst of *E. histolytica/E. dispar* complex. All the microscopy positive isolates appeared to be infected with cyst of *E. histolytica/E. dispar* complex were cultivated and maintained successfully in HSr + s medium for DNA extraction and identification by the PCR method. The PCR study showed that 16 isolates (69.56 %) of the *Entamoeba* samples were *E. dispar* while 7 samples (30.43%) of those microscopy positive samples were not amplified and none of them showed *E. histolytica* pattern.

Conclusion: High frequency rate of *E. dispar* in this study were in high agreement with the estimation rate of these entamoebas worldwide.

Keywords: Entamoeba histolytica, Entamoeba dispar, *PCR, Iran*

Introduction

Amebiasis is still one of the major health problems in tropical and subtropical areas, and is characterized by low socioeconomic status and poor hygiene that favors the indirect fecal-oral transmission of the infection (1). The acceptance of *Entamoeba histolytica* and *Entamoeba dispar* as distinct species had had a major impact on our views of amebiasis, in particular its clinical management and epidemiology (2- 4).

Differential diagnosis of these two species is of great clinical and epidemiological importance, but except in the case of haematophagous trophozoites in acute dysentery, it is not possible to differentiate *E. histolytica* from *E. dispar* by microscopy. Different methods, such as analysis of isoenzyme electrophoretic patterns, specific DNA probes, PCR-based methods and typing with monoclonal antibodies, must be performed to differentiate the non-pathogenic *E. dispar* from the pathogenic *E. histolytica* (5). Several microscopy-based studies on the prevalence of the *E. histolytica/ E. dispar* complex were performed In Iran, but this estimate predates the formal separation of *E. histolytica* and *E. dispar* and is now being reassessed (6).

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Current data indicate that *E. dispar* is perhaps 10 times more common than *E. histolytica*; however, in some parts of the world, such as Japan and Mexico, the rates of *E. histolytica* infection are high (7). Data from some parts of Iran showed that 92.1% of the isolates were *E. dispar* and 7.9% were *E. histolytica* or mixed infections (6). A local prevalence study of an infection disease in a community is an initial step toward the introduction of the proper interventions for controlling the disease in that region. This study represented the distribution of *E. histolytica* and *E. dispar* in urban and rural areas of Gonbad City in the north of Iran.

Materials and methods

Study area

According to the previous study of *E. histolytica/E. dispar* results with a 95% confident a sample size of 500 stool isolates were needed to obtain (8, 6). From February 2005 to July 2006, five hundred and sixty four fecal samples were collected from primary health care centers of Gonbad City; northern of Iran.

Microscopy and culture

The stool specimens were examined by light microscopy (Direct slide smear, Iodine, Formal-ether concentration and Trichrome staining) to distinguish *E. histolytica/ E. dispar* complex and differentiate them from other non-pathogenic intestinal amoebae (9). The positive *E. histolytica/ E. dispar* samples were cultured in HSr+s medium (10). After three or four subcultures, trophozoites were harvested by centrifugation at 3000 rpm for 5 min and washed three times with phosphate buffered saline (pH 7.2). The trophozoites were transferred to 1.5 ml microcentrifuge tubes and stored at -20 until DNA was extracted.

Extraction of genomic DNA

Genomic DNA was extracted with phenol-chloroform method. At the first step the sediment resulting from trophozoites were suspended in 500µl of lysis buffer, containing 1% sodium dodecyl sulfate (SDS) and 2 µl proteins k. The mixture was incubated at 60 °C for 2h, and then boiled for 15 min. DNA was extracted once with phenol-chloroform and then precipitated with absolute ethanol (11). The DNA was resuscitated in 50 µl distilled water, and stored at -20 °C until PCR amplification. The DNA concentration was determined by measuring optical absorbance at 260 and 280 nm.

Polymerase chain reaction

Two sets of oligonucleotides primers, HSP1-2 and DSP1-2 were used for PCR amplification. These primers amplify a region of about 340bp and 430bp of the locus 1-2 gene for *E. histolytica* and *E. dispar* respectively (Table 1) (12, 13). Polymerase chain reaction was carried out in 50 µl reaction mixture containing 0.2 µg of DNA, 1.5 µm concentration of each primer, 1.5 mM Mgcl2, 0.1 µg of bovine serum albumin per µl, a 200 µM concentration of each deoxynucleoside triphosphate (dNTP) and 1.5 U of taq DNA polymerase (CinnaGen inc, Iran) in a Techne PCR machine (FTGRAD5D, England) with the following cycling parameters: (i) Taq activities at 95 °C for 5 min; (ii) 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s and (iii) post extension at 72 °C for 5 min(12). PCR products were electrophoresed in 1.2% agarose gel (Fermentas, #R0491). The results were visualized after staining with ethidium bromide in a UV transilluminator (UVIdoc Deluxe GAS 9000, England).

Table1: Oligonucleotide primers

Primer name	primer sequence 5´to 3´
HSP1 (forward)	GAGTTCTCTTTTTATACTTTTATATGTT
HSP2 (Reverse)	ATTAACAATAAAGAGGGAGGT
DSP1 (Forward)	TTGAAGAGTTCACTTTTTATACTATA
DSP2 (Reverse)	TAACAATAAAGGGGAGGG

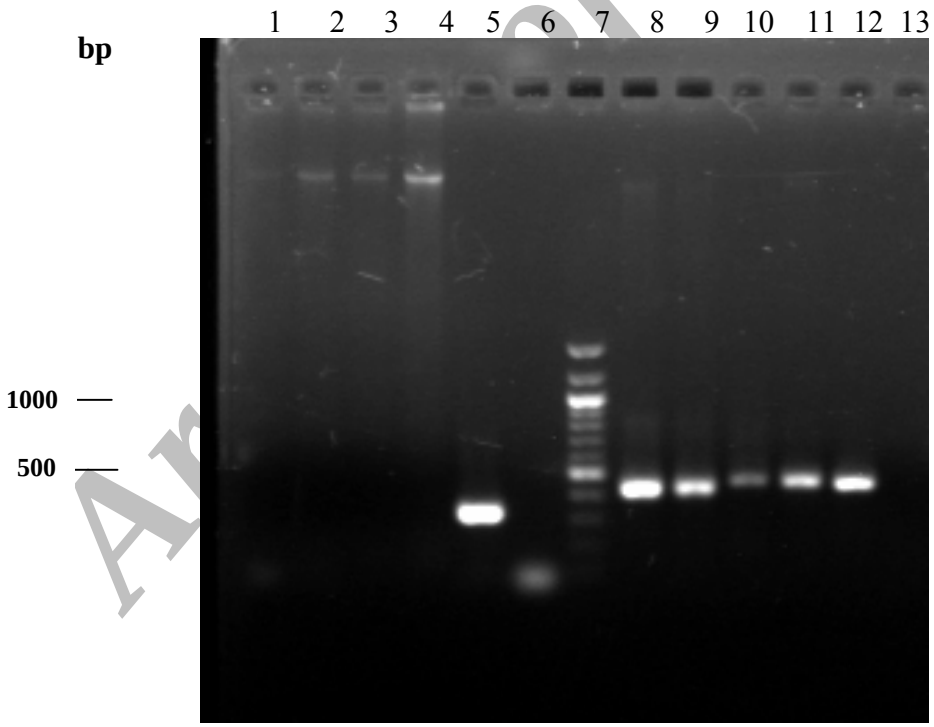
Results

Out of the 564 individuals (74% male and 26% female) referred to the primary health care centers of Gonbad City, 23 (4.07%) specimens were appeared to be infected with *E. histolytica/ E. dispar* complex by microscopic exams. After culturing in HSr+s medium and extraction of DNA, the PCR technique was carried out to differentiate the *Entamoeba* isolates. *E. dispar* was detected and confirmed from 16 samples (69.56 %); while 7 isolates (30.43%) were PCR negative with both of two primer sets. (Table 2, Fig. 1 and 2). Trophozoites of the amoeba with ingested red blood cells were seen microscopically in two of the bloody specimens. However we could not cultivate or maintain these two samples for confirmation by the PCR method.

Table 2: Frequency of the protozoan parasites *Entamoeba histolytica* and *Entamoeba dispar* among 564 individuals according to the PCR results in the primary health care centers of Gonbad, Iran.

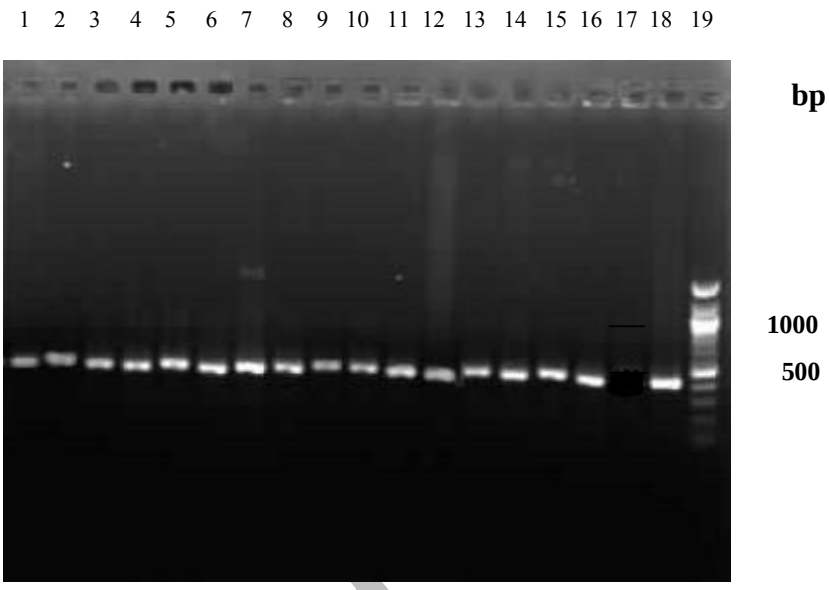
Intestinal parasite	No. of infectied individuals	Frequency (%)
Entamoeba dispar	16	69.56
No amplyfied	7	30.43
E. histolytica	-	-
Total	23	100

Fig. 1: Agarose gel electrophoresis of four positive *Entamoeba dispar* isolates in compare with their *Entamoeba histolytica* negative result



Lane 1-4: *E. histolytica* negative result with HSP1-HSP2 primers
Lane 5: *E. histolytica* positive control (HM1: IMSS) with a band of about 340 bp
Lane 6: *E. histolytica* Negative control (D.W)
Lane 7: 100 bp marker
Lane 8-11: *E. dispar* positive result with DSP1-DSP2 primers
Lane 12: *E. dispar* positive control(AS 16 IR) with a band of about 420 bp
Lane 13: *E. dispar* negative control (D.W)

Fig. 2: Agarose gel electrophoresis of 16 positive *Entamoeba dispar* isolates



Lane 1-16: *E .dispar* positive isolates with DSP1-DSP2 primers
Lane 17: *E. dispar* negative control (D.W)
Lane 18: *E. dispar* positive control (AS 16 IR) with a band of about 420 bp
Lane 19: 100 bp marker
HSP1-HSP2 and DSP1.DSP2 primers were used for PCR amplification of *E. histolytica* and *E. dispar* respectively. All the *E. dispar* positive PCR results are shown in this figure.

Discussion

Infection with *E. histolytica* is a severe health problem in many tropical and subtropical areas of the world, especially in developing countries such as Iran. Most of epidemiological studies for *E. histolytica* infection were performed before of the redescription of two species: *E. dispar* and *E. histolytica*. There is a clear need to perform new epidemiological studies to distinguish these two species of *Entamoeba* and to find true prevalence of *E. histolytica* species (3, 4).
Microscopy is not a sensitive and reliable technique for diagnosing intestinal amebiasis as well as differentiation of *E. histolytica* from *E. dispar*. It is now known that most of human cases of infection with *E. histolytica/ E. dispar* are actually *E. dispar*. *E. dispar* is non-pathogenic, and requires no treatment. Because of this, differential diagnosis of the pathogen *E. histolytica* from the commensally *E. dispar* is of the utmost importance (3, 5).
High frequency rate PCR results of *E. dispar* (69.56%) together with seven cyst passer *E. histolytica/ E. dispar* complex microscopy positive, but the negative PCR result (30.43%) in this study were in close agreement with the estimation rate of these entamoebas worldwide (4). However it seems that the *Entamoeba* in Gonbad is a little more prevalent (4.07%) in comparison with the results in some other regions of Iran so far (6, 14). The 7 negative PCR isolates (30.43%) with both two sets of *E. histolytica* and *E. dispar* primers might be due to lack of enough DNA template, PCR error or misdiagnosis with some other Entamoeba species like *Entamoeba moshkovski*. However, this speculation should be proven by the further development of molecular diagnosis for other nonpathogenic *Entamoeba* species commonly found in

humans, such as *E. coli* and *E. hartmanni* (15). Mixed infections of *E. histolytica* and *E. dispar* were not observed in this study. Since we could not maintain the two injected red blood cell trophozoites in bloody stool specimens for culturing and PCR confirmation they were deleted from the positive results.

This result clearly indicates the difficulty faced by technicians in morphologically differentiating of the cysts of *Entamoeba* and other species by using microscopy for routine diagnosis.

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