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PRELIMINARY COMPARATIVE STUDY OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* BY PCR TECHNIQUE IN IRAN

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ABSTRACT

It has been suggested that two distinct species exist within what was originally known as *Entamoeba histolytica* and *E. dispar*. These are *Entamoeba dispar* for the nonpathogenic and *E. histolytica* for the pathogenic form. Differentiation of these two organisms is of great clinical importance since they are morphologically indistinguishable and both forms can infect the human intestinal cavity. A study was carried out to differentiate *E. histolytica* from *E. dispar* by polymerase chain reaction (PCR) using two sets of primers (p11 plus p12 and p13 plus p14) specific for either species of ameba. The extracted DNA was used for the identification of the species in the stool and culture media by PCR. A total of 16 samples, cysts and trophozoites, were analyzed. In all, 15 samples reacted with *E. dispar* primers, resulting in the expected 101-bp PCR products; however, none of these reacted with *E. histolytica* primers. Only one sample reacted with *E. histolytica* primers. Because of high sensitivity of the PCR method and the high risk of laboratory contamination during processing and extracting DNA and its polymerization, and because of many existing cultures of *E. histolytica* in the same laboratory, the chance of contamination can not be ruled out in the single case of *E. histolytica*. This preliminary study could be an introduction for a PCR-based epidemiological study to determine the importance of *E. histolytica* in Iran.
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INTRODUCTION

Amebiasis is currently defined as infection with the protozoan parasite *Entamoeba histolytica*.[1] On the basis of biochemical and immunological findings and the introduction of deoxyribonucleic acid (DNA)-based methodologies for *Entamoeba* research during the recent years has confirmed the 2 species concept, separating *Entamoeba dispar* from *E. histolytica*.[2] This has brought to an end a long-lasting debate between *Entamoeba* cognoscenti and paved the way for more sophisticated studies on the epidemiology, diagnosis and treatment of human amebiasis.[3] In addition, although *E. histolytica* is pathogenic only to humans, both ameba species are highly similar in genetic background, cell biology, and host range (for both, humans are the only relevant host), the comparison between *E. histolytica* and *E. dispar* provides an interesting area of research for identifying pathogenicity factors of an intestinal protozoan parasite in various geographical conditions.[4] In different epidemiological records in Iran, by routine stool examination based techniques, the prevalence rate of *E. histolytica* has been reported to be from 8% to 30%,[5] but using this technique it is not possible to differentiate *E. histolytica* from *E. dispar*.

MATERIAL AND METHODS

Parasites and culture condition

By light microscopy, *E. histolytica* cysts were identified and obtained by formalin-ether concentration technique from stool specimens in Hamadan. The pellet was washed 4 times by PBS, centrifuged, and frozen at -20°C. Trophozoites isolated from patients in Tehran were cultured in Robinson medium xenically.[6] Thereafter, these cultures were passaged many times in this medium before transporting to Keio University School of Medicine, Japan, where they were cultured monoxenically with *Crithidia fasciculata*[7] and finally axenic culture yield by YIGADHA-S medium.[8-11]

Extraction of DNA

Genomic DNA was extracted from cysts of stool samples according to a modified version of the method of Rivera et al.[12] Briefly, about a 70 μL volume of pellet resulting from the formalin ether sedimentation procedure was placed in a 1.5 mL plastic Eppendorf tube and was then resuspended in 1 mL distilled water and centrifuged for 30 s at 2000 g in a microfuge. This washing step was repeated three times. The supernatant of the last wash was decanted and the pellet was resuspended in a small volume (50-100 mL) of a solution containing 100 mM TRIS (pH 8) and 25 mM ethylene diamine tetra-acetic acid (EDTA). The tubes were left at -80°C for 10 min and then in water at room temperature for 2 min. This procedure was repeated 6 times to rupture the cysts.

After the last treatment, the solution was mixed with 200 μL of a solution containing 200 μg/mL proteinase K, 100 mM TRIS (pH 8), 1% sodium dodecyl sulfate (SDS), and 25 mM EDTA. This step was performed for the pellet of trophozoite that has been prepared from centrifuging the monoxenic culture for 30 s at 2000 g. The mixture was incubated at 60°C for 24h in a shaking condition and boiled for 10 min. The DNA was extracted three times with phenol-chloroform-isoamyl alcohol (25: 24: 1 by vol.) and then precipitated with 3 M sodium acetate and absolute ethanol. The resulting DNA was resuspended in 10 mM TRIS (pH 7.4)/1 mM EDTA (TE buffer) and stored at -20°C until use. To increase the purity of the DNA samples, 50 μL of 25% polyethylene glycol (PEG 6000) in 2.5 M NaCl was added to the 50 μL of DNA suspended in TE buffer and stored at 0°C overnight. Thereafter, the suspension was centrifuged at 15,000 rpm for 10 min at 4°C and the DNA was reextracted using the usual phenol-chloroform extraction procedure as described elsewhere.[13]

PCR of DNA extracted from cysts and trophozoites

Genomic DNA segments were amplified by PCR. The reaction mixture contained 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 500 μg/mL bovine serum albumin (BSA), 0.1% sodium cholate, 0.1% Triton X-100, 0.2 mM dNTP, 25 p*M* each of the two primers, 2.0 U of *Tth* DNA polymerase (TaKaRa Biomedicals, Ltd., Japan), and 0.2 μg genomic DNA as the template in a final volume of 50 μL. The mixture was overlaid with one drop of light mineral oil (Sigma Chemical Co.). The reactions were amplified using the hot-start technique for 35 cycles in an automated PCR machine TSR 300 (Iwaki Glass, Tokyo, Japan). PCR was carried out using two sets of primers, p11 plus p12 for *E. histolytica* and p13 plus p14 for *E. dispar,* as described elsewhere.[13-15] Thermocycling conditions for the these primers were denaturation (94°C) for 1 min (3 min in cycle 1), annealing (59°C) for 1.5 min, and polymerization (72°C) for 1.5 min (7 min in cycle 35). Aliquots (8 μL) of the amplified products were subjected to electrophoresis in 2% LO3 (TaKaRa Biomedicals) agarose gels and the presence of specific bands was visualized with UV light after ethidium bromide staining.

RESULTS

To determine whether the DNA sequence coding the 30,000-M antigen is specific to pathogenic isolates of *E. histolytica,* genomic DNA derived from pathogenic and nonpathogenic isolates were analyzed by PCR, using the four oligonucleotide primers (sequences indicated in Table I). Incubation of genomic DNA of the pathogenic strain with four different pairs of primers (p11 plus p13, p11 plus p14, p12 plus p13, and p12 plus p14) yielded, after 30 PCR cycles, four differently sized products, as expected from the cDNA structure. Of the 16 samples collected, 7 were loose and contained mucus, 9 were loose diarrhea without mucus, and none contained blood.

When the DNAs extracted from the samples were used as templates for PCR amplification using *E. dispar* primers p13 plus p14, the expected 101-bp PCR product resulted from 15 collected samples (Fig. 1). On the other hand, the *E. histolytica*-specific primers p11 plus p12 did not affect the PCR reactions; hence no band was detected. These results indicate that all of the 15 samples microscopically diagnosed as *E. histolytica* were *E. dispar*. Only in one sample a distinct band was observed after ethidium bromide staining of the amplified DNA extracted from the stool sample.

DISCUSSION

Amebiasis is currently defined as infection with the protozoan parasite *Entamoeba histolytica.*[1] It has long been known that many people apparently infected with *E. histolytica* never develop symptoms and spontaneously clear the infection. This was interpreted by many workers as indicating a parasite of variable virulence. However, in 1925 Emile Brumpt suggested an alternative explanation, that there were in fact 2 species, one capable of causing invasive disease and one that never causes diease, which he called *E. dispar.* Brumpt's hypothesis was dismissed by other work-

Table I. Oligonucleotide primers used for PCR

Primer	Sequence	Direction	Corresponding cDNA nucleotides (bp)
p1 5	TAAAGCACCAGCATATTGTC 3	Sense	107-126
p2 5′	GTGAAGTTATTGGAGTGAGT 3	Sense	274-293
p3 5′	GATGACATATCCTCTTCTTG 3	Antisense	458-439
p4 5′	TTAATTCCATCTGGTGTTGG 3	Antisense	637-618

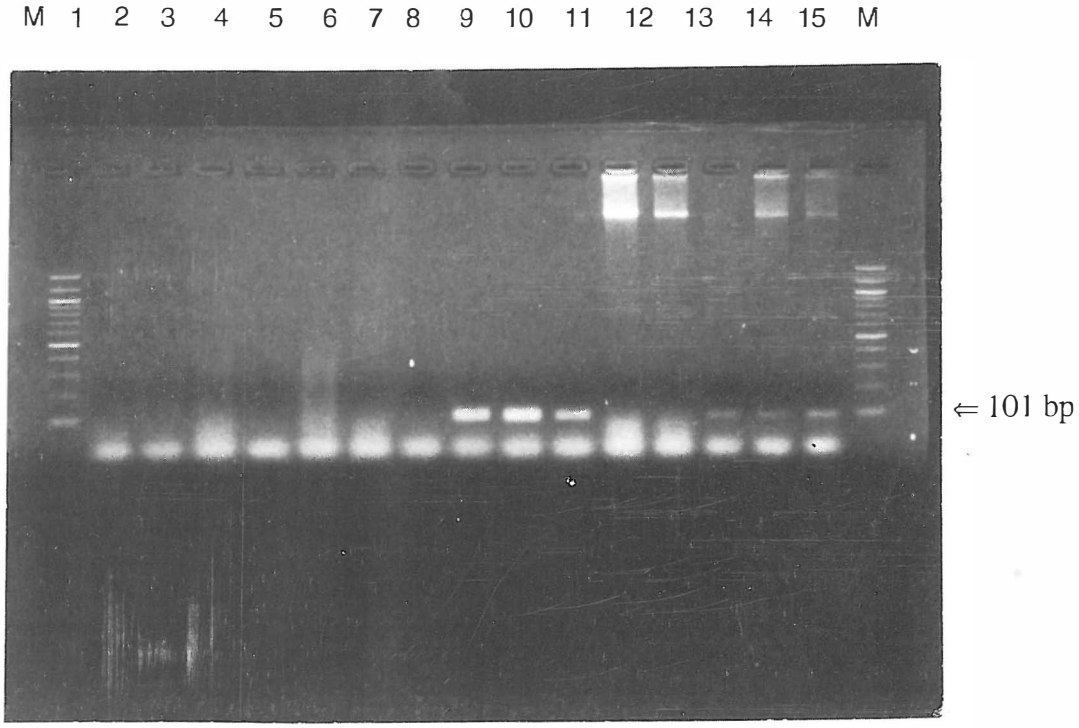

Fig. 1. Agarose-gel electrophoresis of PCR products amplified by *E. dispar* primers p13 plus p14 and *E. histolytica* primers p11 plus p12. Template DNA were extracted from cysts collected after formalin-ether concentration technique and xenic and axenic culture's media from positive stool samples. Amplified products were subjected to electrophoresis in agarose gels and visualized under UV after ethidium bromide staining (Lanes 1-12 test samples; Lanes 8-10 from axenic cultures, Lanes 13-15 from xenic cultures, M DNA size marker-100-bp ladder). Arrow indicates the position and size of PCR products.

ers.[3] In the 1970s, data started to accumulate that gave support to Brumpt's hypothesis of the existence of 2 distinct organisms within what was being called *E. histolytica*. Biochemical, immunological, and genetic data continued to accumulate and in 1993 a formal redescription of *E. histolytica* was published, separating it from *E. dispar*.[3]

This is the first report of PCR-based *E. histolytica/E. dispar* diagnosis from Iran. This report presents an application of PCR for field diagnosis and differentiation of E. histolytica from E. dispar using template DNA directly extracted from stool samples. The DNA-extraction method of Rivera et al. (1996) was slightly modified. The use of a sonicator for the lysis of cysts was omitted. Also, the use of PEG (polyethylene glycol) for repurification of the extracted DNA was added to the protocol and proved to be effective in getting rid of residual fecal debris after phenol-chloroform-isoamyl alcohol extraction, which may affect the PCR. The assay is sensitive and can be performed in less than 2 days.[10] Also, the use of formalin-fixed stools for DNA extraction has an advantage in terms of safe handling and storage of samples.

Although the possibility of the presence of *E. histolytica* in the survey area could not be ruled out, nonpathogenic *E. dispar* predominated in the collected samples.

These results indicate that all of the 15 samples microscopically diagnosed as *E. histolytica* were actually *E. dispar*.

However, the only sample diagnosed as *E. histolytica* is still questionable, because at the time of performing PCR, there were many *E. histolytica* cultures in the same laboratory, and because of high sensitivity of the PCR technique, and perhaps because of low experience of the workers, contamination of the sample may have occurred. We conclude, with high confidence, that all of the samples from Iran were *E. dispar,* but this requires an extensive PCR-based epidemiological study to be undertaken in other areas of Iran in order to understand the accurate distribution pattern of *E. histolytica/E. dispar* in Iran.

REFERENCES

1. Anonymous: Amebiasis. Weekly Epidemiological Record. 14: 97-99, Apr. 4, 1997.
2. Tannich E: *Entamoeba histolytica* and *E. dispar:* comparison of molecules considered important for host tissue destruction. Trans R Soc Trop Med Hyg 92: 593-595, 1998.
3. Diamond LS, Graham Clark C: A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. J Euk Microbiol 40(3): 340-344, 1993.
4. Graham Clark C: *Entamoeba dispar*, an organism reborn. Trans R Soc Trop Med Hyg 92: 361-364, 1998.
5. Sheiban P, Rezaian M: Study on intestinal protozoa in seven villages in Bandar Abbas 1978. Iranian Journal of Public Health 10 (1-4): 45-53, 1981.
6. Robinson GL: The laboratory diagnosis of human parasitic amoeba. Trans R Soc Trop Med Hyg 62(2): 285-294, 1968.
7. Kobayashi S, Imai E, Tachibana H, Fujiwara T, Takeuchi T: *Entamoeba dispar*: cultivation with sterilized *Crithidia fasciculata*. J Euk Microbiol 45(2): 3s-8s, 1998.
8. Diamond LS, Harlow DR, Cunnick CC: A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans Roy Soc Trop Med Hyg 72(4): 431-432, 1978.
9. Graham Clark C: Axenic cultivation of *Entamoeba dispar* (Brumpt 1925), *Entamoeba insolita* (Geiman and Wichterman 1937) and *Entamoeba ranarum* (Grassi 1879). J Euk Microbiol 42(5): 590-593, 1995.
10. Diamond LS: Entamoeba, giardia and trichomonas. In: Taylor AER, Baker JR, (eds.) *In vitro* Methods for Parasite Cultivation. New York: Academic Press, Ch. 1, 1987.
11. Kobayashi S, et al: YIGADHA-S medium for axenic cultivation of *Entamoeba dispar*. (Unpublished data)
12. Rivera WL, Tachibana H, Agnes Silva-Tahat MR, Uemura H, Kanbara H: Differentiation of *Entamoeba histolytica* cysts and *E. dispar* DNA from cysts present in stool specimens by polymerase chain reaction: its field application in the Philippines. Parasitol Res 82: 585-589, 1996.
13. Tachibana H, Ihara S, Kobayashi S, Kaneda Y, Takeuchi T, Watanabe Y: Differences in genomic DNA sequences between pathogenic and nonpathogenic isolates of *Entamoeba histolytica* identified by polymerase chain reaction. Journal of Clinical Microbiology 29 (10): 2234-2239, 1991.
14. Tachibana H, Kobayashi S, Masataka T, Ihara S: Distinguishing pathogenic isolates of *Entamoeba histolytica* by polymerase chain reaction. The Journal of Infectious Diseases 164: 825-6, 1991.
15. Sanuki JI, Asai T, Okuzawa E, Kobayashi S, Takeuchi T: Identification of *Entamoeba histolytica* and *E. dispar* cysts in stool by polymerase chain reaction. Parasitol Res 83 (1): 96-8, 1997.