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Original Article

Identification of *Entamoeba histolytica* by Molecular Method in Surface Water of Rasht City, Iran

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Abstract

Background: This study was conducted with the aim of determining surface water contamination with cysts of *Entamoeba histolytica* using PCR in Rasht City, Northern Iran.

Methods: In this cross-sectional study, 49 water samples including 18 rivers and 6 wetlands were collected from different regions near the city of Rasht in autumn of 2012. After filtration using 0.22 µm nitrate cellulose membrane filters, the samples were examined using microscope and PCR method.

Results: In microscopic examination, four samples of the 49 samples were positive for cysts of *E. (histolytica / dispar / muschkovskii)*. By using PCR method and molecular analysis, one sample was positive for *E. histolytica*.

Conclusion: In the molecular analysis, contamination by *E. histolytica* was proved in the waters of Rasht City. Further investigations including more samples and necessary preparations must be applied to prevent contamination.

Keywords: *Entamoeba histolytica*, PCR, Surface water, Iran

Introduction

Entamoeba histolytica is an enteric anaerobic protozoan parasite with about 50 million infections and over 100,000 deaths worldwide annually (1-4). Developing countries have the highest prevalence of amoebiasis because of human faeces have not been properly separated from food and water supplies. However, socio-economic factors, including poor education, poverty, overcrowding, and unsanitary conditions are also involved in fecal-oral transmission (5). The travellers to endemic areas with low standards of hygiene and sanitation are at risk (6). *E. histolytica* may also be transmitted by food like uncooked vegetables, and salads. Contaminated hands of food handlers are important in transmission, too. The swimming pools are a potential source, although it has not been proved (7).

The cysts of *E. histolytica* are very resistant and can survive for several months in water with temperature of 0 °C, 3 days at 30 °C, 30 minutes at 45 °C, 5 minutes at 50 °C, and are extremely resistant to chlorination (8).

The genus *Entamoeba* contains six species (*E. histolytica, E. dispar, E. moshkovskii, E. poleki, E. coli,* and *E. hartmanni*) in the human intestinal lumen (9-13). *E. moshkovskii* is a free-living amoeba found in anoxic sediments (10) and *E. dispar* is considered as a commensal of the human gut. Although *E. histolytica* is proved a pathogen, we still cannot definitely determine that other two species do not cause diseases (11, 12). *E. histolytica, E. dispar,* and *E. moshkovskii* are morphologically similar, but have differences in genetic and biochemistry characteris-

tics (9-13). Since these three species cannot be differentiated by microscopy that is the most frequently used diagnostic method predominantly in tropical countries where resources are limited and can only be differentiated by the use of molecular methods such as the polymerase chain reaction based methodologies (1, 9, 14).

There are few studies on surface water contamination with *Entamoeba* and the most studies have been done on fecal samples in epidemiological surveillance. In Turkey two out of six water samples (32%) collected from the Ankara River were positive for *E. histolytca* by PCR (15). Based on a study in Thailand, 27% of surface and wastewater samples were positive for *Entamoeba* spp**.** (16). Using direct method and Gram staining on water samples in Mazandaran showed the contamination rate of *E. histolytica* and *E. coli* were 2.3%. and 0.7%, respectively (17) and Mahmoudi et al., detected *Acanthamoeba* species in 14 out of 27 samples by PCR method in surface water of Rasht, Guilan, Iran (18).

Considering the high level of ground water in the northern parts of Iran, the lack of adequate sanitation in rural areas, integration of surface water with domestic and industrial wastewater, especially in the rainy season, and also the fact that contaminated water is one of the transmission ways for *E. histolytica*, this study was conducted with the aim of determining surface water contamination with cysts of *E. histolytica* using PCR method in Rasht City.

Materials and Methods

In this cross-sectional study, 49 water samples were randomly taken from 18 rivers and 6 wetlands from different regions near Rasht City in autumn of 2012. Rasht City, in the southern of the Caspian Sea and capital of Guilan province, is one of the wettest regions in Iran, which can also be very humid. It is Seven meters below sea level and 15 km inland from the Anzali Lagoon. The samples were collected from 30 cm depth in one-liter bulk and transferred to the laboratory in sterile containers. After centrifugation and filtration using 0.22 µm nitrate cellulose membrane filters, the samples were examined and analysed using microscope in direct method. Positive samples for *Entamoeba* spp. were examined by PCR method and sequencing. Genomic DNA of *E. histolytica* (HM-1: IMSS) was kindly provided by Dr. Haghighi, Department of Parasitology, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Freeze-Thaw method was used for lysing cysts wall before DNA extraction. In the first phase, cysts were subjected to five freeze-and-thaw cycles to facilitate the breakage of cyst wall, followed by 20–25 minutes of sonication (30-second pulse followed by 30-second rest) electrical shock (seven shocks every 15 s) was given to cysts by Sonificator system (Hielscher, Germany). Then, DNA was extracted using a DNA isolation kit (DNP kit, Cina gene, Iran) and Phenol- Chloroform extraction method.

PCR primers were designed based on small-subunit rRNA (ribosomal RNA) of *E. histolytica* using Invitrogen site. Primers sequences were as follows: forward primer 5´CCCGAGAATAGAAAACTCTT3´ and reverse primer 5´TCAAGTATAGTGCACCATCT 3´. PCR amplifications were performed in a final volume of 25 µl containing one-time PCR buffer 2.5 µl, 1.5 mM $MgCl_2$ 0.8 µl, 200 µM of each dNTP, 2 U Taq DNA polymerase (Takapoo Zist, Iran), 1 µl of each primer (10 mM, Takapoo Zist) and DNA Template 2.5 µl(100-200 ng). Reactions were carried out in a Thermocycler (Eppendorf, Germany) PCR System and set as follows: 35 cycles contain denaturation at 94 ˚C, annealing at 43.5 ˚C, extension at 72˚C, every stage for 30 s and finally the PCR products were analyzed on 1.8% agarose gel after electrophoresis. PCR generates 220 bp amplicon. The Sequencing was used on PCR product (by Pishgam co., Iran) for controlling of the specificity of the result for *E. histolytica.*

Results

In microscopic examination, four samples of the 49 samples were positive for cysts of *Entamoeba (histolytica / dispar / muschkovskii)*. These three species cannot be differentiated by microscopy and can only be differentiated by the use of molecular

methods. By using PCR method, one sample was positive for *E. histolytica*. Just as we expected, in one sample in addition to positive control that was Genomic DNA of *E. histolytica* (HM-1: IMSS), had a band with 220 bp weight (Fig.1).

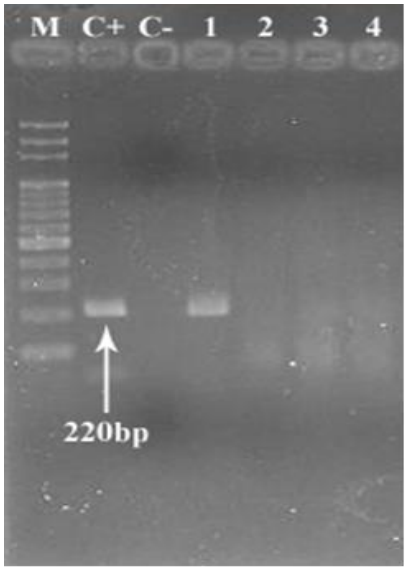

Fig. 1: PCR amplification of samples DNA with the *Entamoeba histolytica* specific primers
Lane M: molecular marker (100 bp) ladders, C+: positive control (*E. histolytica* DNA), C-: negative control (H2O), Lane 1: amplified product (220 bp) indicating positive sample, Lanes 2-4: Positive Samples in microscopic examination that were not amplified by PCR.

In molecular analysis and sequencing (by Pishgam co., Iran) as shown in Table 1., the gene sequence had 94%, 94% and 93% homology with 18s ribosomal RNA(rRNA), 5.8s rRNA (in plasmid) and small-subunit 1 gene *E. histolytica*, respectively.

Discussion

The differentiation between the amoeba species is not possible using light microscopic methods and WHO has put emphasis on the need to develop improved techniques for the species-specific diagnosis of *E. histolytica* infection (3).
Many epidemiological surveys on the prevalence of intestinal amoeba based on microscopic methods were performed in Iran and almost all of them show a high prevalence of infection in different parts of Iran. It is essential to note that the majority of them have not used molecular methods (19-23). The recently recognized distinction among the *E. histolytica, E. dispar,* and *E. moshkovskii* has led to some confusion in epidemiological studies of amoebiasis (24).
A study in stool samples by direct and formalin-ether concentration methods in Iran proved the prevalence of infection with *E. histolytica/ E. dispar* was 0.78%, 3.9% and 4.6% for the central, northern and southern part of Iran, respectively (21).

Table 1: Sequences producing significant algnments (by Pishgam co., Iran)

Gene	Accession Number*	Homology (%)
Entamoeba histolytica rRNA**(18s rRNA)	X65163	94
E.histolytica plasmid genes for 5.8s rRNA and heoly sins HLY1, HLY5mc1 HLY5mc2 HLY4	Z29969	94
E. histolytica ss*** 1 gene	Y11271	93

*GenBank, ** ribosomal RNA, ***small subunite

A molecular method for differential diagnosis of *E. histolytica* and *E. dispar* (PCR-RFLP method) showed that in different regions of Iran, 92.1% of the isolates were *E. dispar* and 7.9% were *E. histolytica* or mixed infections. In the northern areas, 5.9% and 94.1% of isolates were *E. histolytica* and *E. dispar*, respectively (20). Many studies using molecular methods confirm that *E. histolytica* is a rare species in Iran and *E. dispar* is the predominant species (12, 20, 21, 24-30).
The only molecular study on amoeba in Iran suggesting that *E. histolytica* as more prevalent than *E. dispar,* was conducted by PCR, where 10 of 11 positive samples in microscopic examination were

E. histolytica and only one of them was *E. dispar* (31).

Water is a possible source for transmission of *Entamoeba* to human host. Cysts can survive for prolonged periods in the environment, because of the protection by their cell wall (32, 33).

Bakir et al. indicated that two out of six water samples (32%) collected from the Ankara River in Turkey were positive for *E. histolytca* by PCR (15).

Phuc et al., suggested in northern of Vietnam where Livestock and domestic sewage are used in agriculture, infection with *E. histolytica* depends on hygiene-related behaviors and socio-economic factors (34).

In Mazandaran (Iran) by direct method and Gram staining, 197(19.9%) out of 989 samples were contaminated with parasites. From 197 parasitic contaminated samples, 53 cases (26.9%) were pathogenic parasites. The contamination rate of *E. histolytica* was 2.3%. Overall, 100 cases (50.8%) were nonpathogenic and the contamination rate of *E. coli* was 0.7% (17). Furthermore, Mahmoudi et al., detected *Acanthamoeba* species in 14 out of 27 samples by PCR method in surface water of Rasht, Guilan, Iran (18).

In this study, by microscopic examination, four samples of surface water of Rasht were positive for *Entamoeba*, but we had not any suggestion about the species. By PCR method in these four samples, one sample was positive for *E. histolytica.* We had a positive control for *E. histolytica,* but had not any positive controls for *E. dispar and E. moshkovskii,* therefore could not identify the species of other three samples. For confirmation the result of PCR, we used sequencing on the PCR production. In sequencing, the gene sequence had 94%, 94% and 93% homology with 18s rRNA, 5.8s rRNA (in plasmid) and small-subunit 1 gene *E. histolytica*, respectively that is a confirmation for PCR examination.

Conclusion

Contamination by *E. histolytica* was proved in the surface water of Rasht City and this is the first report of detection of *E. histolytica* in surface water in Iran by molecular method.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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