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ORIGINAL ARTICLE

Characterization of *Entamoeba histolytica* and *Entamoeba dispar* in fresh stool by PCR

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ABSTRACT

Aim: The aim of this study was to differentiate *Entamoeba dispar* from *Entamoeba histolytica* by PCR directly from fresh stool.

Background: Microscopy does not allow for the differentiation of *Entamoeba dispar* from *Entamoeba histolytica*. Several PCR-based methods have been described and used successfully for this purpose, but the methods for DNA extraction from stool samples are usually time-consuming and problematic due to inhibitory factors in feces.

Patients and methods: From a total of 1700 stool samples collected and examined by microscopy, 22 samples (1.3%) were microscopically positive for the *E. histolytica /E. dispar* complex. The DNA of these samples was extracted directly from fresh stool and PCR was carried out using two sets of species-specific primers from a short tandem repeat (STR) in the D-A locus.

Results: Of these, 21 samples (95.45%) were diagnosed as *E. dispar* and only one sample (4.55 %) was found to be *E. histolytica*. In this study, by improving the DNA extraction from fresh stool, we were able to efficiently differentiate *E. histolytica* and *E. dispar*.

Conclusion: To avoid unnecessary treatment of patients not infected with *E. histolytica*, the development of effective techniques, such as direct DNA extraction from stool, is recommended.

Keywords: *Entamoeba histolytica, Entamoeba dispar, fresh stool, PCR, locus D-A.*

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INTRODUCTION

Although stool microscopy is a simple and extremely economical procedure that can be done in any laboratory equipped with a light microscope using inexpensive reagents (1), the identification and differentiation of pathogenic *Entamoeba histolytica* from non-pathogenic *Entamoeba dispar* in stool by this method are imprecise (2). Furthermore, this technique depends greatly on the experience and skills of the microscopist (1) and has low sensitivity (2), requiring preparation and examination of multiple samples (3). Epidemiological data on amebiasis are mainly based on microscopic detection of the *E. histolytica/ E. dispar* complex without the differentiation between the two species (4).

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Several polymerase chain reactions (PCR)-based methods have been described and used successfully, but methods for DNA isolation from cysts in stool samples are time-consuming and problematic due to inhibitory factors in feces (5). On the other hand, the direct application of fresh stool is preferable because culturing is laborious and not always successful (5). In theory, microbial culture is not necessary when PCR is applied, if DNA extraction from the stool specimens provides appropriate and sufficient material for analysis. In practice, however, this is not straightforward, and most studies still rely on cultured cells. As improvements in extraction methods develop, the analysis of DNA derived directly from stool is likely to become more widespread (6). Several reports show the presence of the *E. histolytica/E. dispar* complex by DNA extraction directly from fresh stool. For example, in Australia, the presence of the *E. histolytica/E. dispar* complex was investigated by microscopy and PCR on DNA extracted directly from stool samples (7) and another study in Italy reported direct DNA extraction from formalin-fixed stool samples (8).

Early studies of amebiasis in Iran have reported that the incidence of *Entamoeba* species varies from 2.2 to 30% in urban and rural communities, respectively. However, these studies, which did not differentiate *E. histolytica* from *E. dispar* (9, 10), applied PCR-RFLP to samples from northern, central and southern Iran. They showed that 92.1% of the isolates were *E. dispar* and just 7.9% were *E. histolytica* or mixed infections (11). In another Iranian study using direct fecal sample DNA extraction, all asymptomatic patients that were passing cysts were infected with *E. dispar* (12). Due to the clinically important difference between these two species (13), the aim of this study was to determine the prevalence of *E. histolytica* and *E. dispar* by PCR with genomic DNA extracted from fresh stool in all samples found positive for ameba by microscopy.

PATIENTS and METHODS

Study area and stool samples:

From August 2006 to September 2007, a total of 1700 samples were collected at health care centers of Tehran, Iran, in order to determine the prevalence of *E. histolytica* and *E. dispar* among patients with gastrointestinal complaints, such as abdominal pain, flatulence, tenesmus, diarrhea or dysentery (14).

Microscopic examination of the samples:

A single fresh stool specimen was collected from each patient without fixative. The presence of parasites was determined by microscopic examination of fresh stools using direct slide smear, formalin-ether concentrated specimens and trichrome stain (15).

DNA preparation:

Total genomic DNA from cysts and/or trophozoites was purified from stool samples using DNA extraction solution (DNG-plus ™, Cinnagen, Iran), according to the manufacturer's instructions. The DNA concentration of each sample was determined spectrophotometrically, by measuring the optical absorbance at 260 and 280 nm. The extracted DNA was then stored at -20 °C until used.

PCR analysis:

Identification of *E. histolytica* and exclusion of *E. dispar* were verified by PCR using the following two sets of specific primers from a noncoding short tandem repeat (STR) in locus D-A, also known as locus 1-2: Hsp1 (GAG TTC TCT TTT TAT ACT TTT ATA TGT T) and Hsp2 (ATT AAC AAT AAA GAG GGA GGT) for *E. histolytica* and Dsp1 (TTG AAG AGT TCA CTT TTT ATA CTA TA) and Dsp2 (TAA CAA TAA AGG GGA GGG) for *E. dispar* (16, 17), known to amplify ~340 and 430 bp fragments for *E. dispar* and *E. histolytica*, respectively. Amplification consisted of 35 cycles of 45 seconds at 93 °C, 30 seconds at 55 °C, and 60 seconds at 72 °C, with a

final extension of 5 min at 72 °C. The PCR products were isolated by electrophoresis on 2% agarose gels containing ethidium bromide and the gels were photographed under ultraviolet light (UVIdoc, UVItec, Cambridge, UK). *E. histolytica* strain HM-1: IMSS (17) and *E. dispar* strain AS16IR (18) were used as positive controls.

RESULTS

By light microscopy examination of each sample, among the 1700 patients with GI complaints, 787 (46.3%) of whom were male and 913 (53.7%) female, 217 (12.8%) cases were infected with only one species of parasite and 28 (1.6%) samples were infected with two or more species, while no parasite was detected in 1455 (85.6%) of the samples, for a total of 245 (14.4%) patients positive for intestinal protozoa and/or helminthes.

Among the protozoa, the most prevalent species was *Entamoeba coli* (77, 4.5%), followed by *Giardia lamblia* (51, 3%), *Blastocystis hominis* (39, 2.3%), *Endolimax nana* (23, 1.4%), *Iodamoeba bütschlii* (21, 1.2%) and *Cryptosporidium* spp. (2, 0.6%).

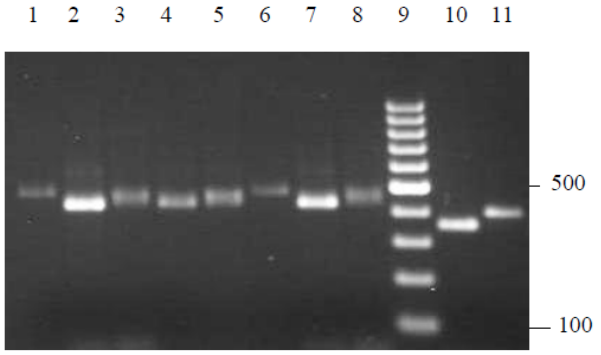

Figure 1. Agarose gel electrophoresis of *Entamoeba histolytica* and *Entamoeba dispar*. Lane 1-7: *E. dispar*-positive isolates with Dsp1-Dsp2 primers; Lane 8: *E. dispar*-positive control (AS16IR); Lane 9: 100 bp marker; Lane 10: *E. histolytica*-positive control (HM-1: IMSS); Lane 11: Iranian *E. histolytica*-positive isolate with Hsp1-Hsp2 primers

Of the 1700 patients, 22 (1.3%) were positive for *E. histolytica/E. dispar* complex. From these, the total genomic DNA was extracted. In 21 samples (95.45%), PCR for STR D-A locus amplified a fragment of ~430 bp identifying these as *E. dispar*. One patient (4.55 %), who suffered from abdominal pain, vomiting and diarrhea, showed a band of about 360 bp, indicating the presence of *E. histolytica* (Fig.1).

DISCUSSION

The use of PCR for the diagnosis of *E. histolytica* is helpful (2), since microscopy has low sensitivity (2) and cannot be used to differentiate it from *E. dispar* (13). To provide proper treatment, the development of efficient techniques, such as direct DNA extraction from stool is recommended by the World Health Organization (13). The extraction method used in this study was originally developed for stool samples, providing fast and easy purification of total DNA from fresh and frozen stool samples.

Of the 22 samples positive for the *E. histolytica/E. dispar* complex by microscopy, only one patient required treatment for *E. histolytica*. Of course, within the differential diagnosis viral or bacterial pathogens known to cause such clinical symptoms must be considered. Our results, along with those of other similar studies performed in northeastern (19), northern, central and southern Iran (11), reveal that *E. dispar* is much more prevalent than *E. histolytica* among patients in Iran. The difference in the size of the *E. histolytica* PCR product from the sample from the Iranian patient versus that of the HM1: IMSS strain is due to genetic polymorphism within the STR D-A gene (6, 14, 20).

In the present study, the highest rate of infection among protozoa was *G. lamblia*, similar to other studies in Iran (10, 12).

In this study, an improvement in the DNA extraction technique from fresh stool allows for a

more efficient and precise detection of the pathogen, *E. histolytica*, as there is no point to identify microbes that do not cause these symptoms, such as *E. dispar*. Thus, this method quickly guides the differential diagnosis toward more likely causes of this disorder.

This extraction method was successfully applied to a PCR assay for the detection of DNA in fresh stool samples, which showed high specificity and low detection limits. Using this approach, there is no need to culture, allowing for faster and more appropriate patient treatment. This report describes a very simple method for the distinction of *E. histolytica* and *E. dispar* in unpreserved fresh fecal samples and this PCR-based method offers technical advantages for routine detection invasive *E. histolytica* (21, 22).

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