



Study of *Entamoeba Histolytica* from Stool Using Polymerase Chain Reaction Amplification

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ABSTRACT

Entamoeba histolytica infection is the third-greatest parasitic disease responsible for death in the world. Amoebic infections result either in a harmless colonization of the intestine, or in an amoebiasis with invasion and damage of the intestine, liver, lung, and brain. These distinct manifestations are due to the existence of *Entamoeba histolytica* alone or with *Entamoeba dispar* as a complex of two different, but morphologically identical species. One that is a nonpathogenic commensal in the intestine of humans, *E. dispar*, and the other that is capable of inducing cell and tissue damage. Due to genomic DNA differences between pathogenic and nonpathogenic of these protozoan infections, we used a polymerase chain reaction (PCR) method that diagnosed and differentiated the two conditions. We report here a DNA extraction protocol using non-fixed stool samples. We characterized 60 of 65 stool specimens from patients with amoebiasis. Among them, 45 (75%) were infected only with the nonpathogenic species, *E. dispar*, while 15 (25%) displayed a mixed infection with both the pathogenic nonpathogenic species, i.e., *E. dispar* and *E. histolytica*. The PCR protocol showed a specificity of 1.00 and a sensitivity of 0.95. The molecular approach is therefore reliable and applicable in the identification of pathogenic *E. histolytica* infection. Our results provide important data for the Iraqi Health Care System and a need to address the emerging problem of amoebic infection in Iraq.

Keywords: *human, Entamoeba Histolytica, PCR, diagnosis*

INTRODUCTION

Intestinal protozoan infections are closely related to a lack of proper sanitation and environmental contamination with faecal matter. *Entamoeba histolytica* prevalence is higher in specific environment that occur most often in developing countries (1-3). Amoebiasis is a potentially severe and life threatening infection caused by enteric protozoa (3-5), most commonly *Entamoeba histolytica*, which is distributed worldwide. *Entamoeba histolytica* infection is the third greatest parasitic disease responsible for death in the world after malaria and schistosomiasis. (6-7). It affects approximately 180 million people, of whom 40,000 to 110,000 die each year (8). Amoebic infections result either in a harmless colonization of the intestine, or in amoebic invasion and ulceration of the intestine, and damage of other host tissues. This assertion derives from extensive microbial, pathological, immunological, and molecular data that

indicate they have a high degree of divergence and are, in fact, two separate species.(4-7)

A clinical diagnosis of amoebiasis can be confirmed by microscopic identification of characteristic cysts or trophozoites in the stool. However, microscopic examination has several limitations,(9-11) the most important being the inability to distinguish *E. histolytica* from *E. dispar*. In addition, multiple samples often have to be examined and the presence of cysts of different species such as *Entamoeba*, *Iodamoeba*, or *Endolimax* can make diagnosis difficult. The epidemiology of *Entamoeba* can be further studied by serological testing, culturing trophozoites and determining isoenzyme patterns by gel electrophoresis. However, these techniques are laborious, expensive, and time consuming, and are not practical for routine diagnostic laboratories and with serological testing, it may be difficult to distinguish past from present infections.(12-15). Molecular biology has helped to fill



this gap. Identification of *E. histolytica* by polymerase chain reaction (PCR) was first used in 1991 (4). Since its discovery, PCR and then real-time PCR have been increasingly used for amebiasis diagnosis and showed to provide rapid, sensitive, and specific results,

MATERIALS AND METHODS

Samples Collection:

Stool samples were collected from individuals who sought medical attention for abdominal discomforts and diarrheal diseases, at the parasitology services of the hilla teaching hospitals from January to June 2015. All specimens were studied by routine procedures used for microscopic examination of feces in the Laboratory. To confirm infection with microorganisms of the *E. histolytica* and or *E. dispar* complex, each sample was analyzed using the enzyme test. This test is a commercial enzyme immunoassay kit, and was used according to the manufacturer's instructions, based on the described procedure(16).

Samples were divided into 3 groups:

- 1) positive group: an *E. histolytica*/ *E. dispar*-positive group with 60 individuals who tested positive by both microscopic examination and the enzyme test.
- 2) A negative control group : including samples from 25 individuals found to be negative by microscopic examination and the enzyme test.
- 3) A cross reaction control group with 20 patients infected with other parasites, including *Blastocystis hominis* (5 patients), *Entamoeba coli* (5 patients), *Giardia lamblia* (5 patients), *Endolimax nana* (5 patients). All patients in this group were confirmed negative for *E. histolytica* and or *E. dispar* by enzyme test.

Extraction of DNA from stool samples.

All procedures were performed using sterile, disposable plastic tubes and pipette tips. DNA was extracted according to the following protocol. Feces (0.5 grams) were placed in a 1.5-ml micro centrifuge tube, washed once with 1 ml of phosphate-buffered saline solution (pH 7.5), and filtered through gauze. The feces samples must be washed with PBS before lysis of cyst and trophozoites to eliminate soluble

In this study, we used a PCR- based approach for the detection and characterization of the two species of the *E. histolytica*/*E. dispar* complex. This PCR methodology was applied to amplify the two species-specific DNA fragments using two pairs of specific primers.

contaminants that affect the specificity of the PCR and yield of amplification. The filtered supernatant was centrifuged at 3000 X g for 5 min, resuspended in 500 µl of lysis-supporting buffer (LSB, pH 8.0), and added to a 2-ml capped tube containing 500 µl of phenol. The mixture was centrifuged at 12,000 X g for 20 min. The aqueous layer was recovered, extracted with chloroform: alcohol (25:1), and the DNA was precipitated with one volume 500 µl of isopropanol. The pellet was resuspended in 100 µl of TE buffer (pH 8.0). Isopropanol was used to selectively precipitate DNA without the carbohydrates that are abundant in *Entamoeba* and could interfere with the amplification reactions. This protocol resulted in the isolation of DNA of sufficient quality and quantity for sensitive and accurate PCR amplification. The PCR method performed for amplification and detection was as described by Acuna-Soto and others(16) using Mastercycler gradient thermal cycler (Eppendorf). The amplification reactions were performed using 10 µL of DNA extract in a volume of 40 µL reaction mixture that contained a 1× of master mix from Applied Biosystems, 29.25 µL of H₂O, 3 µL of MgCl₂ (25 mM), 1 µL each of forward and reverse primer (0.5 µM) and 1,75 U of Taq polymerase. The thermal cycling conditions consisted of 1 cycle of 4 min at 95°C, 30 s at 55°C, and 30 s at 72°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and last cycle of 30 s at 95°C, 30 s at 55°C, and 5mn at 72°C.

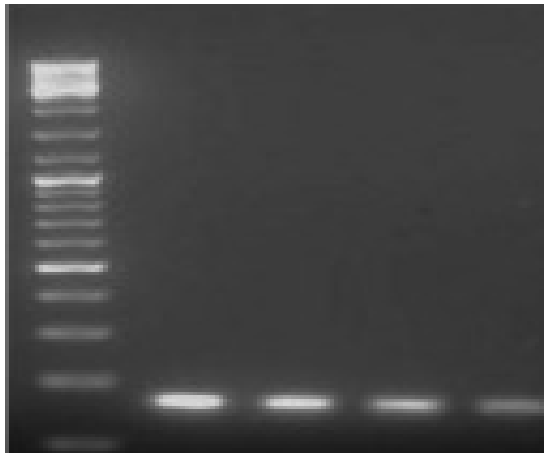
The primers for *E. histolytica* and *E. dispar*: Eh -196F (5'-AAA TGG CCA ATT CAT TCA ATG A-3') Ed-185F (5'-GTA TTA GTA CAA AGT GGC AAT TTA TGT-3') Ehd-294R (5'-CAT TGG TTA CTT GTT AAA CAC TGT GTG-3'). Four controls were included in all experiments: 1) all reagents except DNA template, 2) control DNA from *E. histolytica*, 3)

control DNA from *E. dispar*, and 4) a mixture of control DNA from *E. histolytica* and *E. dispar*. Amplified products were visualized with ethidium bromide after electrophoresis on 10% acrylamide gels.

RESULTS AND DISCUSSION

We characterized 60 of 65 stool specimens from patients with amoebiasis. Among them, 45 (75%) were infected only with the nonpathogenic species, *E. dispar*, while 15 (25%) displayed a mixed infection with both the pathogenic nonpathogenic species, ie, *E. dispar* and *E. histolytica*.

Primer specificity. Amplification was specific for each primer pair. The *E. histolytica* primers (EhP1/2) amplified DNA from the HM1-IMSS strain but not from *E. dispar* whereas the *E. dispar* primers (EdP1/2) amplified DNA from *E. dispar* but not from HM1-IMSS. When parasites from both control strains were mixed and specific DNAs were amplified using a mixture of the two primer pairs, the two 96 and 132 bp fragments were visualized after electrophoresis and staining with ethidium bromide. No interference was noted between the two amplification systems.



In our study, and through using PCR technique it confirmed that A total of 60 out of 65 cases examined samples were infected with *E. histolytica*/*E. dispar* and or *E. dispar*/*E. histolytica*. Among them, 45 (75%) were infected only with the nonpathogenic species, *E. dispar*, while 15 (25%) displayed a mixed infection with both the pathogenic nonpathogenic species, ie, *E. dispar* and *E.*

histolytica. Acrylamide gels were used to ensure proper differentiation of the amplified products, which differ in length by just 36 nucleotides. Visualization was accomplished *via* ultraviolet illumination.

Detection limit. both *E. histolytica* and *E. dispar* DNA were detected by the PCR, even at the minimum parasite concentration tested (100 parasites/0.5 grams of feces)(fig 1). This indicates that up to 10-1 pg of DNA could be detected by this procedure as only 10 µl DNA (100 parasite/100 µl of TE) was used for amplification.

PCR specificity and sensitivity. No products were detected when samples from the negative control group and the cross-reaction control group were tested by the PCR. This represents a maximum specificity (1.00) and no collateral cross-reactions. Results of the PCR with samples from infected individuals showed a sensitivity of 0.95 and indicated circulation of both *E. histolytica* and *E. dispar*.

Figure 1. Results of the polymerase chain reaction (PCR) amplifications products of *E. histolytica*. Representative ethidium bromide-stained agarose gel pictures showing PCR amplification products of *E. histolytica* DNA extracts recovered from feces seeded with various counts of oocysts/cysts .left amplicons were the DNA ladder; . Right amplicons were the the lower detection limits i.e., lowest number of oocysts/cysts pre sent per extract.

histolytica. Other investigators have also found that infection with *E. dispar* is more common than infection with *E. histolytica*(17-22). Evidence of the inverse proportion has been reported by and others(23) who targeted the same specific and tandemly repeated DNA sequences described in the current study and found *E. histolytica* as the predominant population. Similarly,



the occurrence of mixed infections with both *E. histolytica* and *E. dispar* has been reported,(24-28).

Our study confirm that molecular diagnostic approaches is superior to all others laboratories methods in detection and differentiating both the pathogenic and nonpathogenic species of *E. histolytica* *E* and *dispar* in stool samples.(25,27,29) moreover, the improvements and simplification of PCR procedures directly from stools make it superior to others related stool tests .The protocols are accurate and simple.

Several reports,(29-33) found a clear association between *E. histolytica*/*E. dispar* and *Entamoeba coli* in terms of coinfection, although no *E. histolytica*/*E. dispar* mixed infections were detected. They did not discount the possibility of a competitive phenomenon

in vivo between *E. histolytica* and *E. dispar*, but referred to an in vitro study (34) showing that only a minuscule amount of *E. histolytica* can ultimately outgrow *E. dispar* in culture in a given period of time. In contrast, others (35) reported that some pathogenic amoebic zymodemes outgrow others. These findings suggest that an adequate animal model of amoebiasis needs to be developed as a prerequisite to clarify this phenomenon (35).

In conclusion, our data indicates that molecular approach is reliable and applicable in the identification of pathogenic *histolytica* infection. Our results provide important data for the Public Health-Care System and a need to address the emerging problem of amoebiasis in Iraq.

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