Differentiation of *Entamoeba histolytica* from *Entamoeba dispar* by nested multiplex polymerase chain reaction

Original Article

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ABSTRACT

Background: Amoebiasis is a parasitic disease caused by the intestinal protozoon *Entamoeba histolytica*. Microscopic examination fails to differentiate *E. histolytica* from the morphologically identical nonpathogenic *Entamoeba dispar*. To avoid unnecessary treatment of individuals infected with nonpathogenic *E. dispar*, it is essential to differentially diagnose infections caused by pathogenic from nonpathogenic *Entamoeba* spp.

Objective: The aim of this study was to assess the efficacy of nested multiplex PCR (NM PCR) technique as a diagnostic method for differentiating infections caused by *E. histolytica* and *E. dispar.*

Materials and methods: Stool samples collected from patients with and without symptoms of amoebiasis were screened for *E. histolytica*/*E. dispar* trophozoites/cysts by microscopic examination. NM PCR was performed on a total of 52 samples targeting the genus-specific 16S-like ribosomal RNA gene for simultaneous, differential detection of *E. histolytica* and *E. dispar*.

Results: NM PCR detected *E. histolytica* at 439 bp and *E. dispar* at 174 bp, and it was positive in 31 out of 32 cases with a sensitivity of 96.85%. From those, 17 (32.7%) samples were positive for *E. histolytica*, 12 (23.1%) for *E. dispar*, and three (5.7%) for both species. In addition, NM PCR diagnosed *E. dispar* in one of the negative controls with a specificity of 95%.

Conclusions: NM PCR is useful for the specific detection of *E. histolytica* and *E. dispar* in stool samples.

Key Words: *Entamoeba dispar, Entamoeba histolytica*, nested multiplex polymerase chain reaction.

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INTRODUCTION

Amoebiasis is a parasitic disease caused by the intestinal protozoon *Entamoeba histolytica*. It is considered the third cause of death due to parasitic infections in humans after malaria and schistosomiasis, and was reported to be responsible for about 40,000-100,000 deaths/year in Australia[1]. It is worldwide in distribution, and is considered a serious health threat in tropical and subtropical developing areas; it is also considered a problem in travelers and immigrants in the developed world[2]. The prevalence and severity of *E. histolytica* infection vary from one geographical area to another and from one case to another, with the highest prevalence found in regions with low hygienic conditions[3,4].

Six of the morphologically identical *Entamoeba* spp. (*E. histolytica, E. dispar, E. moshkovskii, E. polecki, E. coli,* and *E. hartmanni*) can live in the human intestine. Only *E. histolytica* causes pathological lesions, whereas the others are considered as nonpathogenic[5] while *E. moshkovskii* is debated. In 1997, the WHO declared *E. histolytica* as a pathogenic species and *E. dispar* as a nonpathogenic species[6].

Traditionally, the laboratory diagnosis of *Entamoeba* spp. is based on their morphology on microscopic examination of stool[7]. However, morphological identification fails to differentiate *E. histolytica* from the identical nonpathogenic *E. dispar*[8]. Stool culture followed by isoenzyme analysis allows the differentiation, but it requires several weeks and needs special laboratory facilities, making it impractical for use in the routine diagnosis of intestinal amoebiasis[8]. Isoenzyme analysis for detecting the N-acetyl galactosamine/galactose (Gal/GalNAc) lectin of *E. histolytica* or *E. dispar* has been reported to give excellent sensitivity in endemic areas[9]. Stool antigen assays have been reported to be more sensitive and specific than microscopy. The coproantigen enzyme-linked immunosorbent assay (ELISA) technique has been suggested for screening *Entamoeba* spp. in routine diagnostic procedures and epidemiological studies. Although more reliable and specific than microscopy, it does not differentiate the species[10,11].

Additional methods for species differentiation have been used to avoid unnecessary treatment of individuals infected with the nonpathogenic species[12]. PCR demonstrated excellent sensitivity and specificity

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compared with microscopy. It has been endorsed by several studies[9,13] as the method of choice for clinical and epidemiological studies of amoebiasis. Multiplex PCR is a molecular biology technique used for the amplification of multiple targets in a single PCR reaction[14]; therefore, it is considered more cost-effective and less laborious than other PCR methods[15].

In the present study, NM PCR was evaluated as a diagnostic method for the simultaneous differential detection of *E. histolytica* and *E. dispar* in stool samples.

MATERIALS AND METHODS

Study design: In this descriptive analytical study, fresh stool samples were collected without preservatives in sterile containers from persons with stool analysis requests attending the Outpatient Clinics of Zagazig University Hospitals during the period between April 2014 and April 2015.

Stool samples were screened microscopically for the presence of *E. histolytica*/*E. dispar* cysts/trophozoites complex or any other intestinal parasites. According to the results of the stool analysis, a total of 52 stool samples were selected for testing by NM PCR and were classified into the following groups: group 1 (symptomatic group; G1) included 16 *E. histolytica*/*E. dispar*-positive samples for cysts/trophozoites from patients showing symptoms of amoebiasis (dysentery, tenesmus, abdominal pain and tenderness, alternating bowel habits, flatulence, nausea, vomiting, or fever); group 2 (asymptomatic group; G2) included 16 *E. histolytica*/*E. dispar*-positive samples from asymptomatic patients; and group 3 included 20 samples negative for *E. histolytica*/*E. dispar* cysts/trophozoites and negative for any other parasitic infections (negative-control group; G3). About 2 g of each sample was aliquoted into 1.5-ml screw-cap tubes and stored at -20°C until DNA extraction and NM PCR targeting the 16S-like rRNA gene.

Stool examination: Naked-eye macroscopic examination of collected samples determined consistency, color, odor, and presence of blood, mucous, or pus. For microscopic examination, all samples were subjected to saline and iodine wet-mounts and formol–ether concentration technique within 2 h after fecal sample collection as previously described[16,17]. The results of the stool analysis were recorded either as positive or as negative for *E. histolytica*/*E. dispar*, according to detection of cysts or trophozoites of either species.

Genomic DNA extraction: DNA extraction was carried out using Favor Prep stool DNA Isolation Mini Kit (Cat. No. FASTI001; Favorgen Biotech Corporation, Pingtung, Taiwan), according to the manufacturer's guide, with modification. The thermal shock procedure was carried out (cycling of samples by deep-freezing in liquid nitrogen for 5 min, followed by immediate transfer into a 95°C water bath for 5 min (repeated for five cycles), and incubation for 1 h at 95°C and for 10 min at 56°C. The purified DNA was measured for concentration and purity and kept at -20°C until further use.

Nested multiplex polymerase chain reaction: A primary PCR was performed targeting the 16S-like rRNA gene for the detection of *Entamoeba* genus. Used primers (Table 1) included the forward primer EF and the reverse primer ER amplifying the genus-specific gene, which is ~800 bp. Subsequently, the primary PCR products were subjected to secondary PCR for *Entamoeba* spp.-specific characterization. Amplification was achieved using primer sets EHF and EHR to detect the *E. histolytica* fragment segment at 439 bp and primer sets EDF and EDR to detect the *E. dispar* fragment segment at 174 bp.

The reaction components and the cycling conditions were selected according to Ngui *et al.*[12] with modification in the form of 12.5 µl master mix, 200 nM from each primer, and 3 µl of the template DNA for the primary reaction and 1 µl for the secondary reaction in a total volume of 25 µl and 56°C annealing temperature for the primary and 48°C for the secondary reactions. The amplified products were visualized after 1.5% agarose gel electrophoresis with ethidium bromide staining. Control samples without (negative control) and with *Entamoeba* spp. genomic DNA (positive control) were included in each PCR run. Two standard strains, *E. histolytica* HM-1:IMSS and *E. dispar* SAW760, were used as positive controls.

Table 1: Oligonucleotide primers used in NM PCR

	Primer names	Primer sequence
Genus-specific primers (used in the first PCR)	EF (forward primer)	5′-TAA GAT GCA GAG CGA AA-3′
	ER (reverse primer)	5′-GTA CAA AGG GCA GGG ACG TA-3′
Species-specific primers (used in the second NM PCR)	EHF (forward primer)	5′-AAG CAT TGT TTC TAG ATC TGA G-3′
	EHR (reverse primer)	5′-AAG AGG TCT AAC CGA AAT TAG-3′
	EDF (forward primer)	5′-TCT AAT TTC GAT TAG AAC TCT-3′
	EDR (reverse primer)	5′-TCC CTA CCTATT AGA CAT AGC-3′

Statistical analysis: The results were calculated, tabulated, and statistically analyzed using statistical computer program SPSS, version II, for Windows 7[18]. A *P*-value of less than 0.05 was considered to be statistically significant.

Ethical consideration: A description of the objectives and methodology of the study was given to the patients or parents/guardians before sample collection, and a written or thumb-printed informed consent was obtained. The study was approved by the Committee of Research, Publications, and Ethics of the Faculty of Medicine, Zagazig University, Egypt.

RESULTS

The present study was conducted on 52 stool samples to detect and differentiate *E. histolytica* from *E. dispar* infections by NM PCR. *E. histolytica* was detected at 439 bp and *E. dispar* at 174 bp (Figure 1). Of 32 microscopy-positive samples for *Entamoeba* spp. (Table 2), 31 samples were successfully amplified and characterized as *Entamoeba* spp. on the basis of amplicon size with a sensitivity of 96.9% (Table 3). *E. histolytica* was detected in 17 /32 (53.12%) samples, *E. dispar* in 11/ 32 (34.38%) samples, and three (9.37%) samples were positive for both (Table 2). Examination of 20 microscopy-negative samples confirmed that 19 samples were negative for *E. histolytica* infection, and one sample was positive for *E. dispar* (Table 2) with 95% specificity (Table 3).

In G1, of 16 symptomatic cases, 12 (75.0%) samples were positive by NM PCR for *E. histolytica*, two (12.5%) samples for *E. dispar*, one (6.25%) sample for both, and one (6.25%) sample was negative. Of the 16 positive samples from G2 of asymptomatic cases, five (31.2%) samples were positive by NM PCR for *E. histolytica*, nine (56.3%) samples for *E. dispar*, and two (12.5%) samples for both. Of the 20 microscope-negative samples in G3, one sample was diagnosed as *E. dispar* positive (Table 4). The detection rate of *E. histolytica* was higher (75%) in symptomatic G1 than in nonsymptomatic G2 (31.2%), whereas the detection rate of *E. dispar* was higher (56.3%) in G2 than in G1 (12.5%), and this relationship was statistically significant ($P<0.05$) (Table 4).

DISCUSSION

E. histolytica, the causative agent of human intestinal and extra-intestinal amoebiasis, is a protozoon responsible for significant morbidity and mortality mainly in developing and some of the developed countries[19,20]. The diagnosis and epidemiology of amoebiasis have become more complex with the introduction of the *Entamoeba*

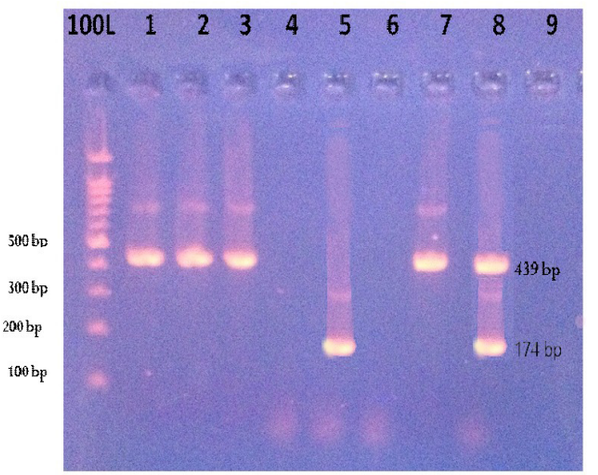

Figure 1: Agarose gel electrophoresis of NM PCR products amplified by *E. histolytica*-specific primers (EHF and EHR) and *E. dispar*-specific primers (EDF and EDR). Lane 100L: 100 bp DNA marker ladder. *E. histolytica* PCR products at 439 bp. *E. dispar* PCR product at 174 bp. Lanes 1–3: *E. histolytica* positive samples. Lanes 4, 6: Negative samples. Lane 5: *E. dispar*-positive sample. Lane 7: *E. histolytica* positive control. Lane 8: mixed infection of *E. histolytica*/*E. dispar*. Lane 9: Negative control.

Table 2: Differential detection of *E. histolytica* and *E. dispar* in stool specimens by NM PCR

	Results of NM PCR [n (%)]				
	Positive				Negative
Results of microscopy	*E. histolytica*	*E. dispar*	*E. histolytica* and *E. dispar*	Total	Total
Positive (N=32)	17 (53.13)	11 (34.38)	3 (9.37)	31 (96.87)	1 (3.12)
Negative (N=20)	0 (0)	1 (5.0)	0 (0)	1 (5)	19 (95)
Total (N=52)	17 (32.69)[a]	12 (23.08)[a]	3 (5.77)[a]	32 (61.54)[a]	20 (38.46)[a]

NM: nested multiplex, [a] Percentage from total

Table 3: Sensitivity, specificity, and positive and negative predictive values of NM PCR for *Entamoeba* spp. complex in relation to microscopic examination

Screening tests (PCR)	Results
True positives (TP)	31
False negative (FN)	1
False positives (FP)	1
True negatives (TN)	19
Sensitivity [TP/(TP+FN)] (%)	96.9
Specificity [TN/(TN+FP)] (%)	95
PPV [TP/(TP+FP)] (%)	96.9
NPV [TN/(TN+FN)] (%)	95

NPV: negative predictive value, **PPV:** positive predictive value.

Table 4: Results of NM PCR in the different groups studied

Groups	*E. histolytica* [n (%)]	*E. dispar* [n (%)]	Mixed [n (%)]	Negative [n (%)]	Statistical analysis
G1 (N=16)	12 (75)	2 (12)	1 (6.25)	1 (6.25)	χ^2=6.055 P=0.014
G2 (N=16)	5 (31.2)	9 (56.3)	2 (12.5)	0 (0)	
G3 (N=20)	0 (0)	1 (5)	0 (0)	19 (95.0)	
Total (N=52)	17 (32.7)[a]	12 (23.1)[a]	3 (5.7)[a]	20 (38.5)[a]	

G1: symptomatic group, **G2:** asymptomatic group, **G3:** noninfected control group, **[a]:** Percentage from total.

complex, which included pathogenic *E. histolytica* and nonpathogenic *E. dispar*[21]. For the assessment of the true prevalence of pathogenic *E. histolytica* and the accurate diagnosis of amoebiasis, it is important to detect and distinguish *E. histolytica* from nonpathogenic species[8]. Microscopic examination of stool samples was the most commonly used diagnostic test, particularly in areas with limited resources; however, it cannot differentiate between these three species[22]. Differentiation between the morphologically identical pathogenic and nonpathogenic species can be achieved genetically, biochemically, and immunologically[5], as well as by performing isoenzyme analysis, but the procedure requires to be preceded by stool culture, which is time consuming and laborious with a sensitivity of only about 50%[8]. Even the commercial ELISA-based method for specific identification and detection of *E. histolytica* in fecal specimens[23] has shown poor sensitivity and specificity in many studies because of cross-contaminations with other parasites[24,25]. In the last decade, molecular-based diagnostic tests have gained importance in the diagnosis of many infectious diseases including amoebiasis to overcome the problems of conventional methods with the advantages of increased sensitivity, specificity, and simplicity[26,27]. Several PCR assays designed to differentiate *E. histolytica* from *E. dispar* have been described[15,28,29]. Most of them targeted either the small-subunit ribosomal RNA gene or specific episomal repeats species. Accordingly, the present study was conducted to assess the application of NM PCR to recognize and differentiate *E. histolytica* and *E. dispar* directly from stool samples.

In the present study, NM PCR diagnosed 31 out of 32 samples collected from symptomatic patients, and failed to diagnose one *Entamoeba*-positive sample with 96.9% sensitivity. Similarly, El Sobky *et al.*[30] reported a sensitivity of NM PCR of 96.4% compared with trichrome-stained preparations (75%). The negative result by NM PCR in the present study in one microscopy-positive sample could be explained by the presence of DNA from an *Entamoeba* spp. other than *E. dispar*/*E. histolytica*. It was suggested by Ngui *et al*.[12] and Fallah *et al.*[31] that the samples detected by microscopy but not PCR may belong to other *Entamoeba* spp. such as *E. coli*, *E. hartmanni*, and *E. polecki*. The results of the present study are comparable with those of Fallah *et al.*[31] who recorded positive PCR in 25 out of 31 stool samples diagnosed by microscopic methods. A slightly lower sensitivity (94%) was reported by Khairnar and Parija[8] whose results showed negative NM PCR in 12 *Entamoeba* spp.-microscopy-positive stool samples. Failure to detect pathogens in stool samples by PCR may be explained by the complexity of the specimens for direct PCR testing due to the presence of PCR inhibitors such as heme, bilirubin, bile salts, and complex carbohydrates, which are often coextracted along with pathogenic DNA[9]. Therefore, optimization of the fecal

DNA extraction procedure is critical to the success of PCR. Stool samples positive by microscopy and negative by NM PCR could also contain a low number of parasites, below the PCR detection limit[12].

In their study, Khairnar and Parija[8] reported that NM PCR results were negative for the 35 negative-control stool samples with 100% specificity. In the present study, the NM PCR result was positive for one sample, which showed negative results by microscopy. This may be explained by possible contamination of the sample by DNA from other sources[31], resulting in a decreased specificity of 95% (Table 3). In the study by Gachuhi *et al.*[32], microscopic examination identified 21.3% of their patients to be infected with *E. histolytica*/*E. dispar* complex, whereas multiplex-PCR detected and distinguished 24.9% of their stool samples to be infected with either/or both species. They explained their findings by the higher sensitivity of multiplex PCR over microscopy in detecting *Entamoeba* spp. infection with 100% specificity.

In the present study, *E. histolytica* infection diagnosed by NM PCR proved to be higher compared with *E. dispar* – that is, 32.7% of the examined stool samples. On the other hand, *E. dispar* was diagnosed in only 23%. *E. histolytica* was more prevalent in samples collected from symptomatic cases complaining of gastrointestinal tract troubles (75%) than in samples from the asymptomatic cases (31.2%). The opposite occurred with *E. dispar* infection, which was more prevalent in asymptomatic (56.3%) than in symptomatic patients (12.5%). These findings oppose the results recorded by Khairnar and Parija[8] who reported monoinfection by *E. dispar* in 49.5% and *E. histolytica* in only 7.4% of the cases examined. Herbinger *et al.*[11] detected *E. dispar* in 88.3% and *E. histolytica* in 9.7% of the cases. Nohýnková *et al.*[33] reported that 95.6% were positive for *E. dispar,* whereas only three (4.4%) patients were positive for *E. histolytica*. Fallah *et al.*[31] reported monoinfection by *E. dispar* in 54.8% and *E. histolytica* in 25.8%, and Gachuhi *et al.*[32] reported monoinfection by *E. dispar* in 20.1% and *E. histolytica* in 2.4%. From Menoufia governorate in Egypt, *E. dispar* was detected in 41.7%, *E. histolytica* in 25%, and mixed infection occurred in 33.3% of the cases analyzed by multiplex PCR[30]. In the present study, mixed infection by both species was found in 5.7% of cases, and reports of coinfection in different studies varied from 2.5[32] to 18.8%[8]. No coinfection was reported by Fallah *et al.*[31]. Compared with other reported records, the unusually high detection rate of *E. histolytica* in our symptomatic group may be attributed to the fact that most of the cases were from neighboring rural areas where such infections are more common.

In a study comparing the sensitivity of NM PCR with microscopy and the TechLab *E. histolytica* II ELISA kit, ElBakri *et al.*[25] reported 19.2% (23/120) *Entamoeba* spp. infection by NM PCR in the United Arab Emirates. Of those, 10% (12/120) included monoinfection with *E. histolytica* and 2.5% (3/l20) with *E. dispar*. They also detected mixed infections by both *E. histolytica* and *E. dispar* in 3.3% (4/120) of cases. However, the TechLab ELISA kit failed to detect *E. histolytica* in any of the *E. histolytica* PCR-positive samples, which was attributed to the low antigen levels in the fecal samples, below the detection limit of the kit, or because the assay recognizes only the amoebic adhesion of vegetative forms, normally present in diarrheal fecal specimens during an acute amoebic infection, and not the cyst-stage antigen. In addition, polymorphism in the lectin antigen used in the *E. histolytica* II ELISA may also explain the failure of this test. Nested PCR microscopy was found to have an overall sensitivity of 52.2% and a specificity of 75.2% for detecting the *Entamoeba* complex.

In conclusion, these findings demonstrate that NM PCR is a more sensitive and reliable technique that allows simultaneous, differential detection of *E. histolytica* and *E. dispar*. It is recommend that molecular techniques such as NM PCR should be used for the specific detection and differentiation of *E. histolytica* and *E. dispar* DNA in stool specimens as it is a highly specific, sensitive, rapid, and promising tool for epidemiological studies, particularly for discriminating pathogenic species from nonpathogenic species of *Entamoeba*.

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