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Original Article

Identification of *Entamoeba* Species in Diarrheal Samples Using Sequence Analysis and Nested Multiplex PCR

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Received 10 Dec 2024
Accepted 19 Mar 2025

Keywords:
Entamoeba spp.;
Diarrhea samples;
Nested multiplex PCR assay

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Abstract

Background: Among the many *Entamoeba* species that infect humans, only *Entamoeba histolytica* is considered pathogenic, being responsible for amoebiasis or amoebic dysentery.

Methods: Between June and October 2022, a total of 106 stool samples were collected from children under six years of age presenting with diarrhea at Paiji Hospital in the city of Paiji, Iraq. DNA was extracted from all stool specimens to detect the presence of parasitic organisms.

Results: Of the 106 fecal samples, 4 (3.7%) tested positive for *Entamoeba* spp. using an initial PCR amplification targeting approximately 900 bp of the 18S rRNA gene. Among these, only one sample tested positive for *E. histolytica* using a nested multiplex PCR assay. In this study, neither *E. moshkovskii* nor *E. dispar* was detected. Sequence analysis of the partial 18S rRNA gene revealed that 0.9% of samples were positive for *E. histolytica*, while 2.8% were positive for *E. coli*. The sequences were deposited in GenBank under the accession numbers OP868733.1 for *E. histolytica* and OP868730.1, OP868731.1, and OP868732.1 for *E. coli*.

Conclusion: Children were infected with different species of *Entamoeba*. Molecular methods are essential for distinguishing between *Entamoeba* species due to their significance in accurate diagnosis and appropriate treatment strategies.

DOI: https://doi.org/10.18502/ijpa.v20i2.19032

Introduction

The protozoan *Entamoeba* spp. includes various species, six of which inhabit the gastrointestinal tract: *E. coli*, *E. histolytica*, *E. moshkovskii*, *E. dispar*, *E. poleki*, and *E. hartmanni* (1). Among these amoebae, only *E. histolytica* is considered pathogenic to humans and is responsible for amoebiasis or amoebic dysentery (2,3). The most common symptoms of this disease include diarrhea, cramping, abdominal pain, low-grade fever, colitis, fatigue, stools containing mucus and blood, ulcers, and weight loss (4,5).

E. coli is considered a non-pathogenic protozoan that resides in the human intestinal tract (6). However, in severe *E. coli* infections, affected individuals may experience gastritis and indigestion. In general, symptoms of amoebiasis caused by *E. coli* include flatulence, loose stools, and, in rare cases, colicky abdominal pain (7).

The life cycle of *Entamoeba* spp. is similar across species. It begins with the ingestion of parasite cysts through food or drink contaminated with feces. Once the cysts reach the small intestine, excystation occurs, and trophozoites emerge. The trophozoites migrate to the large intestine and attach to the mucosal layer (8).

Microscopic examination has been widely used in diagnosing amoebic infections; however, it is both insensitive and unreliable for distinguishing between infections caused by *E. histolytica* and non-pathogenic *Entamoeba* spp. (9). It is essential to differentiate between morphologically similar pathogenic species, such as *E. histolytica*, and non-pathogenic species, including *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli*, and *E. hartmanni* (10,11).

Molecular methods based on genomic DNA amplification have proven to be highly sensitive and reliable for distinguishing between *Entamoeba* species (12).

Materials and Method

Sample Collection

A total of 106 fresh stool samples were collected from Paiji Hospital (city of Paiji, Iraq) from diarrheal children under six years of age during the period from June to October 2022. All samples were placed in sterile containers and transferred to the parasitology laboratory at the University of Tikrit. The samples were stored at -20°C for molecular analysis.

Molecular Method

DNA was extracted from all stool samples to detect *Entamoeba* spp. Genomic DNA extraction was performed using the Stool DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. The purified DNA samples were stored at -20°C for subsequent PCR amplification.

Nested Multiplex PCR Assay

The first PCR amplified approximately 900 bp of the 18S rRNA gene using specific primers: E-1 forward (5'-TAAGATGCACGAGAGCGAAA-3') and E-2 reverse (5'-TACAAAGGGCAGGGACGTA-3') for the detection of *Entamoeba* spp. (13). Each first reaction was performed in a 20 µl volume, containing 1 µl of each primer and 3 µl of template DNA. The thermal profile included one cycle of 95°C for 5 minutes to denature the double-stranded DNA, followed by 30 cycles of 94°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), with a final extension at 72°C for 5 minutes. PCR products were separated using 1.5% agarose gel electrophoresis and visualized.

The second PCR amplified 439 bp, 174 bp, and 553 bp for the detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii*, respectively, using three pairs of primers: EH-1 (5'-AAGCATTGTTTCTAGATCTGAG-3') and EH-2 (5'-AAGAGGTCTAACCGAAATTAG-3'); ED-1 (5'-TCTAATTTCGATTAGAACTCT-3') and ED-2 (5'-TCCCTACCTATTAGACATAGC-3'); and

MOS-1 (5'-GAAACCAAGAGTTTCACAAC-3') and MOS-2 (5'-CAATATAAGGCTTGGATGAT-3'). Each second reaction was performed in a 20 µl volume, containing 1 µl of each primer, 3 µl of the first PCR product, and water. The thermal profile for the second PCR included 35 cycles at 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), with a final extension at 72°C for 5 minutes. PCR products were separated using 1.5% agarose gel electrophoresis and visualized.

Sequencing

The 18S rRNA sequences were compared with sequences in the NCBI nucleotide database using BLAST for analysis and identification. Evolutionary analyses were conducted using MEGA6 software (14).

Results

Of the 106 fecal samples analyzed, 4 (3.7%) tested positive for *Entamoeba* spp. by the first PCR amplification of approximately 900 bp of the 18S rRNA gene (Fig. 1). Among these, only one sample (0.9%) was positive for *E. histolytica* using the nested multiplex PCR assay. Neither *E. dispar* nor *E. moshkovskii* was identified in the current study (Fig. 2). Sequence analysis of the partial 18S rRNA gene revealed that 4 samples were positive, including *E. histolytica* (0.9%) and *E. coli* (2.8%). The sequences were deposited in GenBank under the accession numbers OP868733.1 for *E. histolytica* and OP868730.1, OP868731.1, and OP868732.1 for *E. coli* (Fig. 3).

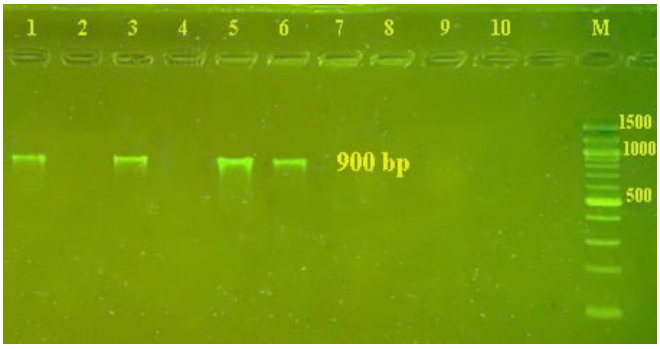

Fig. 1: Agarose gel electrophoresis for the product first PCR Amplify about 900bp of *18s rRNA* gene. lanes 1,3,5,6 positive for *Entamoeba* spp.

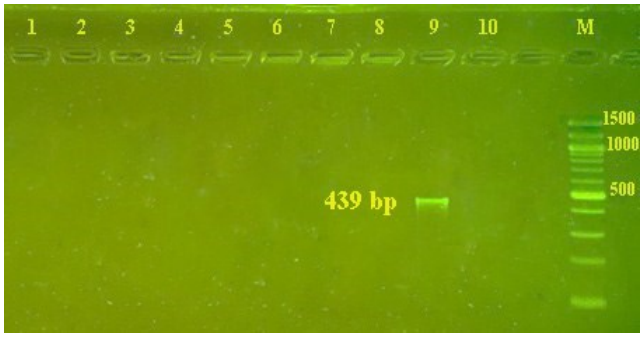

Fig. 2: Agarose gel electrophoresis for the product second PCR amplifying the 439bp, for the detection of the *E. histolytica*. Lane 9 positive for *E. histolytica*.

Table 1: prevelance of species *Entamoeba* in diarrhea children

Collected	First PCR		Nested multiplex PCR		Sequencing assay	
106	Species	Positive sample %	Species	Positive sample %	Species	Positive sample %
	Entamoeba spp.	4(3.7%)	*E. histolytica*	1(0.9%)	*E. histolytica*	1(0.9)
					E. coli	3(2.8)

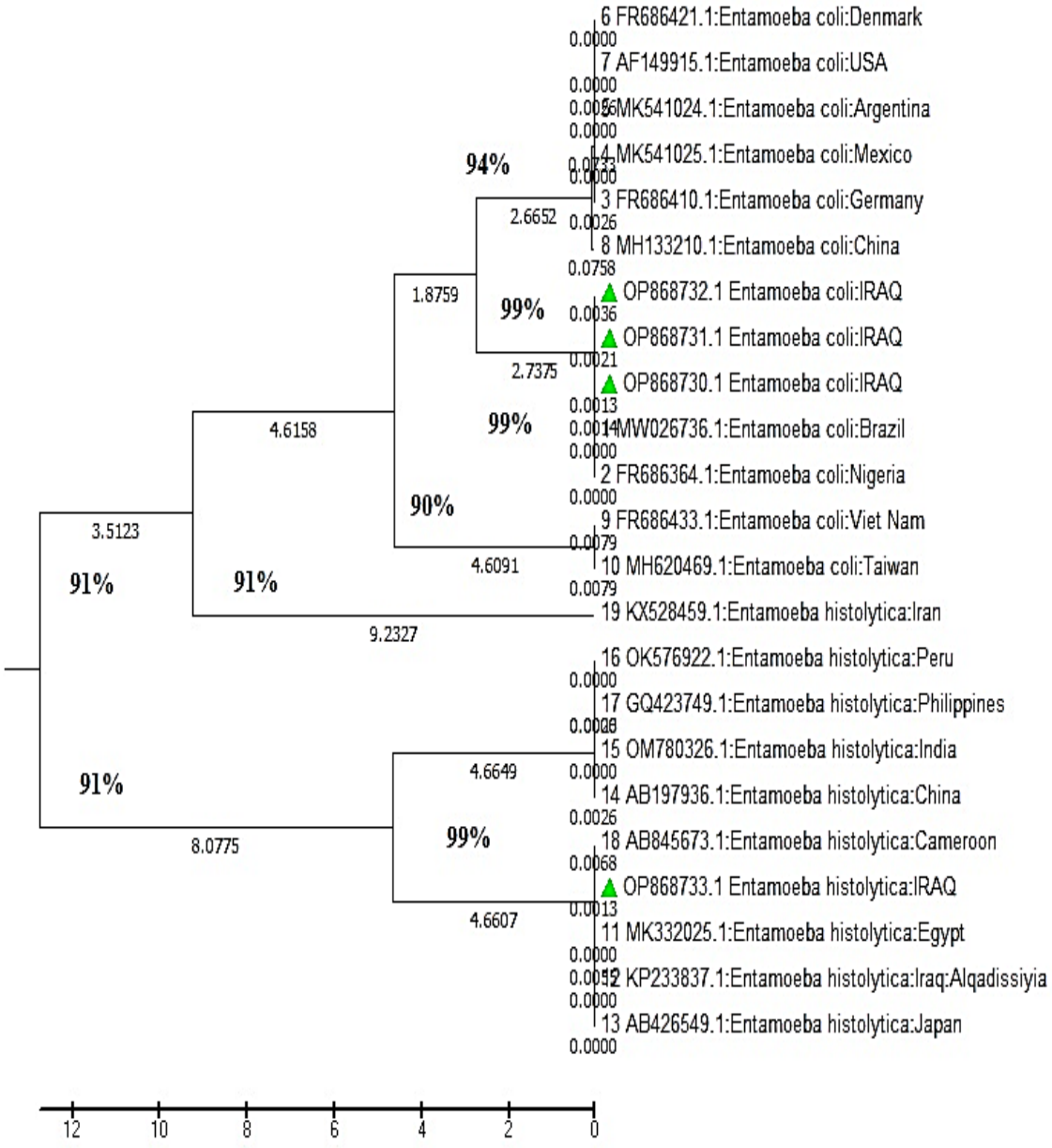

Fig. 3: Phylogenetic tree of *Entamoeba* spp identified in the present study and reference sequences from Gen Bank

Table 2: Genetic rapprochement of *18s rRNA* for the isolate of *Entamoeba* spp. in the present study in comparison with those obtained in the Genbank.

	Accession	Country	Source	Isolation source	Compatibility %
1.	ID: MW026736.1	Brazil	*Entamoeba coli*	Homo sapiens	99
2.	ID: FR686364.1	Nigeria	*Entamoeba coli*	Homo sapiens	99
3.	ID: FR686410.1	Germany	*Entamoeba coli*	Mandrillus leucophaeus	95
4.	ID: MK541025.1	Mexico	*Entamoeba coli*	Homo sapiens	94
5.	ID: MK541024.1	Argentina	*Entamoeba coli*	Homo sapiens	94
6.	ID: FR686421.1	Denmark	*Entamoeba coli*	Homo sapiens	94
7.	ID: AF149915.1	USA	*Entamoeba coli*	--------	94
8.	ID: MH133210.1	China	*Entamoeba coli*	Rhesus macaques	94
9.	ID: FR686433.1	Viet Nam	*Entamoeba coli*	Homo sapiens	90
10.	ID: MH620469.1	Taiwan	*Entamoeba coli*	--------	89
11.	ID: MK332025.1	Egypt	*Entamoeba histolytica*	--------	99
12.	ID: KP233837.1	Iraq:Alqadissiyia	*Entamoeba histolytica*	--------	99
13.	ID: AB426549.1	Japan	*Entamoeba histolytica*	De Brazza's guenon	99
14.	ID: AB197936.1	China	*Entamoeba histolytica*	cynomolgus monkey	99
15.	ID: OM780326.1	India	*Entamoeba histolytica*	Homo sapiens	99
16.	ID: OK576922.1	Peru	*Entamoeba histolytica*	Blueberries	99
17.	ID: GQ423749.	Philippines	*Entamoeba histolytica*	Macaque	99
18.	ID: AB845673.1	Cameroon	*Entamoeba histolytica*	Homo sapiens	99
19.	ID: KX528459.1	Iran	*Entamoeba histolytica*	Homo sapiens	99

Discussion

The presence of *Entamoeba* spp. in stool samples can be detected using microscopic examination, which primarily relies on morphological characteristics such as the number of nuclei and cyst size. However, misdiagnosis may occur when using microscopy due to overlapping morphological characteristics among different *Entamoeba* species (15).

Therefore, molecular methods were employed in this study because of their higher sensitivity and specificity in diagnosing *Entamoeba* spp.

In our study, the prevalence of *Entamoeba* spp. in humans was 3.7% (Table 1). This result is roughly consistent with that of Mahmood and Bakr (16), who reported a 7.4% infection rate in *Entamoeba* spp. in Erbil. However, our findings showed a much lower prevalence compared to Flaih et al. (17), who recorded an infection rate of 52.5%. The discrepancy in prevalence may be attributed to the timing of sample collection; in the current study, samples were collected during the summer when high temperatures likely reduce the viability of infective stages.

Using nested PCR, our study identified a prevalence rate of *E. histolytica* at 0.9%, while *E. dispar* and *E. moshkovskii* were not detected. In contrast, a study in Erbil reported infection rates of 6% for *E. histolytica*, 4.3% for *E. dispar*, and 0.3% for *E. moshkovskii* (16). Higher infection rates were observed in Thi-Qar, where microscopy and PCR detected *E. histolytica* in 55.5% and *E. dispar* in 30% of cases (18). Additionally, other studies reported *E. histolytica* prevalence rates of 0.14% in western Iran (19) and 7.5% in Kenya (20).

Three isolates of *E. coli* and one of *E. histolytica* were deposited in NCBI according to sequence analysis of the partial 18S rRNA gene, with accession numbers OP868730.1, OP868731.1, and OP868732.1 for *E. coli*, and OP868733.1 for *E. histolytica* (Fig. 3). These isolates showed 89–99% homology with *Entamoeba* spp. sequences recorded in GenBank from Brazil (MW026736.1), Nigeria (FR686364.1), Germany (FR686410.1), Mexico (MK541025.1), Egypt (MK332025.1), Iraq: Al-Qadisiyah (KP233837.1), Japan (AB426549.1), and China (AB197936.1), as well as other countries (Table 2, Fig. 3).

In the current study, sequence analysis of the partial 18S rRNA gene revealed the presence of the commensal *E. coli* in 2.8% of diarrhea samples and *E. histolytica* in 0.9%. The presence of *E. coli* in diarrhea samples may be due to co-infections with other pathogenic organisms, including bacteria, viruses, and fungi. In a study conducted in Erbil, *E. histolytica* and *E. coli* were detected with infection rates of 51.7% and 51.2%, respectively (21). Another study reported *E. coli* detection in stool samples using nested PCR with an infection rate of 32.7%, compared to 29.1% via microscopy (7). Furthermore, approximately 70% of patients infected with parasites had *E. coli* (22). The prevalence of *E. coli* in random stool samples was 4.6% (23). Additionally, *E. coli* was detected in Sudan with an infection rate of 7.5% (24) and in Brazil at 11.02% (25). Variations in prevalence rates can be attributed to factors such as differences in study populations, geographical locations, socioeconomic conditions, poor sanitation, personal hygiene practices, and overcrowding (26).

Conclusion

Children are susceptible to infections by both pathogenic *E. histolytica* and non-pathogenic amoebas. Molecular methods are crucial for differentiating between *Entamoeba* species due to their importance in accurate diagnosis and the implications for treatment. This study found a low infection rate of *E. histolytica* compared to *E. coli*, emphasizing the need for enhanced diagnostic techniques to improve disease management and control.

Conflict of Interest

The authors declare that there is no conflict of interests.

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