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Molecular study of amoebopore-c gene of *entamoebahistolytica* isolated from food handlers in Erbil city

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Abstract

Background and objective: *Entamoebahistolytica* is a protozoan parasite with high prevalence rates in tropical and subtropical regions of underdeveloped countries. The outcomes of *Entamoebahistolytica* infection are mostly asymptomatic; only a fraction of those infected develop dysentery and, in rare cases, liver abscesses. Its pathogenicity is due to a huge number of virulence factors. This study investigated the risk of *Entamoebahistolytica* among food handlers, screening, Sequencing, and phylogenetic tree of Ameobopore-c.

Methods: Total of 563 stool samples were collected from food handlers who visit the central laboratory in Erbil City every year. Wet mount microscopic examination was performed to diagnose the *Entamoeba* species, molecular analysis was done for positive samples. FavorPrep Stool for DNA Isolation Mini Kit (Favorgen, Taiwan) was utilized. PCR analysis was done targeting the Amoebopore-c gene using one set of primers to amplify the 705 bp fragment. Gel electrophoresis was performed to visualize the amplified DNA under Ultraviolet light. Fifteen random positive samples of the PCR product were sent to Macrogen in South Korea to determine the DNA sequence of the Amoebopore-C gene present in the *Entamoebahistolytica*.

Results: The study found no statistically significant relationships between infection rates and demographic or socioeconomic characteristics. In fact, that 21 of the 50 microscopic positive cases were also positive by PCR.15 sequences of the amoebapore-C gene revealed that a modification in a single nucleotide leads to a modification in a single amino acid. Alignment of amoebapore C**,** according to NCBI-BLAST Homology Sequence, showed the following identity percentages to samples from different countries: India (99.4%), the USA (99.18% and 99.7%), Iraq (99.38%), and Hamburg (99.84%)

Conclusion: PCR is a highly sensitive method for detecting *Entamoebahistolytica.* The *Entamoebahistolytica* isolates showed high homology in amoebapore-C gene sequences compared to global strains.

Keywords: *Entamoebahistolytica*; Amoebopore-c; Molecular analysis; Food handlers.

Introduction

Entamoebahistolytica causes amoebiasis, an infection of the gastrointestinal tract that might or might not have any signs of illness.(1)

The protozoan parasite *E. histolytica* infects humans and is unicellular. The genus *Entamoeba* comprises a minimum of seven species that can be detected in the feces of humans: *E. dispar, E. mshkoveskii, E. Bangladeshi*, *E. hartmanii, E. polecki*, *E. coli,* and *E. histolytica*. *Entamoebahistolytica* is the sole species that is linked to the disease.(2)

The parasite's life cycle is distinguished by two stages (trophozoite and cysticstage). The former is non-motile and excreted in feces; it can remain in the

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environment for several weeks. Mature cysts typically have four nuclei and a diameter of 20 µm. The trophozoite, the motile form, can range from 10 to 60 µm. Following colonizing the intestines, it mostly produces secretory bloody diarrhea and tissue destruction,[3] where trophozoites burrow into the lining of the intestines, quickly causing ulceration and damage to the tissue. Other organs could become infected as well.[4] The parasite can live in the host's digestive tract for a long time before any symptoms appear; only about 10% of infected people exhibit signs and symptoms of the disease. [5]
Dysentery phenotypes, such as blood and mucus in diarrheal stools, are clinical features of symptomatic amoebic infection. In rare instances, the parasite invades the liver and forms abscesses, which can manifest months or even years after visiting or living in an area where amoebiasis is common. The major virulence factors, such as amoebapore, Gal/GalNAc lectin, and cysteine proteases, have critical impacts on the pathogenesis of *E*. *histolytica*. However, *E*. *histolytica* displays versatility in its ability to adapt to various environmental conditions and exhibit differential impacts on the human host.[6]
There is some evidence that trophozoite virulence variability is influenced by genome plasticity and changes in gene expression that occur in vivo as a result of host invasion. The majority of the molecular pathways that are important for the establishment of an infection remain poorly understood, despite the identification of some virulence factors as critical components in pathogenesis.[7]
The significance of fecal-oral human-to-human transmission is demonstrated by the direct or indirect transfer of intestinal parasites through food, water, or hands. Foodborne illnesses are a major issue in both industrialized and underdeveloped nations, such as the United States, Germany, India, Iraq, etc.[8] Currently, there is no vaccination available to protect against amoebiasis. The most common treatment option is metronidazole, which can lead to serious side effects in humans. [9] Study aims to diagnose and detect pathogenic *Entamoebahistolytica* using routine laboratory procedures, molecular techniques, and screening for the virulence Gene, amebapore-c, sequencing, and a phylogenetic tree for Amebapore-c as a marker for the type of strain of *Entamoebahistolytica* isolate.

Methods

Study site and design

A cross-sectional study was performed between September 2024 and January 2025 on food handlers who came to the central laboratory in Erbil City.

The questionnaire

Everyone who took part in the study filled out a questionnaire that included basic personal information, including their age, gender, and whether they had ever had a medical problem.

Stool sample collection

A fresh stool sample was collected from each participant; in a sterile, airtight plastic container with a screw cap.563 food handlers were surveyed. All fecal samples were examined, macroscopically and microscopically, as soon as they were collected. The positive samples were preserved at -20 °C until the molecular analysis was carried out, and they were stored in special tubes without the use of preservatives.[10]

Laboratory methods

A- Macroscopic examination

Stool samples were examined visually for texture, composition, color, and content; color, for example, brown, yellow, odor, or anything else out of the ordinary; and presence or absence of mucus and blood.[11]

B- Microscopic examination

Microscopic examination was carried out using Lugol's iodine solutions and regular saline in a direct wet-mount fashion. Followed by covering the slide and examining it under 10X and 40X magnifications to identify *Entamoeba*

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species cysts and trophozoites.[11]

C- Molecular method

DNA extraction

Genomic DNA was isolated from feces by utilizing the FavorPrep™ Stool DNA Isolation Mini Kit. NO: FASTI 000(4 preps), FASTI 001 (50 preps), FASTI 001-1 (100 preps). The extracted DNA's purity and concentration were assessed using the Nanodrop spectrophotometer. The absorbance reading at 260 nm to 280 nm is used to measure the DNA purity, as well as its quantity and quality.

Amplification of *Entamoebahistolytica* DNA by PCR

To confirm the presence of *Entamoebahistolytica*, PCR was performed using primers specific to the Amoebopore-C gene,[12] using the isolated genomic DNA from the stool samples as a template." As shown in Table 1.

Thermocycle conditions for the Amoebopore-c gene

The temperature profile consists of the following steps: a 5-minute initial denaturation at 95 °C, 35 cycles of denaturation at 95 °C for 40 seconds, primer annealing at 58 °C for 40 seconds, an extension at 72 °C for 1 minute, and a final 5-minute extension at 72 °C.

PCR amplification reagents

in a 50 µl reaction mixture that included; 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 picomoles (pmol) of forward primers at a concentration of 10 pmol, reverse primers at a concentration of 10 (pmol), DNase-free water, and template DNA, all of which was done using a Bioresearch PTC-200 Gradient thermocycler, presented in Table 2.

Agarose gel electrophoresis

Using1.0- 1.5% agarose gel to separate DNA fragments according to their molecular weight is a standard procedure in nearly all molecular biology experiments, and it was employed here to separate amplified DNA. A DNA-safe dye was used to stain the DNA. For 1minute, a DNA ladder was subjected to 100 volts and then seen under ultraviolet light.

DNA sequencing analysis of the Amoebapore-C gene

Fifteen submitted sequences of the amoebapore-C gene from *Entamoeba histolytica* were dispatched to Macrogen Molecular Company in Korea.

Ethical considerations

The Research Ethical Committee of College of Health Sciences of Hawler Medical University gave its support to the project. Each participant was asked to sign an informed consent form before their sample was collected.

Table 1 Primer set utilized for Amoebopore C-gene

Gene	Primer	Sequence	Product size	Tm
Amoebopore-c	Forward	TGGTGAAAGAAAAACAAGGAAGT	705bp	57°C
	Reverse	ACATGCATGAATCAACCCAC		

Table 2 PCR Amplification Reagents

No.	PCR components	Concentration	Volume (µl)
1	Master Mix	2x	25
2	Forward Primer	10 Pmol	2
3	Reverse Primer	10 Pmol	2
4	DNase Free Water	-	18
5	Template DNA	50ng/µl	3
Total			**50**

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Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 23) was used for data analysis. Each variable has undergone basic frequency and descriptive analysis. A *P* value of less than or equal to 0.05 was deemed statistically significant, and Chi-square and Fisher's exact tests were employed to determine the significance of the responses.

Results

Table 3 shows the results of the microscopy-based analysis of 563 participants' *Entamoeba* species infections, broken down by gender, age, education level, living situation, and place of residence. The infection rate was higher among males (84%) than females (16%); however, the difference was not statistically significant (*P* = 0.670). The age group of 20–30 exhibited the maximum infection rate (40%), with no significant difference (*P* = 0.111). Participants in secondary school had the highest infection rate (50%), however, the disparities among educational levels were not statistically significant (*P* = 0.711). The infection rate was lowest among individuals with medium living conditions (6.4%), although there was no significant link (*P* = 0.464). Urban residents (70%) have higher infection rates compared to rural residents (30%), with the difference being statistically significant (*P* < 0.001).

Table 3 Demographic Characteristics of *Entamoeba* species by direct microscopic examination

Variant		No. of participants (%)	positive by microscope No. (%)	Negative by Microscope No. (%)	*P* value
Gender	Female	79 (14.03%)	8 (16%)	71 (12.61%)	0.670
	Male	484 (85.96%)	42 (84%)	442(78.56%)	
Age_ Group	>19	96 (17.05)	12 (24%)	84 (14.9%)	0.111
	20-30	303 (53.84%)	20 (40%)	283 (50.3%)	
	30 <	164 (29.14%)	18 (36%)	146(25.9%)	
Educational level	Primary school	163 (28.94%)	14 (28%)	149 (26.4%)	0.711
	Secondary school	300 (53.33%)	25 (50%)	275(48.8%)	
	Graduated	100 (17.77%)	11 (22%)	89 (15.8%)	
The living conditions of the individual	Poor	64 (11.37%)	8 (16%)	56 (9.93%)	0.464
	Medium	447 (79.45%)	36 (6.4%)	415 (73.7%)	
	Good	52 (9.24%)	6 (12%)	46 (8.17%)	
Residency	Urban	394 (69.98%)	35 (70%)	359 (63.8%)	<0.001
	Rural	169 (30.02%)	15 (30%)	154 (27.3%)	
Total		**563 (100%)**	**50 (100%)**	**153 (100%)**	

* Chi-Square test, *P*≤0.05 considered statistically significant.

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Table 4 showed that out of the 563 stool samples that were microscopically examined, 120 (21.3%) were from symptomatic individuals and 443 (78.7%) were from asymptomatic individuals. Of the 50 microscopically positive samples, 11 (22%) were from symptomatic individuals and 39 (78%) were from asymptomatic individuals. Microscopic detection of the parasite did not exhibit a statistically significant relation with clinical symptoms (*P* = 0.858).

Table 5 demonstrates that direct microscopy overestimated the infection rate compared to PCR, detecting 50 (8.88%) positive cases while PCR found 21 (42%). The *P*-value (0.000) indicates a significant difference.

Table 4 Clinical implications of *Entamoeba* species detection by direct microscopic

Clinical implication	No. of participants (%)	Positive (No., %)	Negative (No., %)	*P* value
Symptomatic	120 (21.3%)	11 (22%)	109 (21.26%)	0.858
Asymptomatic	443 (78.7%)	39 (78%)	404 (78.84%)	
Total	**563 (100%)**	**50 (100%)**	**513 (100%)**	

* Fisher's Exact Test, *P* ≤0.05 considered statistically significant.

Table 5 Detection frequency of *Entamoebahistolytica* using direct microscopy and the PCR method

Method	Positive No. (%)	Negative No. (%)	Total	*P*-value
Direct microscopic	50 (8.88%)	513 (91.09%)	563	<0.001
Polymerase chain reaction (PCR)	21 (42%)	29 (58%)	50	

* Fisher's Exact Test, *P* ≤0.05 considered statistically significant.

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Figure 1 demonstrates the PCR amplification of the amoeba pore C gene, which targets a 705 bp fragment using specific primers. Of the 50 microscopically positive stool samples, 21 exhibited the anticipated PCR band, which suggests the presence of the gene. The remaining 29 samples did not exhibit any amplification.

Sequence alignment and submission of the Amoebopore-c gene to GenBank

The Amoebopore-c gene sequences were verified for quality using BioEdit v7.0.5. By comparing the query sequences with other biological sequences, the analysis of homology, insertions-deletions, stop codons, and frame shifts was conducted using NCBI-BLAST. All newly found and annotated sequences were uploaded to the GenBank database, and sequences were submitted to the database using the BankIt tool, as shown in Table 6.

Alignment of nucleotide sequences of amoebapore C

A mutation within exon 3 (position 306-566) of the Amoebopore-c gene was identified through sequence alignment presented in Figure 2.

Table 6 The GenBank accession numbers for the *amoebapore-C* gene sequences of *Entamoebahistolytica*

Name	Accession No.	Gene name
Entamoebahistolytica	PV022031	amoebapore C
Entamoebahistolytica	PV022032	amoebapore C
Entamoebahistolytica	PV022033	amoebapore C
Entamoebahistolytica	PV022034	amoebapore C
Entamoebahistolytica	PV022035	amoebapore C
Entamoebahistolytica	PV022036	amoebapore C
Entamoebahistolytica	PV022037	amoebapore C
Entamoebahistolytica	PV022038	amoebapore C
Entamoebahistolytica	PV022039	amoebapore C
Entamoebahistolytica	PV022040	amoebapore C
Entamoebahistolytica	PV022041	amoebapore C
Entamoebahistolytica	PV022042	amoebapore C
Entamoebahistolytica	PV022043	amoebapore C
Entamoebahistolytica	PV022044	amoebapore C
Entamoebahistolytica	PV022045	amoebapore C

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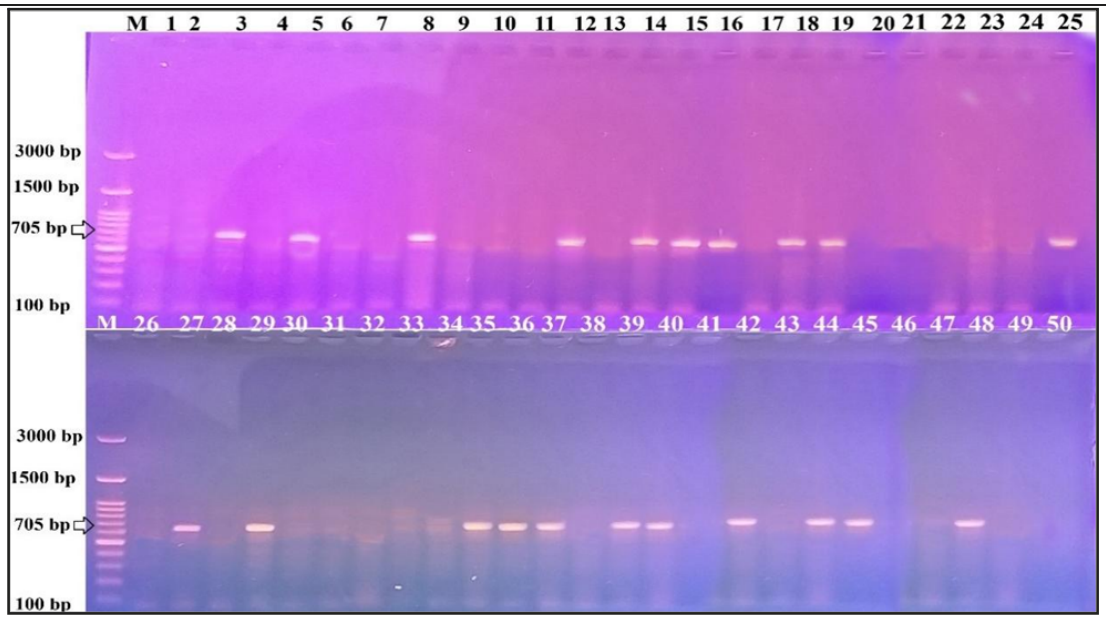

Figure 1 The PCR amplification of a 705 bp fragment of the amoebapore C gene from 50 *Entamoebahistolytica* isolates was demonstrated by agarose gel electrophoresis. Many of the individual DNA samples (lanes 1–50) show distinct bands at around 705 bp, which means that the amplification was successful. As a standard for molecular size, DNA ladders spanning 100 bp to 3000 bp are found in Lane M

Figure 2 Nucleotide sequence alignment of amoebapore C from a variety of *Entamoeba* isolates. The sequence variations are indicated by yellow and blue, and conserved regions are highlighted. This information provides a glimpse into the genetic diversity and potential functional distinctions among the isolates

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Figure 3 shows that the substitution of thymine (T) for adenine (A) at position 313 is the most noticeable variation, leading to the substitution of leucine (L) for glutamine (Q). The reference sequence for this gene is archived in GenBank under accession number X76903, as indicated in Table 7.

Table 7 The amoebapore C of *Entamoebahistolytica* changes positions and quantities of nucleotides in each sample using amino acid codons

Gene name	position sequences	Variant position	Nucleotide changed	Amino acid changed	Codon number changed	GenBank Reference accession number
Amoebapor C	Exon 3 306-566	313	A/T	Q/L	17	X76903

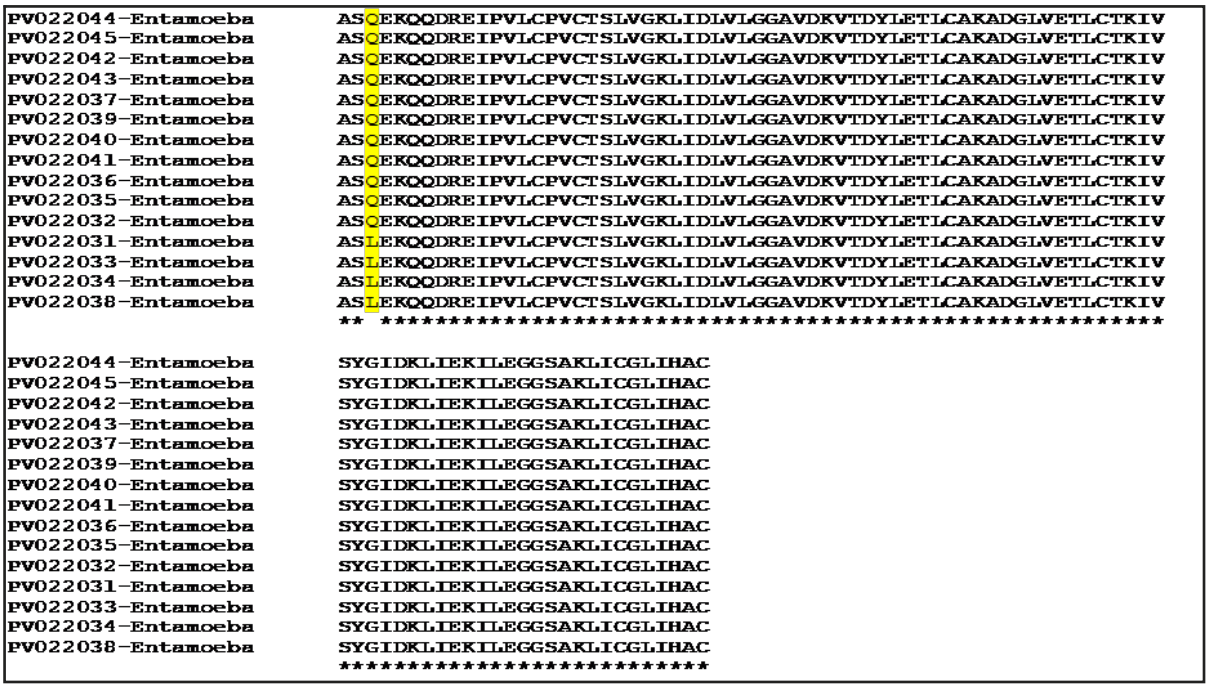

Figure 3 Amino acid sequence alignment for amoebapore C in distinct *Entamoeba* isolates. Yellow highlights conserved residues, which indicate functional or structurally significant sections that are consistent among isolates

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NCBI-BLAST homology results

All of the samples compared to reference sequences in GenBank show high levels of similarity to *E. histolytica* (99.18% to 99.84%). There is widespread genetic similarity across regions, as the strains that have been identified are associated with various countries such as Iraq, India, Hamburg, and the United States. The match rate to X76903 (Hamburg) is 99.84%. As for the other two, 99.18% match the USA-based AY956434, and 99.44% match the Indian-based KP159522 and KP159521, respectively. With a match rate of 99.7% to XM_650937 (USA) and 99.38% to MT951204 (Iraq), as shown in Table 8.

Table 8 Global Reference Sequences for *Entamoebahistolytica* Isolates Analyzed by NCBI -BLAST

Samples	Accession Numbers	NCBI-BLAST Homology Sequence			
		Identical Number (%)	GenBank Accession Number	GenBank Parasite Species Identification	Country
1	PV022031	99.84 %	X76903	*Entamoebahistolytica*	Hamburg
2	PV022032				
3	PV022033				
4	PV022034	99.18 %	AY956434	*Entamoebahistolytica*	USA
5	PV022035				
6	PV022036				
7	PV022037				
8	PV022038	99.44 %	KP159522	*Entamoebahistolytica*	India
9	PV022039				
10	PV022040				
11	PV022041	99.44 %	KP159521	*Entamoebahistolytica*	India
12	PV022042				
13	PV022043				
14	PV022044				
15	PV022045	99.7 %	XM_650937	*Entamoebahistolytica*	USA
		99.38 %	MT951204	*Entamoebahistolytica*	Iraq

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Phylogenetic tree analysis

Phylogenetic study of the amoebapore-c gene revealed that the majority of *Entamoebahistolytica* isolates belonged to a single clade, suggesting that this gene is highly conserved and shared among local strains. There was a cluster of these isolates with GenBank accession codes starting with "PV" next to generic reference sequences. Slight variation in genetic makeup was indicated by a small number of isolates, including KP159521 and MT951204. The presence of *Entamoeba invadens* as an out group in the tree's root system proved that it had evolved separately and was a good candidate for rooting as a separate branch. In addition to demonstrating that the amoebapore-c gene is preserved within *E. histolytica*, the relatively small genetic distance (~1%) between *E. invadens* and the *E. histolytica* group shows how this *Entamoeba* species differs from others, as shown in Figure 4.

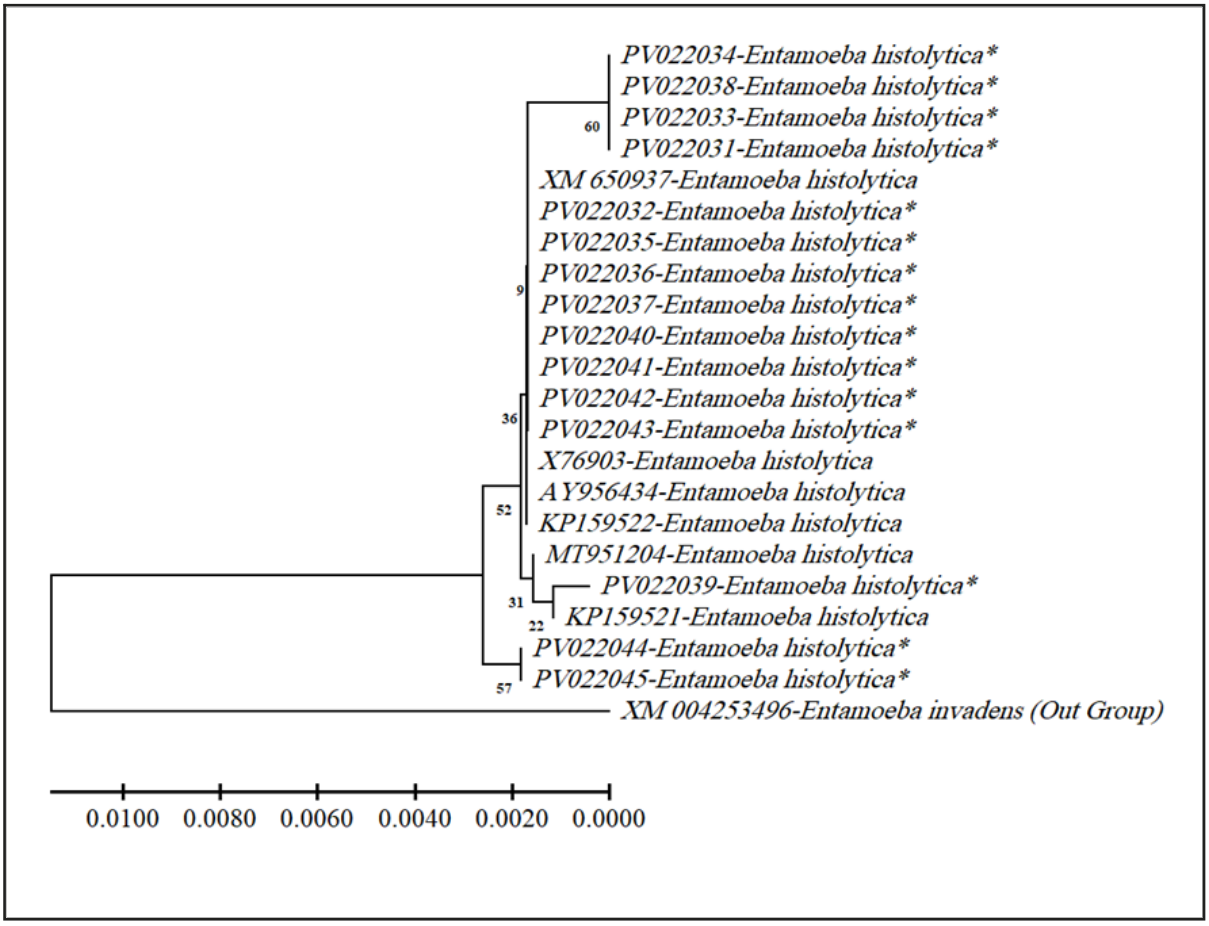

Figure 4 Samples from Kurdistan, Iraq (marked with *) are included in the phylogenetic tree that depicts the evolutionary relationships among *Entamoebahistolytica* isolates. It was constructed using MEGA11's Maximum Likelihood approach using the Tamura-Nei model, and 100 bootstrap replicates were used for validation. The study relied on incomplete amoebapore C gene sequences and used *Entamoeba invadens* as an outgroup

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Discussion

Amoebiasis remains a significant public health concern even though the *Entamoebahistolytica* was discovered over a hundred years ago. Efforts to prevent this are especially fruitless in developing countries where many people lack access to even the most basic forms of sanitation.[13] The city of Erbil in northern Iraq has a higher prevalence among males, probably because they are more heavily involved in the restaurant sector. Similar patterns were observed in investigations conducted in (Al-Qadisiyah, Iraq), (Iran), and (Irbid, Jordan).[8,14,15] The microscopic inspection of stool samples in this study indicated no statistically significant differences in infection rates based on age, gender, education level, or housing situation. Consistent with earlier research from (Libya), (Basra, Iraq), and (Erbil, Iraq),[16-18] however, they contradict the information provided by (Al-Kufa, Iraq), (Dohuk, Iraq),[19,20] Which exhibited substantial variation about demographic factors. Notably, residence appeared to be a significant influence; infection rates were 70% higher in urban than in rural areas (30%). This discovery agrees with the outcomes of (Duhok, Iraq),[21] in areas where the infection rate was higher among urban residents, which may be attributable to factors including lower levels of personal cleanliness, more frequent contact with polluted water and food, and higher population density. Nevertheless, this is in stark contrast to a study conducted by (Iran),[8] in which the prevalence rates in rural and urban areas were not significantly different. Additionally, there was no statistically significant difference between symptomatic and asymptomatic persons when examining the microscopy results (P = 0.858). Interestingly, 78% of the positive instances were discovered among the asymptomatic participants. That asymptomatic carriers could be pivotal in the transmission process is supported by this and other findings from (Al-Qadisia, Iraq), (Erbil, Iraq).[18,22]

Because it cannot differentiate *E. histolytica* from morphologically identical non-pathogenic species like *E. dispar*, this restriction highlights the diagnostic challenges of microscopy. In 21 out of 50 samples, *E.histolytica* was effectively detected using PCR targeting the 705 bp segment of the Amoebapore-C gene. This method outperformed direct microscopy in terms of sensitivity and specificity. This difference is statistically significant (P = 0.000), proving that PCR is the superior diagnostic method, in line with research conducted in (Diyala, Iraq), (Egypt), and (India).[25-27] Although PCR has many benefits, it is still not widely used in routine diagnostics in many endemic regions because of its high cost.

A pore-forming protein called amoebapore-C is encoded by the amoebapore-C gene. It is essential for the pathogenicity of *E. histolytica*.[23] A point mutation was found in 15 PCR-amplified isolates. The mutation involved the replacement of adenine (A) with thymine (T), which resulted in the substitution of leucine (L) for glutamine (Q). Evidence that single-nucleotide polymorphisms can change protein characteristics is provided by this mutation, which affects protein function, as reported in a study from Switzerland.[28] This finding contrasts with a study in (Babylon, Iraq),[23] It revealed no changes in the amoebapore-C gene and showed 99.68% to 100% identity with a U.S.A reference strain (AY956434.2). The local *E. histolytica* isolates shared a high level of sequence identity with global strains, according to homology analysis using NCBI-BLAST. Specifically, 99.84% of the sequences were identical to those of a German isolate (Hamburg, X76903), 99.44% to those of two Indian isolates (KP159521, KP159522), 99.18% to 99.70% with those of two U.S.A isolates (XM_650937, AY956434), and 99.38% to an Iraqi isolate (MT951204).Based on these findings, it is clear that the amoebapore-C gene is highly conserved among *E.histolytica* strains worldwide. Phylogenetic analysis provided

more evidence in favor of these findings by showing that the local *E*. *histolytica* isolates were quite similar genetically. On the other hand, there is a genetic difference of about 1% between the *E*. *histolytica* cluster and the out group *E. invadens*. This contrasts with a study in (Babylon, Iraq),[23] which found that the genetic difference between the reference strain and *E*. *histolytica* isolates was approximately 0.5 percent lower.

Conclusion

The study concluded that PCR is the gold standard for detecting *Entamoeba histolytica*. A single nucleotide mutation, which causes glutamine (Q) to leucine (L), modifies the structure and function of the protein and may influence *Entamoeba-histolytica* pathogenicity." Using NCBI-BLAST homology sequencing, this study confirmed that the samples matched strains from several countries, including Iraq, India, Germany, and the United States.

Competing interests

The authors declare that they have no competing interests.

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