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Research Article

Genetic diversity and phylogenetic positioning of *Entamoeba histolytica* isolates from stool samples of patients from few Hospitals at Diyala governorate

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Abstract
Entamoeba histolytica is a protozoan parasite that causes amoebiasis, an infection primarily affecting the intestines. The study aimed to determine the phylogenetic placement of six isolates of *E. histolytica* that were amplified from three genetic loci, rRNA, cysteine proteinase, and amoebapore C to evaluate the genetic variation pattern of each sequence. DNA extracted from cultured and frozen stool samples using PrestoTM Stool DNA Extraction Kit and Geneaid kits was analyzed for *E. histolytica* diversity in amoebiasis patients at Baquba General Hospital from March-June 2023. Polymerase chaing kreaction (PCR) and sequencing of virulence genes (rRNA, cysteine proteinase, amoebapore C) followed by phylogenetic analysis revealed genetic variation and potential implications for pathogenicity. The conducted sequencing reactions confirmed the precise identification of the examined samples, which were determined to be attributed to variable isolates of *E. histolytica*. Aligning the rRNA sequences of samples A1 and A2 with the most closely related rRNA sequences of *E. histolytica* (GenBank acc. no. OP925909.1) revealed the presence of two nucleic acid variants (212C>T in A1, and 427G>C in A1 and A2) not present in the reference sequences. Aligning the cysteine proteinase sequences of samples B1 and B2 with the most closely related genomic DNA sequences of *E. histolytica* (GenBank acc. no. X91642.1) revealed the presence of four nucleic acid variants (95A>T and 96A>T in B1 and B2, and 246A>T and 247A>T in B2) not present in the reference sequences. Translation of nucleic acid sequences to amino acid residues showed that both 95A>T and 96A>T variants caused a missense effect of p.46E>V, while both 246A>T and 247A>T variants showed another missense effect of p.97T>S. Aligning the amoebapore C sequences of samples C1 and C2 with the most closely related genomic sequences of *E. histolytica* (GenBank acc. no. X76903.1) revealed their entire similarity. It was inferred from the tree that the investigated protozoan samples occupied distinct phylogenetic positions and were suitable for the vicinity of various clinical isolates that were isolated from several locations worldwide. However, the rRNA-based tree provided ample genetic diversity with more effective discrimination compared with the other used loci.

Keywords: Amoebapore C, Cysteine proteinase, *Entamoeba histolytica*, Genetic diversity, Phylogenetic positioning, rRNA

INTRODUCTION

Entamoeba histolytica is a pathogenic protozoan belonging to the family Entamoebidae, causing animal-parasitic diseases. It is a unicellular organism. *E. histolytica* was originally recognised and described in the literature by the doctor F. Aleksandrovich Lösch from St. Petersburg in 1875 (Lübbert *et al*., 2014). The species name "*histolytica*," given by Schaudinn, is related to the tissue destruction caused by this parasite in its host. Dobell (Dobell, 1919) concurred with this designation, noting that this amoeba releases "a powerful cytolytic enzyme" that breaks down tissues instead of causing damage through pseudopods. *E. histolytica* causes a deadly illness known as amoebiasis (Khalaf and Rashid, 2018). Entamoeba infections are widespread, particularly in areas with less developed infrastructure and limited sanitation (Ngui *et al*., 2011). Yet,

evidence now suggests that *E. dispar* and *E. moshkovskii*, once considered harmless commensals, may also contribute to certain types of infections, albeit less severe (Oliveira *et al*., 2015). It is the third most common parasite cause of death in underdeveloped nations, following malaria and schistosomiasis (Carrero *et al*., 2020). According to the World Health Organisation (WHO), almost 500 million individuals worldwide fall ill each year due to amoebosis-induced infectious dysentery, resulting in 100 thousand deaths (Misra and Srivastava, 2020).

The primary causes of infection are food and water contaminated with human faeces carrying *E. histolytica* cysts. Human cyst carriers are the primary reservoir of the illness. Transmission occurs by the ingestion of faecal matter, contact with carriers, and sexual intercourse (Mahmud *et al*., 2013). *E. histolytica* completes its life cycle within a single human host. It transforms into trophozoites, capable of invading and damaging various tissues, including the intestines, liver (the most frequent target), lungs, skin, and even the brain (Bakr *et al*., 2022).

E. histolytica main pathogenic ability is its potent cytolytic capacity, meaning it can break down and kill host cells (Pal *et al*., 2022). 20 years ago, a protein called amoebapore was identified in *E. histolytica*. It forms pores in target cell membranes, causing depolarization and, ultimately cell death. Amoebapore Isoforms: Three types of amoebapore (A, B, and C) were found in different ratios (21:9:1). Their properties and structures were studied extensively (Lozano-Mendoza *et al*., 2023). Amoebapore shares similarities with other proteins found in immune cells, like natural killer (NK) cells, suggesting a similar mechanism of action (Christofyllakis, 2021). Amoebapore resides within cytoplasmic granules of the trophozoite stage (active form) and is thought to be inserted into target cell membranes upon contact without needing specific receptors (Espinosa-Cantellano *et al*., 2022).

Cysteine proteases (CPs) stand out as crucial virulence factors for *E. histolytica*, significantly contributing to its ability to damage tissues. Unlike the amoeba pore-forming peptide, their abundance in parasite secretions suggests an active role in extracellular activity. Notably, EhCP1 and EhCP5, absent in the non-pathogenic *E. dispar* (Roy *et al.,* 2022), hint at their specific involvement in *E. histolytica* virulence. Moreover, overexpressing EhCP5 not only enhances its own activity but also that of other CPs like EhCP1 and EhCP2, suggesting an "enzyme-converting" function and a significant impact on overall proteolytic capacity (Argüello-García *et al*., 2023). *E. histolytica* high cysteine protease (CP) activity, absent in its non-pathogenic cousin, fuels its virulence. These CPs act like molecular machetes, dismantling the host's defenses by degrading protective mucus, evading immune components, and weakening the epithelial barrier. They even contribute to neuronal damage. Notably, the "enzyme-converting" EhCP5 amplifies the destructive power of other CPs, highlighting their crucial role in *E. histolytica* arsenal for tissue invasion, nutrient acquisition, and cell death (Lozano-Mendoza *et al*., 2023).

Phospholipase C (PLC) is a key virulence factor of *E.histolytica* (Castellanos-Castro *et al*., 2020). This enzyme plays a significant role in disrupting host cell membranes, triggering inflammatory responses, weakening the immune system, facilitating cell adhesion, and influencing cellular signaling pathways. All of these effects contribute to damaging host cells and helping the parasite survive and thrive (Nakada-Tsukui *et al*., 2019).

The study aimed to determine the phylogenetic placement of six isolates of *E. histolytica* that were amplified from three genetic loci, rRNA, cysteine proteinase, and amoebapore C to evaluate the genetic variation pattern of each sequence.

MATERIALS AND METHODS

A total of 450 stool samples from patients with diarrhea and abdominal pain were collected from various hospitals in Diyala Governorate, Iraq, between November 2022 and November 2023. The participating hospitals included (Al-Batoul Maternity and Children's Teaching Hospital, Baquba Teaching Hospital, Jalawla General Hospital).

Ethical approval

Ethical approval was obtained from the Scientific Research Ethics Committee at the University of Diyala under 7/18/4788 on August 2022.

Molecular diagnosis

DNA extraction and electrophoresis

DNA was extracted from cultured and frozen stool samples using the PrestoTM Stool DNA Extraction Kit Quick Protocol. A total of 93 out of 450 microscopically diagnosed samples underwent DNA purification and extraction using the Extraction kit provided by Geneaid, Taiwan. The purified DNA samples were then analyzed using DNA electrophoresis technique, which separates DNA fragments based on size. The DNA samples were loaded onto an agarose gel. An electric current was then applied to the gel, which caused the DNA fragments to migrate through the gel at different rates depending on their size. The DNA fragments were then visualized using a DNA stain, such as ethidium bromide This research used specific primers tables 1 to target three key virulence factors of *E. histolytica* (Active Cysteine proteinase, amoebapore C, and Phospholipase C) for both microscopic and molecular identification. By amplifying and sequencing three gene fragments

(rRNA, cysteine proteinase, and amoebapore C) from various *E. histolytica* isolates, the study explored genetic diversity within the parasite population. Identified variations in protein-coding regions were translated and analyzed for potential functional changes. A comprehensive phylogenetic tree was also constructed to understand how these variants are related and distributed geographically. This combined approach offers valuable insights into the population structure of *E. histolytica* and the potential for functional adaptations, highlighting potential areas for further investigation and understanding of its virulence (Ahmad, 2021).

Polymerase chain reaction (PCR) amplicon sequencing

Bidirectional Sanger sequencing of PCR amplicons was performed by Macrogen Inc. (South Korea). Only high-quality chromatographs from ABI files, free of PCR or sequencing artifacts, were used for downstream analysis as shown in Table 1. This ensured the accurate identification of virtual positions and characteristics of the obtained fragments through comparison with reference sequences (Obaid *et al*., 2023).

Sequencing data analysis

PCR product sequences were edited, aligned, and analyzed against reference sequences using BioEdit software. Observed variations were assigned numerical identifiers in amplicons and their corresponding reference genome positions. Snap Gene Viewer annotated detected variants, and all analyzed sequences were submitted to National Center for Biotechnology Information (NCBI) for unique accession numbers.

Amino acid translation of coding region variations

Coding region sequences from PCR products were edited, aligned, and analyzed against reference sequences using BioEdit. Observed variations were assigned numerical identifiers in both amplicons and corresponding reference genome positions. Additionally, amino acid sequences of targeted proteins were retrieved from the Protein Data Bank.

GenBank deposition

PCR product sequences were trimmed to specific regions and then aligned and analyzed alongside their reference sequences in the database using BioEdit v7.1 sequence alignment editing software (DNASTAR, Madison, WI, USA). Observed variations in each sequenced sample were assigned unique numerical identifiers corresponding to both PCR amplicons and their corresponding positions within the reference genome. Nucleotide positions within PCR amplicons and their corresponding locations within the reference genome were systematically numbered. SnapGene Viewer v4.0.4 software (https://www.snapgene.com/snapgene-viewer) annotated each detected variation within the parasite sequences. All analyzed sequences were submitted to NCBI to obtain a unique accession number for each studied sequence.

Phylogenetic tree construction

For each gene fragment, neighbour-joining trees were constructed using NCBI-BLASTn for sequence comparison and iTOL for visualization. Sequences were color-coded for enhanced differentiation as per Alwan *et al*. (2023).

RESULTS AND DISCUSSION

The results of the PCR assay, as shown in Fig. 1 and Table 2 indicated that *E. dispar* was the most prevalent species, followed by *E. histolytica* and *E. moshkovskii*. The chi-squared test showed a significant difference in the prevalence of the three species ($P \leq 0.0001$). *E. histolytica* was detected in 40.86% of samples, *E. dispar* was detected in 44.09%, and *E. moshkovskii* was detected in 11.83% of samples. The results of this study suggest that PCR is a sensitive and specific method for diagnosing amebiasis. The assay can be used to differentiate between the three species of Entamoeba, which can be helpful for the diagnosis and treatment of amebiasis.

The present study's findings diverged from those of research conducted in Anbar, which revealed that *E. histolytica* was the most prevalent infection, accounting for 85% of cases, followed by *E. dispar*, which constituted 15% of all Entamoeba-positive samples. The results of Table 2, which examined the virulence factors of *E. histolytica* samples, revealed no statistically significant differences.

Virulence factor results of the detection of Amoebapore C showed its presence in only 32 samples, at a rate of

Table 1. Showing specific primer of virulance gees (Khairnar & Parija, 2007)

Primer	Sequence		Product size
E. histolytica	5TATCTGGTTGATCCTGCCAG- ' 3'- 35'-CTATTGGAGCTGGAATTACC- '	Forward Reverse	310bp
Amoebapore C	3'- TATCTGGTTGATCCTGCCAG-5' 5'-CTATTGGAGCTGGAATTACC-3'	Forward Reverse	300bp
Cystine proteinase	3'- GACTTTAAGAGTTGGGCTGC-5' 5'- GGAGTAACATGAGTTAAACC-3'	Forward Reverse	584 bp
Phospholipase C	3'- GATTTAGTCCTTGGTGGAGC-5' 5'- GCCAAATAGATTAATGATTG-3'	Forward Reverse	295bp

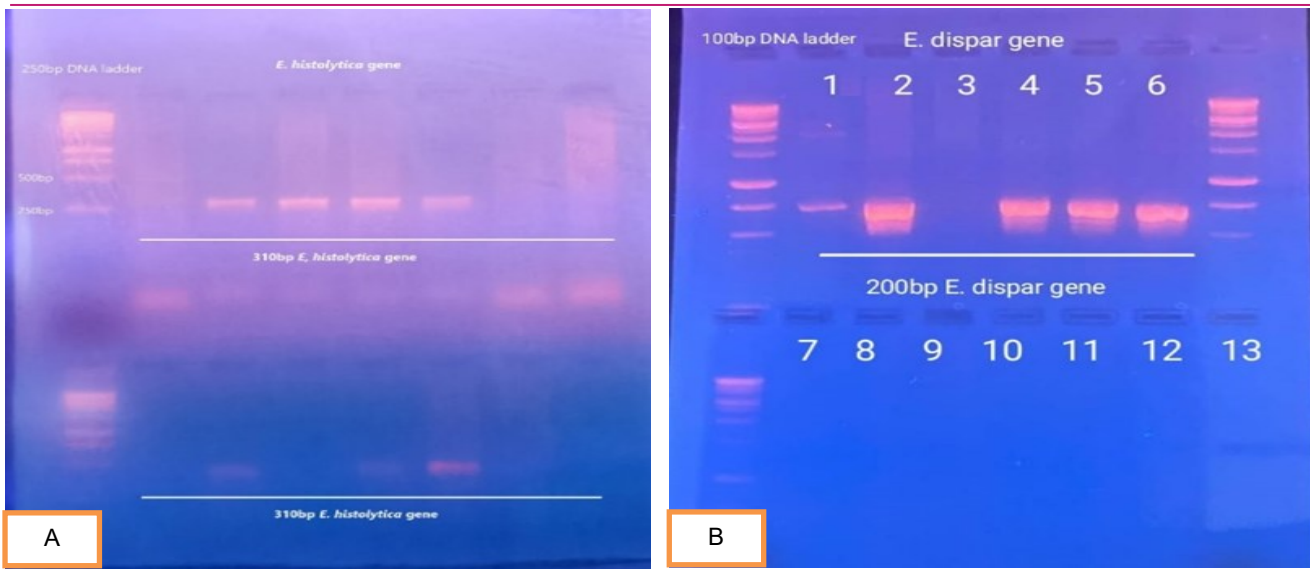

Fig. 1. *Agarose gel electrophoresis of PCR products from E. histolytica, E. dispar, and expected product sizes were 310 bp for E. histolytica (A) and E. dispar (B) and 200 bp for E. moshkovskii. Electrophoresis was performed on a 1.5% agarose gel at 70 V, 60 A, in 1x TBE buffer for 1 hour. A 1000 bp ladder using as a size marker*

Table 2. Sample distribution according to PCR positive genes

PCR positive genes	Positive		Negative		Total	
	No.	%	No.	%	No.	%
E. histolytica	38	40.86	55	59.14	93	100.00
E. dispar	41	44.09	52	55.91	93	100.00
E. moshkovskii	11	11.83	82	88.17	93	100.00
χ^2	26.867				0.000	
P-value	≤0.0001**				1.000	

**significant association at p≤0.01.

84.21%, as shown in Fig. 2 and Table 3.This indicates that the presence of this gene was significantly related to virulence. These results agree with Al-Damerchi and Al-Ebrahimi (2016), who found that only 58 samples were positive for Amoebapore C. Additionally, (Al-Kaeebi and Al-Difaie, 2016) found that the aforementioned virulence factor was present in all samples; this agrees with the higher prevalence of virulence genes in the positive group as noted in present study. Studies have shown that trophozoite form *E. histolytica* that lacks Amoabapore C is less virulent Lazar *et al*. (2023). Virulence factor results of the detection of Cysteine proteinase showed its presence in only 25 samples, at a rate of 65.79% only, as shown in (Fig. 3 and Table 3). The cysteine proteinase, a crucial virulence factor released by the parasite, breaks down host tissues, eliminates host cells upon contact, triggers apoptosis in specific host cells, and distinguishes the Trophozoite of pathogenic species like *E. histolytica* from non-pathogenic species. This aligns with previous research demonstrating the significant role of cysteine proteinase in mediating interactions between parasite and host, aiding in nutrient acquisition, promoting tissue invasion, and defending against the host's immune response (Rostami *et al*., 2017).

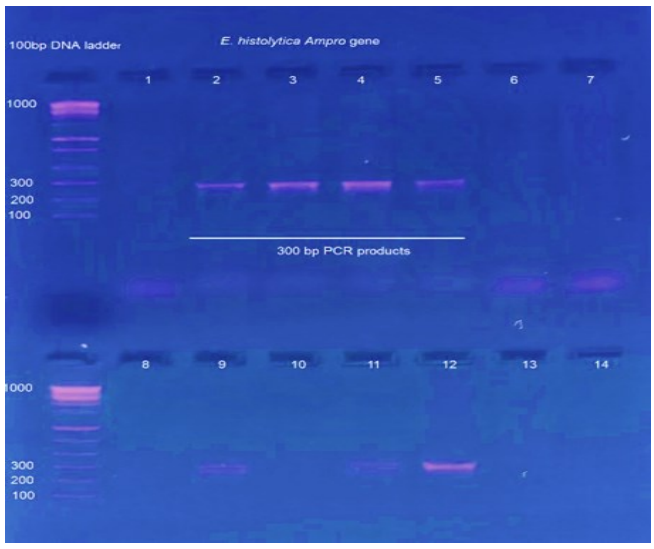

Fig. 2. *PCR product of Amoebapore C for E. histolytica. The gel image shows the PCR product of the Amoebapore C gene in E. histolytica. The expected band size is 300 bp, which is consistent with the observed band size in the gel. The PCR product was electrophoresed on a 1.5% agarose gel at 70 V, 60 mA, and 1x TBE buffer for 1 hour. A 1000 bp DNA ladder was used as a size marker*

Virulence factor results of the detection of Cysteine proteinase showed its presence in only 29 samples, at a rate of 76.32% only (Fig. 4 and Table 3). Six samples (A1-C3) were analyzed to partially amplify and sequence rRNA, cysteine proteinase, and amoebapore C

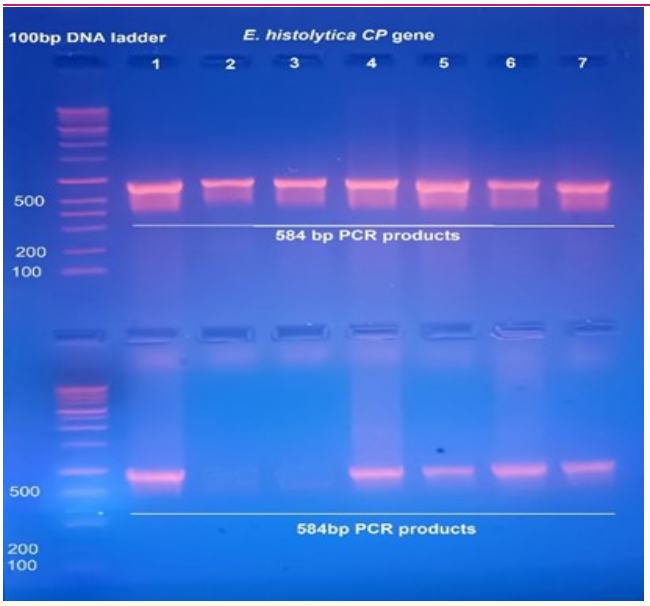

Fig. 3. *PCR product of Cysteine proteinase for E. histolytica,the band size 584 bp , the product was electrophoresis on 1.5% agarose , 70 Vol, 60 amp, 1x TBE buffer for 1.0 h, ladder 1000 bp*

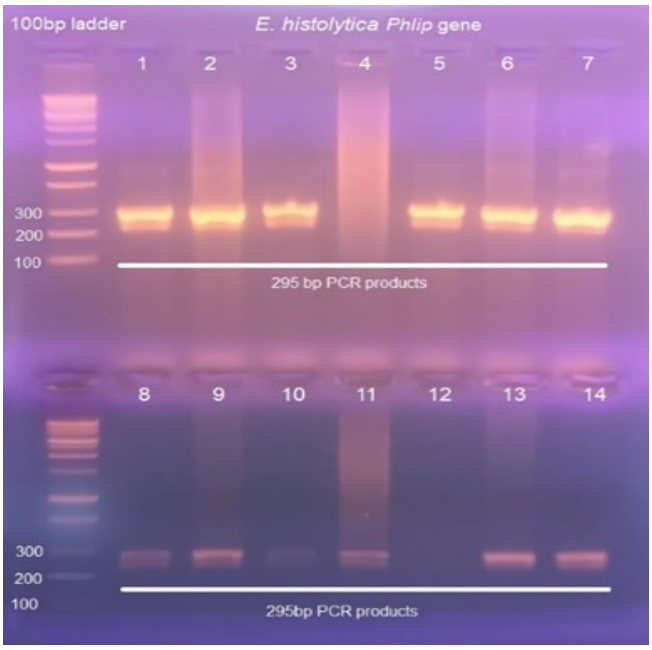

Fig. 4. *PCR amplification of the cysteine proteinase gene in E. histolytica. Lane 1 shows the 1000 bp DNA ladder. Lane 2 shows the PCR product with a single band at 295 bp, corresponding to the expected size of the cysteine proteinase gene. The PCR product was electrophoresed on a 1.5% agarose gel at 70 V, 60 A for 1 hour in 1x TBE buffer*

genes of Entamoeba histolytica. BLASTn analysis against NCBI databases revealed approximately 99% homology between sequenced amplicons and the respective reference sequences for each gene, confirming the presence of *E. histolytica* with accurate fragment identification. The investigated Entamoeba histolytica sequences were deposited in GenBank under accession numbers PP275726 (A1), PP275727 (A2), PP315668 (B1), PP315669 (B2), PP315670 (C1), and PP315671 (C2).

Phylogenetic trees construction

To elucidate the evolutionary relationships and genetic diversity of investigated *E. histolytica* isolates, a comprehensive phylogenetic tree was constructed using rRNA, cysteine proteinase, and amoebapore C gene sequences. These trees, visualized as rectangular and circular cladograms to offer complementary insights, revealed distinct clades and provided valuable information on the spread, transmission, and population structure of *E. histolytica* strains.

Phylogenetic analysis of rRNA amplicons

Phylogenetic analysis of 38 aligned nucleic acid sequences identified four distinct Entamoeba clades (*E. histolytica, E. dispar, E. moshkovskii, and E. bovis*). Notably, the investigated A1 and A2 sequences clustered within the *E. histolytica* clade, indicating high rRNA specificity for this species. This largest clade encompassed diverse *E. histolytica* strains and suggested an Indian origin (GenBank X64142.1) for our samples (Fig 5), highlighting their wide distribution. Distinct positioning of A1 and A2 within the *E. histolytica* clade (due to point mutations) and increasing distance from this clade towards the root for other clades (*E. dispar, E. moshkovskii,* and *E. bovis*) reinforce the utility of rRNA for species delineation and evolutionary studies. Overall, this study demonstrates the valuable role of rRNA sequencing in accurately identifying and differentiating Entamoeba species, contributing to a better understanding of their evolution and facilitating improved clinical management.

Phylogenetic analysis of cysteine proteinase amplicons

The total number of aligned nucleic acid sequences in this comprehensive tree was twenty two. In the con-

Table 3. Sample distribution according to *Entamoeba histolytica* virulence genes

***E. histolytica* virulance genes**	**Positive**		**Negative**		**Total**	
	No.	%	No.	%	No.	%
Amoebapore C	32	84.21	6	15.79	38	100.00
Cystine proteinase	25	65.79	13	34.21	38	100.00
Phospholipase C	29	76.32	9	23.68	38	100.00
χ^2	3.503				0.000	
P-value	0.173				1.000	

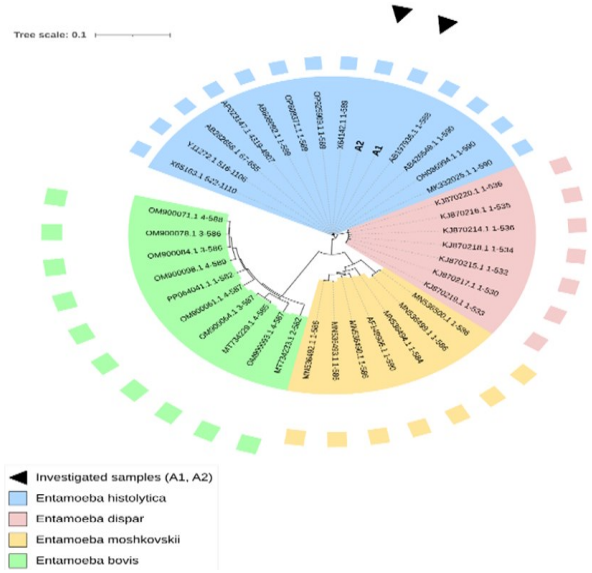

Fig. 5. *Comprehensive rectangular (in branch A) and circular (in branch B) cladogram phylogenetic tree of the rRNA, for two samples of E. histolytica (A1 and A2). The black-colored triangle refers to the analyzed protozoan sequences, while coloured squares represent various genotypes of E. histolytica. All the mentioned numbers referred to the GenBank accession number of each referring species. The numbers at the top portion of the tree refer to the degree of scale range among the comprehensive tree-categorized organisms*

structed cladogram, the incorporated samples were clustered into only three phylogenetic clades within the genus Entamoeba; a species represented each clade. These clades are *E. histolytica, E. dispar*, and *E. invadens*. As in the case of the rRNA-based tree, our investigated protozoan sequences of B1 and B2 were found to be suited in close positions in the clade of *E. histolytica*. This clade represents the major clade within the constructed tree, which consists of eleven sequences belonging to various strains of *E. histolytica*. In the clade of *E. histolytica*, the B1 sample was positioned near a strain isolated from Germany (GenBank X91642.1). Whereas the B2 sample was suited in the vicinity to various strains that were collected from Japan (GenBank AK419958.1, AK418827.1, AK420462.1, AK419074.1, AK419832.1, AK420694.1, AK420337.1, and AK421265.1). The reason for the different positioning between B1 and B2 samples is attributed to the presence of 246A>T and 247A>T only in the B2 sample. Accordingly, both 246A>T and 247A>T variants are the reason for changing the geographical distributions of the B2 sample compared with the B1 sample. Therefore, it was inferred from the tree that the European and Asian sources are the most likely sources from which B1 and B2 samples might have originated, respectively. However, these sorts of geographical distributions may refer to a lower potential of biological diversity of the amplified cysteine proteinase samples compared with the multinational sources of the amplified ribosomal sequences (Fig. 5).

Given the distinct positioning of the *E. histolytica* clade apart from other clades, it is reasonable to assert that the presently employed cysteine proteinase sequences hold significant potential for elucidating the protozoan's phylogenetic position without ambiguity with other closely-related species. This observation underscores

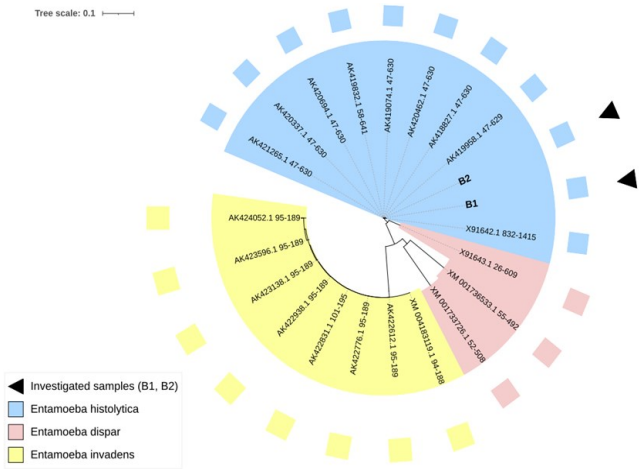

Fig. 6. *Phylogenetic analysis of cysteine proteinase sequences from two E. histolytica samples (B1 and B2) revealed a cladogram with two distinct branches: a rectangular branch (A) and a circular branch (B). The analyzed protozoan sequences (black triangles) cluster with various E. histolytica genotypes (colored squares). GenBank accession numbers identify each species, and the scale bar indicates the evolutionary distance between organisms*

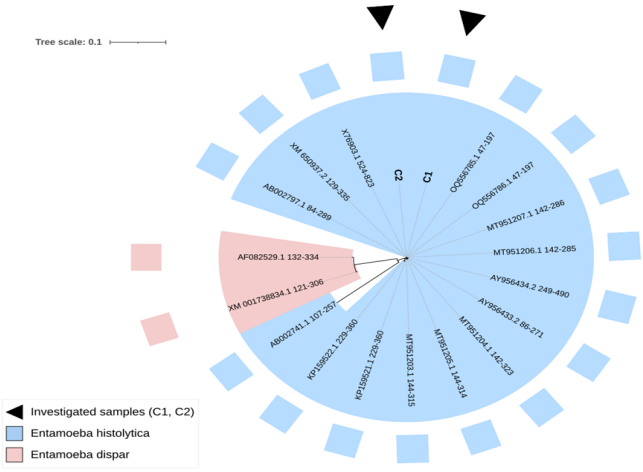

Fig. 7. *Phylogenetic analysis of amoebapore C in E. histolytica samples C1 and C2. This comprehensive tree includes both rectangular (branch A) and circular (branch B) cladogram topologies. Black triangles indicate the analyzed sequences, while colored squares represent E. histolytica genotypes. GenBank accession numbers identify each reference species, and scale range values are displayed at the top of the tree*

the high specificity of cysteine proteinase sequences in precisely delineating the identity of the currently examined samples. Away from the *E. histolytica* clade, two additional clades were positioned. As in the case of the rRNA-based tree, *E. dispar* represents the relatively closest clade to our investigated samples and is made of only three sequences. Next E. dispar clade, E. invendens is positioned, which is made of eight strains. No other *Entamoeba* species were found to be similar or have considerable similarity to the cysteine proteinase sequences. Owing to its proximity to the tree's roots, the tree constructed based on cysteine proteinase indicated that the *E. histolytica* clade serves as the common phylogenetic ancestor for the other included species. In contrast, the *E. invadens* clade, positioned further away from the roots, represents the most recent clade. This data unequivocally confirms the specificity of the cysteine proteinase fragment utilized to elucidate the origins of *Entamoeba* species. However, this specificity is less diverse than the ribosomal-based phylogenetic tree.

Conclusion

The present study investigated *E. histolytica* isolates from human stool for their phylogenetic diversity. The amplified rRNA locus revealed two nucleic acid variants (212C>T in A1 and 427G>C), while the cysteine proteinase harbored four variants (95A>T, 96A>T, 246A>T, and 247A>T), leading to two missense mutations (p.46E>V and p.97T>S). Due to its superior discriminatory power compared to cysteine proteinase and amoebapore C genes, the rRNA fragment emerged as a powerful tool for identifying and characterizing *E. histolytica* in clinical samples, owing to its high sensitivity, specificity, affordability, and rapid turnaround time.

Conflict of interest

The authors declare that they have no conflicts of interest.

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