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Research Article

Molecular Detection using Polymerase Chain Reaction and Phylogenetic Tree Analysis of *Entamoeba histolytica* Isolates from Diarrheal Stool Samples of Patients in Nassiriyah City, Iraq

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Abstract

Each year, around 50 million people—roughly 10 percent of the global population—get infected with *E. histolytica*. The 18S ribosomal RNA gene sequences for five novel *E. histolytica* isolates have been discovered. Using the 18SrRNA gene sequence, the current study examined the phylogenetic connections between five local isolates of *E. histolytica* and seven worldwide isolates of Entamoeba spp. The 501 bp 18S rRNA gene was amplified using polymerase chain reaction, and the multiple sequence alignment and adjacent phylogenetic tree analysis were performed using the ClustalW multiple sequence alignment online analysis. The findings of the phylogenetic analysis of these gene sequences showed that the local isolations of *E. histolytica* were more closely related to two isolations from Iraq (MW029814.1) and (KP233838.1) than to isolates from other nations. This is the first study on the use of molecular phylogeny to categorise *E. histolytica*, which is discovered in Nassiriyah City, Iraq. Accession Nos. (OR122459.1, OR122460.1, OR122461.1, OR122462.1, and OR122463.1) are isolates that are distinct and novel strains.

Keywords: Diarrhea, *E. histolytica*, Phylogenetic tree analysis

Introduction

Although parasitic diseases have decreased noticeably in developed and industrialised countries

due to improvements in diagnostic techniques and increased awareness of their treatment and control, they continue to be a major health concern in many other countries around the world(Al-Rumaidh & AL-Aboody, 2017). *E. histolytica* is an intestinal protozoan parasite with the capacity to bind to and destroy epithelial tissue. Bloody diarrhoea, fever, abdominal discomfort, colitis, malaise, exhaustion, gas, and weight loss are just a few of the symptoms this parasite-caused illness frequently exhibits(Hadi et al., 2019). The *E. histolytica* parasite has two types in its life cycle: trophozoites and cysts. When cysts are consumed by tainted food or drink, infection results. Trophozoites arise from cysts in the small intestine, colonize the colon, and then attach to the large intestine in the mucosal layer after emerging from the cysts. Intestinal and extra-intestinal infections are brought on by *E. histolytica* (López-López et al., 2017). Some trophozoites, however, may be expelled in feces without having the capacity to survive (Samie et al., 2012).

E. histolytica parasites can cause hepatic abscesses in extra-intestinal infections because they can penetrate the intestinal wall and enter the liver through the portal circulation. Untreated, these abscesses have the potential to be lethal. Furthermore, the abscesses could extend to the brain and lungs, among other organs. (Al-Dalabeeh et al., 2020). Amoebiasis is thought to afflict almost 500 million people worldwide (Guevara et al., 2019). Previous research has estimated that there are 50 million cases of amoebiasis each year and that 100,000 people die from the disease (Gomes et al., 2014; Hameed & Khalaf, 2022; Saidin et al., 2019). As a result, among human parasitic illnesses, amoebiasis is the third leading cause of death(Al-Dalabeeh et al., 2020; Nasrallah et al., 2022). It has been established via significant research that amoebae comprise a very varied and polyphyletic group. To better understand *E. histolytica*'s biology and find possible targets for biochemical research or the creation of a vaccine to treat amoebiasis, researchers have concentrated on molecular approaches to examine gene signaling and virulence (Malaa et al., 2019). This study aims to molecular detection of *E. histolytica* and clarification the relationships among local isolates in Nassiriyah city and isolates in Genebank-NCBI, through concentrated on examining the 18S rRNA genes by phylogenetic trees analysis.

Material and methods

Samples collection and examination

Samples collected from August 2022 to October 2022, 90 stool samples were taken from patients

suffering from diarrhea in Nassiriah City. The age range is one day to seven years. Using a sterile stool cup, about 2 g of fresh stool samples were taken from each patient and sent to the lab in an hour, then applying the wet mount, stool samples were placed using a wooden stick on a slide. For semi-solid and diarrheal samples, a drop of normal saline (0.85%) was used to emulsify the stool. After that, a cover slide was placed over them, and a microscope with 40x lenses was used to examination.

DNA extraction

Human stool was used to obtain genomic DNA using a stool DNA extraction kit (Bioneer, Korea). Utilizing Nanodrop (Thermo Scientific, UK), the separated DNA was examined. After that, the samples are stored at -20°C until the next test.

Conventional PCR and phylogenetic analysis

5µL of DNA template; 1.5µL of F and R oligonucleotide primers for 18SrRNAgene; 5µL of master mix; and 7µL of PCR water; a set of oligonucleotide primers as shown in table (1). The AccuPower® PCR PreMix Kit includes all the ingredients required for a PCR reaction, including dNTPs, Taq DNA polymerase, TrisHCl, pH 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye. After that, each PCR tube was placed into an Exispin vortex centrifuge and spun for three minutes at 3000 rpm, after which it was put in a PCR thermocycler (Biorad, USA, T 100, Thermal Cycler). Steps in PCR: initial denaturation, 95°C, 5 min, 1 cycle; denaturation, 95°C, 30 sec, 30 cycles; annealing, 58°C, 30 sec, 30 cycles; extension, 72°C, 1 min, 30 cycles; final extension, 72°C, 5 min, 1 cycle; cold, 4°C, forever. Using agarose gel electrophoresis (2%) and Gel documentation, the amplification products were evaluated. After that, sequencing was carried out using the UPGMA tree. Multiple alignments and BLAST were used for the sequence analysis. The software of MEGA 6.0 was used to conduct phylogenetic analysis.

Table 1. The oligonucleotide primers are used in this study

Primer	Sequence of Primer		Product Size
18SrRNAgene*	F	GGGGAGTATGGTCACAAGGC	501bp
	R	TGTGTACAAAGGGCAGGGAC	

**E. histolytica* (GQ423750.1)

Results and discussion

Ninety stool samples were examined using the PCR method as part of the current research. Positive samples of *E. histolytica* at agarose gel electrophoresis with m.w. 501bp are shown in (Figure 2). From the 90 samples that were analyzed by PCR, the ratio of infected children was determined by 74.44% (67) positive samples and 25.56% (23) different causes, as shown in (Figure 1).

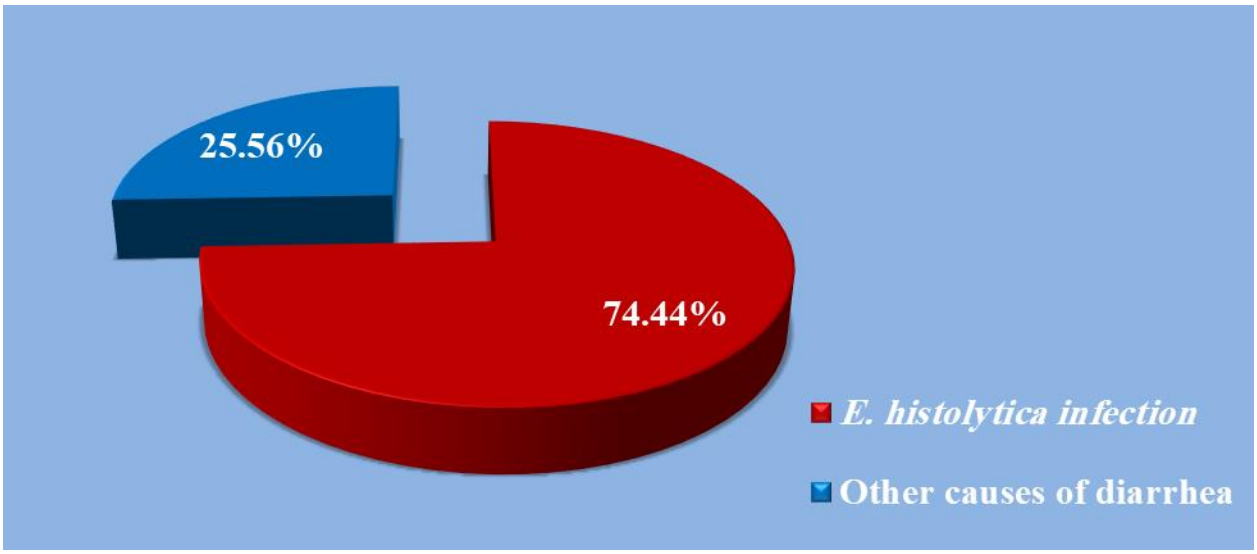

Figure 1. shows the percentage of *E. histolytica* infections determined by PCR

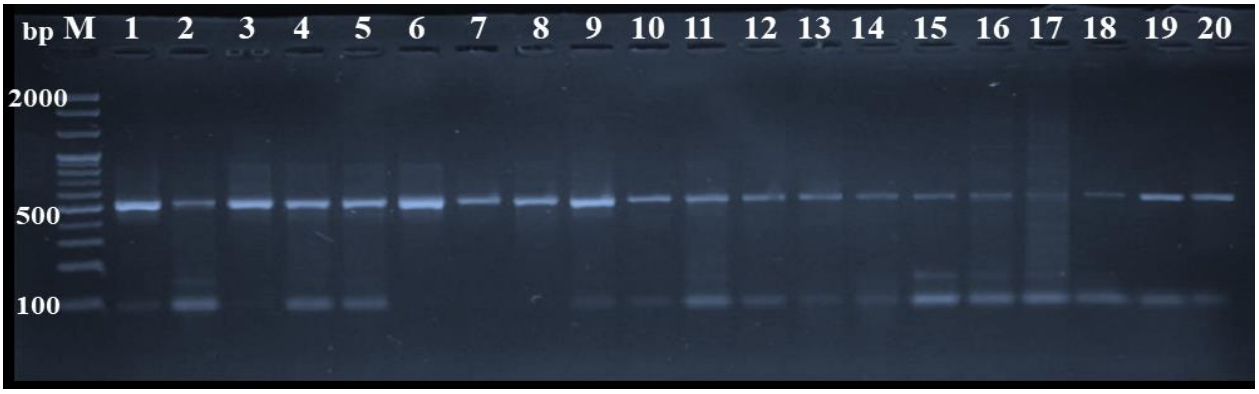

Figure 2. PCR product for the 18SrRNA gene in *E. histolytica* isolates using an agarose gel electrophoresis picture. M (Marker ladder, 100 bps to 2000). A few positive *E. histolytica* stool samples at 501 bp prduct size in Lanes 1–20

Overall, five *E. histolytica* positive samples were sequenced and entered into GenBank under the accession codes OR122459.1, OR122460.1, OR122461.1, OR122462.1, and OR122463.1. As in table (2) 99% identity was found between *E. histolytica* from local isolate and *E. histolytica* with accession numbers MW029814.1 and KP233838.1 after sequencing analysis with BLAST and

multiple alignments. The local parasite isolates (Nos. 1 through 5) were found to be closely connected to the GenBank with the accession codes MW029814.1 and KP233838.1, with overall genetic alterations of 0.01% by the analysis of the phylogenetic tree (Figures 3, 4).

Table 2. shows the proportion of identity in NCBI-BLAST homology sequence between local isolates and NCBI-BLAST closed genetic related isolates:

Local isolate	Accession number of local isolate	Homology sequence identity (%), NCBI isolates		
		Accession number	Related country	Identity (%)
Entamoeba histolytica IQD Human isolate No.1	OR122459.1	MW029814.1	Iraq	99.89%
Entamoeba histolytica IQD Human isolate No.2	OR122460.1	KP233838.1	Iraq	99.56%
Entamoeba histolytica IQD Human isolate No.3	OR122461.1	MW029814.1	Iraq	99.89%
Entamoeba histolytica IQD Human isolate No.4	OR122462.1	MW029814.1	Iraq	99.89%
Entamoeba histolytica IQD Human isolate No.5	OR122463.1	MW029814.1	Iraq	99.88%

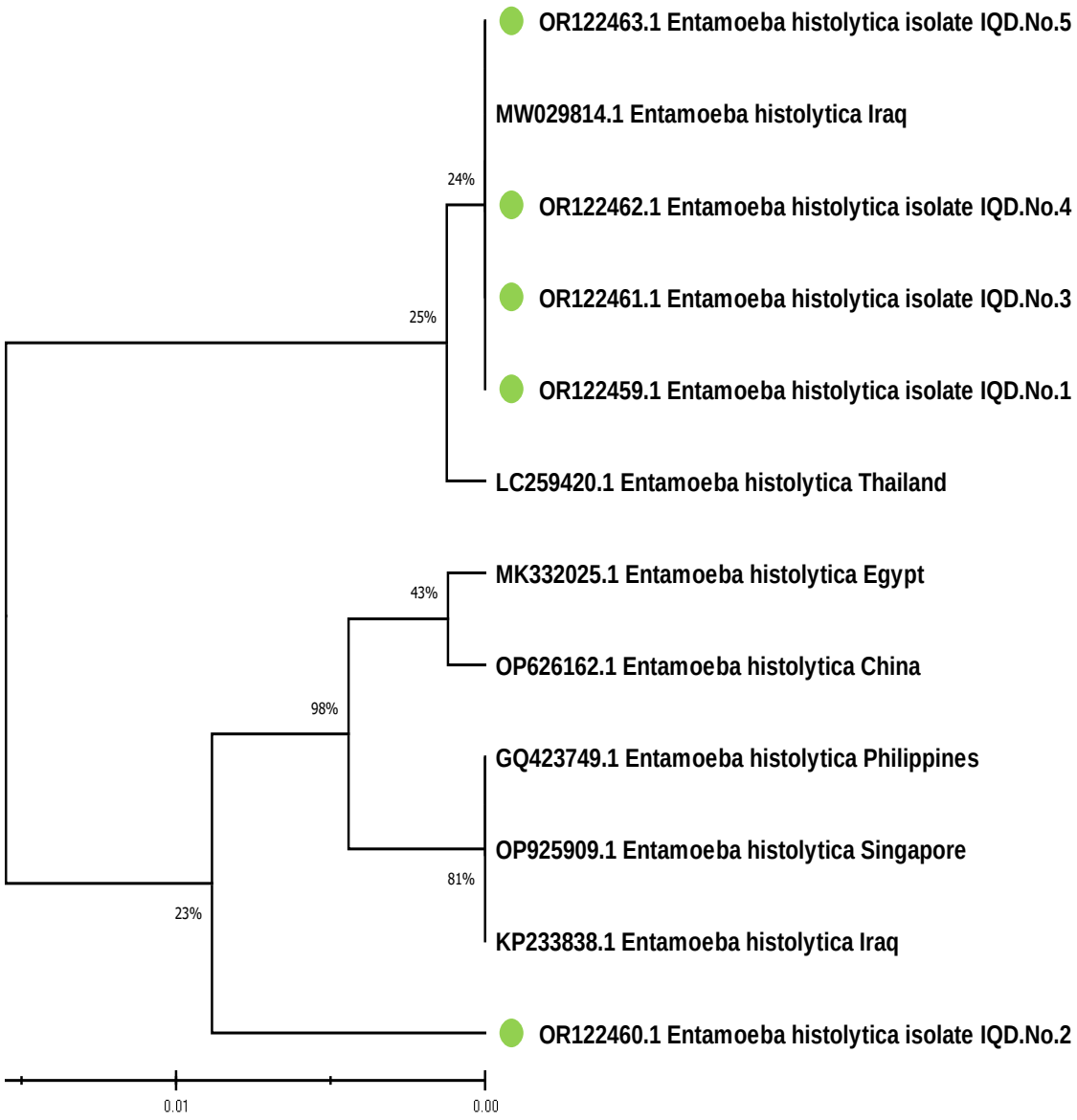

Figure 3. The small subunit ribosomal RNA (18SrRNA) gene partial sequence based on phylogenetic tree analysis applied for genetic relationship study in local *E. histolytica* IQD Human No. 1 - No. 5 isolates. Using the Unweighted Pair Group technique with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version), the phylogenetic tree was built. The local *E. histolytica* IQD Human Nos. 1–No. 5 isolates revealed a closed relationship, at total genetic alterations (0.01%), with the NCBI-BLAST *E. histolytica* (MW029814.1, KP233838.1) Iraq isolates.

Figure 4. Multiple alignment sequence study of the small subunit ribosomal RNA gene in the NCBI-Genbank linked *E. histolytica* isolates and the local *E. histolytica* IQD Human No. 1 and No. 5 isolates. Using the ClustalW alignment programme (online), the multiple alignment analysis was built. The results of the alignment analysis revealed substitution mutations in the small subunit ribosomal RNA gene across isolates and nucleotide alignment similarities indicated by the symbol (*).

This study agrees with the 75.0% rate reported by (Ngui et al., 2012). According to Roy et al., the PCR rate was 72.0% in 2005 (Roy et al., 2005). Moreover, the West Bank's Shareef (2022) indicates a rate of 74%. The present study's result contradicts those of (Ngosso et al., 2015)

Show rate in Dar Es Salaam is 33.3% (Shareef, 2022). Salim (2018) reported a 5.0% *E. histolytica* rate in Thi-Qar (Ngosso et al., 2015). In Thi-Qar, Alkhuzaey & Aboody (2019) found a 38.1% *E. histolytica* rate (Salim, 2018). This discrepancy may be explained by the epidemic of the *E. histolytica* in some areas, and a decrease in others.

Gastrointestinal protozoa, such as *E. histolytica* continue to be a major cause of enteric illness in developing nations in addition to viral and bacterial infections. *E. histolytica* has a substantial association with infections that cause persistent diarrhea, particularly in children (Muhsen & Levine, 2012). Poor hygiene and sanitation practices, poverty, and limited or nonexistent access to dependable water sources and healthcare facilities are the major risk factors for these gastrointestinal disorders (Berkman et al., 2002). A fundamental molecular technique, polymerase chain reaction (PCR), can solve several specificity and sensitivity problems that are often associated with the detection of protozoan infections The molecular identification of protozoan infections is accomplished using a variety of PCR-based techniques (Wang et al., 2004).

It is standard procedure to directly observe feces under a light microscope to look for intestinal protozoan parasites. However, this approach is less sensitive and also requires knowledge (McHardy et al., 2014). Therefore, molecular technologies offer useful data that aid in studying taxonomy, population genetics, and epidemiology. Additionally, these techniques have several benefits for diagnosing intestinal parasites, including excellent specificity and sensitivity (Sow et al., 2017). The 18S rRNA gene is the main constituent of the eukaryotic RNA transcription unit in all parasitic species. According to (Hamzah et al., 2006), several different species that provide a large number of databases concerning sequence comparison had their sequences in this area analyzed. Additionally, because to this region's high level of conservation, phylogenetic analysis of the intestinal protozoan depends on it (Malaa et al., 2019). To construct a phylogenetic tree with the Iraqi isolates of Entamoeba spp. stored in GenBank, the sequences of the 18S rRNA region for *E. histolytica* isolates were employed. The isolates of the *E. histolytica* under study (no1–no5) were close to *E. histolytica* (MW029814.1 and KP233838.1).

Conclusion

The authors conclude from the present study the following:

1. *E. histolytica* are the major parasite associated with diarrhea in children in Nassiriah City.

2. Molecular detection and phylogenetic tree analysis can be used to directly diagnose gastrointestinal infection in people with *E. histolytica*.
3. Phylogenetic tree analysis is useful for quantifying genetic changes in whole DNA and detecting potential mutations.

Ethical approval

The ethical clearance was obtained with oral informed consent from every study participant.

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