

Molecular study for the virulence factors Gal/GalNAc lectin and Amoebapores genes of *Entamoeba histolytica* isolated from infected patients in Thi-Qar province/Iraq

Zeina Talib Shnawa

Zainab Abdali Mohammad

Department of Biology/College of Education for Pure Science/Thi-Qar University

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Abstract:

The parasite *Entamoeba histolytica* is a major public health concern and globally important. Due to the widespread occurrence of the *E. histolytica* infection in Thi-Qar province, this study aimed to detect *E. histolytica* by using polymerase chain reaction (PCR), and to study the pathogenicity of *E. histolytica* in humans through detecting the virulence factor Gal/GalNAc lectin and Amoebapores genes and comparing the local isolates of these genes with global genetic sequencing. The diagnosis of *E. histolytica* parasites in human stool is based on the diagnostic 18s rRNA gene. About 572 stool samples were collected from patients who suffer from diarrhea and complain of abdominal pain in the Al-Hussein Teaching Hospital, Bint Al-Huda Educational Hospital for Women and Children, and Mohammed Al-Musawi Hospital in Thi-Qar Province during the period from the beginning of December 2023 to the end of May 2024.

The results of the current study showed 100 out of 572 stool samples were infected with *E. histolytica* parasites according to microscopic examination, with a prevalence 17.48%. The molecular diagnosis recorded that 64 out of 100 samples were positive for the diagnostic 18s rRNA gene, with a prevalence 64%. The virulence factor Gal/GalNAc lectin was positive in 50 out of 50 examined samples. The results also showed the presence of the gene for the virulence factor Amebopore in 34 out of 50 samples infected with the *E. histolytica* parasite, at a rate of 68%. The genetic sequence of Gal/GalNAc lectin and Amoebapores genes was performed and compared with the genetic sequence of virulence factors registered globally at the NCBI. The study recorded that the Gal/GalNAc has the highest degree of genetic homology (98.68% - 99.14%) and the lowest genetic variability (0.0020% - 0.0180%). The current results showed that the multiple genetic sequences of this gene for three local isolates of the *E. histolytica* parasite showed a high genetic correlation between the three local isolates and the global strain from the United States of America, with the serial number USA (M59850.1). The local isolate with the serial number (PQ049161) was the most identical with a percentage of 99.14% and the least heterogeneous with a percentage of 0.16%. Also, the virulence factor gene Amoebopore C showed high genetic similarity (99.71%-99.42%) with low genetic variability (1.67%-1.90%). The sequencing analysis of three local isolates of *E. histolytica* parasite also showed a high genetic linkage between the three local isolates and the global strain from India with the serial number ALA1

(KP159522.1). The local isolate with the serial number PQ049148 was the most identical, with a percentage of 99.71%, and the lowest percentage of variability 0.29%. These findings suggest a high degree of genetic similarity between the local Iraqi *E. histolytica* isolates and the reference strain, potentially indicating similar virulence profiles.

Keywords: *E. histolytica*, Gal/GalNAc lectin, Amoebapore, PCR technique

1- Introduction:

Entamoeba histolytica is a protozoan parasite and the principal causative agent of intestinal amebiasis in humans [1], [2]. Amoebic dysentery caused by *E. histolytica* is considered one of the most common parasitic diseases worldwide after malaria and schistosomiasis [3]. Infection with this parasite may be asymptomatic or may progress to severe clinical manifestations such as amoebic liver abscess or amoebic colitis [2]. Amoebic colitis is one of the leading causes of severe diarrhea worldwide [4]. If left untreated, some cases of amoebic dysentery may result in death. Global estimates indicate that approximately 50 million people are infected annually, with nearly 100,000 deaths reported worldwide [2]. Clinical symptoms vary among patients depending on the severity of infection and host immune response [5]. The pathogenicity of *E. histolytica* is attributed to several virulence factors that enable invasion, colonization, attachment to host tissues, evasion of the host immune system, and suppression of immune responses. The parasite's ability to adapt to environmental conditions further enhances its virulence. Tissue destruction primarily occurs through parasite attachment to host cells followed by cellular degradation mechanisms [3].

Gal/GalNAc lectin is a major virulence factor of *E. histolytica* and a key cell-surface molecule involved in parasite adhesion to colonic mucosal glycoproteins, red blood cells, and neutrophils [6]. *E. histolytica* trophozoites bind to intestinal epithelial cells via Gal/GalNAc lectin, which recognizes galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) residues on host cell membranes. Following attachment, the parasite initiates cytotoxic mechanisms including apoptosis, phagocytosis, and trogocytosis, resulting in host cell death and tissue invasion [7]. Due to its essential role in adhesion and immune evasion, Gal/GalNAc lectin has been proposed as a promising vaccine candidate [6].

Amoebapores represent another important virulence factor of *E. histolytica*. These small peptides consist of approximately 77 amino acids and induce cytotoxic effects by forming ion channels in host cell membranes, leading to membrane disruption and cell lysis [8], [9]. Amoebapores belong to the saposin-like protein family and are localized within the cytoplasmic granules of trophozoites. Three forms have been identified: Amoebapore A (AP-A), Amoebapore B (AP-B), and Amoebapore C (AP-C), which exhibit optimal activity at acidic pH (5.2) [8]. Amoebapore-mediated calcium ion leakage and enzyme entry into host cells ultimately lead to programmed cell death and tissue destruction [9].

2. Materials and Methods

Collection of stool samples:

About 572 stool samples were collected from patients who suffer from diarrhea and complain of abdominal pain in the Al-Hussein Teaching Hospital, Bint Al-Huda Educational Hospital for Women and Children, and Mohammed Al-Musawi Hospital in Thi-Qar Province during the period from the beginning of December 2023 to the end of May 2024. The stool samples were examined directly under microscopy and the positive samples kept in a refrigerator - 20°C in a stool container for use in molecular examinations.

Molecular diagnosis:**Extraction of the genomic DNA:**

The parasite's DNA extracted from the positive fecal samples of microscopic examination using a special Presto™ Stool DNA Extraction Kit, supplied by Geneaid, and this was done in accordance with the company's instructions. Genomic DNA extracted from stool samples was examined using a Nanodrop spectrophotometer (THERMO. USA), which examines the concentration and purity of DNA by reading the absorbance at (260-280) nm.

The Primers:

The diagnostic primers of *E. histolytica* based on small subunit ribosomal 18s rRNA gene and virulence factor gene calreticulin were designed in this study using NCBI-GenBank and primer 3 plus design. These primers provided from ScientificResercher.Co.Ltd, Iraq, table (1).

Table (1): The primers used in current study.

Primers	Sequence 5'-3'		Product size	Genbank reference code
ssrRNA gene	F	ACGGGAGAGGTTGAAAATCCA	695bp	AB426549.1
	R	TGCGGCCCAAGATGTCTAAG		
Gal/GalNAc lectin gene	F	ACAACCTACCGATGATCCATCA	393bp	AF501280.1
	R	ACACGACAATTACCACATGCA		
amoebapore gene	F	ACAGAAGTGGGAGTAGGTTTTGT	408bp	X76903.1
	R	ACTGGAATTTCTCTGTCTTGTGT		

PCR master mix preparation:

The PCR master mix was prepared by using (Go taq **Green PCR Master Kit**) and this master mix done according to company instructions.

Table (2). Components of the multiplex PCR mixture.

PCR Master mix	Volume
DNA template 5-50ng	5µL
Forward primer (10pmol)	2µL
Reverse primer (10pmol)	2µL

Green Master mix	12.5 μ L
PCR water	3.5 μ L
Total volume	25 μ L

PCR Thermocycler Conditions:

The thermal conditions of PCR reaction were prepared according to table (3).

Table (3): thermal conditions of multiplex PCR.

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	5min.	1 Cycle
Denaturation	95°C	30sec.	1 Cycle
Annealing	59°C ₁ 58°C ₂	30sec.	35 Cycle
Extension	72°C	2min.	35 Cycle
Final Extension	72°C	5min.	1 Cycle
Hold	4°C	Forever	-

1. Gal/GalNAc lectin gene 2. Amoebapore gene

PCR product analysis:

The PCR products were analyzed by agarose gel electrophoresis and visualized by using UV Transilluminator.

3-Results:

Infection with amoebiasis:

The results of the current study showed that 100 out of 572 (17.48%) were infected with amoebiasis by microscopic examination.

The results of the current study showed that 64 (64%) samples out of 100 DNA samples were positive for *E. histolytica*, with a prevalence 64% according to the polymerase chain reaction (PCR) technique using special primers based on the 18s rRNA gene, as in Figure 1.



Figure 1: Agarose gel electrophoresis of PCR product shows the analysis of small subunit ribosomal RNA gene in *E. histolytica* from Human stool samples. M: marker (2000-100bp). The positive *E. histolytica* samples showed a small subunit ribosomal RNA gene at a (695bp) PCR product.

Virulence factors Gal/GalNAc lectin and Amoebapore genes of *E. histolytica* by PCR:

The virulence factor Gal/GalNAc lectin was positive in 50 out of 50 examined samples, with a prevalence 100% by using PCR technology. The results also showed the presence of the gene for the virulence factor Amoebapore in 34 out of 50 samples infected with the *E. histolytica* parasite, at a rate of 68%. Table (6), figures (2 and 3).

Table (4): The prevalence of virulence factors Gal/GalNAc lectin and Amoebapore in stool samples according to the polymerase chain reaction (PCR) technique.

Virulence factors	No. Examined samples	No. Positive samples	No. Negative samples	Prevalence %
Gal/GalNAc lectin	50	50	-	100
Amoebapore	50	34	16	68

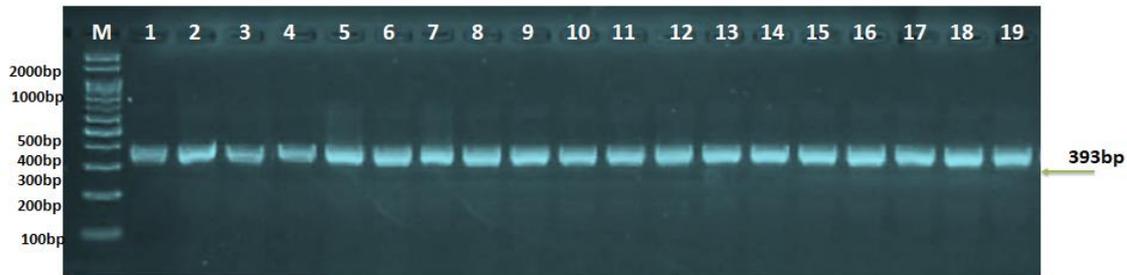


Figure 2: Agarose gel electrophoresis showed the PCR product analysis of virulence factor Gal/GalNAc lectin gene in *E. histolytica* positive samples. Where M: marker (2000-100bp). The positive *E. histolytica* virulence factor Gal/GalNAc lectin gene were showed at (393bp) PCR product.



Figure 3: Agarose gel electrophoresis showed the PCR product analysis of the virulence factor amoebapore gene in *E. histolytica* positive samples. Where M: marker (2000-100bp). The positive *E. histolytica* virulence factor amoebapore gene was shown at (408bp) PCR product.

Sequencer of virulence factors Gal/GalNAc lectin and Amoebapore genes of *E. histolytica*:

DNA sequencing was used to investigate the genetic characteristics of the virulence factor, Gal/GalNAc and amoebapore genes in local *E. histolytica* isolates from Iraq (IQN) compared to human isolates and reference strains from GenBank. These genes were analyzed using phylogenetic and sequence homology approaches. The Phylogenetic analysis using the UPGMA method in MEGA 6.0 software revealed a close association between the Iraqi isolates (IQN.1-No.3) and established reference strains for each analyzed gene. The genetic sequence of Gal/GalNAc lectin and Amoebapores genes was performed and compared with the genetic sequence of virulence factors registered globally at the NCBI. The Gal/GalNAc and Amoebapore C genes. Both genes demonstrated high similarity (98.68% - 99.14% and 99.10% - 99.71% identity, respectively) and low genetic variations (0.0020% - 0.0180% and 0.29% - 0.58% respectively) compared to their reference strains.

1. Gal/GalNAc gene:

The results of the current study revealed that the multiple gene sequences of the Gal/GalNAc virulence factor of three local isolates of *E. histolytica* (IQN.1-No.3) showed a significant genetic correlation between the three local isolates and the global strain isolates from the United States of America with the serial number USA (M59850.1). The local isolate (IQN. -No.3) with the serial number (PQ049161) was the most identical with a percentage of 99.14% and the least variable with a percentage of 0.16% compared to the local isolate (IQNNo.1-No.2)) with the serial numbers (PQ049159, PQ049160) respectively, which showed a percentage of identity (99.12%, 99.13%), which falls within the minimum genetic variations (0.0120-0.0020%). Table (7), Figure (4, 5).

Table (5) :the NCBI-BLAST Homology Sequence identity and genetic variation analysis between *Entamoeba histolytica* isolates and NCBI-BLAST closed genetic related *E. histolytica* isolate.

No.	Local isolate and gene	Accession number	Homology sequence identity (%)			
			Genetic related isolate with accession No.	Country related	Genetic variation (%)	Identity (%)
1	IQN.No.1 Gal/GalNAc lectin	PQ049159	<i>Entamoeba histolytica</i> USA strain M59850.1	USA	0.18%	99.12%
2	IQN.No.2 Gal/GalNAc lectin	PQ049160	<i>Entamoeba histolytica</i> USA strain M59850.1	USA	0.17%	99.13%
3	IQN.No.3 Gal/GalNAc lectin	PQ049161	<i>Entamoeba histolytica</i> USA strain M59850.1	USA	0.16%	99.14%

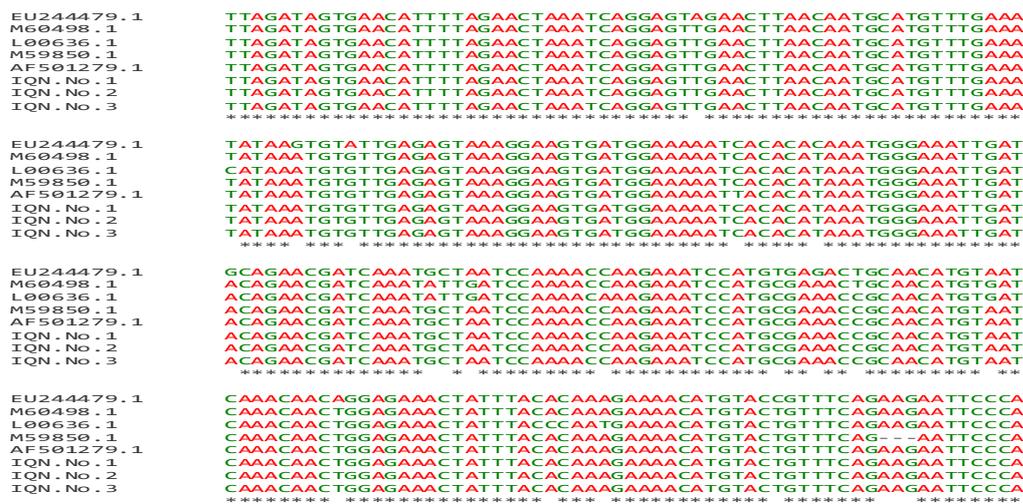


Figure (4): Multiple sequence alignment analysis of virulence factor Gal/GalNac gene in local *Entamoeba histolytica* IQN. Human isolates and NCBI-Genbank genetic related *Entamoeba histolytica* isolates. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in Gal/GalNac gene between isolates.

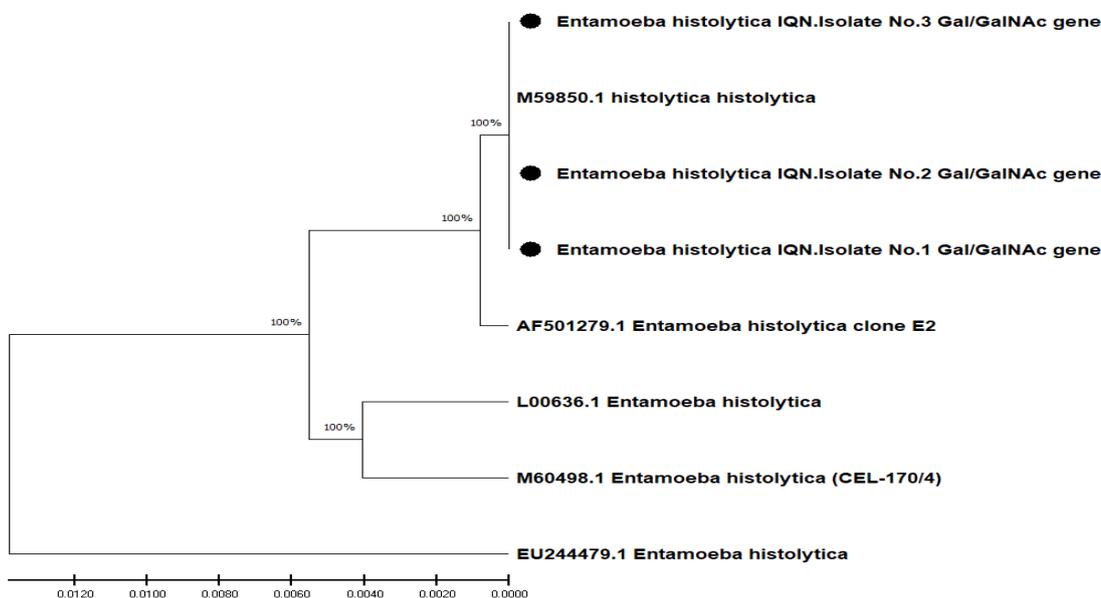


Figure (5): Phylogenetic tree analysis-based virulence factor Gal/GalNac gene

partial sequence in local *Entamoeba histolytica* IQN Human isolates that used for genetic relationship analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The *Entamoeba histolytica* IQN.1-No.3 isolates showed closed related to NCBI-BLAST *Entamoeba histolytica* USA strain (M59850.1) at total genetic changes (0.0120-0.0020%).

2.Amoebapore C gene:

The results of the multiple gene sequence analysis of the virulence factor Amoebapore C of three local isolates of *E.histolytica* (IQN.1-No.3) showed a high genetic correlation between the three local isolates and the global strain isolates from India with the serial number ALA1 (KP159522.1). The local isolate (IQN. -No.2) with the serial number PQ049148 was the most identical with a percentage of 99.71% and the lowest percentage of variation with a percentage of 0.29% compared to the two local isolates (IQN.1-No.3) which showed a percentage of identity 99.42% and a percentage of variation 0.58% which falls within the minimum genetic variations (0.0120-0.0020%). Table (9). Figure (6, 7).

Table (6) : NCBI-BLAST Homology Sequence identity and genetic variation analysis between *Entamoeba histolytica* isolates and NCBI-BLAST closed genetic related *Entamoeba histolytica* isolate.

No.	Local isolate and gene	Accession number	Homology sequence identity (%)			
			Genetic related isolate with accession No.	Country related	Genetic variation (%)	Identity (%)
1	IQN.No.1 amoebapore C	PQ049147	Entamoeba histolytica strain ALA1 KP159522.1	India	0.58%	99.42%
2	IQN.No.2 amoebapore C	PQ049148	Entamoeba histolytica strain ALA1 KP159522.1	India	0.29%	99.71%
3	IQN.No.3 amoebapore C	PQ049149	Entamoeba histolytica strain ALA1 KP159522.1	India	0.58%	99.42%

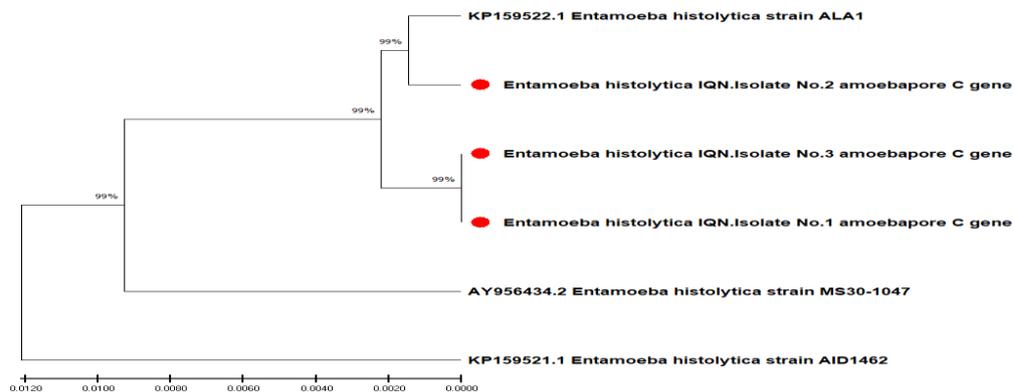


Figure (6) : Analysis of the multiple gene sequence alignment of the virulence factor Amoebapore C in local isolates of the *Entamoeba histolytica* IQN parasite. Which confirms the existence of a close genetic relationship between the local isolates and the global isolates registered in the World Gene Bank using the ClustalW alignment tool (online) and (*) indicates nucleotide variations.

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KP159521.1      GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
AY956434.2     GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
IQN.No.1       GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
IQN.No.3       GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
KP159522.1     GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
IQN.No.2       GTTATTTCATGTTTATTCTTTTGATAAATTTATTGGTAATTTTTAAGAAAAGTTGAAAC
*****

KP159521.1     ATATTTAAGAAGAACTATGAGTAAACCTTCTATTATTGTATGAAGTATTTGTTCTCC
AY956434.2     CTA TTTAAGAAGAACCA TGAGTAAACCACTTATTATT TTTATGAAGTATTTGTTCTCC
IQN.No.1       ATATTTAAGAAGAACTATGAGTAAACCA TTTTATTATT TGTATGAAGTATTCGTTCTCC
IQN.No.3       ATATTTAAGAAGAACTATGAGTAAACCA TTTTATTATT TGTATGAAGTATTCGTTCTCC
KP159522.1     ATATTTAAGAAGAACTATGAGTAAACCA TTTTATTATT TGTATGAAGTATTTGTTCTCC
IQN.No.2       ATATTTAAGAAGAACTATGAGTAAACCA TTTTATTATT TGTATGAAGTATTCGTTCTCC
*****

KP159521.1     TTTGTGTTTTTGATTTATGTTTAGCTTCTCAAGAAAACTACAAGACAGAGAAATCCAG
AY956434.2     TTTGTGTTTTTGT TTTATGTTTAGCTTCTCAAGAAAAACAACAAGACAGAGAAATCCAG
IQN.No.1       TTTGTGTTTTTGT TTTATGTTTAGCTTCTCAAGAAAAACAACAAGACAGAGAAATCCAG
IQN.No.3       TTTGTGTTTTTGT TTTATGTTTAGCTTCTCAAGAAAAACAACAAGACAGAGAAATCCAG
KP159522.1     TTTGTGTTTTTGT TTTATGTTTAGCTTCTCAAGAAAAACAACAAGACAGAGAAATCCAG
IQN.No.2       TTTGTGTTTTTGT TTTATGTTTAGCTTCTCAAGAAAAACAACAAGACAGAGAAATCCAG
*****

KP159521.1     TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
AY956434.2     TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
IQN.No.1       TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
IQN.No.3       TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
KP159522.1     TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
IQN.No.2       TTC TTTGTCC TGT TTTG ACATCAC TTGTTGG AAA GTTGATTGATTTAGTCC TTGGTGGAG
*****

KP159521.1     CAGTTGATAAAAGTAACTGATCATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
AY956434.2     CAGTTGATAAAAGTAACTGATATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
IQN.No.1       CAGTTGATAAAAGTAACTGATATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
IQN.No.3       CAGTTGATAAAAGTAACTGATATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
KP159522.1     CAGTTGATAAAAGTAACTGATATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
IQN.No.2       CAGTTGATAAAAGTAACTGATATCTTGAAACACTCTGTGCTAAAAGCAGATGGTCTTGT TG
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Figure(7): Phylogenetic tree analysis-based virulence factor amoebapore C gene partial sequence in local *Entamoeba histolytica* IQN Human isolates that were used for genetic relationship analysis. The phylogenetic tree was constructed using the Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA 6.0 version. The *Entamoeba histolytica* IQN.1-No.3 isolates showed close relatedness to NCBI-BLAST *Entamoeba histolytica* strain ALA1 (KP159522.1) at total genetic changes (0.0120-0.0020%).

4-Discussion

Intestinal parasitic infections remain a major public health problem worldwide, particularly in developing regions. In the present study, microscopic examination revealed that 100 out of 572 stool samples were positive for amoebiasis, with a prevalence rate of 17.48%.

Diagnosis of *E. histolytica* infection is often challenging due to the presence of asymptomatic carriers and the existence of morphologically indistinguishable non-pathogenic species such as *Entamoeba dispar* [1]. Molecular analysis confirmed that 64% of microscopy-positive samples were infected with *E. histolytica*. This prevalence was lower than that reported in Al-Diwaniyah Province (74%) [10], but higher than values reported in other studies from the same province (47%) [11], Basra Province (30%) [12], and Bangladesh (4.63%) [13]. A study conducted in Thi-Qar Province reported a prevalence of 68%, which is close to the findings of the current study [14], whereas studies from South Africa reported lower prevalence rates of approximately 8.5% [15]. These variations may be attributed to differences in environmental conditions, diagnostic methods, DNA extraction protocols, parasite load, and sample preservation procedures [16].

The Gal/GalNAc lectin gene was detected in all examined *E. histolytica*-positive samples in the present study, indicating a prevalence of 100%. Similar findings were reported in previous studies where this virulence factor was detected in 55 out of 61 samples [17] and in 84 out of 112 samples (75%) [10]. However, these findings contrast with a study conducted in Thi-Qar Governorate that reported a much lower prevalence of Gal/GalNAc lectin (22.91%) [4].

The amoebapore gene was detected in 68% of *E. histolytica*-positive samples in the current study. This detection rate was lower than that reported in Al-Diwaniyah Governorate (80.35%) [10], but higher than rates reported in other studies (34.72%) [4]. Studies conducted in Babylon Governorate reported detection of the amoebapore-C gene in all PCR-positive samples [18], while another study reported that 93% of samples were positive for amoebapore-C [17]. Amoebapore-C plays a crucial role in inducing necrosis and programmed cell death in host cells [9].

Sequencing analysis revealed high genetic similarity between local Iraqi isolates and global reference strains of *E. histolytica*. Similar findings were reported in Thi-Qar and Babylon Governorates, confirming close genetic relationships with strains registered in GenBank [4], [18]. These results support the reliability of molecular diagnosis and highlight the importance of virulence gene sequencing in epidemiological and molecular studies [19].

Conclusions:

The study revealed a high prevalence of virulence factors Gal/GalNAc lectin and Amoebapores genes in local Iraqi isolates of *E. histolytica* parasite and a high degree of genetic similarity between the local *E. histolytica* isolates and the reference strain, and similar virulence features with the presence of a certain percentage of genetic variation.

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