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Article

Detection of Molecular Typing of the Intestinal Parasite *E. histolytica* in Humans

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Abstract: This study was conducted on patients visiting Tikrit Teaching Hospital and some private laboratories in Tikrit from the beginning of January 2023 to the end of December 2023. A total of 220 stool samples were collected, with a percentage of 22.72% positive for *Entamoeba histolytica*, based on 50 positive microscopic tests. The results demonstrated the success of the Polymerase Chain Reaction (PCR) technique in amplifying the gene responsible for encoding the Galactose/N-acetyl-D-galactosamine lectin enzyme from *E. histolytica* samples indicating the method's effectiveness and efficiency in diagnosing the targeted genetic sequence.

Keywords: Molecular Profiling, *E.histolytica*, Entamoeba, Parasite.

1. Introduction

Entamoeba histolytica is classified as an intestinal protozoan parasite and is a common cause of diarrhea and malabsorption in humans. It leads to chronic inflammation, weight loss, and stunted growth, potentially resulting in amoebic colitis and liver abscess (Ghosh et al., 2019). According to (Paniker and Ghosh, 2018). *E. histolytica* is classified, based on genetic, immunological, and biochemical methods, into the phylum Sarcomastigophora and the class Gymnamebia.

The PCR technique is one of the most important methods used for the accurate diagnosis of amoebic species and has been approved by the World Health Organization (Hamzah et al., 2010). This technique possesses high sensitivity and accuracy in distinguishing between different strains of the same species, as well as in identifying the genetic composition and its relationship to the virulence of pathogenic and non-pathogenic species, which aids in selecting appropriate treatment. Furthermore, PCR contributes to providing accurate epidemiological information regarding the distribution and prevalence of this genus. This type of technology is used to avoid errors that typically occur during diagnosis using microscopy, serological methods, and other techniques (Paniker and Ghosh, 2018). The PCR technique has proven effective in detecting the gene responsible for the small subunit ribosomal ribonucleic acid (rRNA) which differentiates between *E. dispar*, *E. moshkovskii* , and *E. histolytica*, offering specificity and sensitivity close to 90-100% (Bhat, 2018)

The success of parasitic infection depends on several factors, including dose quantity, parasite virulence, bowel motility, host diet, the presence or absence of intestinal bacteria in the natural flora, and the ability of the amoeba to adhere to the

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mucosal cells in the colon. Effective penetration of epithelial cells in the colon is influenced by amoebic movement and the enzymes it possesses, such as histolysin and lytic enzymes, which damage mucosal membranes. Lectins mediate the adherence of the active form to host cells, such as the mucosal cells in the colon (Mahmud et al., 2017).

Studies in Iraq have shown varying levels of *E. histolytica* infection, ranging from 2.92% to 65.25% among diarrhea patients (Shakir and Hussein, 2014). In Ramadi, Al-Jnabee and Al-Quqaili conducted a study in 2015 to assess the prevalence of *E. histolytica*, utilizing the PCR technique to examine 75 stool samples. This study aims to investigate Entamoeba histolytica the causative agent of amoebic dysentery in the intestines of humans in Salah al-Din City, and to diagnose it using PCR technology.

2. Materials and Methods

Samples collection:

The current study included the collection of stool samples from children lying down and visiting Tikrit Teaching Hospital and some external laboratories in the city center who suffer from symptoms of diarrhea, fever, loss of appetite, and others, as 220 samples were collected for the period from the beginning of January 2023 to the end of December 2023, and then the samples were kept after microscopic examination in the freezer (Deep freeze) at 20 degrees Celsius until molecular tests were performed.

Microscopic examination:

The samples were examined macroscopically and it was observed whether the stool sample was bloody or mucous, as the availability of blood and mucus is an indication of the occurrence of infection, so samples containing blood and mucus are given special care, and in such a case the sample should be taken from the areas churned with blood and containing mucus. Stool samples were examined no later than one hour from the time of their arrival at the laboratory. The samples were observed macroscopically to determine the nature of the feces (solid solid, semi-solid Semisolid, Liquid Liquid) and to indicate the presence of mucus mucus or Blood in the sample. All stool samples were examined microscopically, a swab of feces was taken on a clean glass slide, and several drops of phlegmatic saline solution 0.9% were added, with the use of drops of iodine – lokel solution (logols lodine) to stain the nuclei of the parasite cysts, the slide was examined using a microscope and the magnification force was greater 40 x for the diagnosis, where two swabs were prepared for each sample, and it was examined in a laboratory

Method of purification of samples and isolation of parasite bags with saline phosphate solution:

Some stool samples marked with a small amount can be purified by the PBS solution method (Latifah et al., 2005).

Diagnosis of the parasite using molecular methods:

DNA extraction from stool samples: DNA was isolated from stool samples according to the QIAamp® Fast DNA Stool Mini Extraction Kit protocol.

Primer preparation: Primers were prepared by the company Macrogen in lyophilized form. The primers were dissolved in water free of the nuclease enzyme to give a final concentration of 100 microl/ microl as a stock solution (stock solution). A working solution for these primers was prepared by adding 10 microliters of primer stock solution (work solution) (stored in the freezer -20 degrees Celsius) to 90 microliters of nuclease-free water to obtain an effective primer solution of 10 microl / microl.

Reaction steps and temperature protocols:

Calculation of concentrations for the first cycle:

10	**Number of samples**
56	**Badi hybridization temperature**
2 µl	**Sample size**

bp896	**The length of the product of the polymerase chain enzyme reaction**
40 دورة	**The number of cycles in the polymerase chain reaction**
E1	**Front padding**
E2	**Rear initiator**

Components of the polymerase chain reaction enzyme mixture for one sample:

10µl	**Master Mix**
µl 1	**Forward primer**
µl 1	**Reverse primer**
µl 4	**Nuclease Free Water**
µl 4	**Template DNA**
µl 20	**Total**

Temperature protocol of polymerase chain reaction:

Number of Sessions	The time	Temperature range	steps
1	**05:00**	**95**	**Denaturation Initial**
40	**00:30**	**95**	**Denaturation**
	00:30	**56**	**Annealing**
	01:00	**72**	**Elongation**
1	**07:00**	**72**	**Final extension**
1	**10:00**	**10**	**Hold**

Calculation of concentrations for the second cycle of the sequenced polymerase enzyme:

10	**Number of samples**
46	**Badi hybridization temperature**
2 µl	**Sample size**
bp439	**The length of the product of the polymerase chain reaction**
40 دورة	**The number of cycles in the polymerase chain reaction**
Eh1	**Front padding**
EH2	**Rear initiator**

Components of the mixture components of the polymerase chain reaction mixture of one sample:

10µl	**Master Mix**
µl 1	**Forward primer**
µl 1	**Reverse primer**
µl 4	**Nuclease Free Water**
µl 4	**Template DNA**
µl 20	**Total**

- Temperature protocol of polymerase chain reaction:

Number of Sessions	The time	Temperature range	Steps

1	05:00	95	**Denaturation Initial**
40	00:30	95	**Denaturation**
	00:30	46	**Annealing**
	01:00	72	**Elongation**
1	07:00	72	**Final extension**
1	10:00	10	**Hold**

Electrophoresis:

After PCR amplification, Agar Gel electrophoresis was adopted to confirm the presence of amplification.

The stage of preparation of acarose:

- 100 ml of X TAE1 is poured into a beaker.
- 1.5 g (1.5%) of acarose after weighing by the sensitive scale was added to TAE.
- The solution was heated to a boil (using a microwave) until all the jelly particles dissolved.
- A microliter of ethidium bromide (10 mg/mL) was added to the acarose.
- The acarose has been stirred in order to mix and avoid bubbles.

The stage of casting acarose:

- The special mold for casting the acarose was prepared after placing the Comb comb to make pits inside the acarose depending on the number of samples.
- The melted acarose is poured and left to cool and Harden at room temperature for 15 minutes
- After hardening the acarose and carefully removing the combs, the hardened acarose is placed in the electric Relay device and a tar solution is added until the acarose is immersed in the transformer.

The stage of additions:

- 8 microliters of loading dye were added to the first hole of the acarose to determine the size of the resulting DNA after electrophoresis
- 8 microliters of polymerase chain reaction product were added to the pits
- The negative pole was connected to the near pole of the drill and the negative pole at the far end of the drill.
- The electric relay was switched on at a voltage of 100V/mAmp for 75 minutes.

The results of the diagnosis of the histolytic type of Amoeba:

Nested PCR has been developed to increase both the sensitivity and the quality of the polymerase chain reaction as it uses two pairs of amplification primers and two consecutive cycles of the reaction, usually one pair of primers is used in the first round of the polymerase chain reaction from 15 to 30 cycles, then the products of the first round of amplification are subjected to a second round of amplification using the second set of primers, which the increased sensitivity of the total number of cycles increases while the increased specificity arises from the correlation of the prefixes used in the cycle The second of the sequences is present only in the products of the first round (McPherson et al., 2021).

Gene sequencing:

Ten samples of the diagnosed samples from the results of the second cycle using the Sanger sequence cross - linked polymerase chain reaction reaction technique using ABI3730XL, automated DNA sequencing, were sent by the companymacrogen Corporation-Korea and the results were received by e-mail.

Statistical analysis:

The program statistical analysis system-SAS (2010) was used in the statistical analysis of the studied data to study the influence of various factors in the studied qualities, and the significant differences between the averages were compared with the test of the least significant difference (LSD), and the percentage differences between them were compared with the chi-square test).

3. Results

220 stool samples of children attending Tikrit Teaching Hospital and a number of external laboratories in the city center were examined using the direct swab method and the number of positive infections was 50 (22.72%), as shown in Table (1).

Table (1) shows the percentage of the number of positive samples for infection with the e parasite histolytica

Number of negative samples	Number of positive samples	Total number of samples
170 (%77.27)	**50 (%22.72)**	**220**

The percentage of the current study is similar to the findings of Ahmed (2020) in Mosul governorate by (20%), Hussein (2016) by (26.41%) in Dhi Qar governorate and al-Emam (2015) by (23.37%) in Babylon governorate. While the percentage was lower than what was achieved by Abdullah (2017) by (46.6%) in Erbil and Dohuk governorate, Hamad (2012) by (30%) in Erbil governorate, Jaffar (2021) in Wasit governorate by (47.7), Al-zubadi (2021) by 34.3% in Najaf governorate and Al-Tufaili (2020) by 43.29 the percentage was higher in Kufa than Kadir (2018) recorded by 9.7% in Tikrit. The difference in the incidence rates recorded in the current and previous studies may be due to the difference in the level of personal hygiene, geographical location, climatic conditions, as well as the level of poverty, the type of living conditions and the availability of clean water (Zeb et al., 2018) as it was noted that people in crowded places with an inadequate sanitary and environmental system are more susceptible to infection than others (Al-Taei, 2019) and different diagnostic methods (Mezeid et al., 2021), sample size and duration of the study (Dincer et al., 2017), climatic conditions and a large population all play an important role in infection (Dhubyan Mohammed Zaki, 2021). The e parasite was diagnosed. histolytica in 10 stool samples taken from patients of Tikrit Teaching Hospital and private laboratories, who suffered from bloody diarrhea or abdominal pain, vomiting with nausea and high body temperature, as the diagnosis was made by direct microscopy and PCR polymerase chain reaction technique (Banisch et al., 2015). . The results of DNA extraction from 10 faecal samples of people infected with histolytic amoeba using maximum PCR premium kit (i-Taq) 20 µlrxn (Cat. No. 25025) (Figure (1).

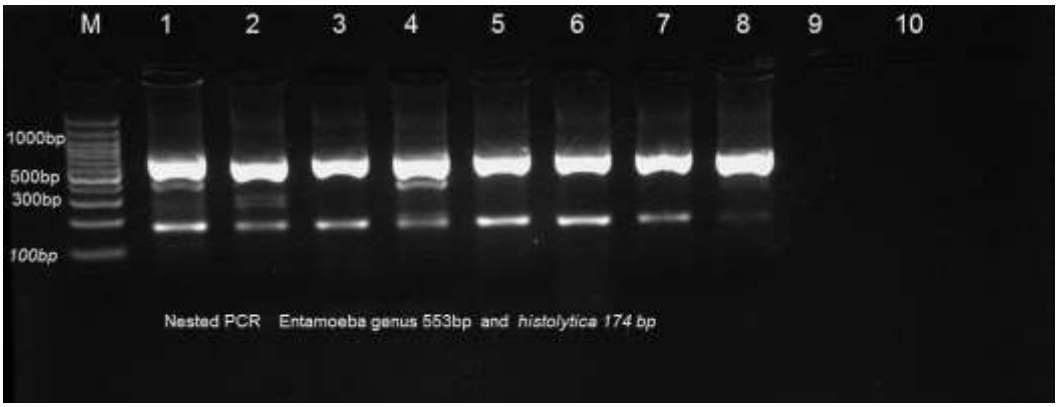

Figure (1) electrophoresis of DNA extracted from 10 faecal samples belonging to the parasite E.histolytica is based on acarose gel with a concentration of 1% and a potential difference of 70 volts, a current of 60 amperes for one hour.

To measure the purity of samples in a Nanodrop device, we put 1-2 µl of miniature DNA DNA into the base. Then the lid is closed and we press the measurement start button, then after completion we check the concentration and purity of DNA DNA.

Table (2) shows the result of Nanodrop

Sample ID	Nucleic acid conc. (ng/ml)	260/280 purity

1	32.64	**1.672**
2	46.14	**1.531**
3	27.19	**1.82**
4	84.1	**1.871**
5	32.46	**1.952**
6	63.18	**1.753**
7	44.04	**1.521**
8	28.1	**1.621**
9	39.9	**1.435**
10	53.18	**1.703**

The results after sequencing of the nitrogenous bases of the genes used in the study showed that the samples were identical to the globally diagnosed isolates after the matching process was performed through sequence analysis of nucleotides by using the Bio edit alignment program for comparison with the isolates recorded globally in the gene Bank, the relevant sequences were obtained with the samples in the (NCBI) www.ncbi.nlm.gov/nucleotide). The isolation in the current study was 90% similar to the globally recorded isolation, the type of change in nitrogenous bases (Transversion) was as shown in Table (3), as studies were conducted showing the sequence of nitrogenous bases of the complete gene of the e parasite.histolytica shows that 99% of Amoeba genes contain cysteine protease genes (Bruchhaus et al., 2003). Another study was also conducted and showed by sequencing the nitrogenous bases the presence of the phospholipase gene responsible for the cyst in the cyst phase (Cyst) (Ehrenkaufer et al., 2013).

Table (3) shows the analysis of the sequence results of the e parasite. Histolytica

Entamoeba histolytica						
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Source**	**Identities**
4	DELEITION	227	-\G	OL771810.1	Entamoeba histolytica isolate H211110-028_M01_27_E1.ab1770 small subunit ribosomal RNA gene, partial sequence	(77%)
	Transversion	228	T\G			
	Transition	229	T\C			
	Transversion	230	G\T			
	Transversion	231	G\T			
	Transition	232	G\A			
	Transition	233	G\A			
	DELEITION	242	-\A			
	Transversion	243	C\A			
	Transversion	244	G\C			
	MULTI	(268-573)	MULTI			
8	Transversion	64	T\A	JQ963443.1	Odontura aspericauda isolate OAO-A1	(92%)
	Transversion	44	C\A			
	Transversion	43	T\A			
		38	A\G			

Transversion	37	T\G	5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Entamoeba histolytica isolate H211110-028_M01_27_E1.ab1770 small subunit ribosomal RNA gene, partial sequence
Sequence ID: OL771810.1Length: 770Number of Matches: 1
Range 1: 208 to 573GenBankGraphicsNext MatchPrevious Match
Alignment statistics for match #1

Score	Expect	Identities Gaps	Strand
195 bits(105)	2e-44	288/376(77%)	13/376(3%) Plus/Plus

```
Query 1   CACCAGGAGTGGAGCCTGC-TTGGGGTTTGACTC-CGACGGGAAACCTCACCCGGCCCGG 58
          ||||||||||||||||||     ||||||||  ||||||||||||||||||||||||
Sbjct 208 CACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGG 267

Query 59  ACACGGAAAAGATTCGCAGATTGATAGAACTTTCTCGATTCCGTGGTCCGTGGTGCAAGA 118
          ||||||| | ||||| |||||||||||| |||||||||||||||||  |||||||| |
Sbjct 268 ACACGGACAGGATTGACAGATTGATAGCTCTTTCTCGATTCCGTGGGTGGTGGTGCATGG 327

Query 119 ACGGCTCTTAGCTGGCGCAGCGATTAGCCTGAATGAGTCATCATAACTGTCGAGACTGTG 178
          || ||||||| ||| | |||||||| | ||| || | ||  |||||  ||||||| ||
Sbjct 328 CCGT-TCTTAGTTGGTGGAGCGATTTGTCTGGATAATTCCG-ATAACGAACGAGACTCTG 385

Query 179 GG-TGTCCGGGGCTAGTTACAGCGTTTCCCCGGGCGGAGGGCGTCCCCCCGCTTCTTAGAG 237
          | ||| |||||||| |||  |||| |||| |||||||||| ||||||||||
Sbjct 386 GCATG-CTAA-CTAGTTAC-GCGACCCCCGA-GCGGTCGGCGTCCCCCAACTTCTTAGAG 441

Query 238 TCACGGGTGGGGGGCCCAGCCGTCCATAGATTGAGCTATAACAGTTCTGACATGACCTCCC 297
          || |||| | |||| || |||||||||| |||||||| |||| ||| |||
Sbjct 442 GGACAAGTGGCGTTC-AGCCACCCG-AGATTGAGCAATAACAGGTCTGTGATGCCCTTA- 498

Query 298 GATGTAAGCGGCAGCACTCGCTCTACACTGACTGGCTCAGCGTGTGCCGACACTAACGCC 357
          ||||| | ||| |||| ||| ||||||||||||||||||||||||||||| || || |||||
Sbjct 499 GATGTCCGGGGCTGCACGCGCGCTACACTGACTGGCTCAGCGTGTGCCTACCCT-ACGCC 557

Query 358 CGCCGGCGCGGGGAAC 373
          || |||||||||| |||
Sbjct 558 GGCAGGCGCGGGTAAC 573
```

After the samples were aligned with each other and the best mathematical pattern was found, and then the genetic variation between the samples was found by the MEGA-X program, the internal genetic tree was analyzed between the samples, after the samples were aligned with the matching samples recorded in the Genebank and the best mathematical pattern was found, and then the genetic variation between the samples was

found by the MEGA - X program, the genetic tree was analyzed between the samples by (Tree UPGMA) (Unweighted Pair Group Method with arithmetic Mean), the the results showed the similarity of the samples under study with the samples recorded globally, the samples were jq963443. Registered by Jebur (2021) in Baghdad governorate.

4. Conclusion

The study on Entamoeba histolytica, a major intestinal parasite causing various gastrointestinal issues, focused on diagnosing the parasite in patients from Tikrit, Iraq, using both microscopic and molecular techniques. Specifically, PCR (Polymerase Chain Reaction) was employed to enhance diagnostic accuracy by differentiating between pathogenic and non-pathogenic strains of the parasite. The results demonstrated a 90% genetic similarity between local isolates and those globally recorded, affirming the reliability of PCR in diagnosing amoebiasis. The study's findings align with prior research in Iraq, though incidence rates varied across regions, influenced by factors such as hygiene, living conditions, and diagnostic techniques. The study highlights the importance of molecular methods like PCR in providing precise epidemiological insights, aiding in the selection of effective treatment strategies, and enhancing our understanding of the genetic diversity of E. histolytica.

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