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Molecular Surveillance and Phylogenetic Analysis of *Entamoeba Histolytic* Isolated from Children in Kirkuk Province, Iraq

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Abstract

The current study included the microscopic diagnosis of Entamoeba histolytic parasite, (140) stool samples were collected from children ranging in age from one to fourteen years with diarrhea from the Children's Hospital in Kirkuk from September 2020 to August 2021 using direct examination methods. The percentage of total infection was (23.57%). The current study also included the use of the polymerase chain reaction (PCR) method to diagnose amoeba infection, and the genetic sequencing method was used to determine the type of amoeba. Where 28 positive samples were subjected (through microscopy) to molecular examination by diagnosing the 18s ribosomal RNA gene, and the results showed that 19 out of 28 samples were (positive for amoeba) and 17 of 19 samples were (positive for *Entamoeba histolytica*). Molecular characterization and genetic analysis of *E.histolytica* was carried out and it was found that the closest recorded isolates belong to isolates from Baghdad, Diwaniyah and Dhi Qar (from Iraq) and to South Africa, Egypt, India and Iran. Also, (11) samples were sent to the International Gene Bank (NCBI) and approval was obtained to register the study samples that were subjected to molecular characterization and given them the serial numbers, OL771805, OL771806, OL771807, OL771808, OL771809, OL771810, OL771811, OL771812, OL771813 and OL77.

Keywords: *Entamoeba histolytic***,** children ,molecular

Introduction

Intestinal parasitic infection remains a public health problem in many societies [1]Especially in developing countries, where its spread is closely related to inadequate sewage treatment and environmental pollution with feces [2] It is also more prevalent in hot and humid environments and is associated with overpopulation, especially among children in rural areas[3].

Amoeba disease is still an important health problem in the twenty-first century, causing deaths between 40,000 and 100,000 after malaria and bilharzia[4]. Amoebiasis is a worldwide disease caused by a protozoan parasite called Entamoeba spp.[1]. It is an internal parasite or coexist in animals and humans[5].

The species *Entamoeba histolytica* is a single-celled, non-flagellated pseudopodium parasite

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that causes proteolysis and tissue lysis (hence its name).Food and water contaminated with bags are the main source of infection transmission [1]

Amoebic colitis and the progressive development of diarrhea are the symptoms of the intestinal form, while amoebic liver abscess is the most common symptom of the extra intestinal form[6].

Microscopic examination of *Entamoeba histolytica* in stool samples cannot differentiate *Entamoeba histolytica* from other species[7].

Whereas, the molecular methods, the most important of which is the Polymer Chain Reaction (PCR) technique, is one of the important methods used to diagnose Entamoeba species, which has been approved by WHO [8].These methods are characterized by high sensitivity and accuracy to distinguish the strains of one species and to identify the pathogenic and non-pathogenic ones, and it is also used to know the genetic structure and its relationship to the virulence of the pathogenic species, which contributes to choosing and determining the appropriate treatment[9].

The DNA Sequencer method is defined as the process of determining the exact arrangement of nucleotides within a DNA molecule, and it includes any method or technique that is used to determine the arrangement of the four bases adenine, guanine, cytosine and thymine in the DNA strands, and the emergence of rapid DNA sequencing methods has greatly accelerated in research [8].

Material and Methods

Microsocial examination:

140 stool samples were collected from children attending the Children's Hospital in Kirkuk governorate who suffer from severe diarrhea and diarrhea accompanied by blood for the period from the beginning of October 2020 until the end of October 2021.

The direct smear method was used by taking a quantity of stool (especially mucous from it to increase the possibility of the parasite's appearance) by means of a wooden stick and placing it on the glass slide (microscopically slide) and adding a drop of glass physiological solution and a drop of iodine dye, and then Examine the slide under the microscope.

The samples were placed in a sterile plastic box for microscopic examination, and then the samples were kept in the freezer (Deep freeze) at -20°C until the molecular tests are conducted.

Genetic methods

Primers

Table 1. The sequence of primers with annealing temperature

Primer Name	Seq.	Annealing Temp. (ºC)
E1	5`-TAAGATGCACGAGAGCGAAA-3`	56
E2	5`-GTACAAAGGGCAGGGACGTA-3`	
EH1	5`-AAGCATTGTTTCTAGATCTGAG-3`	46

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Methods and Workflow

DNA Extraction

Genomic DNA was isolated from Stool samples according to the protocol of QIAamp® Fast DNA Stool Mini Extraction Kit as the following steps:

- From each stool sample 180-220mg was mixed with 1ml InhibitEX Buffer in 1.5ml micro centrifuge tube, then vortexed thoroughly until the sample was homogenized.
- All samples were Incubated in water bath at 70oC for 5min.
- After incubation all tubes were centrifuged at 13,000rpm for 1min to pellet stool particles.
- For protein break down ,15µl from Proteinase K was added to new 1.5ml micro centrifuge tube and all supernatant from previous step was transferred to this new tube.
- For cell lysis 200µl from AL Buffer was added to all samples, then mixed thoroughly using vortex until it formed a homogenized solution.
- To complete cell lysis all samples were incubated in water bath at 70oC for 10min.
- After incubation, the 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from inside the lid.
- For DNA purification, 200µl of ethanol (96-100%) was added to samples and mixed by plus vortex for 15s.
- All mixes were carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1 min.
- After centrifuge, the QIAamp Mini spin column was placed in a clean 2 ml collection tube and 500µl of AW1 Buffer was added then centrifuged at 8000 rpm for 1 min.
- After centrifuge, the QIAamp Mini spin column was placed in a clean 2 ml collection tube and 500µl of AW2 Buffer was added then centrifuged at 12000 rpm for 3 min.

For DNA elution the QIAamp Mini spin column was placed in a clean 1.5 ml micro centrifuge tube and 100µl of AE Buffer was added, incubated at room temperature for 5 min, and then centrifuged at 10000

Quantitation of DNA

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted QuantiFlour

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Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Reaction Setup and Thermal Cycling Protocol
PCR Component Calculation for First Run

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	10
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease Free Water					4
PCR Product		ng/µl		ng/µl	4
Total volume					20
Aliquot per single rxn	16 µl of Master mix per tube and add 4 µl of Template				

PCR Program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	40
Annealing	56	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

PCR Component Calculation for Nested Run

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	10
Forward primer	10	µM	1	µM	1

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Reverse primer	10	µM	1	µM	1
Nuclease Free Water					4
DNA		ng/µl		ng/µl	4
Total volume					20
Aliquot per single rxn	16 µl of Master mix per tube and add 4 µl of Template				

PCR Program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	40
Annealing	46	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

Solutions

1 X TAE buffer, loading dye, DNA ladder marker, Ethidium bromide (10mg / ml).

Preparation of agarose

- 100 ml of 1X TAE was poured into a beaker.
- 1.5 gm (for 1.5%) agarose was added to the buffer.
- The solution was heated to boiling (using Microwave) until all the gel particles were dissolved.
- 1µl of Ethidium Bromide (10mg/ml) was added to the agarose.
- The agarose was stirred in order to get mixed and to avoid bubbles.
- The solution was left to cool down at 50-60°C.

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Casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was left to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

DNA loading

PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was turned on at 100v/mAmp for 75min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

Standard Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analysed using genius software**.**

Results & Discussion

28 positive microscopic specimens were subjected to molecular analysis

Geneticexaminations:

The speed and low cost of direct stool swab made the diagnosis of amoeba disease one of the routine methods used in most laboratories, however, where some studies have indicated that nearly half of the suspected cases of tissue amoeba are false positive results and the results must be confirmed by additional examination 10. Polymerase chain reaction is the preferred method for clinical and epidemiological studies in developed countries and has been strongly endorsed by the World Health Organization. The diagnosis of the parasite under study by polymerase chain reaction is of high accuracy, sensitivity and specificity11, and *E. histolytica* can be identified in a variety of clinical samples, including faeces, tissues and liver abscess aspirates. The polymerase chain reaction of the (18S rRNA) gene is about 100 times more sensitive than the best ELISA kit currently available 12

Diagnosis of Amoeba spp.:

The molecular results, using the traditional technique of polymerase chain reaction and using the (E1) and (E2) primers, showed that eighteen positive samples with a percentage of (67.9%) out of twenty-eight samples that were subjected to the molecular examination by the appearance of the DNA bundle after the electrophoresis The size is (896bp) as shown in the figure .1

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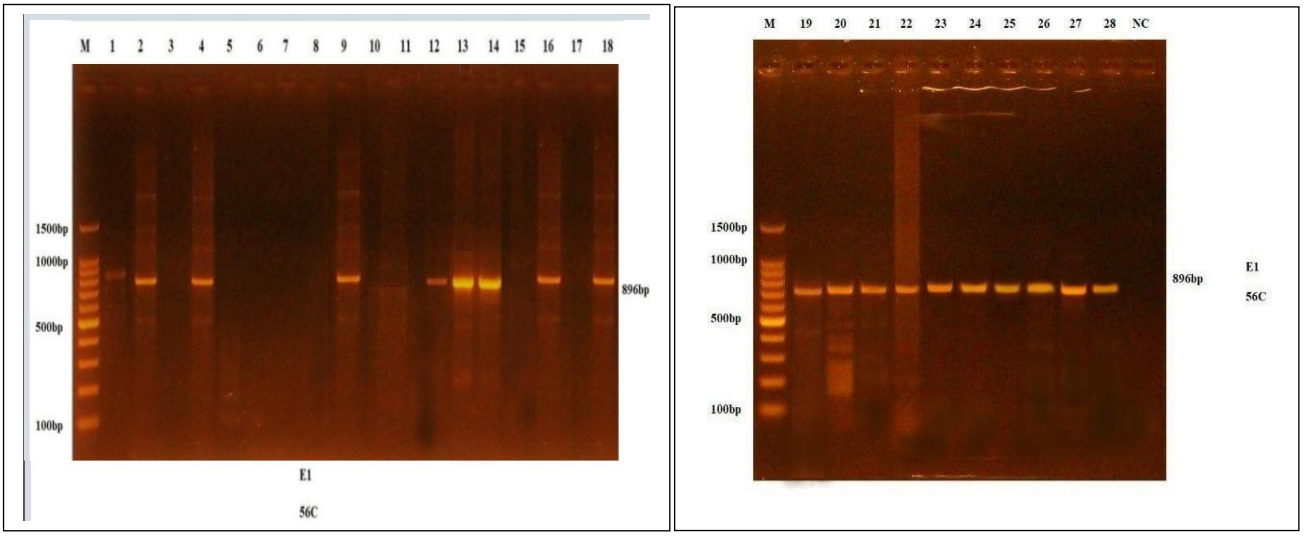
Figure 1.Agarose gel electrophoresis of PCR products (896bp) of the amoeba parasite with ethidium bromide dye, M represents volume guide 100-1500 bp



Through this study, it was found that nine samples with a percentage of 32.1% gave a negative result in the polymerase chain reaction test, although it was positive by microscopic examination, and similar cases of this result were recorded in other regions of the world in a study conducted by Haque (1998), where it was found that 5 Negative samples out of 80 were positive by microscopic examination13, Furrows (2004) recorded that there are 5 negative samples out of 101 positive samples by microscopic examination 14, and Samie (2006) recorded 67 negative samples out of 103 positive samples by microscopic examination 15, and Santos (2007) recorded 16 negative samples out of 27 positive fecal samples by microscopic examination 16, and Fotedar (2007) recorded 21 negative samples out of 110 positive samples by microscopic examination 12.
The difference in the infection rate between diagnosis by microscope and diagnosis by polymerase chain reaction is due to the fact that the diagnosis by microscope is inaccurate and insensitive, in addition to that it depends mainly on the skill of the technician or the examining person 17has proven that a parasite examination Tissue amoeba in stool by direct wet microscopy alone can lead to significant false-positive results. To obtain a reliable diagnosis of a parasite and to avoid unnecessary treatment of this parasite, at least one more specific examination is required 10. False-negative results can also be obtained due to the presence of DNA polymerase inhibitors in stool samples, and failure to amplify a single diagnostic sequence due to inhibitors in the sample or potential mutations in the primer-binding region may lead to false negative results 18 or It may be due to the period between sampling and DNA extraction and the use of the polymerase chain reaction technique 14 in addition to climatic conditions, geographical identification, nature of places, the degree of contamination by the causative agent, and the variation in the spread of the parasite in different countries 19.
The emergence of the difference in the results of the current study using the polymerase chain

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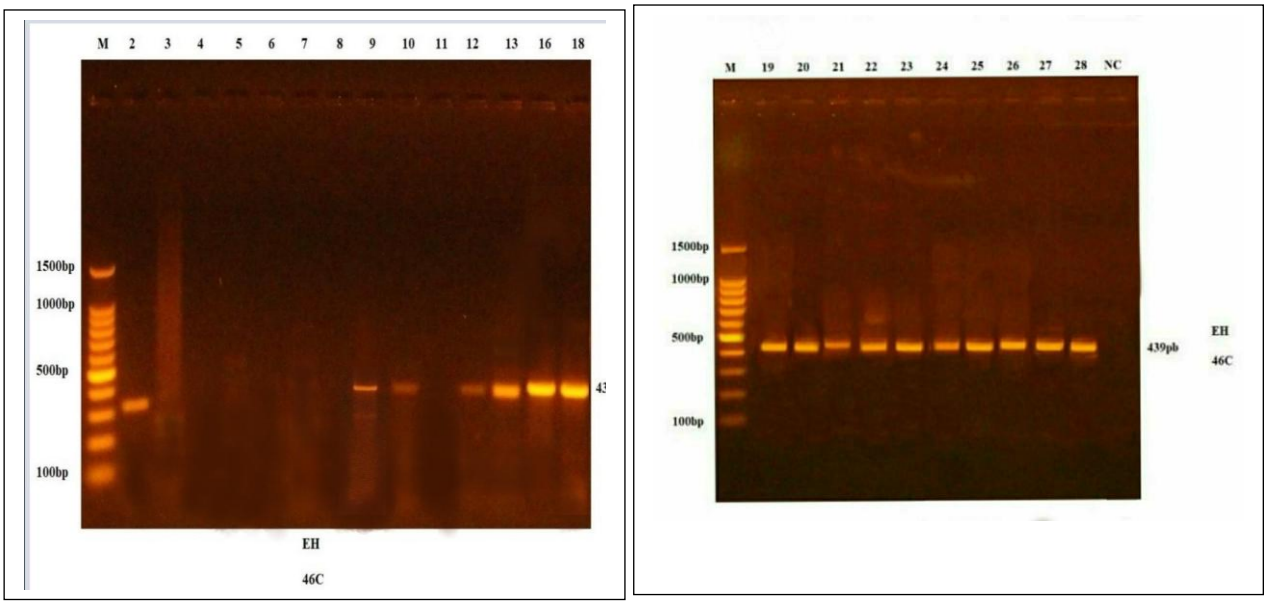
reaction technique with the results of previous studies may be attributed to the difference in the methods of extracting DNA, and also due to the difference in the amount of parasites present in the excrement, and also due to the irregular spread of the parasite in most countries of the world due to different climatic conditions health and other reasons 20

Diagnosis of *E.histolytica* :

Nested polymerase chain reaction (Nested PCR) has been developed to increase both the sensitivity and specificity of the polymerase chain reaction as it uses two pairs of amplification primers and two consecutive cycles of reaction, usually one pair of primers is used in the first round of 15-30 cycles of polymerase chain reaction, The products of the first round of amplification are then subjected to a second round of amplification using the second set of primers, which is targeted to an internal sequence of the sequence amplified by the primer pair in the first cycle. Second round of sequences found only in first round products 21 .

Agarose gel electrophoresis of PCR products (439bp) of tissue amoeba parasite with ethidium bromide dye, M represents volume guide 100-1500 bp



The appearance of two negative samples in the product of the overlapping polymerase chain reaction is due to the use of one type of primer specific to the tissue amoeba species only. The reason may be due to the fact that the negative sample belongs to another type of amoeba genus. In a study conducted by Mahmood (2020) using the nested polymerase chain technique and using more than one primer, the infection rate of tissue amoeba was 57/70 (81.4%), the percentage of infection with *E.dispar* 41/70 (58.6%), and the infection rate of E. moshkovskii3/70 (4.3%) in

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Erbil governorate 22, and also the researcher ElBakri (2013) indicated in his study The infection rate of tissue amoeba was 10%, the percentage of infection with *E. dispar* (2.5%), the percentage of infection with *E. moshkovskii* (2.5%), and the percentage of joint infection with *E. histolytica/E. dispar* (3.3%) in the United Arab Emirates 23 .

Gene sequencing results

After sending the products of DNA replication by PCR technology to Macrogen Corporation in Korea for the purpose of identifying and sequencing the nitrogenous bases of the 18s ribosomal RNA gene for 11 samples of DNA extracted from stool samples diagnosed microscopically using sanger sequencing technology, the sequences were analysed in BLAST (Basic) program Local Alignment Search Tool) and by selecting the blastn tool after it was processed using Chromas program to remove interference between nitrogenous bases and Mega-X program, then the results were compared with the data recorded at the National Canter for Biotechnology Information (NCBI) belonging to the same isolates registered globally http://www.ncbi.nlm.nih.gov/). The results of the genetic sequence analysis were confirmed when comparing the alignment of nitrogenous bases with the globally registered isolates in the NCBI Gene Bank, which have the numbers MZ905641.1, MK332025.1, MF421529.1, KC853037.1, MH752556.1, KP233840.1, Y11272.1, KP233840. 1. All samples that sent belong to the parasite *E. histolytica* .

Eleven samples were registered in the NCBI Gene Bank, as shown in Tab.2

Tab.2. Shows access numbers for the cloned samples in the NCBI Gene bank

Accession	Accession Sequence ID	Release Date
OL771805	H210824-030_K02_18_E1.ab1515	Dec 13, 2021
OL771806	H210824-030_I02_16_E1.ab1660	Dec 13, 2021
OL771807	H210824-030_C15_13_E1.ab1503	Dec 13, 2021
OL771808	H210419-027_G19_1_EH1.ab1134	Dec 13, 2021
OL771809	H210824-030_A15_1_E1.ab1658	Dec 13, 2021
OL771810	H211110-028_M01_27_E1.ab1770	Dec 13, 2021
OL771811	H211110-028_K01_26_E1.ab1690	Dec 13, 2021
OL771812	H211110-028_I01_24_E1.ab1686	Dec 13, 2021
OL771813	H211110-028_G01_23_E1.ab1366	Dec 13, 2021

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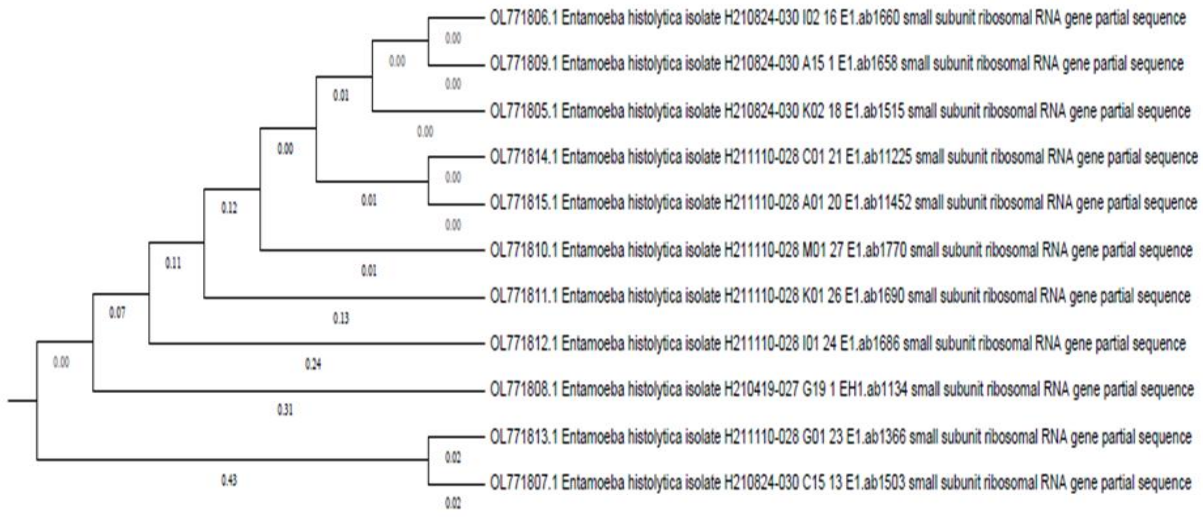
OL771814	H211110-028_C01_21_E1.ab11225	Dec 13, 2021
OL771815	H211110-028_A01_20_E1.ab11452	Dec 13, 2021

Genetic variance and genetic tree analysis between extracted samples and the closest match:

After the samples were aligned with the corresponding samples registered in the gene bank, finding the best mathematical pattern, and then finding the genetic variance between the samples using the MEGA-X program, the genetic tree between the samples was analysed using the Tree UPGMA (Unweighted Pair Group Method with Arithmatic Mean).), the results showed the similarity of the samples under study with the samples registered globally. The samples were MT296779 recorded by faraj(2020) in Baghdad and MW426074. Recorded by Jebur (2021) in Baghdad Governorate, KC853037 recorded by samie (2013) in North Africa, KP233840 recorded by Alobadi (2015) in Diwaniyah Governorate, MK332025 recorded by Azzam (2018) in Egypt is genetically closer to samples OL771815. OL771806. , OL771809 and the most distant genetically were samples KY823425. Recorded by Bahrami (2018) in Iran. As for the sample Y11272 by Roy (1997) in India, it was genetically closest to the sample OL771811.1

Fig.3 . show the genetic tree analysis between extracted samples and the closest match



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Conclusions:

The ability of E.histolytica to genetic variation that may affect the virulence and pathogenesis of the parasite.

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