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Original Article

Genetic Detection of Genes Encodes Some Enzymes in *Entamoebahistolytica* in Diarrhea Children in Iraq

Saleh Ahmed, S[1], Ghanim Abdulwahhab, I[1 *], Salman Alagely, H[2]

1. College of Education, University of Tikrit, Tikrit, Iraq
2. Biotechnology Research Center, Al Nahrain University, Baghdad, Iraq

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Corresponding Author: dr.en79@tu.edu.iq

Abstract

Entamoebahistolytica is a protozoan, an anaerobic intestinal parasite that causes about 50 million infections and a mortality rate of more than 100,000 worldwide. For diagnosis, two hundred samples of children with diarrhea signs were evaluated using staining and polymerase chain reaction techniques. The current study recorded 11 positive cases of *E. histolytica*, which were diagnosed by polymerase chain reaction (PCR) out of a total of 51 positive cases diagnosed microscopically for pediatric children arriving at Tikrit General Hospital in Tikrit city and the nearby areas. The percentage of positive cases reached 21.57% for the PCR assay, as significant differences appeared compared to the microscopic examination. The results showed that the parasite infection rates differed between males (54.9%) and females (45.1%). The percentage of infected numbers in the age group less than one year was about 43.1%, while the percentage of disease control and prevention programs f infected people in the age group 1-2 years was (31.4%). The results showed that the percentage of infected age group between (2-3) years was 15.7%. The recorded data showed that 5.9% and 3.9% of the participants were infected in the age group of3-4 and over four years old, respectively. The genes encoded in Cysteine proteinase five and Phospholipase were diagnosed using the PCR technique. The concordance with the current study isolate and 90% match globally. In conclusion, the methods of detection of *E. histolytica* appeared differences in positive results for this parasite.

Keywords: *Entamoebahistolytica*, Cysteine proteinase 5, Phospholipase

1. Introduction

Entamoebahistolytica is a protozoan, an anaerobic intestinal parasite that causes about 50 million infections and a mortality rate of more than 100,000 worldwide annually. Which colonizes and invades the intestines, causing amoebiasis, colitis, and may also amoebic liver abscess (ALA) (1). Amoebic infection is the third most common cause of death among parasitic diseases, after malaria and schistosomiasis (2). This infection is widely reported in developing countries such as India and Bangladesh, tropical African countries, and in some areas of Brazil and Mexico. Cases of infection are increasing in developing and advanced countries such as the USA and European countries due to global travel and the migration of people from endemic areas (3). Amoebiasis is often asymptomatic; in the acute form of the disease, symptoms such as muscle cramps, abdominal pain, watery or bloody diarrhea, and weight loss appear. The migration of the amebole from other organs, such as liver abscess, pneumonia, purulent pericarditis, and even cerebral amoebiasis, have been reported (1, 2).

The polymerase chain reaction is a molecular technique that detects the presence of target microorganisms by increasing the number of genetic materials. This technique is considered the first to

distinguish between *Entamoebahistolytica* and *Entamoebadispar* (4). The polymerase chain reaction is essential in isolating and increasing the number of genes. The short DNA sequences are amplified using specialized primers, DNA Taq polymerase, DNA template, and dNTPs by PCR, and this reaction consists of several cycles. Each cycle includes three phases (5). This technique has become the most prevalent in genetic study laboratories worldwide. The discrepancies that appear in the results of tests are due to changes in the sequence of the nitrogenous bases of the DNA strand of the same species due to mutations such as deletion, insertion, or nitrogenous bases substitution, which leads to a change in the cross-linking site of the DNA template (6). This study aimed to detect the *E. histolytica* in children early by PCR.

2. Materials and Methods

2.1. Sample Collection

Two hundred samples were obtained from children with diarrhea at Tikrit Hospital, Iraq. They include age groups from one month to 5 years. The blood sample was collected for hematological analysis such as blood group, Rh factor, ESR (Erythrocyte Sedimentation Rate), and C-reactive protein test.

2.2. Stool Examination

All stool samples were examined microscopically. The stool was taken on a clean glass slide, and several drops of 0.9% physiological saline were added, using drops of iodine-Lugol solution to stain the nuclei of the cysts. The slide was examined under the power of 10 X and 40 X for diagnosis (7).

2.3. DNA Extraction

DNA extracted from *Entamoebahistolytica*from stool samples following the manufacturer instructions Quick-DNA™ Fecal/Soil Microbe Miniprep Kit.

2.4. PCR Detection

The primers were used from IDT (Integrated DNA Technologies Company, Canada) as shown in tables 1 and 2. Also, Maxime PCR PreMixkit (i-Taq) 20µlrxn Cat. No.25025) was used in this study. PCR reaction with a final volume of 25µl included Premix5µl, Forward primer 10 picomols/µl(1 µl), Reverse primer 10 picomols/µl (1 µl), DNA template 2µl, distill water 16µl and the program cycles for each gene as shown in tables 3 and 4, in addition, it was electrophoresis by using Safe red stain and SiZer DNA Markers Ladder 1000bp (intron/ Korea) to detect PCR product for each gene. It was sequencing PCR products by the Sanger technique, which was sent to Macrogen/Korea.

Table 1. Sequence of cysteine proteinase 5 primer of *Entamoebahistolytica*

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'-TTTCAATACTTGGGTTGCAAAT- 3'	58	52	885bp
Reverse	5'-GCAGCTCCTGAAGCAATACC- 3’	57	53	

Table 2. Sequence of Phospholipase primer of *Entamoebahistolytica*

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- TGCTGATTTGGCTCTTGGGA -3'	61	55	420 bp
Reverse	5'- CCAAGCCCTCTTTCCCCAAA-3’	62	55	

Table 3. Program cycles for Cysteine proteinase 5 in polymerase chain reaction for *Entamoebahistolytica*

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	5 min	1 cycle
2	Denaturation -2	94°C	1 min	
3	Annealing	54°C	1 min	35 cycle
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	7 min	1 cycle

Table 4. Program cycles forPhospholipaseprimerpolymerase chain reaction for detection of *Entamoebahistolytica*

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	5 min	1 cycle
2	Denaturation -2	94°C	30 sec	
3	Annealing	58°C	30 sec	35 cycle
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	7 min	1 cycle

2.5. Statistical Analysis

The data of this study were analyzed according to analysis variance by using SPSS software statistical program (SPSS for windows ver.20). The difference in mean values was considered significant at ($P \leq 0.05$).

3. Results and Discussion

The current study recorded 11 positive cases of the *E. histolytica* parasite, which were diagnosed by polymerase chain reaction (PCR) out of a total of (51) positive cases diagnosed microscopically for pediatric patients referred to Tikrit General Hospital in Tikrit city and the nearby areas. The results showed that the percentage of positive cases of infection with the tissue amoeba parasite *E. histolytica* reached (21.57%) for the polymerase reaction assay, as significant differences appeared when compared with the microscopic examination, as shown in table 5.

Table 5. Comparison of microscopy and polymerase chain reaction for the number of positive cases in *E. histolytica*

PCR	Microscopic examination				Total
	Positive	Percentage	Negative	Percentage	
Positive	11	21.57	0	0	11
Negative	40	78.43	149	100	189
Total	51	100	149	100	200

Chi-Square=34.0 *P*-Value=0.001
Significant differences under a probability of 0.05

The results were higher than those reported by Mozer, Abdulwahhab (8) in the city of Tikrit, where the infection rate was about (9.3%). The results of the current study were similar to a study conducted by Ahmed (9) in Duhok, 21.67, 17.03, and 10.68%, respectively, while it was higher than the results recorded in the city of Erbil, that recorded infection rates of 7.52% and 2.33%, respectively (9).

Incidence of differences in the infection rate with *E. histolytica* in the mentioned studies may be due to differences in the time and the difference in geographical locations and the number and methods of the samples tested, as well as the difference in the percentage of parasites examined in the diagnosis. The standard of living as well as its increase in popular areas and housing in some unqualified areas leads to an increase in infection rates, as well as contamination of drinking water and its low levels, slow movement, and not being appropriately treated have a role in the increase in the rate of infection with the amoeba parasite, as well as the lack of attention to personal hygiene *E. histolytica* the cleanliness of foods, especially vegetables, and the absence of health control in restaurants (9).

3.1. Distribution of Prevalence of *E. histolytica* based on Gender

The current study shows that parasite infection rates vary between males and females, and the infection rate in males was (54.9%), while the infection rate in females was (45.1%), as shown in table 6.

Table 6. The percentages prevalence of *E. histolytica in* infected samples by gender

	Positive	Percentage
Males	28	54.9
Females	23	45.1
Total	51	100.0

Chi-square=1.8 *P* value=0.159

The results of this study did not show any significant differences at the level of probability (0.05). The results of the current study agree with the results of what was also stated by Pham Duc, Nguyen-Viet (9), and Green and Sambrook (10), and this can be explained that the nature and behavior of males have a significant role in their injury, as a lot of activity and movement characterizes them. Exercising them makes them more vulnerable to infection. In addition, males are the most active group in society, prompting them to eat ready-made foods without ensuring they are clean, while females care more about hygiene and are less mobile than males. The reason for compatibility may be due to the environment, as males are more effective and in contact with the external environment than females (11).

3.2. Distribution of *E. histolytica* Infections based on Age

The current study showed the relationship between people infected with the parasite *E. histolytica* by age groups less than (5 years). The percentage of infected numbers in the age group less than one year was about (43.1%). In contrast, the percentage of infected people in the age group 1-2 years was (31.4%), the age group between (2-3) years was (15.7%), the age group 3-4 years recorded 5.9%, and the age group over four years old recorded 3.9%. The results of this study did not show any significant difference at the level of probability (0.05).

The results of the statistical analysis showed that there were no significant differences at the level of probability (0.05), as most injuries occur in children under the age of one and a half years due to the failure of some mothers to adhere to basic general hygiene, and in addition to that the contamination of breastfeeding tools, and in addition to that, children who Under the age of two years, their immunity is low and they are more easily susceptible to infection. Repeated exposure to the parasite for young age groups (children) may stimulate their immune system and be more developed, leading to a decrease in infection rate (12).

3.3. Genetic analysis

The polymerase chain reaction (PCR) technique is used to duplicate a specific region within the DNA of the tissue-state amoeba parasite using special primers for the encoded gene (for Cysteine proteinase 5) and (for Phospholipase). After passing the amplified DNA products, the result appeared in the electrophoresis device. The molecular DNA coded gene (for Cysteine proteinase 5) is about 885 base pairs, and the gene coded (for Phospholipase) is about 420 base pairs compared with the standard size guide for Ladder DNA as shown in figures 1-4.

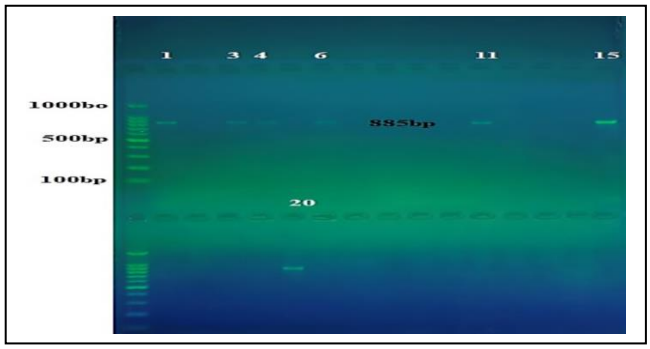

Figure 1. PCR product of the gene (Cysteine proteinase 5) for samples (1, 3, 4, 6, 11, 15, and 20) and the size of the gene was 885 base pairs, ladder (1000bp)

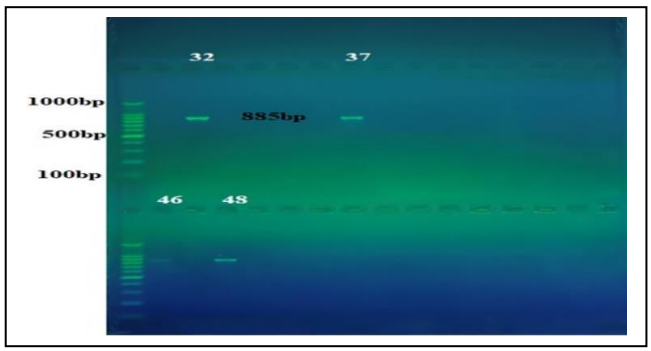

Figure 2. PCR product of the gene (Cysteine proteinase 5) for samples (32, 37, 46, and 48) and the size of the gene was 885 base pairs, ladder (1000bp)

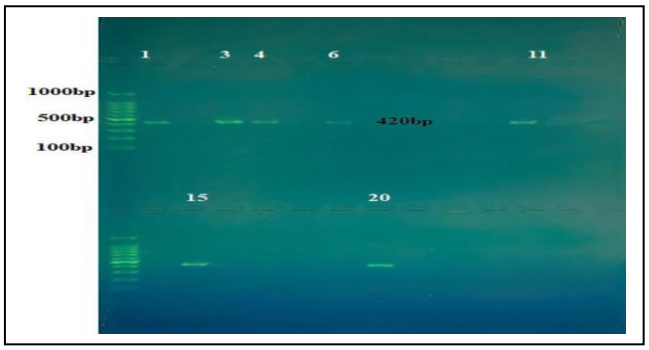

Figure 3. The result of the polymerase chain reaction PCR for the gene (Phospholipase) for samples (1, 3, 4, 6, 11, 15, and 20) and the size of the gene was 420 base pairs ladder (1000bp)

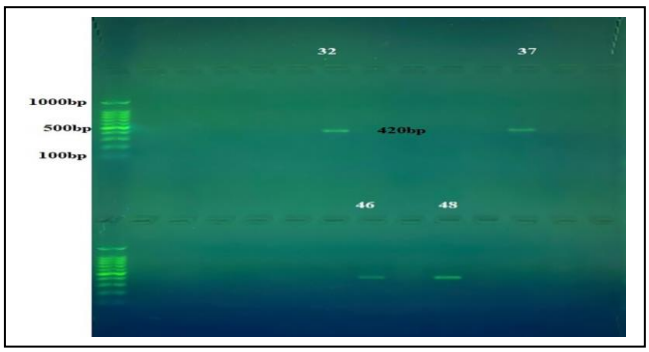

Figure 4. PCR product of the phospholipase gene for samples (32, 37, 46, and 48) and the size of the gene was 420 base pairs, ladder (1000bp)

Results after sequencing the nitrogenous bases of the genes used in the study showed that the samples were identical to the globally identified isolates after performing the matching process through nucleotide sequence analysis by using the Bioedit alignment program to compare with the globally registered isolates in GenBank. Relevant sequences with samples were obtained from the NCBI database (www.ncbi.nlm.gov/nucleotide). The isolate in the current study was 90% similar to the globally recorded isolate. The type of change in nitrogen bases was (Transversion) as shown in tables 7 and 8.

Table 7. The analysis of sequence results for the parasite *E. histolytica*

No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Transversion Transition Transversion	852 861 862	C/A G/A A/C	ID:XM_649902.2	*Entamoebahistolytica* HM-1:IMSS hypothetical protein, conserved (EHI_198890)	90%

Table 8. The concordance with the current study isolate and 90% matching with the globally registered isolate with ID number XM649902.2 (*Entamoebahistolytica* HM-1:IMSS Clone 6 2001, chromosome 16 DNA, nearly complete genome)

Score	Expect	Identities	Gaps	Strand
41.9 bites (45)	0.021	27/30 (90%)	0.30 (0%)	Plus/Plus

Query 786 TTTCCATCCAATGAATAAAACTGAAATAAA 815
Subject 275001 TTTACATCCAATACATAAAACTGAAATAAA 275030

The PCR technique was used to detect DNA for rapid diagnosis and to avoid the low sensitivity of other traditional tests. By using molecular methods, parasites can be diagnosed and genotyped. It also allows overcoming the difficulties faced by direct examination that does not allow determining the type of parasite that causes infection (12, 13).In other studies, the Cysteine proteinase five genes were identified in tissue-soluble amoeba isolates in samples of children with diarrhea, where 61 out of 186 samples were positive for this gene using the polymerase chain reaction (PCR) technique. This is in agreement with another study conducted for the detection of *E.histolytica* and *E.dispar* using the polymerase chain reaction (PCR) technique, in which Cysteine proteinase five was diagnosed in *E.histolytica* but not in *E.dispar*, and it was noted that the polymerase chain reaction (PCR) method showed high specificity and sensitivity (13). In another study, the gene encoding Phospholipase was diagnosed in children infected with the *E. histolytica* parasite Amoeba. The diagnosis was made using the polymerase reaction technique in all samples examined microscopically, and the presence of the parasite was confirmed in this sample. Other studies showed the importance of using the polymerase reaction technique in detecting parasites. In a study, the polymerase reaction technique was used to diagnose the parasite Giardia in vegetables, where a direct wet swab with iodine dye was used in microscopic examination. The prevalence rate was 1.7%, an average of 4 positive samples out of 230. In addition, the polymerase chain reaction (PCR) was used to detect the 16SrRNA gene of the Giardia parasite, as it was detected in an average of 5.9% of the samples. Another study also used the polymerase reaction technique to detect the Cryptosporidium parasite by detecting the HSP-70 gene, as it was found in 10 out of 102 microscopically (14).

The methods of detection of *E. histolytica* appeared differences in positive results. Moreover, the infection in males more than in females in the age less than one year more infected than in other ages. The isolate in the current study was 90% similar to the globally recorded isolate. The present results are essential in the preparation of disease control and prevention programs.

Authors' Contribution

Study concept and design: I. G. A.
Acquisition of data: S. S. A.
Analysis and interpretation of data: H. S. A.
Drafting of the manuscript: I. G. A.
Critical revision of the manuscript for important intellectual content: I. G. A.
Statistical analysis: S. S. A.
Administrative, technical, and material support: S. S. A.

Ethics

The human study was approved by the ethics committee of the University of Tikrit, Tikrit, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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