Confirmation between Microscopy and Pcr Technique for Differentiation between *Entamoeba Histolytica* and *Entamoeba Dispar* among Children in Duhok City, Iraq

Original Article

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ABSTRACT

Aims: The current study aimed to confirm between microscopy and PCR techniques to differentiate between *Entamoeba histolytica* and *Entamoeba dispar* and to determine the prevalence of amoebiasis among children in Duhok City, Iraq.

Materials and Methods: We conducted this study from June to August 2021. A total of 300 fresh stool samples were collected from children with a history of diarrhea/dysentery from both sexes.

Results: Depending on the microscopic examination of stool samples, the prevalence rate of amoebiasis among children in the Duhok Province was high (34.0%). A higher prevalence rate was recorded according to gender in girls than in boys (18.0% & 16.0%) respectively, and among the age groups reported a high prevalence rate in the age groups between (4-6 years & 7-9 years) (15.67% & 12.33%), respectively. The lowest infection rate was recorded in the age groups (1-3 years and 10-12 years) (4.0% and 2.0%). This variation was statistically not significant at $p < 0.05$. Finally, the PCR results show that similar results were recorded positive for *Entamoeba histolytica* (92.0%). By microscopy, just a few samples were positive with *Entamoeba histolytica*, and by PCR, they were negative for *Entamoeba histolytica* and positive for *Entamoeba dispar* (8.0%), and one sample was found positive with *Entamoeba histolytica and Entamoeba dispar*.

Conclusion: To lower the prevalence rate of infection among the community by supplying clean water, a suitable sanitation system, and supporting efforts to educate people about health.

Key Words: Amoebiasis, *entamoeba histolytica*, *entamoeba dispar*, microscopy, PCR, prevalence.

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INTRODUCTION

Entamoeba histolytica is an amoebic protozoan parasite that inhibits the large intestine of humans[1]. *Entamoeba histolytica*(E. histolytica) is the causative agent of amoebiasis and is considered the main protozoan parasite that causes high morbidity and mortality globally, especially in developing nations. Globally, about 50 million cases of intestinal amoebiasis and around 100,000 cases of death occur per year[2]. Several species of Amoebae infect the human intestine, including *E. histolytica, E. coli, E. dispar, E. hartmanni, E. moshkovski, and E. polecki*. All species mentioned above are non-pathogenic for humans, except *E. histolytica* a pathogenic species that colonizes the large intestine of humans and causes a disease named amoebiasis[3, 4].

Amoebiasis is transmitted to humans during ingestion of the infected stage (mature cyst) of *E. histolytica*. The disease may be asymptomatic in the light infection or symptomatic and characterized by abdominal pain, vomiting, and diarrhea, and in severe cases, accompanied by dysentery. The disease is more common in children than adults[5] *E. histolytica* also causes amoebic colitis, and in severe cases, if not treated, trophozoites of *E. histolytica* may invade the intestinal wall and reach the many organs like the liver, lung, brain, uterus, etc., causing extra-intestinal amoebiasis[3, 5].

The diagnosis of amoebiasis is by microscopic examination of stool samples for searching of both stages: trophozoite and cyst, and this method of examination cannot be helpful in the differentiation between the infections with *E. histolytica* and *E. dispar* if the examiner had no experience. However, there are some available methods, such as ELISA and PCR, that are more sensitive than microscopy for differentiation between species of Entamoeba[6- 8]

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AIM OF THE WORK

This aimed to differentiate between E. histolytica and E. dispar in Duhok City, Iraq.

MATERIAL AND METHODS

Study area and sampling:

A cross-sectional study was carried out from June to August of 2021. A total of 300 stool samples were collected from children with various age groups, ranging between 1 and 12 years of both sexes. That study was conducted on children who attended the Hevi Pediatric Hospital in Duhok City with a history of diarrhea or dysentery.

1. Microscopic examination: a fresh stool sample was collected from each child and examined directly by the direct stool method for searching for *Entamoeba* trophozoites and by the concentrated flotation method for searching for *Entamoeba* cysts.[9, 10] The positive stool samples were stored at -80 °C for PCR study.
2. PCR Technique: whole genomics DNA was extracted from 50 positive stool samples by microscopy (25 diarrheal samples and 25 dysenteric samples for confirmation) using the Stool Genomic DNA Extraction (Beijing Solarbio Life Science, China). Three primers were used in the present study: the first primer and the second primer were specific primers for the detection of *E. histolytica*,[11] while the third primer was used for the detection of *E. dispar*,[12] as mentioned in (Table 1). All molecular kits, including the extraction kit, primers, DNA leader, and agarose gel from Beijing Solarbio Life Science, China.

The PCR amplification and PCR reactions were carried out according to the protocol[13] as mentioned in (Table 2). PCR conditions consist of conditions are: cycle 1 for (3 minutes) at (94°C), 30 cycles for (30) seconds at (94°C), (30) seconds at (55°C), and (30) seconds at (72°C), and the final stage includes 1 cycle of (7) minutes at (72°C). Finally, the PCR products were run on gel electrophoresis (agarose gel 2.0%). The formed bands were identified depending on specific size: a band with 439 bp for E. histolytica and a band with 174 bp for *E. dispar*, and a DNA ladder with a size of 100 bp was used.

Table 1: Primers were used in this study.

Specificity	Locus	Sequence (5' –3')	Amplicon Size (bp)	References
E.histolytica	16S rRNA gene	5'AAGCATTGTTTCTAGATCTGAG 3'	439 bp	(11)
		5'AAGAGGTCTAACCGAAATTAG 3		
E.histolytica	16S rRNA gene	5'ATGCACGAGAGCGAAAGCAT-3′	439 bp	(12)
		5'GATCTAGAAACAATGCTT CTCT-3′		
E.dispar	16S rRNA gene	5'GAAACCAAGAGTTTCACAAC 3'	174 bp	(11)
		5'CAATATAAGGCTTGGATGAT 3'		

Table 2: All components were used in the PRC reaction as follows.

No.	Components	Volume
2	Forward Primer	1.0 µl
3	Reverse Primer	1.0 µl
4	Template DNA	2.0 µl of DNA sample
5	Taq DNA polymerase	0.3 µl
6	dNTPs	1.0 µl
7	Taq Buffer	2.2 µl
8	MgCl2	2.5 µl
9	Then complete the volume to 25µl with water (nuclease-free)	15.2 µl
Total		25.0 µl

SpecifStatistical Analysis:

The statistical analysis was performed to analyze the risk factors, including genders and age groups for amoebiasis, using SPSS (25.0), and the chi-square test was used to analyze the variables used in the current study at $P < 0.05$ considered statistically significant[14].

RESULTS

1.Morphological Results:

It was clear from (Table 3) that one hundred-two stool samples out of 300 were found positive for amoebiasis, and the prevalence of infection among children was 34% depending on the direct stool and flotation stool methods.

Table 3: Number of stool samples were collected from children who attended Hevi Pediatric Hospital.

Variable	Categories	Positive No.	Prevalence%
Sex	Female	170	56.67
	Male	130	43,33
Intestinal Amoebiasis	Positive	102	34.0
	Negative	198	66.0
	Total	300	100.0

Statistically non-significant at $p < 0.05$

It was obvious from (Table 4), that the infection rate among genders was higher in girls than the boys (18.0% & 16.0%) receptively. This present difference is statistically not significant.

Table 4: The infection rate of amoebiasis microscopically according to gender.

Gender	Total Stool No.	Positive Cases by Microscopy	Rate of amoebiasis %
Girl	170	54	18.0
Boy	130	48	16.0
Total	300	102	34.0

Statistically not significant at $p < 0.05$

(Table 5) shows the prevalence rate of infection among different age groups. There was a high prevalence rate of infection in the age groups between 4-6 years and 7-9 years (15.67% and 12.33%), respectively. The lowest infection rate was recorded in the age groups between (1-3 years and 10-12 years) (4.0% and 2.0%). This variation was statistically not significant at $p < 0.05$.

Table 5: The infection rate of amoebiasis according to the age group.

Age	Total Stool No.	Positive Cases	Rate of amoebiasis %
1-3 years	50	12	4.0
4-6 years	130	47	15.67
7-9 years	90	37	12.33
10-12 years	30	6	2.0
Total	300	102	34.0

Statistically not significant at $p < 0.05$

2. Molecular Results:

It is clear from (Table 6): we selected fifteen positive samples by microscopy to confirm them by PCR technique, from which 46 samples were identified by PCR technique as *E. histiolytica* and only 4 samples were identified as *E. dispar* (92.0% and 8.0%), respectively.

Table 6: Differentiation between E. histolytica and E. dispar by PCR technique.

Total samples were positive microscopically	Species	No. of positive cases by PCR	%
50	E. histolytiica	46	92.0
	E. dispar	4	8.0
	Total	50	100.0

During the current study, two types of primers were used for the identification of *E. histolytica*, and both primers were given multiple bands in the same sample among different children as mentioned in (Figure 2). That means that the child can be infected with several strains of *E. histolyitca* at the same time of infection.

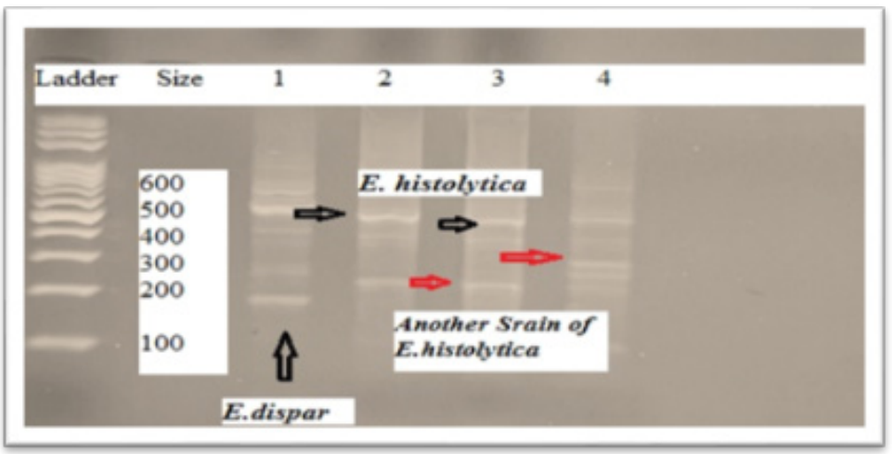

Fig. 1: The amplified PCR products for *E.histolytica* with used loading DNA marker (100 bp) & lanes 2, 3 & 4 samples with size (439 bp) on agarose (1.5%), *E.dispar* on lane 1 with size (174 bp) and lanes (2, 3), and 4 another strains of *E.histolytica* with the size (200 bp).

DISCUSSION

In the current study, the amoebiasis was diagnosed by direct stool and concentration flotation stool methods. Then the positive cases for amoebiasis were confirmed by PCR technique for differentiation between *E. histolytica* and E. dispar. This study was carried out according to our knowledge; this study is the first to identify *E. histolytica/E. dispar* by PCR technique in Duhok City. A high prevalence of amoebiasis among children was recorded in the current study (34.0%); this finding is similar to the results of the studies done by[9, 15]; they also recorded a higher prevalence rate of infection in children.

Depending on the direct stool and concentration flotation stool methods, 102 stool samples were identified for cysts of E. histolytica, and the prevalence of amoebiasis among children was 34.0%). The concentration stool method was used because it has some advantages, such as increasing the number of parasites and decreasing the number of debris from the stool sample. Therefore, concentration stool methods (flotation and sedimentation) are regarded as the ideal standard method for the searching of all stages of protozoa in the stool sample and have one disadvantage: they destroy the trophozoite stage of protozoa.

The current study reported a higher prevalence rate of infection among girls than boys (18.0% & 16.0%), respectively. This change among genders statistically is not significant, and this may be due to several girls who participated in this study being higher than the number of boys. The same data recorded by[16] was recorded in Erbil City, Iraq. On the other hand, this data disagreed with the data of ***Hasan et al.***[17], who published that the prevalence rate of infection was higher in boys (67.43%) than girls (32.56%) and the data of ***Nyenke et al.***[18]. This variation of infection through gender may be related to the behavior status of boys; boys are spending more time outside the home than the girls, and they are more susceptible to infection; they eat and drink from the street, all of which increase the susceptibility to infection in boys[19, 20].

This study also recorded the highest infection rates of amoebiasis found among children in age groups (4-6 years and 7-9 years) (15.67% and 12.33%), respectively; the lowest infection rate was found in the age groups between (1-3 years and 10-12 years) (2.0% and 4.0%), respectively. The present study findings agreed with the results of a study done by ***Al Saqur et al.***[21] This high prevalence rate in these age groups is related to several factors, such as the low level of education among these age groups. These age groups are highly active and waste most of their time playing outside the home.

The PCR results show that similar results were recorded positive for E. histolytica (90.0%). By microscopy, just a few samples were positive with *E. histolytica*, and by PCR, they were negative for *E. histolytica* and positive for E. dispar (8.0%), and one sample was found positive with mixed species *E. histolytica/E. dispar*. This obtained finding agrees with the results of Hamzah[22] and this result is strongly near the microscopic examination; this depends on the experience of the examination; did he/she use both direct and concentration stool methods; and did he/she prepare more than stool smears for examination? The present findings are in agreement with the findings of the study done by ***Santos et al.***[23] A study done by ***Ekou et al.***[24], who strongly reported that the use of microscopic examination and PCR technique in the identification of Entamoeba species has the same results. The current study highlighted in the future that working on sequencing methods for searching for new strains of *E. histolytica*.

The presence of multiple bands for *E. histolytica* may be due to the presence of changes on allelic chromosomes between polymorphism and homologous loci, the presence of the repeat loci at various regions in the genome of *E. histolytica*, or the presence of different strains of E. histolytica in the area, and a child could be infected with multiple strains of E. histolytica at the same time, and this led to the appearance of the several bands[25,26].

CONCLUSION

It is concluded, depending on the microscopic examination of stool samples, that the prevalence rate of amoebiasis among children in the Duhok Province was higher, especially among girls than in boys (18.0% & 18.0%), respectively. According to the age groups, a high prevalence rate was reported in the age groups (4-6 years & 7-9 years) (15.67% & 12.33%), respectively. The lowest infection rate was recorded in the age groups (1-3 years and 10-12 years) (4.0% and 2.0%). This variation was statistically not significant at $p < 0.05$. Finally, the PCR results show that similar results were recorded positive for E. histolytica (92.0%). by microscopy Just a few samples were positive with *E. histolytica* microscopically and by PCR were negative for *E. histolytica* and positive for *E. dispar* (8.0%), and one sample was found positive with mixed species *E. histolytica/E. dispar*.

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ETHICS

The study proposal was approved by the ethics committee of the College of Health Sciences/ University of Duhok, Duhok, Iraq. Reference No.30102024-9-25

CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

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التأكيد بين تقنية المجهر وتقنية PCR لتمييز بين الانتاميبا الهيستوليتكا والانتاميبا الديسبار بين الأطفال في مدينة دهوك، العراق

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الأهداف: تهدف الدراسة الحالية إلى تأكيد التقنيات الميكروسكوبية وPCR للتمييز بين انتاميبا هستوليتكا وانتاميبا ديبار، وتحديد انتشار الأميبا بين الأطفال في مدينة دهوك، العراق

المواد والأساليب: أجرينا هذه الدراسة من يونيو إلى أغسطس ٢٠٢١. تم جمع ما مجموعه ٣٠٠ عينة براز طازجة من أطفال لديهم تاريخ من الإسهال/الدسنتاريا من كلا الجنسين.

النتائج: اعتمادًا على الفحص المجهري لعينات البراز، كانت نسبة انتشار الأميبا بين الأطفال في محافظة دهوك مرتفعة (٣٤,٠٪). تم تسجيل معدل انتشار أعلى حسب الجنس للفتيات مقارنة بالأولاد (١٨,٠٪ و١٦,٠٪ على التوالي)، وبين الفئات العمرية، تم الإبلاغ عن معدل انتشار مرتفع في الفئات العمرية بين (٤-٦ سنوات و٧-٩ سنوات) (١٥,٦٧٪ و١٢,٣٣٪ على التوالي). تم تسجيل أدنى معدل عدوى في الفئات العمرية (١-٣ سنوات و١٠-١٢ سنة) (٤,٠٪ و٢,٠٪). لم يكن هذا الاختلاف ذو دلالة إحصائية عن $p < ٠,٠٥$. أخيرًا، تظهر نتائج الـ PCR أن نتائج مماثلة تم تسجيلها إيجابية لانتاميبا هيستوليتكا (٩٢,٠٪). من خلال المجهر، كانت هناك بعض العينات الموجبة لانتاميبا هيستوليتكا، ومن خلال الـ PCR، كانت سلبية لانتاميبا هيستوليتكا وإيجابية لانتاميبا ديسبار (٨,٠٪)، ووجدت عينة واحدة إيجابية مع انتاميبا هيستوليتكا وانتاميبا ديسبار

استنتاج: لتقليل معدل انتشار العدوى بين المجتمع من خلال توفير مياه نظيفة ونظام صرف صحي مناسب ودعم الجهود لتعليم الناس حول الصحة.