DETECTION OF*ENTAMOEBAHISTOLYTICA* IN STOOL SPECIMENS AMONG DIARRHEAL CHILDREN

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ABSTRACT

This study was aimed to detect the spreading of *E.histolytica* among children with diarrhea, to achieve that,221 stool specimens were collected from diarrheal children of all ages and both gender, in Baghdad city at a period extended from early September 2019 to the end of February 2020. The collected specimens were examined directly by the light microscope for detecting the presence of *E.histolytica*. DNA was extracted from positive microscopically stool specimens, then examined by PCR to confirm the diagnosis of *E. histolytica*, by targeting the 18S ribosomal RNA(18S rRNA) gene. The result showed that 78 (35.3%) of diarrheal cases were caused by *E. histolytica* according to the microscopically direct smear method, while 143 (64.7%) were other diarrheal causes. The infection with *E. histolytica* rate in males (59%) was higher than in females (41%), however, this variation was not significant (P = 0.973). *E. histolytica* was considerably more prevalent in the age group<1-3 years (62.9%)and lower rate in the age group 10-12 years (3.8%). PCR examination results confirmed the presence of *E. histolytica* in 70 (89.7%) of 78 samples that were positive by microscopic examination which were 44 (62.8%) and 26 (37.2%) males and females respectively, this variation was significantly. Patients in the age group less than 1-3 years had the most infection with *E. histolytica.*

Keywords : diarrhea, amoebiasis,PCR, DNA extraction, microscopic examination.

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التحري عن طفيلي *Entamoeba histolytica* في عينات الخروج بين الاطفال المصابين بالإسهال

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المستخلص

تهدف هذه الدراسة معرفة انتشار طفيلي *E. histolytica* عند الاطفال المصابين بالاسهال ، ولذلك تم جمع 221 عينة براز من الأطفال المصابين بالاسهال من جميع الأعمار وكلا الجنسين في الفترة من أوائل ايلول2019 حتى نهاية شباط 2020 من مدينة بغداد.تم فحص العينات التي جمعت مباشرة باستعمال المجهر الضوئي للكشف عن وجود الاميبا*E. histolytica*.تم استخلاص الحمض النووي (DNA) من عينات البراز التي أظهرت نتيجة موجبة في الفحص المجهري (الحاوية على الطفيلي)، ثم اختبرت بواسطة تقنية تفاعل البلمرة المتسلسل (PCR)لتأكيد التشخيص المجهري للطفيلي *E. histolytica* ، عن طريق استهداف جين(18S rRNA).أظهرت النتائج أن 78 (35.3%) حالة من حالات الإسهال كان سببها الطفيلي*E. histolytica* (حسب طريقة اللطخةالمباشرة)بيمنا تعود 143 (64.7%) حالة لمسببات إسهال أخرى.وكانت نسبة الإصابة عند الذكور(59٪)أعلى منها عند الإناث (41%)،لكن هذا الاختلاف غير معنوي (P = 0.973).كما كان هذا الطفيلي أكثر انتشارًا في الفئة العمرية أقل من 3–1سنوات (62.9%) ومعدل أقل في الفئة العمرية 10– 12(3.8%).أكدت نتائج فحص تقنية تفاعل البلمرة المتسلسل (PCR)وجود طفيلي *E. histolytica* في 70(89.7٪) عينة من 78 عينة كانت إيجابية في الفحص المجهري والتي كانت 44 (62.8٪) و 26 (37.2٪) من الذكور والإناث على التوالي ، وكان هذا الاختلاف معنوياً. المرضى في الفئة العمرية أقل من 1–3 سنوات هم الأكثر إصابة*E. histolytica* .

الكلمات المفتاحية:الإسهال،الزحار الأميبي،تقنية التضخيم PCR،استخلاص DNA،الفحص المجهري

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INTRODUCTION

Diarrhea is one of the most common problems in health and is defined as the passage of loose or watery stools with an increased number of times of defecation, three or more are passed in 24 hours (30). Although diarrhea is prevalent in all ages, they are often the most severe and deadly among children(38), caused by the consumption of contaminated food or drink by various pathogens, including bacteria, fungi, viruses, protozoa, or helminths(25). Parasites are one of diarrhea causative agent, usually characterized by intermittent diarrhea and lasts more than one week(17). Children are more susceptible to protozoa infections, which have a detrimental effect on their cognitive capacity and physical development due to fat and vitamin B12 malabsorption, deficiency of vitamin A and nutrition deficiencies too (21). *Entamoeba histolytica* is a unicellular, protozoon anaerobic parasite caused amoebiasis, and it is the most common protozoan intestinal parasite of humans(23).Amoebiasis can be asymptomatic or have obvious symptoms as infected individuals may show a wide range of clinical signs, such as bloody diarrhea, fever, and abdominal pains, owing to invasive infection(21).As well as extra-intestinal disease including liver disease(1).Amoebiasis is a potentially severe and life-threatening infection, and it is considered the third most common cause of death (after malaria and schistosomiasis) among parasitic diseases(18). Parasitic infections are an acute problem, despite the development in health care because no vaccines are available to prevent it(35). *E. histolytica is* diagnosed based on microscopic examination of stool samples. Although it a faster and easier method, but its sensitivity is limited and requires an experienced observer to accurately distinguish between the *Entamoeba* species. For this reason, considered to be insufficient(28). Molecular methods are necessary for differentiating these amoebae. Nested multiplex PCR assay is considered one of the most modern methods has been developed for the rapid detection and identification of *Entamoeba* species(12, 33).Previous studies have demonstrated the prevalence of *E.histolytica,* Hadi(15)clarified that infection with *E. histolytica* was 29.2% out of 720stool samples from diarrheal patients with different age groups rang from one year to more than 21 years. Kumarand his team(18)examined 656 stool specimens from patients with gastrointestinal symptoms independent of age, *E.histolytica* was identified in only12.2% of them. Another study(4) performed in Bangladesh examined 423fecal samples from diarrheal children, they found only 74 samples (17.5%) were positive for *E.histolytica*. Due to the prevalence of intestinal parasites that cause diarrhea, this study aimed to detect *E.histolytica* among children with diarrhea by microscopic examination and confirm the detection by nested polymerase chain reaction (PCR).

MATERIALS AND METHODS

Stool specimens collection

A total of 221 stool specimens were collected from diarrheal children from hospitals in Baghdad city, Central Teaching Hospital of Peadiatric, Al-kadhimiya Hospital for Children and Al-alwya Hospital for Children, from early September 2019 to the end of February 2020.Children's ages are ranged between 4 days to 12 years from both genders, male and female. fresh fecal samples were collected by sterile containers and examined microscopically then stored at -20 °C for molecular analysis(8).

Microscopic examination

The fresh feces were examined microscopically to detect the trophozoites and cyst of *E.histolytica* by direct wet mount method**.** Small drop of physiological saline (0.9%) was putted on slide and mixed well with a small portion of feces by wooden stick, and covered with a cover slide, then examined under power enlarge 40X and 100X(5, 24).

Fecal DNA extraction

Genomic DNA was extracted from stool samples(180 mg)using QIAamp® Fast DNA Stool Mini Extraction Kit Qiagen / Germany according to the company protocol, then stored it at -20ºC until used for the molecular analysis(3).

Nested multiplex PCR assay

This assay was performed to detect the *Entamoeba* genus in the first round using specific primers, designed by (39):*Entamoeba*-1 as the forward primer, and *Entamoeba*-2 as a reverse primer (Table 1). The specific primers

were amplified the region900 bp of 18S small subunit ribosomal RNA gene rRNA gene(18S rRNA)(12). The primary PCR was performed in a 20 µl reaction volume: 10 µl of the master mix, 1µl of each primer(forward and reverse), 8µl of DNA template, and no addition of nuclease-free water. While the second round of PCR for *E. histolytica* detection based on amplifying the region 439 bp of 18S rRNA gene, by specific primer that mentioned in (Table1), designed by (39):(*E. histolytica*-1as forward primer and *E. histolytica*-2as reverse)and performed in 20µl reaction volume:10 µl of the master mix, 1µl of each primer(forward and reverse),3µl PCR product and 5 µl nuclease-free water(39). Amplification was carried out by *E.histolytica*-1(F), *E. histolytica*-2 (R)primers in both runs. The negative control in both runs prepared using the same ingredients without a DNA template. Program of PCR used for both rounds was illustrated in(Table2).

Table 1. Primers used in this study

Primer	Sequence (5'-3')	Product Size (bp)
***Entamoeba*-1**	**5`-TAA GATGCA GAG CGA AA-3`**	**900bp**
***Entamoeba*-2**	**5`-GTA CAA AGG GCA GGG ACG TA3`**	
***E.histolytica*1(F)**	**5`-AAG CATTGT TTC TAG ATC TGA G-3`**	**439 bp**
***E. histolytica*2(R)**	**5`-AAGAGG TCT AAC CGA AAT TAG-3`**	

Table 2. PCR program for first and second rounds

Gene	PCR steps	No. Cycle	Temperature	Time(M:S)
18S rRNA (First round)	**Initial Denaturation**	**1 Cycle**	**95 ºC**	**05:00**
	Denaturation	**30 Cycle**	**95 ºC**	**00:30**
	Annealing		**56 ºC**	**00:30**
	Extension		**72 ºC**	**00:30**
	Final extension	**1 Cycle**	**72 ºC**	**07:00**
	Hold		**10 ºC**	**10:00**
18S rRNA (Second round)	**Initial Denaturation**	**1 Cycle**	**95 ºC**	**05:00**
	Denaturation	**30 Cycle**	**95 ºC**	**00:30**
	Annealing		**48ºC**	**00:30**
	Extension		**72 ºC**	**00:30**
	Final extension	**1 Cycle**	**72 ºC**	**07:00**
	Hold		**10 ºC**	**10:00**

The PCR products were analyzed by agarose gel electrophoresis, 2% Agarose gel was prepared by add 2 gm agarose to the 1X TAE buffer in Erlenmeyer flask. The agarose solution was microwaved until all the gel particles were dissolved (15 minutes),then allowed to cool down at 50-60°C.1µl of ethidium bromide (10mg/ml) was added, later the agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes, then the comb was carefully removed. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.Finally,10µl of PCR products were loaded to the well. Electrical power was turned-on at 100v/mAmp for 75min.PCR products were visualized by UV Transilluminator(12, 27).

Statistical analysis: Statistical analysis was performed with Excel application (version 2013) and Statistical Package for the Social Sciences (SPSS) (version 25). Chi-square test used for independent and goodness of fit, P value $\leq$ 0.05 was considered statistically significant and P-value $\leq$ 0.01 considered High statistically significant (36).

RESULTS AND DISCUSSION

Demographic characteristics of diarrhea infected children=In this study, 221patient children enrolled, involved 91 (41.2 %) females and 130 (58.8%) males. The distribution of ages was between 4 days to12 years, patient ages were grouped into four groups; <1-3 y, 4-6 y, 7-9y and 10-12y, which constituted 174 (78.73%), 27 (12.23%), 10 (4.52%) and 10 (4.52%) respectively (Table3).

Table 3. Demographic Characteristic to Distribution of diarrhea infected children.

Group	Patients (n=221) N (%)
Age Range	4 day to 12 year
Gender: Male	130 (58.8%)
Female	91 (41.2%)
Age Groups: <1-3 y	174 (78.73%)
4-6 y	27(12.23%)
7-9y	10 (4.52%)
10-12y	10(4.52%)
Sample Size	141*

*** sample size (141) or more measurements/surveys are needed to have a confidence level of 95% that the real value is within ±5% of the measured/surveyed value.**

Detection of *Entamoeba histolytica* by microscopic examination: The result showed that 78 (35.3%) of diarrheal cases were caused by *E. histolytica* according to the microscopically direct smear method, while 143 (64.7%) were other diarrheal causes. This negative result was high significantly (P = <0.0001)as illustrated in(Table 4).This result agreed with Ped and others(11) results which found that 14 (35%) of diarrheal children were infected with *E. histolytica*, after examined 40 stool samples using light microscope. Also, a study performed in Erbil(24) which examined 200stool specimens from children aged<1- 12 years and reported that 60 (34.69%) cases were positive for *Entamoeba histolytica*. While, this study approach approximated with previous studies conducted by Jawad (16)that demonstrated the prevalence of *E. histolytica in* 23.37% of 492 patients with in all ages. Oliewi and Al-Hamairy(27)showed that of 987 stool samples of patient suffering from diarrhea, which were examined by a direct wet mount method, 261 (26.4%) samples were positive for *E. histolytica.* In another study conducted in Baghdad(31) they found that among 240 diarrheal children younger than5 years, 99 (41.25%) cases have *E.histolytica.* Kavili(17)clarified the prevalence of *E. histolytica* infections in 42.1% amongst the pupils ranged from 6 to 12 years old. In other hand, the results of the present study disagree with several previous studies. Shlash(34)in Najaf Governorate found that 75% (90) of diarrheal children (120) were infected with *E. histolytica* of 120 diarrheal children. A study conducted on780 diarrheal patients with age ranged from 4 months to 70 years using light microscope for detecting intestinal parasites, found that only 12.17% of diarrheal cases were caused by *E. histolytica*(7,28). Jasim(19) found that of 2177diarrheal children, only 377(17.3%) cases were positive for *E. histolytica* infection. These differences in *E.histolytica* prevalence are presumed due to the differences in geographic locations, although may indirectly indicate local sanitation and living conditions(15).Other reasons may be affected such as the methodology used in these studies, such as limited sample size ,sensitivity precision of the laboratory examination, or disease stage(7, 31).It must be taken in to consideration that the period was between September to the end of February, and the previous studies indicated that the rate of infection with *E.histolytica* is higher in the summer season, because the high temperature during the summer months activated the cyst of this parasite(7, 31), (37), (2).

Table 4. Detection of *Entamoeba histolytica a*mong diarrheal children by microscopic examination and distribution according to gender

	Patients(n=221)				p-value
	Positive		Negative		
Microscopic	Count	%	Count	%	>0.001
	78	35.3%	143**	64.7%	
Value of Chi-Square= 241.0					
Male	46	59%	84	58.7%	
Gender					0.973^{NS}
Female	32	41%	59	41.3%	
Value of Chi-Square= 0.001					

**** The correlation is significant at the P < 0.01 level (Highly Significant)(microscopic detection)**
Data presented as Chi-square independence test. NS=Non-significant(distribution according to gender.)

Distribution of the patients according to gender: (Table 4)Showed that the infection of diarrheal cases related to *E. histolytic* in males were46(59%) and in females were 32 (41%). However, the percentage of infected males were higher than females, but this variation was not significant(P = 0.973).These results were agree with Kumar and others(18) which found that more males infected with *E. histolytic* as compared with females but it was not statistically significant (p>0.05). Jawad(16)showed that no significant differences were found between infected males and females patient show ever, the rate of infection in males was more than females, While disagree with Ntulumeand others(26) showed that more females had infections with *E. histolytica* as compared with the males. Another study(34) in Al-Najaf Governorate found that more females (87.8%) infected with amoebiasis than males (59.2%).The non-significant differences between the genders might be due to that both males and females children play in the garden and may come in contact with contaminated soil, water and food, so the possibility of getting infected with *E. histolytica* by contamination food and water is the same.

Distribution of the patients according to the age groups: The study showed that the *E. histolytica* was considerably more prevalent in the age group <1-3 years (62.9%) and lower in the age group 10-12 years(3.8%), this variation was high significant (P = <0.0001),as summaries in (Table 5). The results of the present study was agree with Ahmed's study 2016(4) which found higher rate of infection in age group (13-24) months and (25-36)months after examined 185 diarrheal children. Mathurin and his team(20)which reported that the age group 0-24 months and 5-36 months showed high rate of *E. histolytica* infection while, fewer infections were detected in the older children. Otherwise, the current study disagrees with Hamad and Ramzy(24) they found that the children less than 1 year old had a lower rate of *E. histolytica* infection. The high prevalent in the age group <1-3 may be related to formula-fed, pacifier, poor health hygiene of mothers and low education level(9).Children in the age group 10-12 years although went school and socialize with children, more involved in both outdoor activities and feeding but they become more conscious especially, with parental guidance

Table 5. Distribution of *Entamoeba histolytica* infected patients according to the age groups based on microscopic examination

Patient samples		Microscopic				*p* value
		Positive		Negative		
		Count	%	Count	%	
Age Groups (Year)	<1-3	49	62.9	125	87.4	<0.0001**
	4-6	21	26.9	6	4.2	
	7-9	5	6.4	5	3.5	
	10-12	3	3.8	7	4.9	
Value of Chi-Square= 26.2 df=3						

Data presented as Chi-square independence test. ** The correlation is significant at the P < 0.01 level (Highly Significant).

Detection of *Entamoeba histolytica* by PCR technique: In first round used *Entamoeba*-1, *Entamoeba*-2primers (Table1) and *E. histolytica*-1, *E. histolytica*-2 in the second round but the result showed another bands in second round (Figure1A), so use *E. histolytica*-1as , *E. histolytica*-2 primers in both rounds(39).The bands detected in70 (89.7%) samples out of 78 (positive by microscope)while , 8 (10.3%) samples are negative by PCR(Figure1B) and(Figure 2).The negative result by PCR examination of some microscopy positive samples may due to the high accuracy of the PCR compared with the microscopic examination(28) or may due to that light microscope not differentiate all the species of *Entamoeba* because Some *Entamoeba* spp. are morphologically identical

in both their cyst and trophozoite stages ,for instance *E. histolytica*, *E. dispar*, and *E. moshkovskii*(21, 30).This result agree with Rodulfo and othersfound that only89.7% of *E. histolytica* microscopically posative cases were by posative by PCR analysis. Alkhuzaey and Al-aboody(6)found59 (61.5%) positive and 37 (38.5%) negative *E. histolytica* samples, after reexamined 96 microscopically positive stool samples, by PCR technique. A study in Jordan analyzed 70 positive (examined by microscope) stool specimens using PCR. They found that all specimens were negative for *E.histolytica* detection(11) Roshdy and others(29) elucidated that out of 37 samples characterized as positive by microscope, only 20samples were positive when examined by PCR. While a study of Ngosso and others(25) demonstrated that among 144 samples positive for *E. histolytica* by microscope, only 48(33.3 %) samples were positive for *E. histolytica* by PCR technique.

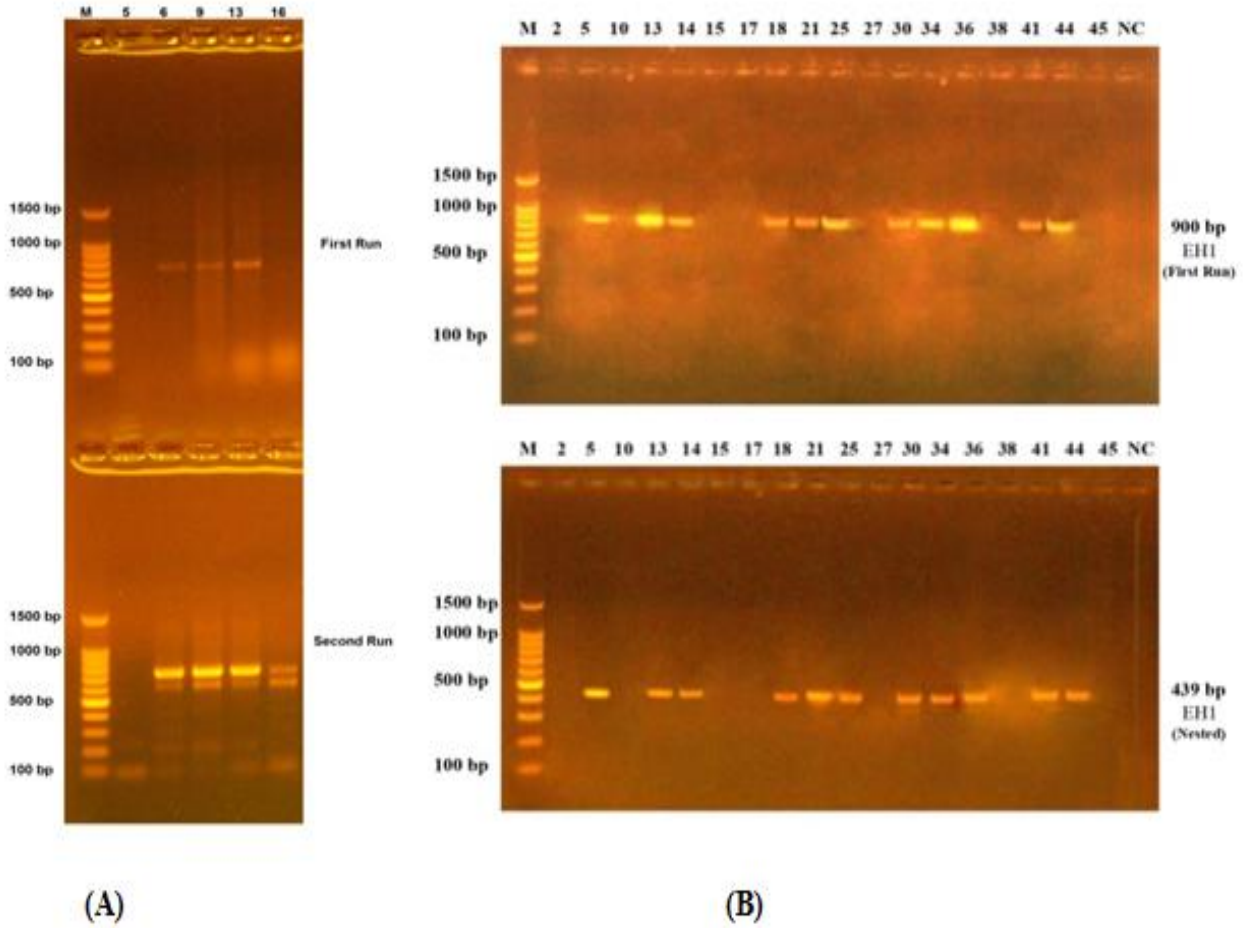

Figure1.AAgarose gel electrophoresis image that show PCR product analysis18S rRNA gene900 bp) in *Entamoeba* genus in first round, while second round of PCR not the region of 18S rRNA gene(439 bp)for *E. histolytica.*

(B)Agarose gel electrophoresis image that show PCR product analysis for 18S rRNA gene(900 bp) in *Entamoeba* genus in first round, while second round of PCR for *E. histolytica* detection based on amplify the region of 18S rRNA gene(439 bp). M (Marker 100bp. Lane (2-45) some positive for *E. histolytica* and some negative samples(2,10,15,17,27,38) no bands were appear. Lane NC: negative control (all PCR mixture with the substitution of water for DNA template)

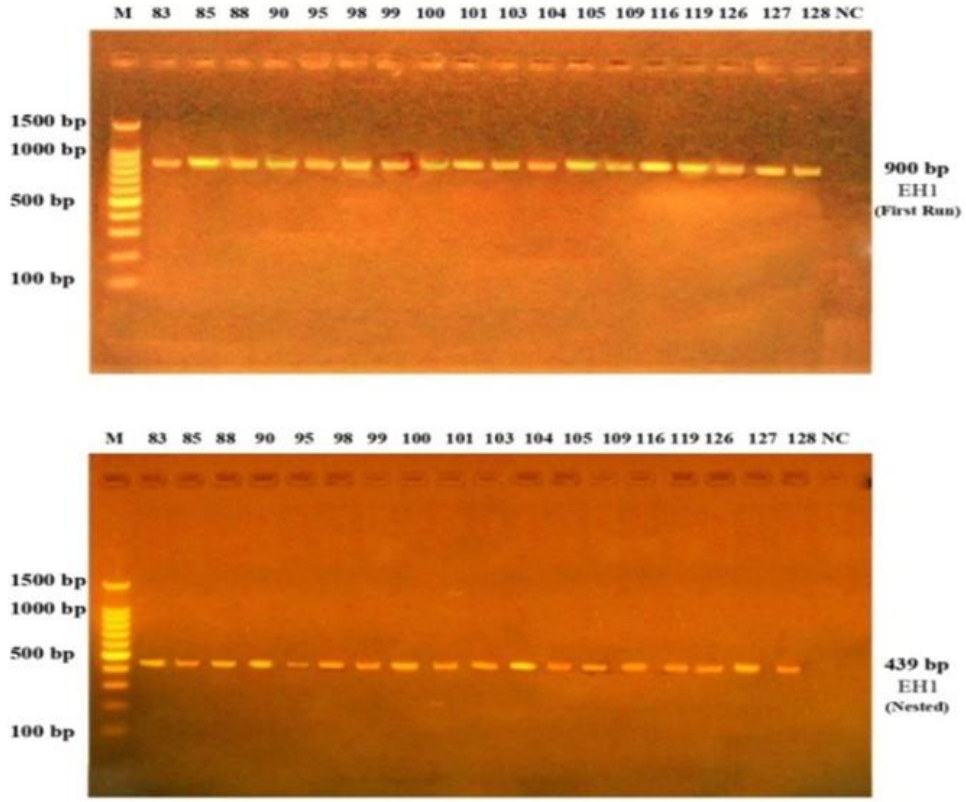

Figure 2.Agarose gel electrophoresis image that show PCR product analysis for 18S rRNA gene(900 bp) in *Entamoeba* genus in first round, while second round of PCR for *E. histolytica* detection based on amplify the region of 18S rRNA gene(439 bp). Lane M (Marker 100bp. Lane (83-128) some positive *E. histolytica* stool samples Lane NC: negative control (all PCR mixture with the substitution of water for DNA template).

-Distribution of the patients according to gender: (Table6) showed the gender distribution of *E.histolytica* infected patients based on PCR technique examination. The infection with *E. histolytic* was 44 (62.8%) in males and 26 (37.2%)in females. The rate of males cases was higher than females, and this variation was statistically significant (P = 0.039). These results agreed with Alkhuzaey and Al-aboody(6) which found that more males(52.5%) infected with *E. histolytic* as compared with females(47.5%) according to PCR analysis.

Distribution of patients according to the age groups: The results of the present study showed that the patients' samples in the age group<1-3 years had a higher rate of parasitic diarrhea (65.7%), while a lower rate of parasitic diarrhea (4.3%) was found in the age group 10-12year. The variation between these two groups was not significant (P = <0.0001),as showed in (Table 7). This result disagrees with Alhamiary's (5) study which reported that low rate of *E. histolytica* infection in less than one year, while higher infections were detected in the older children.

Table 6.Distribution of *Entamoeba histolytica* infected patients according to the gender based on PCR examination

Patient samples		PCR		*p* value
		Positive	Negative	
Gender	Male	44 (62.8)	2 (25.0)	0.039
	Female	26 (37.2)	6(75.0)	
Value of Chi-Square= 4.253df=1				

Data presented as Chi-square independence test. NS=Non-significant P < 0.05 level (Significant),

Table 7.Distribution of *Entamoeba histolytica* infected patients according to the age gropes based on PCR examination

Patient samples		PCR(N=78) Positive Count	%	Negative Count	%	*p* value
Age Groups (Year)	<1-3	46	65.7	3	37.5	0.317
	4-6	17	24.3	4	50.0	
	7-9	4	5.7	1	12.5	
	10-12	3	4.3	0	0	
Value of Chi-Square= 3.532df=3						

CONCLUSION

In conclusion, the study showed that some diarrheal cases were caused by *E. histolytica*, while most cases were other diarrheal causes. The highest infections with these parasitic diarrhea as in the age group (<1-3year), which was more than other age groups. In addition to that the nested multiplex polymerase chain reaction technique was effective for accurate diagnosis of intestinal amoebiasis and for knowing the true prevalence of pathogenic *E. histolytica* in the community, by distinguish the *E histolytica* from other non-pathogenic species of *Entamoeba* to avoid unnecessary treatment for other non-pathogenic species, because of the high sensitivity and specificity of the modified PCR assay.

REFERENCES

1. A. Castellanos-Gonzalez, A. White, P. Melby and B.Travi. 2018. Molecular diagnosis of protozoan parasites by recombinase polymerase amplification. ActaTropica182(8):4–11. https://doi.org/10.1016/j.actatropica.2018.02.002

2. A.Alanazi. 2017. The prevalence of intestinal parasitic protozoan among patients in Ad-Dawadimi general hospital, Saudi Arabia. Tropical Biomedicine 34(2): 453–460

3. A.Evangelopoulos, G. Spanakos, E. Patsoula, N.Vakalis and N. Legakis.2000. A nested multiplex PCR aassay for the simultaneous detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in faeces. Annals of Tropical Medicine & Parasitology 94(3):233–240. https://www.tandfonline.com/doi/full/10.1080/00034983.2000.11813534

4. Ahmed, H. Khanum, S. Uddin, P. Barua, T. Arju, M. Kabir and R. Haque. 2016. *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* spp. infection in children in an Urban Slum area of Bangladesh.Bioresearchcommun.2(1): 5–10

5. Ahmed.Alhamiary.2016. Epidemiological and diagnostic study for diarrheic parasites (*Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium* spp.) Pharmaceutical, Biological and Chemical Sciences7(1): 438–447

6. Ali. Alkhuzaey and B. Al-aboody.2019. Co-infection of Intestinal Parasites and *Helicobacter Pylori* in Patients with Chronic Diarrhea in some Districts of Thi-Qar Province

7. Ayoub.Bazzaz , O.Shakir and R. Alabbasy. 2017. Prevalence of two gastrointestinal parasites *Entamoeba histolytica* and *Giardia lamblia* within Samarra City, Iraq. Advances in Bioscience and Biotechnology 8(11): 399–410

8. Ayse. Caner, O. Zorbozan, V. Tunalı, M.Kantar, S. Aydoğdu, S. Aksoylar, Y.Gürüzl, and T. Nevin. 2019. Intestinal protozoan parasitic infections in immunocompromised child patients with diarrhea.Japanese Journal of Infectious Diseases. 73(3):187-192

9. Charlie. Cruz, S. Abdurhaman, A. Bakr, C. Alcantara and A. Sannat. 2015. Prevalence of intestinal parasites in Afif, Saudi Arabia: A 5-year restrospective study. Int.J.Curr.Microbiol.App.Sci .4(5) :746–751

10. David. Kavili. 2014. Prevalence of *Entamoeba histolytica* infections among children attending primary schools in Kyuso Zone, Kyuso District, Kitui County, Kenya : 1–64

11. Elaf. Al-Dalabeeh, F. Irshaid, S. Roy, I. Ali and A. Al-Shudifat. 2020. Identification of *Entamoeba histolytica* in patients with suspected amebiasis in Jordan using PCR-based assays. Pakistan Journal of Biological Sciences 23(2): 166–72

12. Fares. Bahrami, A. Haghighi, G. Zamini and M. Khademerfan. 2019. Differential detection of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* in faecal samples using nested multiplex PCR in west of Iran. Epidemiology and Infection 147(96): 1–7.

13. Galgamuwa. Lahiru, D. Iddawela and S. Dharmaratne. 2016. Intestinal protozoa infections, associated risk factors and clinical features among children in a low-income tea plantation community in Sri Lanka. International Journal of Community Medicine and Public Health 3(9): 2452–2458

14. Gehan. Oreby, El. Abeer, H. Sayed, Q. Sahar and M. Lubna.2019. Coproantigen versus microscopic examination for diagnosis of *Giardia lamblia* and *Entamoeba histolytica* infection in children in Banha Teaching Hospital Al-Azhar Journal of Ped. 22(45):388–402

15. Hadi. Sadoon. 2011. A study of prevalence of intestinal parasitic infection in Shatrah District / Thi-Qar Governorate. Journal of College of Education for Pure Science 1(5): 28–39

16. Jawad. 2015. Prevalence of *Entamoeba histolytica* and *Giardia lamblia* parasites among patients attending Al-Emam Ali Hospital in Al-Mashrooh / Babylon Province. Kufa Journal for Veterinary Medical Sciences 6(1): 30–34

17. Khariri, M. Agtini, E. Ariyanti, R. Ekowati, N. Puspandari, M.Maha2020. Parasitic infestation in the incidence of diarrhea among toddlers in Jakarta, Bogor, Banjarmasin and Makassar 22(3):658–662

18. Kumar. Surinder and A. Varsha. 2016. Prevalence of *Entamoeba histolytica* and *Giardia lamblia* infection in a rural area of Haryana, India. International Journal of Current Microbiology and Applied Sciences 5(6): 204–209

19. Mahmoud. Safi, M. Tavalla, M .Mardani, and R. Afrisham. 2016. Prevalence of intestinal parasitic infections among applicants for health cards attending ahvaz east health center during 2012-2013.Asian Pacific Journal of Tropical Disease 6(2): 151–154. http://dx.doi.org/10.1016/S2222-1808(15)61002-7

20. Mathurin. Koffi, N. Martial, K. Thomas, and D. Yao. 2014. Molecular characterization of intestinal protozoan parasites from children facing diarrheal disease and associated risk factors in Yamoussoukro, CtedIvoire. African Journal of Environmental Science and Technology 8(3): 178–184

21. Miichi. Fumika, H. Yoshida, and S. Hamano. 2016. *Entamoeba* encystation: new targets to prevent the transmission of amebiasis. PLoS Pathogens 12(10): 1–13

22. Moshira. Helmy, L. Rashed and H. Abdel-Fattah. 2007. Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR. Journal of the Egyptian Society of Parasitology 37(1): 257–274

23. Nadia. El-Dib. 2017. *Entamoeba histolytica*: An overview. Current Tropical Medicine Reports 4(1): 11–20

24. Narmin. Hamad and I. Ramzy. 2012. Epidemiology of *Entamoeba histolytica* among children in Erbil province, Kurdistan Region-Iraq. Journal of Research in Biology 2(1): 57–62

25. Ngosso, G. Kwengulila, and A. Namkinga. 2015. Identification of pathogenic intestinal parasitic protozoa associated with diarrhea among under-fives children in Dar Es Salaam, Tanzania. International Invention Journal of Medicine and Medical Sciences 2(4): 2408–7246.
http://internationalinventjournals.org/journals/IIJMMS

26. Ntulume, J. Tibyangye, A. Aliero, and B. Banson. 2017. Prevalence of intestinal protozoan infections and the associated risk factors among children in Bushenyi District, Western Uganda. International Journal of Tropical Disease& Health 23(2): 1–9

27. Oliewi. Khadhim, and K. Ahmed. 2016. Epidemiological and diagnostic study for diarrheic parasites (*Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium* ssp.) among diarrheic infected patients by using multiplex polymerase chain reaction in the Babylon Province, Iraq. Research Journal of Pharmaceutical, Biological and Chemical Sciences 7(1): 438–447

28. Rodulfo. Hectorina, B. Ahmar, L. Mora, M. Rodríguez1, and M. Donato. 2012. Nested PCR reveals elevated over-diagnosis of *E.*

histolytica in Barcelona, Venezuela. Invest Clin 53(534): 365–377

29. Roshdy and H. Mohamed. 2017. Molecular diagnosis of *Entamoeba* spp. versus microscopy in the great Cairo. Acta Parasitologica 62(1): 188–191

30. Series. Editor and Y. George. 2011.Diarrhea Diagnostic and Therapeutic Advances

31. Shakir. Mohammed and A. Areej. 2014. Frequency of intestinal parasitic infection among children under 5 years of age in Baghdad province. International Journal of Advanced Research Journal2(8): 332–337.www.journalijar.comInternational Journal of Advanced Research

32. Shirley. Debbie-ann, C. Hung and S. Moonah. 2015. *Entamoeba histolytica* (amebiasis) intestinal and genital infections. Elsevier Inc.6(2):699-706 https://doi.org/10.1016/B978-0-323-55512-8.00094-6

33. Shirley. Debbie-ann, L. Farroji, K. Watanabe, and S. Moonah. 2018. A review of the global burden, new diagnostics, and current therapeutics for amebiasis. Open Forum Infectious Diseases 5(7):1–9

34. Shaimaa. Shlash. 2016. Impact of *Entamoeba histolytica* infection on some haematological and immunological parameters among children in Najaf Governorate.Kufa Journal for Nursing Sciences 6(3): 102–110

35.Stacey. Burgess, C. Gilchrist, T. Lynn, and W. Petri. 2017.Parasitic protozoa and interactions with the host intestinal microbiota. Infection and Immunity 85(8): 1–12

36. Sun. Ji-Qian. 2017. Handbook of Medical Statistics

37. T. Jasim. 2019. The incidence of *Entamoeba histolytica*& *Giardia lamblia* associated with diarrhea among children in ibn Al-Balady Hospital in Baghdad. Iraqi J. Comm. Med21(5): 17–20

38. Waqar.Al-Kubaisy, H. Al-Talib, A. Al-Khateeband and M. Shanshal. 2014. Intestinal parasitic diarrhea among children in Baghdad – Iraq. Tropical Biomedicine 31(3): 499–506

39. Yee. Lau, C. Anthony S. Fakhrurrazi, J. Ibrahim, I. Ithoiand and R. Mahmud. 2013. Real-Time PCR assay in differentiating *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovski*i infections in Orang Asli Settlements in Malaysia. Parasites and Vectors6(1):1–8.