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Molecular Detection of Virulence Factors of some Protozoan Infection Causing Diarrhea in Children in Waist Province

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Abstract

Background: Intestinal parasite infections (IPIs) have significantly declined in recent decades, largely due to hygiene and health education improvements. *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* . This study aimed to achieve two key objectives: To diagnose these three parasites using nested PCR, and identify the most common virulence factors associated with each parasite (*E. histolytica*, *G. lamblia*, and *C. parvum*) in children using multiplex PCR. Material and methods: This cross-sectional study was conducted in Wasit province. A total number of 296 stool samples were randomly collected and examined, 96 samples of them from children (<16 years) affected by diarrhea. Results: Nested PCR results revealed the following positivity rates for the three parasites: 82/96 (85.42%) positive for *E. histolytica*, 17/96 (17.71%) positive for *G. lamblia*, and 34/96 (35.42%) positive for *C. parvum*. Multiplex PCR analysis of virulence factors showed that, out of the 96 patients, 77 were positive for the cysteine proteinase gene, and 72 were positive for the amoeba pore c gene in *E. histolytica*. For *G. lamblia*, 17 out of 96 patients were positive for the cysteine proteinase gene and 17 were positive for the variant-specific surface protein gene. Finally, for *C. parvum*, 34 out of 96 patients were positive for the cysteine proteinase gene, and 34were positive for the 60 kDa glycoprotein gene. Conclusion: These findings suggest that virulence factors play a significant role in parasitic pathogenesis and are frequently involved in the initial pathogen-host interactions.

Keywords: Intestinal parasites, diarrhea in children, n PCR, virulence factors of some parasite ,cysteine proteinase , *18S rRNA*

الكشف الجزيئي عن عوامل الضراوة لبعض عدوى الطفيليات الاولية للاطفال المصابون بالإسهال في محافظة واسط

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الخلاصة:

انخفضت بشكل كبير عدوى الطفيليات المعوية ،بشكل كبير خلال العقود الأخيرة ويرجع ذلك في الغالب الى زيادة الحالة الصحية التعليم الصحي والصرف الصحي الجيد. ، *E. histolytica, G. lamblia,* *C. parvum* and المسببات للعدوى المعوية على التوالي، *G. lamblia,* and *C .parvum* هما اكثر

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الاوليات انتشارا المرتبطة بالإسهال في كل الدول المتقدمة والنامية ،سعت هذه الدراسة الى تحقيق اهداف رئيسية: تشخيص وتحديد هذه الطفيليات الثلاثة باستخدام تفاعل البلمرة المتعدد وعوامل الضراوة الاكثر شيوعا لكل طفيلي عن طريق تفاعل البلمرة المتسلسل المتعدد. تم جمع 296 عينة براز وفحص 96 طفل مصاب بالإسهال في عمر اقل من 16عاما، واشارت النتائج الى انه تم الكشف عن طريق تفاعل البلمرة المتعدد PCR المتداخل ايجابي للطفيليات الثلاثة 82 (85.42%), 17 (17.71%) and 34(35.42%) على التوالي، وكشف عوامل الضراوة لهذه الطفيليات عن طريق تفاعل PCR المتعدد ، بالنسبة *E. histolytica,* كان هناك 72 من اصل 96 مريض لديهم لديهم ameobapore يليه 77 من اصل 96 وجد لديهم cysteine proteinase ،بينما *G. lamblia* كان لدى 17 cystine proteinase ايجابي و 17 variant-specific surface protein gene من اصل 96 مريضا اما *C .parvum* فكان لدى 34 cysteine proteinase ايجابي وكذلك 34 60 kDa glycoprotein ايجابي من اصل 96. تشير الدراسة الحالية الى ان عوامل الضراوة تلعب دور رئيسي في تسبب الطفيليات للإمراض غالبا ما تشارك في التفاعلات الاولية بين مسببات الامراض والمضيف.

1. Introduction

Intestinal parasite infections (IPIs) are among the most neglected tropical diseases, affecting over 3.5 billion individuals and causing significant morbidity in approximately 450 million people [1]. School-age children are particularly vulnerable to IPIs due to their less-developed immune systems, poor personal hygiene practices, and the tendency to play in contaminated environments [2]. Protozoan parasites, such as *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum*, are major contributors to intestinal morbidity in children, especially in underdeveloped regions such as the Middle East [3]. *E. histolytica* , the causative agent of amoebiasis, completes its life cycle within a single human host, transforming into trophozoites that can invade and damage various tissues, including the intestines, liver (the most common site), lungs, skin, and even the brain [4]. A protein called amoebapore, identified in *E. histolytica*, forms pores in target cell membranes, resulting in depolarization and subsequent cell death. [5]. Cysteine proteases (CPs) are recognized as critical virulence factors for *E. histolytica*, significantly contributing to tissue damage [6]. The flagellated protozoan parasite G. lamblia, found in the small intestines of humans, causes diarrhea and abdominal pain [7]. G. lamblia utilizes cysteine proteases to penetrate the mucus layer, microbiota, and epithelium. This allows trophozoites to attach to the small intestinal epithelium through the ventral disk and its cytoskeletal components, preventing their removal from the intestine. Antigenic variation and variable surface proteins also contribute to parasite survival and attachment on to IECs [8]. Cryptosporidium parvum is a primary cause of diarrheal cases in infants and children aged two years or younger, with transmission occurring through contaminated water, food, or insects [9]. Sequence analysis of the 60-kDa glycoprotein (gp60) gene reveals distinct families that are common in children and HIV-positive persons in low- and middle-income countries [10]. This study aimed to identify the most common virulence factors of each of these three parasites (*E. histolytica*, *G. lamblia*, and *C. parvum*) in children by using nested PCR (n PCR).

2. Materials and Methods

The present study involved a cross-sectional study conducted in Wasit province, Iraq. A total of 296 stool samples were randomly collected, and 96 of these samples were from diarrheic children aged less than 16 years. These children were patients at several hospitals, namely Al-Zahra Teaching Hospital, Al-Karama Teaching Hospital, and Al-Kut Maternity and Children Hospital, as well as public health centers in Al-Kut city. Data and samples were collected between December 1, 2023, and May 1, 2024. Fecal samples were collected using sterile containers and immediately transported to the laboratories of the respective hospitals for analysis. Aliquots of 200 mg of each fecal sample were stored directly at -20°C for subsequent molecular analysis by PCR [11]. DNA was extracted from the collected stool samples using the Presto TM Stool DNA Extraction Kit (Geneaid, Korea) [12]. Following the manufacturer's instructions. After extraction, DNA samples were analysed using a Nano Drop spectrophotometer (Thermo-scientific, UK) to assess their concentrations and purities. To target the *18S rRNA gene*, three sets of primers were designed according to published sequences and provided by Bioneer Company (Korea) to detect *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* [13]. The primer sequences used for n PCR targeting the *ssr RNA* gene using NCBI-Genbank (MK332025.1, DQ157272.1, and AF308600.1) and primer 3 plus design were shown in Table 1, 2, 3, and 4:

Table 1: Nested PCR primers for detection *E. histolytica*, *G. lamblia*, and *C. parvum* with their sequences and product size

Primers	Sequence 5'-3'		Product size (bp)
nPCR-ssrRNA gene *Entamoeba histolytica*	F	CGCGGTAATTCCAGCTCCAA	426
	R	ACGACGGTATCTGATCGTCT	
nPCR-ssrRNA gene *Giardia lamblia*	F	TTGAAGGCATTGACGGAGGG	265
	R	ATCACAGACCTGCTATCGCC	
nPCR-ssrRNA gene *Cryptosporidium parvum*	F	TCAATTGGAGGGCAAGTCTG	510
	R	AGGTGCTGAAGGAGTAAGGA	

Table 2: The PCR primers for virulence factor gene in *Entamoeba histolytica* (AY956434.2 and M94163.1) with their sequences and product size

Primers	Sequence 5'-3'		Product size (bp)
Amoeba pore C gene *Entamoeba histolytica*	F	ATTTGTTCTCCTTTGTGTTTTTGTT	301
	R	TGTTTAACATGCATGAATCAACCC	
cysteine proteinase gene PCR- *Giardia lamblia*	F	TCACTTGCAGCTCTTGAAGGA	552
	R	TGGATCTGTAGCAACACCACA	

Table 3: The PCR primers for virulence factor gene in *Giardia lamblia* (U83277.1 and M80480.1) with their sequences and product size

Primers	Sequence 5'-3'		Product size (bp)
cysteine protease gene *Giardia lamblia*	F	AGCCTCCTGATGCCTGTTTC	611
	R	CCGTAGCCCACCATATCGAC	
VSP gene variant-specific surface protein *Giardia lamblia*	F	AAGACGGCTTCTTCCCCAAG	549
	R	CCCCGTACCTGAGTGCTTTT	

Table 4: The PCR primers for virulence factor gene in *Cryptosporidium parvum* (CP141120.1 and MH715474.1) with their sequences and product size

Primers	Sequence 5'-3'		Product size (bp)
cysteine protease gene *Cryptosporidium parvum*	F	CCAAATGTCGATTGCTCACGG	598
	R	CCCCCAATAAATCCTTGTCGG	
60 kDa glycoprotein gene *Cryptosporidium parvum*	F	ACTAGTGCTGCTTCCCAACC	351
	R	TCTTGATGAAGCCTGACCCG	

The amplified DNA samples were then visualized using agarose gel electrophoresis under UV light [15].

Ethical approval

This research received ethical approval from the Council of the College of Medicine, University of Wasit, in July 2023, and from the Wasit Health Directorate (reference number 1201) on November 1, 2023. Prior to the commencement of sample collection, all participants involved in the study were thoroughly informed about its objectives. Informed consent was obtained from each participant through a verbal agreement. This study was conducted in strict adherence to the ethical principles outlined in the Declaration of Helsinki.

Inclusion criteria

The inclusion criteria for this study were as follows: Children aged 16 years or younger, diarrheic children, both inpatients and outpatients, and children residing in both rural and urban areas of Wasit city.

Statistical analysis

Data were entered and analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 26. Descriptive statistics, including frequencies and percentages, were used to summarize the data. The association between categorical variables was assessed using the Chi-square test or Fisher's exact test, as appropriate. Agreement between categorical variables was assessed using Cohen's Kappa test, and disagreement was assessed using McNemar's test. A *p*-value of less than 0.05 was considered statistically significant [16].

3. Results

3.1. Nested PCR

Nested PCR was performed for 96 (32.4%) examined stool samples, the gender ratio was 36 (37.5%) female and 60 (62.5%) males to detect *E. histolytica*, *G. lamblia*, and *C. parvum*. The results indicated positivity rates of 82 (85.42%) for *E. histolytica* as show in Figure (1), 17 (17.71%) for *G. lamblia*, as shown in Figure 2 ,and 34 (35.42%) for *C. parvum,* as shown in Figure 3 . PCR analysis for virulence factors of *E. histolytica* revealed that, among the 96 patients, 72(75%) were positive for the amoebapore c gene, as shown in Figure 4 and Table 1, positive cysteine proteinase gene 77(93.90%) , as shown in Figure 5 and Table 2. For *G. lamblia*, 17 out of the 96 patients were positive for the cysteine proteinase gene, as shown in Figure 6. and 17 were positive for the variant-specific surface protein gene, as show in Figure 7. Finally, for *C. parvum*, 34 of the 96 patients were positive for the cysteine proteinase gene, shown in Figure 8 and Table 3. 33 were positive for the 60 kDa glycoprotein gene, as show in Figure 9.

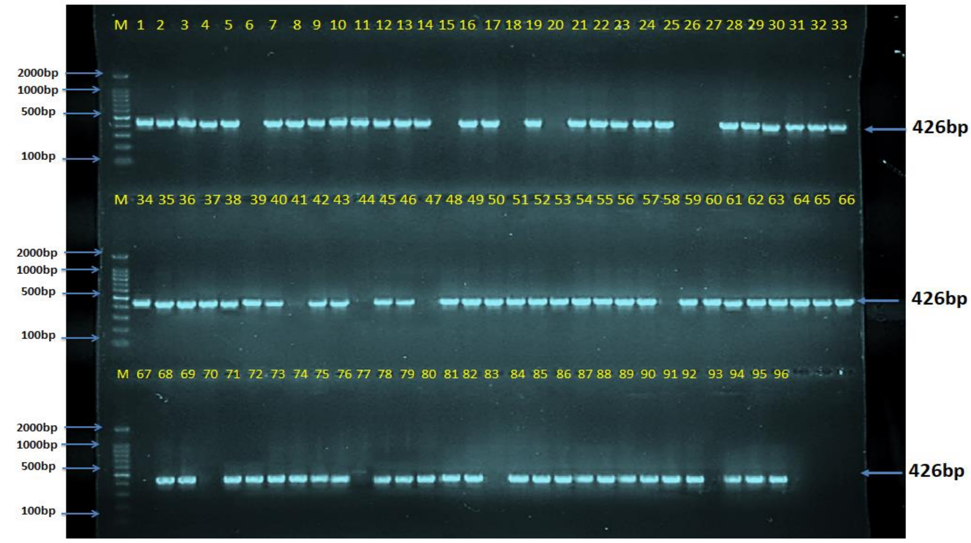

Figure 1: Agarose gel electrophoresis image demonstrating the nested PCR amplification products of the small subunit ribosomal RNA gene for the detection of *Entamoeba histolytica* in human stool samples positive sample (82) and negative sample (14) in (6,15,18,20,26,27,41,44,47,57.70,77,83,93). Lane M: DNA marker ladder (2000-100 bp). Lanes 1-96: Representative samples showing *ribosomal RNA* gene amplification for *E. histolytica*, with a PCR product size of 426 bp , 2% agarose gel was prepared in using 1X TPE.

Figure 2: Agarose gel electrophoresis image demonstrating the nested PCR amplification products of the small subunit ribosomal RNA gene for the detection of *Giardia lamblia* in human stool samples positive this gene (17)(3,9,10,18,20,24,44,49,59,61,62,65,70,73,77, 84,94) and negative (65). Lane M: DNA marker ladder (2000-100 bp). Lanes 1-96: Representative samples showing positive amplification for *G. lamblia*, with a PCR product size of 265 bp , 2% agarose gel was prepared in using 1X TPE.

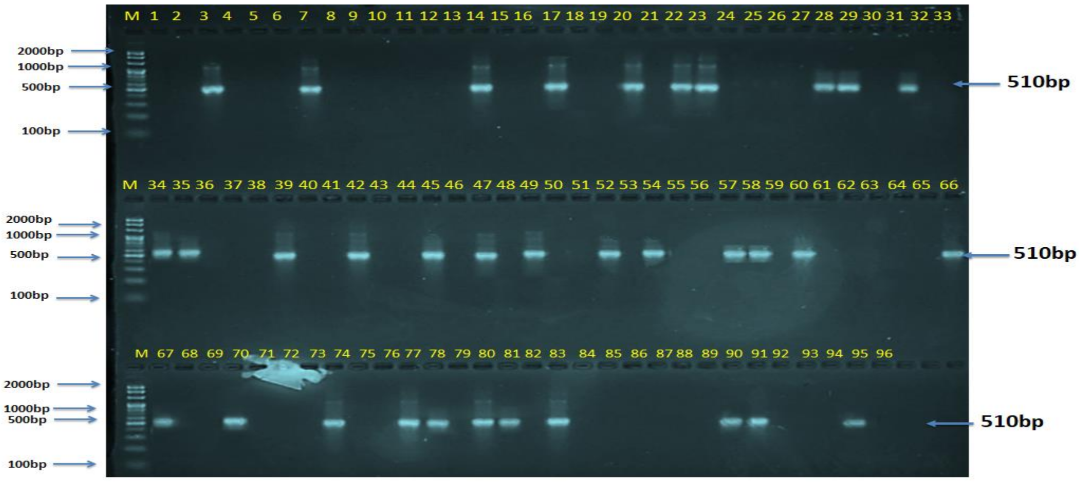

Figure 3: Agarose gel electrophoresis image demonstrating the nested PCR amplification products of the small subunit ribosomal RNA gene for the detection of *Cryptosporidium parvum* in human stool samples in (3,7,14,17,20,22,23,28,29,31,34,35, 39,42,45,47,49,52, 54, 57,58,60,67,70,74,77,78,80,81,83,89,90,91,95).and negative (62), Lane M: DNA marker ladder (2000-100 bp). Lanes 1-96: Representative samples showing positive amplification for *C. parvum*, with a PCR product size of 510 bp , 2% agarose gel was prepared in using 1X TPE.

Table 1: *E. histolytica* virulence factors: amoebapore c gene and cysteine proteinase gene.

Virulence factor		Amoebapore c gene		Total	P-value
		Positive	**Negative**		
Cysteine proteinase gene	**Positive**	61(63.5%)	13(13%)	74(77%)	<0.001
	Negative	7(7.2%)	15(15.6)	22(22%)	
Total		68(70.8)	28(29.1)	96(100%)	

Figure 4: Agarose gel electrophoresis image demonstrating the nested PCR amplification products of the small subunit ribosomal RNA gene for the detection of *Giardia lamblia* in human stool samples positive this gene (17) (3,9,10 ,18,20,24,44,49,59 ,61,62,65, 70,73,77, 84,94) and negative (65). Lane M: DNA marker (2000-100 bp). Lanes 1-96: Representative samples showing amplification for *G. lamblia*, with a PCR product size of 265 bp, 2% agarose gel was prepared using 1X TPE.

Table 2: *G. lamblia* virulence factors: cysteine protease gene and variable surface protein (VSP).

Virulence factor		Cysteine protease gene		Total	P-value
		Positive	Negative		
VSP gene variant-specific surface protein gene	Positive	17(17.7%)	0(0%)	17(17.7%)	<0.001
	Negative	0(0%)	79(82.2%)	79(82.2%)	
Total		17(17.7%)	79(8.2%)	96(100%)	

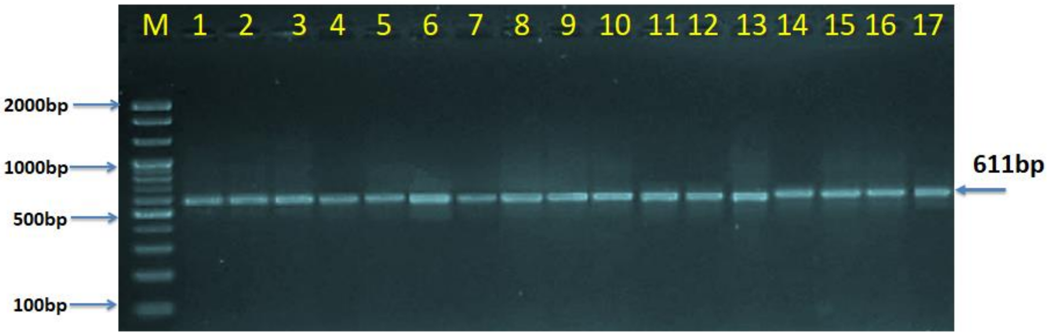

Figure 5: Agarose gel electrophoresis image demonstrating the PCR amplification products of the *Giardia lamblia* cysteine proteinase virulence factor gene in human stool samples positive for this gene(17) and negative gene(65). Lane M: DNA marker (2000-100 bp). Lanes 1-17: Representative samples showing amplification for the cysteine proteinase gene, with a PCR product size of 611 bp, 2% agarose gel was prepared using 1X TPE.

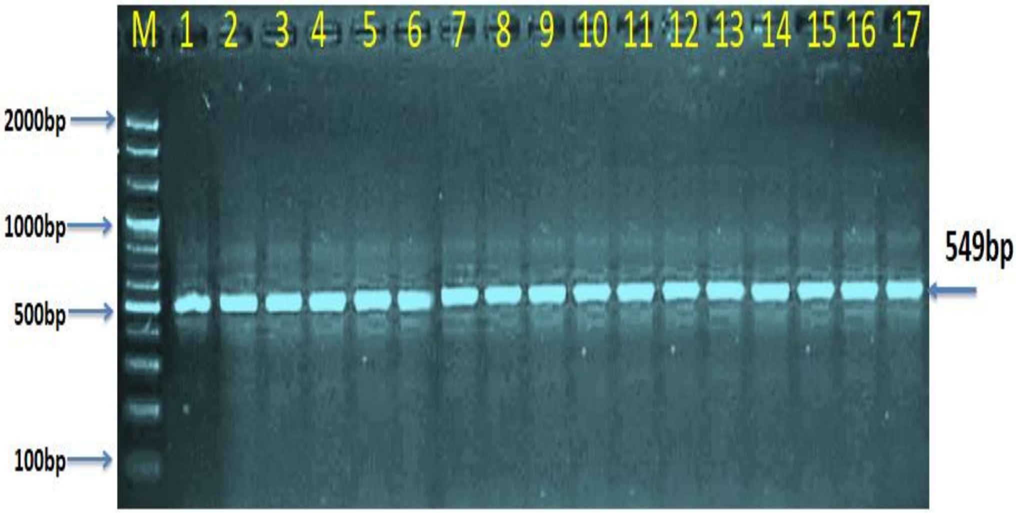

Figure 6: Agarose gel electrophoresis image demonstrating the PCR amplification products of the *Giardia lamblia* variable surface protein (VSP) virulence factor gene in human stool samples positive for this gene (17) and negative (65). Lane M: DNA marker (2000-100 bp). Lanes 1-17: Representative samples showing amplification for the VSP gene, with a PCR product size of 549 bp, 2% agarose gel was prepared using 1X TPE.

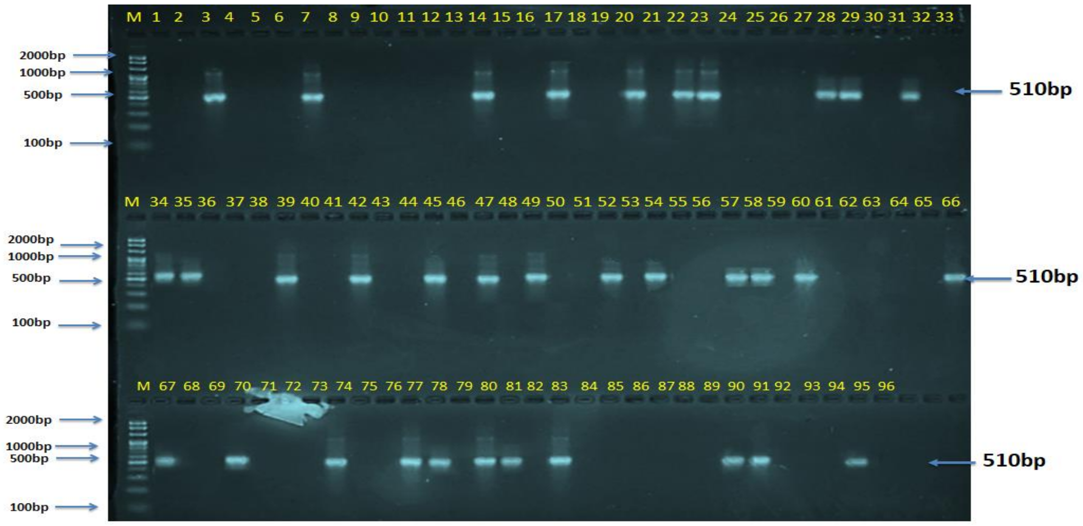

Figure 7: Agarose gel electrophoresis image demonstrating the nested PCR amplification products of the small subunit ribosomal RNA gene for the detection of *Cryptosporidium parvum* in human stool samples in (3,7,14,17 ,20,22,23,28,29,31,34 ,35,39,42,45,47,49,52,54, 57,58,60,67,70,74,77,78,80,81,83,89,90,91,95), and negative (62), Lane M: DNA marker (2000-100 bp). Lanes 1-96: Representative samples showing amplification for *C. parvum*, with a PCR product size of 510 bp, 2% agarose gel was prepared using 1X TPE.

Table 3: *Cryptosporidium* virulence factors: cysteine protease gene and 60 kDa glycoprotein gene.

Virulence factor		Cysteine protease gene		Total	P-value
		Positive	Negative		
60kDaglycoproteine gene	Positive	34(35.4%)	0(0%)	34(35.4%)	<0.001
	Negative	0(0%)	62(64.6%)	62(64.6%)	
Total		34(35.4%)	62(64.6%)	96(100%)	

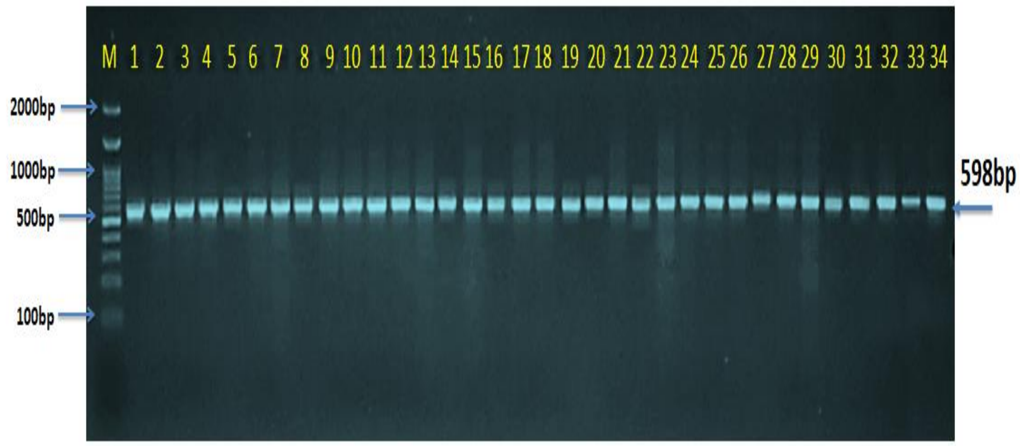

Figure 8: Agarose gel electrophoresis image demonstrating the PCR amplification products of the *Cryptosporidium parvum* cysteine proteinase virulence factor gene in human stool samples positive for this gene (34) and negative (62). Lane M: DNA marker (2000-100 bp). Lanes 1-34: Representative samples showing amplification for the cysteine proteinase gene, with a PCR product size of 598 bp, 2% agarose gel was prepared using 1X TPE.

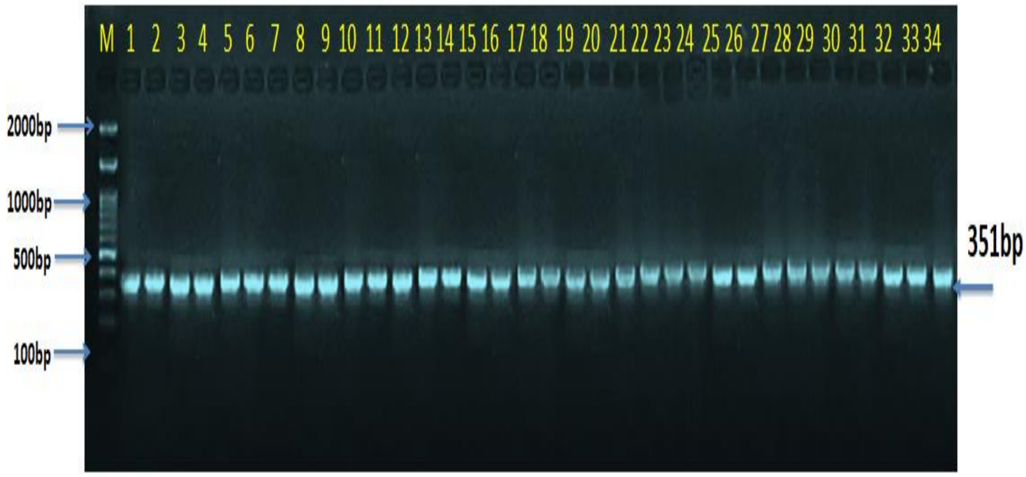

Figure 9: Agarose gel electrophoresis image demonstrating the PCR amplification products of the *Cryptosporidium parvum* 60 kDa glycoprotein virulence factor gene in human stool samples positive for this gene (34) and negative (62). Lane M: DNA marker (2000-100 bp). Lanes 1-34: Representative samples showing amplification for the 60 kDa glycoprotein gene, with a PCR product size of 351 bp, 2% agarose gel was prepared using 1X TPE.

Discussion

Intestinal parasitic diseases are commonly transmitted through unsanitary practices, including including ingesting ova or cysts from unwashed hands and fingernails, consuming contaminated food and water, and skin penetration by larval stages in unsanitary environments [3]. This study's findings are consistent with several previous studies, for example, a study in Nasiriyah city, Iraq, analysing 2,639 stool samples from children under seven years old, reported a 74.44% positivity rate for *E. histolytica* infection using PCR analysis [17]. This is comparable to our finding but should not be taken as a direct comparison. Similarly, a study in northern India, which collected 301 stool samples, found a 6.6% prevalence of *Entamoeba* histolytica, in intestinal cases of infection [18]. The sensitivity of nested PCR, employed in our study, is attributed to the fact that the product of the first PCR is sufficient to provide adequate templates for the synthesis of the second PCR product, which is then detected by ethidium bromide staining [19]. One common inference drawn by all these studies, is the fact that differences in prevalence are attributable to associated risk factors, which include, poverty, poor sanitation, cultural and religious issues, individual personal hygiene practices, unhygienic methods of waste disposal, poor clean or pipe borne water supply, immune status and level of enlightenment about the infection in the community [20].

In contrast, a study in Kirkuk, Iraq, analysing 600 samples, reported a 68.42% positivity rate for *Giardia lamblia* using nested PCR, and a 31.579% negativity rate [21]. This is in contrast to a study by Mostafa in Tikrit, Iraq, which collected 132 samples from children with diarrhea and found that *Giardia* parasite 16SrRNA was detected in an average of only 5.9% of samples using PCR [22]. Another study in Thi-Qar, Iraq, examining 96 stool samples from diarrheic patients using PCR, found that *Cryptosporidium parvum* had the lowest infection rate at 11.9%, whereas *G. lamblia* had the highest infection rate (65.5%) and *E. histolytica* at 22.6% [23]. This suggests variations in the distribution and prevalence of these parasites across regions.

Our findings are also in agreement with a study in Babylon, Iraq, that found a total infection rate of for *E. histolytica*, *G. lamblia*, and *Cryptosporidium parvum* was (31.3%, 28.1%, and 2.1%, respectively) [24]. A study in Turkey using PCR reported 1.3% and 0.7% positive results for *Giardia lamblia.* and *Cryptosporidium* parvum., respectively, and no detection of *E. histolytica* [25]. A study in Iran by Najafi [26] found that 3 out of 170 stool samples were positive for *Cryptosporidium* spp. using PCR. These studies highlight the need for structured molecular studies and effective control measures to prevent the transmission of these parasites, especially to vulnerable children [27].

Our multiplex PCR analysis detected the cysteine proteinase gene in 74 out of 96 patients, and the amoeba pore c gene in 68 out of 96 patients with E. histolytica, which aligns with other studies. A study in Kirkuk, Iraq, found the E. histolytica amoebapore C gene virulence factor in 52% of the examined samples [28]. Similarly, a study in Diyala Governorate, Iraq, detected E. histolytica in 40.86% of samples, with the amoebapore C gene virulence factor present in 84.21% of these samples and the cysteine proteinase in 65.79% (using results from a total of 450 stool samples) [29]. The high prevalence of the amoebapore C gene in studies including ours highlights its significant role in amoebic colitis. In Ethiopia, a study analysing 4804 stool samples from selected elementary schools, found G. lamblia infection in 27.1% of the children [30]. Our findings relating to G. lamblia cysteine proteinase gene and variant-specific surface protein gene were not comparable with existing studies. Regarding Cryptosporidium, a study in Al-Diwaniya, Iraq, analysing 100 stool samples from children, found that all samples contained the *900 glycoprotein gene* [31], whilst other studies using different primers did not give comparable results. Also, another study in Al-Diwaniya found that 100% of samples contained the *Cryptosporidium* Oocyst Wall Protein, Surface Protein of sporozoite COWP factor and the molecular factor of the CP15 (230bp) [32]. In Babylon, Iraq, a study using nested PCR to target the gp60 genes found that 14% (9/50) were positive for *C. parvum* [33]. The rural infections are higher than those in urban areas due to contact with infected domestic animals and the drinking water purification problem [34]. These diverse findings across various geographical regions highlight the complex nature of intestinal parasitic infections and the need for continuous research and targeted interventions. This study aimed to identify the most common virulence factors of each of these three parasites (*E. histolytica*, *G. lamblia*, and *C. parvum*) in children by using nested PCR (n PCR).

Conclusion

The nested PCR strategy offers enhanced sensitivity and specificity for detecting and differentiating parasitic infections in pediatric patients, achieved through the direct extraction of DNA from stool samples. This method can be widely used as a diagnostic method, and we recommend using it frequently. In this study, two virulence factors of each parasite that contribute to the pathogenesis of these parasites were investigated.

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Nil.

Conflict of interests

The authors declare no conflicts of interest among themselves.

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