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Identification and Molecular Characterization of *Giardia lamblia* Among Children with Acute Diarrhea

Enas J. Alhadad, Sara Abdulkareem Raheem[1], Niran Kadhim F. AL-Rubaey[2]

[1]Department of Medical Laboratory Techniques, Institute of Medical Technology AL-Mansour, Middle Technical University, 10066, Baghdad, Iraq.

[2]Department of Microbiology, Hammurabi College of Medicine, University of Babylon, 51002, Babylon, Iraq

enas_jaafer1@mtu.edu.iq; https://orcid.org/0009-0000-8693-1364

Sara-abdulkareem@mtu.edu.iq ; https://orcid.org/0009-0004-1852-888X

dr.nirranfarhood@yahoo.com ; https://orcid.org/0000-0002-9582-9908

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Abstract

Still among the most common intestinal protozoan parasites in children five years or less in resource-poor countries, *Giardia lamblia* can have long-lasting consequences with potential stunted growth and delayed neurodevelopment. **Methods:** This study was conducted to identify *Giardia lamblia* as the causative agent in children less than 6 years suffering from acute diarrhea by traditional microscopical examination, immunochromatography, and multiplex PCR assays, and also to assess the frequency of infection with age, gender, place of residence, and month distributions

Stool samples were collected in Babylon Governorate, Iraq, from November 2023 to April 2024, with a complete sample size of 210 young children, irrespective of gender. G. lamblia isolates were identified by examination of the samples under the microscope and confirmed by the immunochromatography test, and a multiplex PCR assay was done to establish the presence of GLCP6-S1 gene in the isoline. Among the 210 stool samples, 118 (56.2%) is/are positive and 92 (43.8%) is/are negative when viewed under a microscope. All 118 suspected samples were tested for the diagnosis of *G. lamblia* antigen using an immunochromatography test, which revealed that 80 (67.8%) were positive. The PCR assay confirmed that 83 (70.3%) were positive for the *GLCP6-S1* gene in *G. lamblia* isolates. The multiplex PCR assay is the most effective method for detecting *G. lamblia* in stool samples. Also, to prevent the transmission of infection, particularly among children, access to clean drinking water, proper sanitation facilities, and thorough health education on personal hygiene habits are necessary.

Keywords: *Giardia lamblia*; Multiplex PCR; Diarrhea; Rural Area

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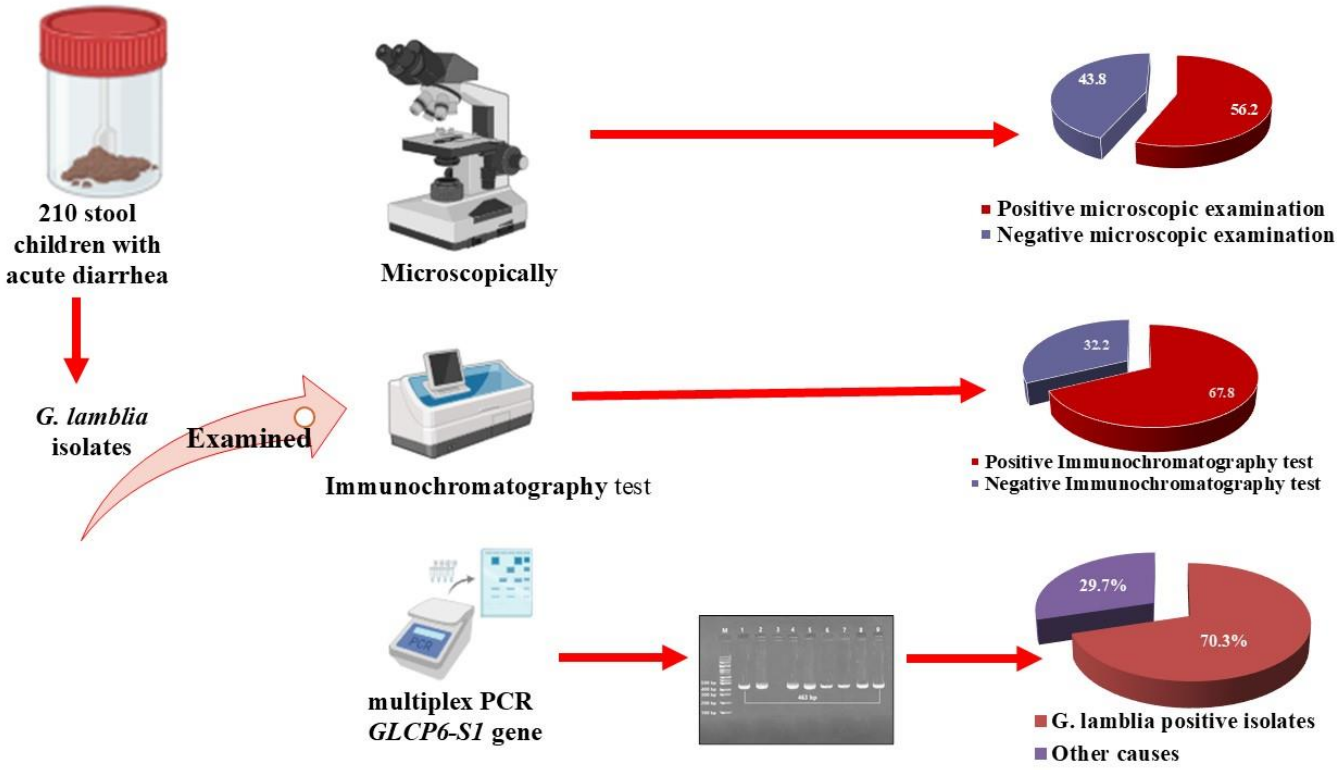
Graphical abstract:



Introduction

Diarrhea is two or more loose, liquid, or watery stools or an increased frequency of bowel movements exceeding normal for one individual during a day. Diarrhea is one of the most widely used clinical manifestations that may be associated with the ingestion of a microbial agent (bacterial, protozoan, or viral). Diarrheal infections are the second most common cause of death among children aged less than five years, mainly in developing countries [1]. Among several parasitic infections, amoebic dysentery and giardiasis are the most common public health problems in tropical and subtropical countries. Giardiasis except patients who belong to low-income countries, infected people in high-income countries, and daycare employees [2]. *Giardia lamblia* is one of the causative agents of diarrhea in developing countries, while it can cause a more or less asymptomatic infestation in developed countries. *Giardia lamblia* is considered an enteric pathogen, causing infections primarily in pediatric populations [3].

Giardia lamblia is a prevalent parasitic flagellate that infects the human intestines. Cysts are infective-stage forms. Ingested cysts undergo excystation, and trophozoites are released. Passive processes, such as adhesion to the epithelium by the ventral disk, appear to be important in attachment and possibly pathogenesis. Unlike trophozoites, cysts are excreted in most, but not all, cases where the augmentations in environmental resistance following differentiation would protect the life cycle from environmental change [4]. The distribution of *G. lamblia* among pediatric populations largely

depends on socioeconomic, hygienic, and research setting factors. The infection has been extensively studied in resource-poor regions, where the infection rates ranged between 1.6% and 60%. In Asia, the rate of *G. lamblia* infection ranges between 2.1% and 40%, whereas in northern Europe and Australia, the prevalence ranges between 2.5% and 46% [5]. The diagnosis of giardiasis is a major part of the management of diarrhea in children. Therefore, attention should be given to the diagnostic methods and approaches of frequently used laboratory techniques for diagnosing *G. lamblia* in children with diarrhea [6]. Various molecular approaches have been used to identify and/or genotype the organism in samples from various sources. The central feature of all of these techniques is the use of a PCR amplification step to generate target sequences for additional analysis. The subsequent analysis may be further characterized by DNA sequencing [7].

The current study aimed to detect *G. lamblia* associated with acute diarrhea in children under 6 years old by traditional microscopic examination supplemented by an immunochromatography test and evaluation of a multiplex PCR assay for identification of the *G. lamblia* genome in stool samples for estimating the prevalence rate of infection based on age, gender, residency, and month variation studies

Materials and Methods

Two hundred and ten stool samples were taken from a randomly chosen group of young children, aged 1 month to 6 years, who had acute diarrhea. The children, of both genders, were returning to different hospitals and primary health centers at Intervals between November 2023 and April 2024 in Babylon Governorate, Iraq.

Clinical features of acute diarrhea cases and some other sociodemographic information, including age, sex, breastfeeding practices, water source, and residence, were acquired from the parents or legal guardians of the child and afterwards recorded. Also, all children shouldn't receive antibiotics or antiparasitic drugs to avoid false negative results. Stool samples have been collected in an appropriate, clean, and sterile plastic container and transferred to the laboratory within 30 minutes to 1 hour on ice in sealed bags.

Laboratory diagnosis

1. **Microscopic examination:** Initially, each stool sample was exposed to direct normal saline on clean, dry microscope slides. A normal saline smear was performed by mixing 2-5 mg of the stool specimen with a little droplet of 0.9% sodium chloride, followed by the addition of one or two drops of 1% eosin solution in the case of the stool being in liquid form. After adding Logol's iodine, the samples were covered with a cover slip. The slide was examined carefully by the light microscope, starting with a 10x magnification for initial screening and later switching to a 40x magnification for identifying the cyst and/or trophozoite of the *G. lamblia* parasite [8].

2. **Immunochromatography test (IC):** Every 210 stool samples were tested to determine the presence of *G. lamblia* antigen by utilizing a commercially available kit, "CERTEST Crypto+Giardia and Entamoeba COMBO CARD TEST," provided by Zaragoza, Spain. The test was conducted following the manufacturer's guidelines. Any samples that tested positive for either method were kept at a temperature of -20°C, and DNA was subsequently extracted from these samples.

DNA extraction: The DNA extraction method was conducted to isolate genomic DNA from *G. lamblia* in positive fecal samples by following the manufacturer's guidelines for the QIAamp DNA

stool mini kit (QIAGEN, Germany) and using inhibitEX tablets to remove PCR inhibitors.

The Cathepsin L-like protease gene (*GLCP6-S*) in *G. lamblia* isolates was amplified by utilizing certain primers with an amplicon size. Both the primer sequence and the protocol for PCR reaction setup were identical to those previously described [9]. The detailed primers' sequences are documented in Table 1. The multiplex PCR amplification reaction was prepared with a 50 µL master mix that contained "10× PCR Buffer, 20 mM $(NH_4)_2$ SO_4, 75 mM Tris-HCl (pH 8.8), 0.3 mM dNTP mix, 3 mM $MgCl_2$, 1 µl DNA template, 1.2 units/µl Taq DNA polymerase, 20 pmol/µl of each primer sense & antisense, and x ddH_2O." PCR products (15 µL) were separated using 1% agarose gel electrophoresis after being stained with ethidium bromide. Next, gels were photographed once they were visualized under a UV transilluminator [10]

Table 1. Oligonucleotide primer sequence used in the present study

Gene	Primer sequence	Product size
GLCP6-S1	Forward: 5′-AATCTGTTGACTTAAGGGAGTA-3′ Reverse: 5′-ATTGAGTCATTATAGGGATTGT-3′	463 bp

Typically, the thermal cycling process begins with an initial denaturation step lasting 10 minutes at a temperature of 94 °C. Afterwards, 35 amplification cycles were carried out, which included "a denaturation for 30 seconds at 94 °C, primer annealing for 1.5 minutes at 55 °C, and extension for 1.5 minutes at 72 °C", as well as a final extension for a duration of 10 minutes at a temperature of 72 °C. This will be followed by a cooling step at a temperature of 20 °C for a duration of 1 minute [11].

Ethical approval:

The study was conducted in compliance with the ethical guidelines derived from the Declaration of Helsinki. Before collecting the sample, the patient's verbal and analytical permission was acquired. It was obtained according to the guidance of the Middle Technical University Medical Ethics Committee, Al-Za'franiya, Baghdad city, P.C.:10074 (MEC No: 43, Approval Date 9/11/2024), which is consistent with the relevant Iraqi legislation.

Results

210 stool samples were taken from a randomly chosen group of young children, aged 1 month to 6 years (144 males and 66 females), who had acute diarrhea. These stool samples were then examined using a microscope to determine if they were positive for *G. lamblia*. The findings indicated that 118 samples (56.2%) exhibited positive results, and 92 samples (43.8%) exhibited negative results, as shown in Table 2.

All positive microscopic examination samples, 118 obtained from patients with acute diarrhea, were subjected to an immunochromatography test. The findings revealed that positive *G. lamblia* antigen was discovered in 80 samples (67.8%), whereas it was not discovered in 38 samples (32.2%), as illustrated in Table 3.

Subsequently, DNA was obtained from all 118 (positive microscopic examination isolates). This DNA was then used as a template to identify the presence of the GLCP6-S1 gene. The results indicated that the gene was detected in 83 out of the 118 stool samples, while it was absent in 35 stool samples (Table 4), and (Figure 1).

According to the PCR assay results, 83 out of 118 samples were positive for *G. lamblia*, and the frequency of *G. lamblia* infection in 118 (positive microscopic examination isolates) represented 70.3%. The results are displayed in Figure 2.

According to Figure 2, *G. lamblia* positive isolates accounted for the greatest rate of acute diarrhea infection, at 70.3%, while the other causes accounted for 29.7% of acute diarrhea infection.

Furthermore, all 83 *G. lamblia* positive isolates from young children with acute diarrhea, aged between 1 month and 6 years of both genders, were taken. The details are shown in Table 5.

Table 5 clearly indicates that the highest prevalence of *G. lamblia* infections occurred in the age groups ranging from 4 to 6 years, accounting for 55.4% of the cases. Both genders had effects, with males (62.7%) being affected more than females (37.3%). Additionally, a higher preponderance of males was seen among children aged 4-6 years

In addition, the findings indicated a greater prevalence of *G. lamblia* in rural areas, accounting for 53 cases (63.9%), compared to urban areas, which accounted for 30 cases (36.1%) (Figure 3).

Additionally, investigate the monthly variation of acute diarrhea infection caused *by G. lamblia* over six months from November 2023 to April 2024. The result of the study showed that in November, the infection rate was 11 isolates (13.3%). Subsequently, there was a decrease in the infection rate, with 8 isolates accounting for 9.6% of the total isolates, in both December and January. However, there was an increase in the infection rate observed in February and March, with 14 isolates (16.9%) and 18 isolates (21.7%), respectively. The infection rate then increased further to reach a peak in April with a total of 24 isolates (28.9%) (Figure 4).

Table 2. Distribution of stool samples based on the results of microscopic examination and gender

Results	No. of samples	Gender		%
		Male	Female	
Positive microscopic examination	118	81	37	56.2
Negative microscopic examination	92	63	29	43.8
Total	210	144	66	100

Table 3. Distribution of stool samples based on the results of the Immunochromatography test

Results	No. of samples	%
Positive Immunochromatography test	80	67.8
Negative Immunochromatography test	38	32.2
Total	118	100

Table 4. Distribution of stool samples based on the results of the PCR assay

Results	No. of samples	%
Positive PCR assay	83	70.3
Negative PCR assay	35	29.7
Total	118	100

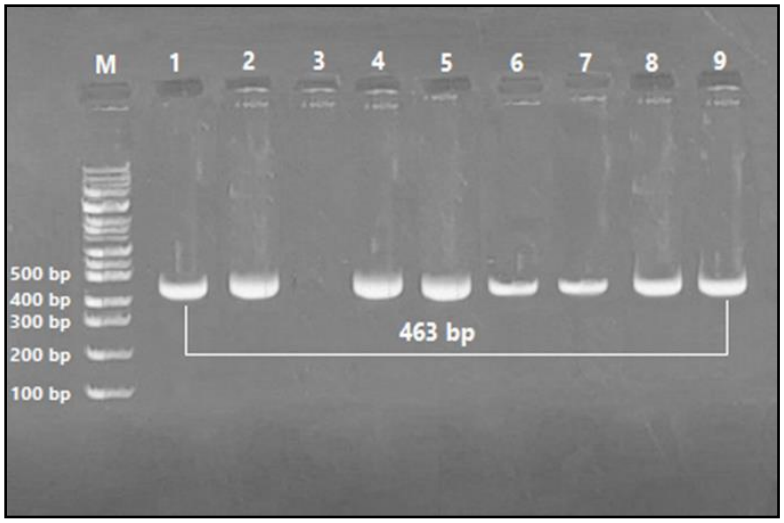


Figure 1. Agarose gel electrophoresis to detect GLCP6-S1 gene in *G. lamblia* isolated from stool samples. Lane M: marker with (100 bp) ladder. Lanes 1-2, 4-9: positive to GLCP6-S1 gene, while lane 3: negative to GLCP6-S1 gene, product size: 463 bp

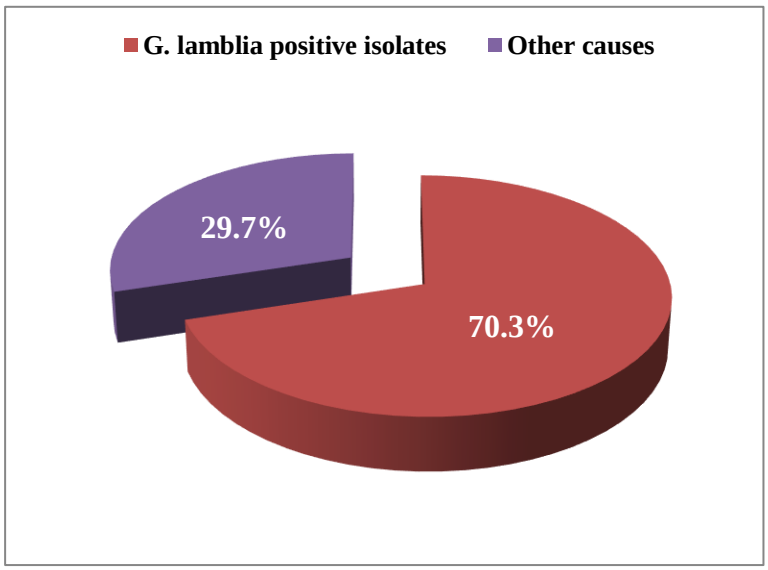


Figure 2. The frequency of *G. lamblia* infection in positive microscopic examination isolates

Table 5. Distribution of patients infected with *G. lamblia* based on age groups and gender

Age groups in years	Patients		Gender	
	No.	%	Male	Female
≤ 2	12 (14.5)		8	4
2-4	25 (30.1)		16	9
4-6	46 (55.4)		28	18
Total	83 (100)		52 (62.7%)	31 (37.3%)

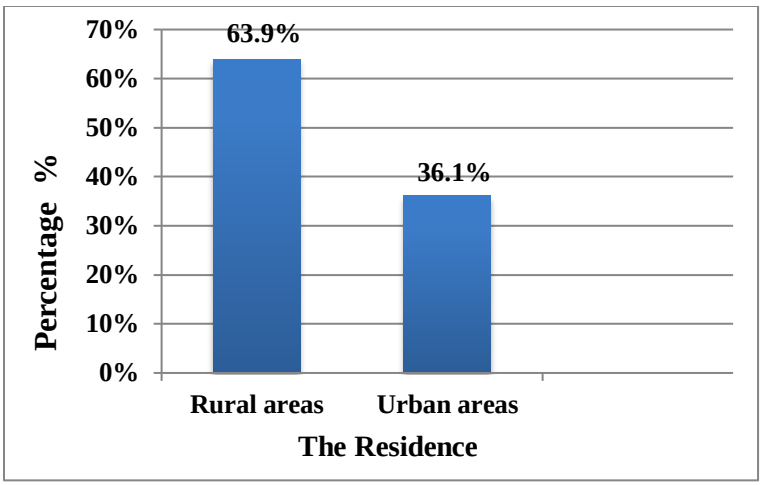


Figure 3. The geographical distribution of patients infected with *G. lamblia* is based on residency

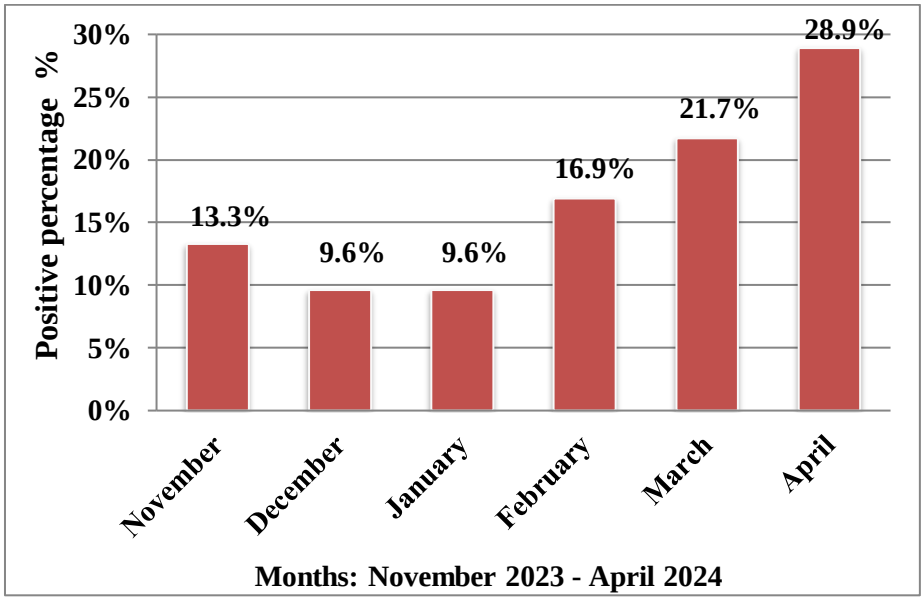


Figure 4. The distribution of patients infected with *G. lamblia* is based on months' variation

Discussion

Giardia lamblia is a parasitic protozoan with flagella that inhabits and multiplies in the small intestine, leading to symptoms such as persistent diarrhea, severe abdominal pain, and compromised nutrient absorption, which can result in weight loss and malnutrition. This microscopic organism is known for its ability to cause intestinal infections, affecting millions of people worldwide [10].

In this study of a total of 210 stool samples, the findings indicated that 118 samples (56.2%) were found positive for *G. lamblia*, and the remaining 92 samples (43.8%) were diagnosed as negative when examined using a microscope. While being investigated using an immunochromatography test. It was found that a positive *G. lamblia* antigen was discovered in 80 samples (67.8%). This result was similar to a study done by Zaboon (2021) in Babylon province, which showed a prevalence of (55.82%) for *G. lamblia* in children when investigated by microscopic examination and a prevalence of (68.33%) by immunochromatography assay [11], However, the findings of the present study are contradictory to the results recorded in Dohuk by Al Saeed and Issa (2006) which showed a prevalence of (38.5%) for *G. lamblia* when investigated by using microscopy [12]. In another past research conducted in Thi-Qar province by Al-Aboody et al (2020), the low prevalence rate was (8.1%) against G. lamblia isolated in patients with acute diarrhea infection when stained and examined with a microscope [13]. In southern Ethiopia, however, Flecha et al (2015) showed a low G. lamblia prevalence level of 10.9%, identified through microscopy and IC test. The differences in possessing the G. lamblia rate can be explained by the alteration in a technique, namely, using several kits to perform the same procedure, geographical area, character of the studied population, or disease stage [14].

Further, according to the PCR assay data given in the paper, it was observed that the prevalence of G. lamblia was 70.3%. This finding is supported by the findings registered by Mathurin et al (2015), who reported positive PCR findings. The high frequency of G. lamblia infection that has been observed in the current study could probably be due to the usage of such a PCR technique in the identification process of this parasite [15]. The nucleic acid dilatation procedure is a highly sensitive technique, and it is more reliable than other methods, such as the microscope or IC test [15]. The world domination in the spread of G. lamblia infection is perhaps an attribute of infection by this parasite. Major modes of transmission of this parasite include the fecal oral route, either directly by direct person-to-person contact or indirectly through the consumption of food and water that has been contaminated with fecal material. Poor quality of living conditions may also play a role in this [16]. In addition, the results revealed that the age group of 4-6 years reported the maximum prevalence of G. lamblia infection, comprising 55.4 percent of all cases. This finding was in line with that of Tiwari et al (2013), which mentioned that infection with G. lamblia occurred commonly in children aged 4-6 years, with a percentage of 38.18 [17]. The high prevalence noticed in the current study may be associated with the fecal-oral pathway, as well as inadequate personal hygiene and poor environmental cleanliness. All these contribute to the high prevalence rate amongst children.

G. lamblia is not only very common but also often of a high prevalence as a worldwide parasitic protozoan that causes a significant contribution to the incidence of diarrhea among children, especially those in countries with poor sanitation and hygiene [18]. In developing countries, children are the most common category of affected because of lower immunity. The notable thing is that children are not the only ones who are susceptible to this disease. As a matter of fact, geriatric individuals and immunocompromised patients (those with AIDS) are also susceptible to giardiasis [19]. The increased

frequency of G. lamblia infection in children of 4-6 years is explained by more frequent contact with contaminated food and water, along with poor personal hygiene in this population group. The same results have also been found by [5] and [2], with the exception that the preschool and early school-aged children were the most infected. This might be attributed to the increased social interactions that they have in kindergarten and in school, where they are easily transmitted. With respect to gender distribution, our findings revealed more males under infection (62.7%) than females (37.3%). The reason behind this difference could be attributed to behavioral factors, which include higher outdoor activity in boys, as earlier studies have shown [1].

The elevated font in the rural (63.9%) as compared to the urban (36.1%) setting can be attributed to l0 % of sanitation levels and access to sanitary water supplies by the residents within the rural areas, as witnessed in the surveillance of [9]. Thus, our results are comparable to those of the existing studies; nevertheless, they also present significant new information on the Iraqi context.

The difference in the prevalence of the G. lamblia infection between months of November 2023 to April 2024 shows that there is a seasonal factor at play. The frequencies of lower infections presented in November-January can be related to cooler temperatures and the absence of contact with contaminated water sources. Conversely, the noticeable rise in February and April may be ascribed to heat, water usage, and perhaps a higher degree of evens outdoors, all of which may improve G. lamblia transmission.

Similar seasonal patterns have been documented in other regions. For example, [14] reported higher giardiasis prevalence during warmer months due to favorable conditions for cyst survival in the environment. Likewise, [9] found a positive correlation between seasonal changes and giardiasis incidence, highlighting the role of environmental and behavioral factors

Therefore, our findings not only align with global trends but also provide the first evidence of seasonal variation in giardiasis prevalence in Iraq, emphasizing the importance of considering climatic and social factors in public health planning

Both genders were affected in this study, with males (62.7%) being affected more than females (37.3%). This result was consistent with other previous local studies conducted in various cities in Iraq by AL-Kubaisy (2014), Al-Saqur (2017), Hussein and Meerkhan (2019), and Al-Sultany and Al-Morshidy (2023) showed that males had a higher prevalence of *G. lamblia* infection than females [20-23]. However, the findings of the current study contradict the results obtained in Baghdad City by Jaeffer (2011), which indicated females were more likely than males to be infected with *G. lamblia* [24].

Furthermore, the results revealed that *G. lamblia* was more common in rural areas (63.9%), in contrast to urban areas (36.1%). This result agreed with the result reported in Babylon province by Al-Sherefy and Al-Hamairy (2022), who observed that *G. lamblia* is more common in rural areas compared with urban areas [25]. In addition to that, Hussein and Meerkhan (2019) in Duhok City concluded that rural regions had a much greater incidence of *G. lamblia* infection (65.90%) than urban regions (34.09%) [22]

Likewise, Tyoalumun et al (2016) and Samie et al (2020) also reported a comparable outcome in that the incidence of G. lamblia infection was lower in urban than in rural areas. According to what Abdullah (2021) said, a reasonable size of protozoan parasites such as G. lamblia may be contaminated in fresh vegetables [28]. Infections of G. lamblia can spread a lot through this contamination. The risk of infection in rural areas is most likely to be attributed to the multifactor nature correlating with the

unsatisfactory sanitation, poor access to clean water, and the use of the waste of animals as an organic fertilizer, as it positively impacts the growth of fruits and vegetables and the environmental contamination by the livestock and wildlife [28].

In addition, the results demonstrated that G. lamblia infection was found in various proportions in samples collected in November 2023 and April 2024. Incidence was highest in April, representing 28.9 percent of cases. Multiple factors, including the socioeconomic status of the population, the condition of the environment, the geographic area, and sanitary conditions, could have played a role in the causative agents of the variation in months over the duration of the epidemic. This outcome concurred with the outcome by Zaboon (2021) in Babylon province, who observed that the highest incidence of G. lamblia infection occurs in April (during the summer season) [11]. The reported upsurge of infection in the period between February and April can be connected to the seasonal aspect, which includes increasing temperature and increased water intake that may favour the spread of G. lamblia. Such an explanation is in line with the findings of other studies that indicated seasonality of incidence of giardiasis [15]. Hence, we can concur with the previous reports so far, and we opine that the local environmental conditions have a great influence on the trend.

Moreover, the other studies conducted earlier in Jordan revealed that G. lamblia was mostly common during the summer season [29], as shown in the previous study conducted in Jordan (done by Jaran 2017).

Change in G. lamblia infection prevalence per month, as occurred in our study, was lower in December and January and then significantly increased and peaked in April (28.9%). This trend can be linked with seasonal fluctuations, whereby cold seasons tend to restrict outdoor lifestyles and lessen exposure to contaminated water sources, warm seasons generate survival of G. lamblia cysts in the environment, and improve human-to-human contact with possibly contaminated food and water. Similar results have previously been cited. As an example, giardiasis incidence is known to be higher in warmer months than in winter, as reported by Al-Saeed and Issa (2006) based in Duhok, Iraq [12]. Likewise, Flecha et al. (2015) in Brazil noted seasonality as the infections peak during warm and rainy seasons [14]. These findings indicate that environmental conditions, e.g., temperature and water quality, are a primary determinant of transmission dynamics of G. lamblia. Thus, our findings correspond to previous publications and give further support to the seasonal effect on the prevalence of giardiasis in Iraq, which requires special measures to be taken during the highest infection risk months.

Conclusions

Multiplex PCR showed the most optimal technique for detecting *G. lamblia* in faecal samples compared to conventional diagnostic methods. Moreover, it is critical to provide clean drinking water, adequate sanitation facilities, and comprehensive health education regarding personal hygiene practices to limit the spread of intestinal parasites within communities and minimize the transmission of *G. lamblia*, particularly among children, this paper demonstrated that Giardia lamblia is a predominant etiological cause of acute diarrhea among young children in Babylon Governorate with a prevalence of 56.2 % by microscopy technique. Molecular detection: On targeting the GLCP6-S1 gene, 70.3 percent of specimens that tested positive under microscopy were confirmed to be infected, rendering the multiplex PCR as the most sensitive and reliable diagnostic algorithm compared with the immunochromatography and conventional microscopy.

Epidemiological data revealed that the highest penetrance of infection was in children aged between 4-6 years and that males (62.7%) were more

implicated compared to females (37.3%). A much greater proportion was found to be prevalent in the countryside (63.9%) than in the city (36.1%), pointing to the impact of the environment and hygienic state. Moreover, seasonal variability was registered in the study, meaning that the highest infection rates were registered in April (28.9%), implying that climatic and environmental factors also play a role in transmission.

To sum up, the best possible method for Giardiasis diagnosis in the clinical and epidemiological investigation should be regarded as multiplex PCR. To minimize the morbidity of giardiasis, preventive interventions, such as the supply of safe drinking water, sanitation, and health education, especially in rural areas, as well as among families with children aged between 4-6 years, are necessary to curb the burden and transmission of giardiasis.

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Conflict of Interest: NIL

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