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Distribution of *Giardia lamblia* Genotypes in Children

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Abstract

The current study aimed to detect *Giardia lamblia* subtypes that infect children under 13 years and determine the association of subtype infection with some risk factors as age, gender, and residence.

Triosephosphate isomerase gene from the positive microscopic samples was isolated. To confirm infection and the sensitivity of this gene, DNA in one negative and some positive samples were confirmed when amplified using β-giardin gene primers.

From the 66 samples positive by microscopy, 65 tested positive by molecular assay of the triosephosphate isomerase gene with 98.4% sensitivity. While 37/65 (56.9%) represented subtype B, 23/65 (35.4%) represented subtype A, 5 (7.6%) represented A+B, while there was no E subtype. A subtype A in asymptomatic children exceeded 10/25 (40%) compared to 13/40 (32.5%) in symptomatic children. The proportion of B subtype among symptomatic children was higher than that of asymptomatic children (57.5% and 56% respectively). There was no significant difference between the subtype presence and gender, age or residence. Subtype A was higher in the watery diarrhoea samples than in the steatorrhea samples. On the contrary, B subtype was higher in the steatorrhea samples than in watery diarrhoea.

Use of the gene triosephosphate isomerase showed a high sensitivity for molecular detection of *Giardia* spp. Children could be infected with both groups A and B subtypes. The study did not record any presence of subtype E. No significant difference between genders, age groups or place of residence was recorded. Symptoms and diarrhoea were insignificantly associated with subtype B.

Keywords: Giardiasis; Molecular detection; Genotyping; Subtype E; Children.

انتشار الأنماط الوراثية لطفيلي *Giardia lamblia* في الأطفال.

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الخلاصة

هدفت هذه الدراسة إلى الكشف عن الطرز الوراثية لطفيلي الجيارديا لامبليا والتي تصيب الأطفال دون سن 13 عاما، ولتحديد ارتباط الإصابة بالطراز الوراثي مع بعض عوامل الخطر مثل العمر والجنس والسكن.

تم عزل جين إيزوميراز ثلاثي الفوسفات من العينات المجهرية الإيجابية، ولتأكيد العدوى وتأكيد حساسية هذا الجين تم تضخيم الحمض النووي في عينة سلبية واحدة وبعض العينات الإيجابية باستخدام بوادئ جين β جياردين.

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من بين 66 عينة إيجابية عن طريق الفحص المجهري، كانت 65 عينة إيجابية عن طريق الفحص الجزيئي لجين إيزوميراز ثلاثي الفوسفات بحساسية (98.4٪)، كان منها 65/37 (56.9٪) تمثل الطراز الوراثيB، 23/65(35.4%) تمثل الطراز الوراثي A و5(7.6%) تمثل الطراز A+B، ولم يظهر الطراز الوراثي Eفي أي من هذه العينات. كانت نسبة ظهور الطراز A في الأطفال الذين لم تظهر عليهم أعراض 25/10 (40٪) مقارنة ب 40/13 (32.5٪) في الأطفال الذين تظهر عليهم أعراض. كانت نسبة الطراز الوراثي B بين الأطفال الذين تظهر عليهم الأعراض أعلى من نسبة الأطفال الذين لا تظهر عليهم أعراض (57.5٪ و56٪) على التوالي. لا يوجد فرق كبير بين وجود التجمع والجنس أو العمر أو الإقامة. كان الطراز الوراثي Aأعلى في عينة الإسهال المائي منه في عينات الإسهال الدهني، على العكس من ذلك، كان الطراز الوراثي Bأعلى في عينات الإسهال الدهني منه في الإسهال المائي.

1. Introduction

Infection with intestinal parasites acts as a medical problem in most developing countries. Intestinal parasites are among the most important pathogens of diarrhoea[1]. *G. lamblia* is one of the most common intestinal parasites that are pathogenic to humans. It is most prevalent because it possesses some features, including that this widespread is not related to specific vectors such as malaria or leishmaniasis [2]. Giardiasis infects a large number of people due to its low infectious dose and it spread in a hot and moderate climate area in the tropical and subtropical regions. In addition, its cysts are highly resistant to various factors. It maintains its vitality for three weeks while it is in the soft, not dry stool [3].

Giardiasis is the most common disease among children, especially school-age children and immunocompetent [4]. Many patients are asymptomatic. Symptoms that it may cause include severe intestinal disorders, acute or chronic diarrhoea, and accompanying symptoms as mal-absorption of fats and sugars and some vitamins such as vitamin A and vitamin B12 [5]. The symptoms are more severe among children in particular. In addition to the above, children may have delayed growth, low weight, low intelligence and delayed mental development[6].
Conventional PCR and the PCR-RFLP methods are used to detect *Giardia* spp. in fecal samples. Real-time PCR reaction and sequencing are used to determine the different genotypes of *G. lamblia* in human and animal isolates [7]. Many molecular studies have been conducted to determine this parasite genotype and sub-genotype in human, animal and aquatic samples. These genotypes differ in their ability to genotyping (due to different ratios of nucleotide replacement within each gene, where some of these genes are at high degree of stability) such as elongation factor 1-α glutamate dehydrogenase (GDH), small subunit rRNA (ss rRNA), Triosephosphate isomerase (TPI) and β-giardin (βG) [8].

G. lamblia possesses several similar morphologically (indistinguishable microscopically) and different genetically genotypes. There are eight groups (A-H) that infect humans and animals, A and B being the most prevalent subtypes as they infect both humans and mammals [9]. Many reports indicate that subtype E infects humans [10] and cats [11], in addition to the rest of the mammals. Subtype A has three sub-subtype: AI infects and transmits between humans and animals (Zoonotic), AII transmits from human to human (Anthroponotic), and AIII infects animals only [12]. Subtype B has two sub-subtypes: BIII and BIV infecting humans and animals[13].

2. Methods

2.1. Patients and Samples

We collected 234 faecal samples from children aged 2 to 12 (3.1± 0.2423) who attended private clinics in Al-Qadisiyah Governorate, of which 183were asymptomatic, 51 symptomatic,

124 males and 110 females). After collection, samples data was recorded before storing the samples at -20°C.

2.2. Microscopic Test

Microscopes with direct smear method were used. One gram of faeces was mixed (by Loop) with a drop of iodine solution. Slide cover was placed over once the homogeneous mixture was ready which was then regularly examined under (10x) and (40x) lenses [14].

2.3. Molecular Analysis

2.3.1. Genomic DNA Extraction

AccuPrep® stool DNA extraction kit, supplied by Bioneer Company, Korea, was used to extract DNA from 200 mg of positive microscopy stool samples.

2.3.2. Primers

Bioneer Company Korea supplied all primers (Table 1) [15].

Table 1: Sequences, types and product sizes of study Primers.

Primer (Subtype)	Sequences	Accession Number	Size Pb
tpi gene	AL3543 5`-AAATIATGCCTGCTCGTCG-3`	U57897	605
	AL3546 5`-AAACCTTITCCGCAAACC-3`		
A	Af: 5`-CGC CGT ACA CCT GTC A-3`	AY368157	332
	Ar: 5`-AGC AAT GAC AAC CTC CTT CC-3`		
B	AssBF: 5` GTT GTT GTT GCT CCC TCC TTT 3`	AY228628	140
	AssBR: 5` CCG GCT CAT AGG CAATTA CA 3`		
E	Ef: 5`-CCC CTT CTG CCG TAC ATT TAT-3`	AY228645	388
	Er: 5`-GGC TCG TAA GCA ATA ACG ACT T-3`		

2.3.3. PCR Programme

The triosephosphate isomerase gene was amplified in two rounds. The first round detected parasite presence, while the second round amplified the first round products to differentiate parasite subtypes (Table 2) [16].

Table 2: PCR master mix component and thermo cycle characterizes to amplify *tpi* gene.

First Round PCR Master Mix	Volume	Thermo Cycle Characterizes		
DNA template (5-50ng/µl)	5µl	Cycle numbers	Temperature	Time
G. lamblia F primer	1µl	An initial denaturation step	95˚C	5 min
G. lamblia R primer	1µl	35 cycles	94˚C	45s
PCR water	13µl		50˚C	45s
Total volume	20µl		72˚C	60s
		Final extension step	72˚C	10 min
Nested PCR Master Mix	**Volume**	**Thermo Cycle Characterizes**		
First round PCR products	2µl	Cycle numbers	Temperature	Time
Subtype A, B and E F primer	1µl	An initial denaturation step	95˚C	10 min
Subtype A, B and E R primer	1µl	35 cycles	94˚C	45s
PCR water	16µl		50˚C	45s
Total volume	20µl		72˚C	60s
		The final extension step	72˚C	10 min

2.3.4. Sensitivity of triosephosphate isomerase PCR

Various concentrations of 10, 50, 100, 500 and 1000 cysts per 100 l µl were prepared. β-giardin gene primers were used to examine all negative and a little bit of positive molecular samples as laid down by Saleh *et. al.* [17] (Table 3).

Table 3: Sequences of β-giardin gene that were used in first and second rounds

First Round Primers		Sequences
G7	F	5`-AAGCCCGACGACCTCACCCGCAGTGC- 3`
G759	R	5- GAGGCCGCCCTGGATCTTCGAGACGAC-3
Second Round Primers		
PGair	F	5`- GAACGAGATCGAGGTCCG-3`
PGair	R	5`- CTCGACGAGCTTCGTT-3`

2.4. Statistical Analysis

Chi-square values at the significance level P≤ 0.05 was calculated using the Statistical Package for the Social Sciences (SPSS), V20 software.

3. Results

This study detected trophozoite or cyst in 66 of the 234 samples examined under the microscope, with a prevalence rate of 28.2 % (Od = 21.958; CI= 10.01 – 48.18). The positive samples in symptomatic patients were 40 (78.4 %) and 26 (14.2 %) in asymptomatic patients (Table 4) (Figure 1).

Table 4: Prevalence of *G. lamblia* in children as microscopic test

Parameters	Asymptomatic	Symptomatic	Total		
Total Patients	183	51	234	Odd ratio	21.954
Negative	157	11	168	95% CI	10.01-48.18
Positive	26	40	66	Z statistic	7.71
Percentage	4.2%	78.4%	28.2%	P. value	< 0.0001

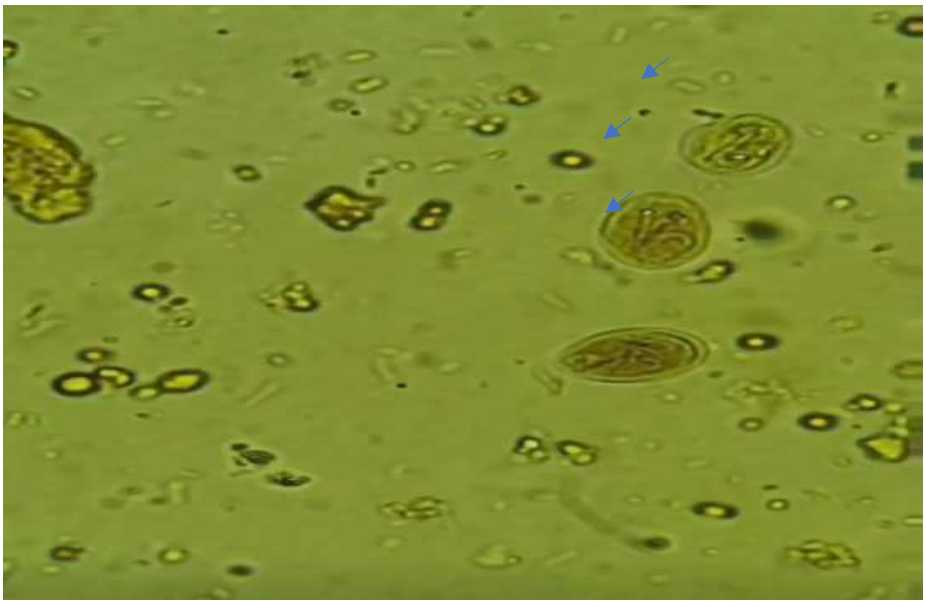

Figure 1: Microscopic image for *G. lamblia* cyst 40x.

Out of the 66 positive microscopic samples, triosephosphate isomerase gene amplification failed only in one asymptomatic sample. Sensitivity test with *β-giardin* gene provided same results. Sixty-five samples tested positive by molecular test (Figure 2), meaning that the sensitivity of the molecular assay using the triosephosphate isomerase gene was 98.4%.

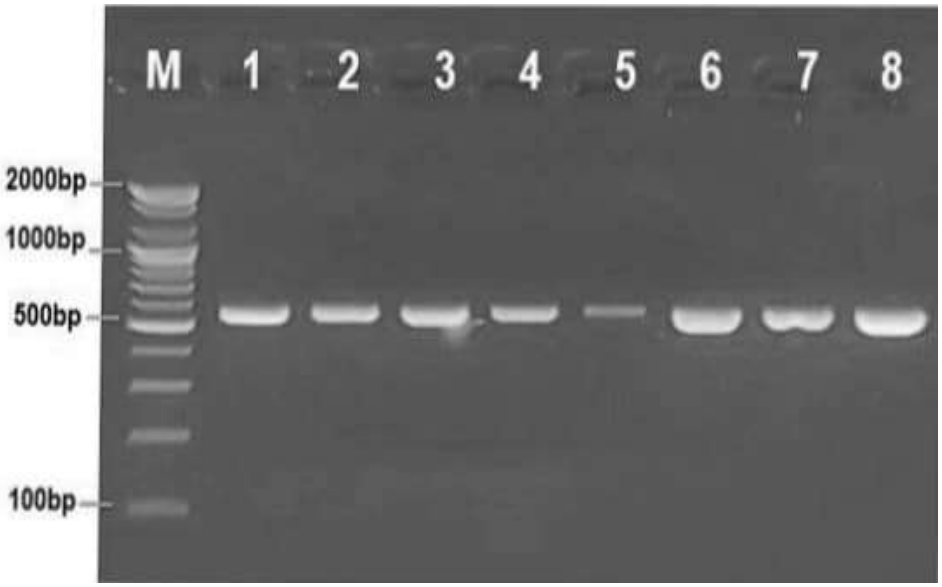

Figure 2: Analysis of triosephosphate isomerase gene (605pb) product by 1.5% agarose- gel electrophoresis.

From the 65 samples, 37 (56.9%) had subtype B, while 23 (35.4%) had subtype A. Subtype A was 10/25 (40%) in asymptomatic children compared to 13/40 (32.5%) in symptomatic children. The prevalence of subtype B was higher in symptomatic children than in asymptomatic children (57.5 % vs. 56 %). The result did not include subtype E (Figure 3, 4, 5).

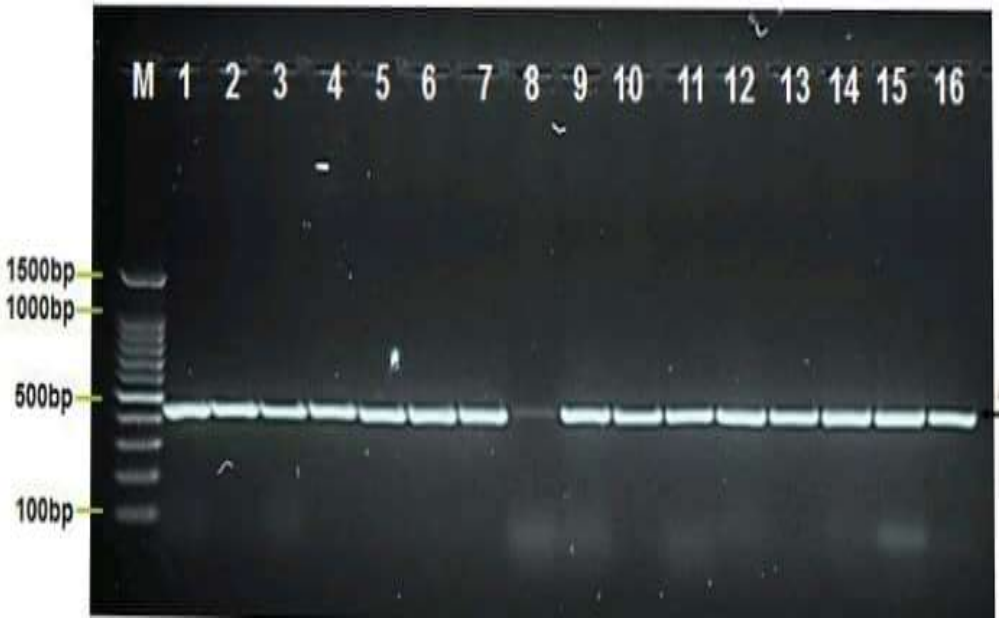

Figure 3: Analysis of subtype A of triosephosphate isomerase gene (332pb) amplification by 1.5% agarose gel electrophoresis.

Figure 4: Analysis of subtype B of triosephosphate isomerase gene (1**40**pb) amplification by 1.5% agarose gel electrophoresis.

Table 5: Distribution of *G. lamblia* subtypes in the study samples.

Subtypes	Symptomatic	Asymptomatic	Total
A B A+B E	13(32.5%) 23(57.5%) 4(10%) 0(0%)	10(40%) 14(56%) 1(**4**%) 0(0%)	23(35.4%) 37(56.9%) 5(7.6%) 0(0%)
Total	40(61.5%)	25(38.4%)	65(100%)
Odd ratio = 0.791		Z statistic= 0.433	
95% CI = 0.27 – 2.28		P. value= 0.66	

In symptomatic children, 6-9 years age group had the highest infection rates (50%) (20/40). In the same group, subtype B had the highest score of 13/40 (32.5 %), and subtype A had the highest score of 6/25 (15 %). The percentage of subtype B in males and females was the highest, with infection rates of 35% and 22.5 %, respectively. Subtype B was more prevalent in rural areas (32.5 %), while Subtype A was more prevalent in urban areas (17.5 %) (Table 6).

Table 6: Relation of *G. lamblia* with the demographic data of symptomatic children

Demographic Data	Subtype A	Subtype B	Mixed A+B	Total	Statistical Tests
Age 2-5 6-9 10-12	 3(7.5%) 6(15%) 4(10%)	 7(17.5%) 13(32.5%) 3(7.5%)	 1(2.5%) 1(2.5%) 2(5%)	 11(27.5%) 20(50%) 9(22.5%)	P. value= 0.22
Gender Male Female	 8(20%) 5(12.5%)	 14(35%) 9(22.5%)	 2(5%) 2(5%)	 24(60%) 16(40%)	Odd ratio=1.03 95% CI = 0.26-4.16 Z statistic = 0.04 p. value = 0.96
Residence Urban Rural	 7(17.5%) 6(15%)	 10(25%) 13(32.5%)	 1(2.5%) 3(7.5%)	 18(45%) 22(55%)	Odd ratio= 1.52 95% CI = 0.38-5.95 Z statistic = 0.59 p. value = 0.55

In asymptomatic children, 6-9 and 10-12 years age had the highest infection rate (36%). Subtype B was the highest in the 10-12 years group with a 24% (6/25) infection rate. Subtype A was the highest in the age group 2-5 years (16%) (4/25). The present study recorded a high infection rate with subtype B among females (32%; 8/25), while subtype A was higher among

males (24%; 4/25). As by residence, subtype A was higher (7/25) in urban areas than in the rural areas, while subtype B was higher in rural than urban areas (8/25) with an infection rate of 28% and 32% respectively (Table 7).

Table 7: Relation of *G. lamblia* with the demographic data of asymptomatic children

Demographic Data	Subtype A	Subtype B	Mixed A+B	Total	P. value
Age 2-5 6-9 10-12	 4(16%) 3(12%) 3(12%)	 3(12%) 5(20%) 6(24%)	 0(0.0) 1(4%) 0(0.0)	 7(28%) 9(36%) 9(36%)	0.576
Gender Male Female	 6(24%) 4(16%)	 6(24%) 8(32%)	 1(4%) 0(0.0)	 13(52%) 12(48%)	Odd ratio = 2.0 95% CI = 0.39-10. 4 Z statistic = 0.82 p. value =0.41
Residence Urban Rural	 7(28%) 3(12%)	 6(24%) 8(32%)	 1(4%) 0(0.0)	 14(56%) 11(44%)	Odd ratio = 3.11 95% CI = 0.56-17.33 Z statistic =1.29 p. value =0.196

In symptomatic children, the current study indicated that the parasite presence was in a higher percentage in fatty diarrhoea, where infection rate was 45% (18/40). In addition, it confirmed the superiority of subtype B in steatorrhea (27.5%, 11/40), while subtype A was superior in watery diarrhoea by 12.5% (5/40) (Table 8).

Table 8: Relation of *G. lamblia* with stool status

Diarrhoea	Subtype A	Subtype B	Mixed A+B	Total	P. value
Water/ Liquid	5(12.5%)	8(20%)	1(2.5%)	1**4**(**3**5%)	0.37
Fatty	4 (10%)	11 (27.5%)	3 (7.5%)	18 (45%)	
Blood	4 (10%)	4 (10%)	0 (0.0)	8 **(**20**%)**	

4. Discussion

The prevalence of *G. lamblia* among children was moderate (28.2%). The study recorded a higher prevalence rate than the results of Bakr *et al.* (21.5%) [18], Al-daoody, *et al*. (4.6%) [19] and Rhadi(6.8%) [20] in Iraq, and Naz *et al*. (2.75%) [21] in Pakistan. This result was lower than those recorded by Al-difaie (57%) in Iraq [22], Alshahethi *et al*. (54.2%) [23] in Yemen and Ghosh *et al*.(33.14%) [24] in India. The reason behind could be different climates of geographical areas, different cultures or due to the sample selection method.

In the present study, the sensitivity of targeting the *tpi* gene was 98.4%, which was very high and helped in diagnosing *G. lamblia* infection, as one sample out of 66 failed to amplify. We obtained same results when using the βg gene which means that the triosephosphate isomerase gene is suitable to diagnosis with high efficiency and ability, especially after the successful detection of a tiny amount of parasites (10 cysts/100 micromoles) [25].

The appearance of symptoms is related to more than one factors such as the infection dose amount, parasite strain [26], the host genetic predisposition and the immune response [27] and the nature of the normal flora [28]. Previous studies by Gozalbo *et al.* and Al-Ani *et al.* indicated that the onset of symptoms in GIT was associated with subtype B presence [29, 30]. Belkessa's *et al.* and Chourab *et al.* studies also confirmed that subtype A presence in asymptomatic children is more comprehensive than B [31, 32]. On the other hand, some other studies have indicated a relationship between the appearance of symptoms and subtype A [33,

34]. While other studies have confirmed the opinion that the prevalence of subtype B is more among the asymptomatic [35, 36].

Studies explain the causes of male infection due to his behaviour in dealing with the environment and to working conditions outside the home[37], and that they are less likely to follow the rules of hygiene, in addition to the males' desire to eat food outside which increases their exposure to parasite cysts [38]. On the other hand, housewives deal with house cleanliness and take care of children, even female workers that are exposed to the same conditions as males [39]. The present study indicated that no significant difference in the infection of males and females, as they both lived under the same conditions. This result is consistent with Tembo *et al.* [40] and Nawaz *et al.*,[41]. On the other hand, Abozahra *et al*., 2021 study indicated that subtype A infects males more [42], as well as, Messa *et al*., confirmed that subtype B infects females more [43].

The main cause behind the relative infection rate in children between 6-10 years of age is they being the most mobile and active, which means they are most in contact with environmental factors. Also, they are less concerned with personal hygiene [44]. Chanu *et al*. believed that subtype B is common in different age groups [45]. On the contrary, Pacheco *et al*., indicated the predominance of subtype A, especially in children under seven years [46]. Elhadad *et al.*, confirmed that children between 6-10 years of age are more susceptible to subtype B infection, while subtype A is more prevalent among younger children [47].

The high rate of infection in the rural areas compared to urban is attributed to many environmental, social and cultural factors. Low economic level of the rural population is generally the main reason [48]. The use of untreated sewage water in the irrigation and drinking, and the presence of dogs and livestock in rural areas that carry the Zoonotic genotype, as well as the spread of domestic insects that are mechanical vectors of parasites, are effective factors in parasites spread [49]. The current study showed the predominance of subtype A in urban areas and subtype B in rural areas. This result is consistent with what Al-Mayali and Al-Ibrahimi found [50]. On the contrary, Hasan *et* al., explained the association of subtype A with the rural environment because subtype B is Anthroponotic, while subtype A is Zoonotic [51]. Nawaz *et al.*, said that the difference in the presence of parasite subtypes in rural or urban is not significant as the results of their studies confirmed the predominance both of A and B subtypes in rural [41, 52].

G. lamblia is one of the most important pathogens that cause diarrhoea, especially among children and immune-compromised [53]. When the parasite invades the intestinal mucosa, it destroys the villi and reduces its ability to absorb electrolytes which causes a loss of a lot of minerals, protein and fats from the body [54]. Loss of essential minerals from the body causes impaired liver function, anaemia and weight loss in children [55]. The current study result is consistent with when Skhal *et* al., who indicated that no significant relationship exists between subtype type and the diarrhoea type [56]. Also, studies of Huchaimi and Mahmoudi confirmed the presence of all sub subtypes in all diarrhoea types [57, 58]. On the other hand, Al-Shehri saw the association of group B with bloody diarrhoea [59]. Other studies indicated the predominance of subtype B in diarrhoea cases among children, [60, 61, 62], while García-Cervantes *et al.*, confirmed relationship between group A and diarrhoea [63].

5. Conclusion

Using gene triosephosphate isomerase is the high sensitivity of molecular detection of *Giardia* spp. Children are infected with both subtypes A and B. The study did not record the presence of subtype E. As well as no significant difference in infection between genders, age

groups or residence was recorded. Symptoms and diarrhoea were insignificantly associated with subtype B.

***Conflict of Interest*:** No conflict of interests is declared.

Ethics approval: All patients' parents were told about the significance and method of the study before obtaining their agreement to participate as volunteers.

Authors' contributions: Musafer H. Al-Ardi did all article parts.

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