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A Comparison between Microscopic and Conventional PCR in the diagnosis of Giardia lamblia among Children in Kirkuk

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A B S T R A C T

Giardia lamblia is flagellated intestinal protozoan parasite reported by (WHO) that infect 450 million children and infants worldwide. A totally 450 stool samples taken from patients with ages 1-12 years old in Kirkuk hospitals. Results showed that a percentage of positive samples *G. lamblia* by microscopic examination was (4.88%) and negative samples was (91.9%). While , Conventional PCR recorded the percentage of infected (5.45%) in males and (4.34%) in females. According to month/years the infected patients in the June/may/February recorded (7.4%, 5.3%, 5%) and lowest infected patients (2.5% - 2.8%) in December and January . The highest infected patients found (5.6%) in Rural area and lowest infected patients found (4%) in Urban area. Molecular *G.lamblia* was detected by PCR using 18S rRNA sequence specific primers. The infected found (25%) in males and lowest infected found (20%) in females. According to age group the infected was (42.9%) in age group (2-4 years) . The infected patients found (28.6%) in Rural area and (12.5%) found in the urban area. Conventional PCR is more specific in the detection of *G. lamblia* infects in children.

Introduction

The high prevalence of intestinal protozoan parasites is a major public health issue that affects more than three billion people worldwide [1-4]. The majority of infected people are children and infants, the World Health Organization (WHO) reported that these parasites infect 450 million children and infants worldwide [5,6]. These intestinal parasites are most commonly dominated by *Giardia lamblia*. They are wide spread and common in developing countries. They cause dangerous diseases that eventually cause public health problems [7].

G. lamblia protozoa are flagellates that can infect a variety of hosts. The prevalence of G. *lamblia* parasite ranges from (2%- 5%) in developed countries, but it is much higher in developing countries, at 30%. Common infection with this parasite occurs as a result of ingesting the parasite's mature cysts via the oral-fecal route (8). G. *lamblia* is common in developing countries due to poor sanitary conditions and inadequate water quality control. According to epidemiological studies, most children under the age of 12 years become infected with this parasite [9]. Common symptoms of Giardiasis including diarrhea, steatorrhea, abdominal cramps, bloating, flatulence, pale greasy and malodorous stools and weight loss are some of the symptoms. The patient may also experience nausea or vomiting at times [4]. This protozoan reproduces in the host's intestinal, causing malnutrition and anemia by interfering with the absorption of vitamins, iron, proteins, minerals, lipids, and carbohydrates [10].

Children who are malnourished are typically the most infected, with impaired growth and weight loss being common symptoms of their infection. In addition to intestinal symptoms, parasitic infection can cause anemia, as well as physical and mental problems like delayed growth. *Giardia duodenal* carries a high degree of genetic diversity that has been grouped into genotypes from A – H [11]. Molecular studies revealed the presence of other secondary genotypes within these major groups [12]. Genotype A is usually divided into secondary genotypes AI, which is found in animal origin, and AII, which is found in human origin, whereas AIII is limited to animals only [13]. The genotype B is divided into secondary genotypes BIII and BIV, which were found in similar proportions in the isolates. Hence, it could be human beings, as well as in animals (such as dogs, cattle, and horses) [14]. Most genotyping studies of the *Giardia duodenal* are more than one genetic marker such as small-subunit ss RNA genes. Triose phosphate is omerase "tpi" and glutamate dehydrogenase “gdh”, β-giardin [15].

Materials and Methods

Collection of Samples:

In this study 450 stool samples were collected from children infected with the *Giardia lamblia* in the city of Kirkuk/Iraq from November 2021 to June 2022. Age groups ranging from less than one year to 12 years old. Samples were collected in sterile plastic containers and examined with a light microscope using the direct wet mount and concentration method.

Direct wet mount preparation

The samples were examined under a light microscope using a direct saline method prepared with 0.9 percent sodium chloride. A small amount of freshly passed stool was taken by a tip of a wood applicator, mixed with a drop of physiological saline or lugol's iodine solution, and put on a glass slide. This slide was examined microscopically by the direct smear process for the presence of cysts or trophozoites [16-17].

Purification of cyst and trophozoites stage

The purification process was used, with some modifications, according to Sheffield and Bjorvatan [18].

Culture medium preparation for *Giardia lamblia*

HSP-1 Medium preparation of *G. lamblia* was carried out according to Meyer’s method [19].

Laboratory growth *Giardia lamblia*

Following the preparation of the HSP-1 culture medium for *Giardia lamblia*.

1. Add 1 ml of the suspension containing 105 cells from the cystic phase to the culture medium, followed by 3 ml of human serum.
2. The inoculated tubes were incubated for three days at 37 °C inclined way.
3. A routine examination was performed on the third day of culture by taking a drop from the culture, placing on a slide, covering it with a cover slip, and examining it under a 400x microscope.
4. A small amount of trophozoites was found in addition to the cysts.
5. After ten days, secondary culturing was performed, and trophozoites were counted in the first days of culturing to ensure the presence of trophozoites after taking 3 ml from the grown culture and 1 ml from a new culture free of antibiotics [20].

DNA Extraction / Purification

Genomic DNA was isolated from Stool samples according to the protocol of QIAamp® Fast DNA Stool Mini Extraction Kit (Qiagen / Germany) [21].

Conventional polymerase chain reaction method of diagnosis

Conventional PCR mixture was used to detect *Giardia lamblia*, as shown in Table 1. The 18S rRNA gene using custom primers designed for this purpose from Macrogen company/Korea, product 542 packet size using the 18S rRNA gene sequence Forward primer with the sequence (5'GGGCTAGAAGGCGATCAGAC3') and Reverse primer with the sequence (5'GGCGCCTACAAGACATTCCT3')

Table 1. Conventional PCR reaction mixture

	PCR master mix	Volume µL
1.	Master Mix (2X)	5
2.	DNA template	3
3.	18S rRNA forward primer 10pmol *G.lamblia*	1
4.	18S rRNA reverse primer 10pmol *G.lamblia*	1
5.	Nuclease Free Water	15
6.	Total	25

Table 2. The steps for the conventional PCR reaction

	Steps	Temperature °C	Hours m: s	Cycle
1.	Initial Denaturation	95	5 min	1
2.	Denaturation	95	30 sec	30
3.	Annealing	58	30 sec	
4.	Extension	72	30 sec	
5.	Final extension	72	7 min	1
6.	Hold	10	10 min	

Statistical analysis

The Chi-square test was used to show the relationship between the incidence of *Giardia lamblia* and its relationship to sex, age group, residence and months of the year [22]. Analyzed by the statistical program SPSS. Chi-Square statistical analysis was used and the value of $P \leq 0.05$ [23].

Results and Discussion

Microscopic examination results

After examining 450 stool samples from children infected with the parasite *Giardia lamblia*, their ages ranged from less than one year to 12 years. It was found that 22 positive samples with percentage of (4. 88%), was recorded in Table (3) showed.

Table 3. *G. lamblia* according to sex by microscopic examination

Gender	No. of infection	No. of positive	Percentage %
Male	220	12	5.45 %
Female	230	10	4.34 %
Total	450	22	4.88 %

X^2 calculated = 0,295, fd 1 , X^2 tabular = 3,841

* There are no significant differences

The current study recorded an incidence of *Giardia lamblia* infection among children in both sexes by 4.88%. The result agreed with the results in the city of Nasiriya, where it recorded an infection rate of 4.8% when examining 500 samples from Al-Batha and Al-Gharraf districts [24].

Besides, the results of the current study agree with the recorded findings in Kirkuk city, *Giardia lamblia* infection percentage of 3% infected patients [25].

The difference in the rate of infection between males and females may be due to the fact that males are the most mobile group and are in contact with the external environment factors while playing and being the working group in the society, this makes them more connected to pathogens than females, where they also eat and drink in public places or from Street vendors and this increases the chances of exposure. However, the distribution of *Giardia lamblia* was also studied on the level of the age of the study participant showed in Table (4).

Table 4. Number and Percentage of *G. lamblia* according to age group by microscopic examination.

Age group / year	No. of sample examination	No. of infected *G.lamblia*	Rate %
< 2 years	100	5	5
2-4	120	7	5.8
4-6	75	4	5.3
6-8	50	2	4
8-10	70	3	4.2
10-12	35	1	2.8
Total	450	22	4.88

X^2 calculated =0.696, fd 5 , X^2 Tabular = 11,070

* There are no significant differences

The current study recorded the highest infection rate of 5.8% in the age group 2-4 years, It agrees with what was recorded in [26]. Where the previous study in AL-Diwaniyah governorate recorded the highest infection rate of 13.87% in the age group 2-4 years. And agree with [27] recorded in Baghdad, where it recorded the highest infection rate of 2.75% in the age group 2-4 years.

The high incidence of infection in the age group 2-4 years under study may be due to the fact that children at this age are more mobile and active and have less awareness of hygiene rules such as washing hands before eating and after using the toilet, and the practice of putting fingers in the mouth, and the fact that children at this age are eager to taste anything, which may increase the incidence of infection in this age group (28). The same factors also involved the other age groups from (4-10). Consequently, the age group of (10-12) years showed a significant decrease ($P \leq 0.05$) in the *G. lamblia* infection where those children are more aware of the healthy life style measurements. Moreover, the infection rate was also analyzed according to the residence of the study participant as Table (5).

Table 5. Number and Percentage of *G.lamblia* according to the location of residence by microscopic examination

Habitation	No. of sample examination	No. of positive	Percentage %
Rural	250	14	5.6
Urban	200	8	4
Total	450	22	4.88

X^2 calculated =0,32, df 1 , X^2 tabular = 3,841

* There are no significant differences

Although, a non-significant difference was recorded with participants according to their residence a difference in the infection rate among rural residents, reaching (5.6%) was recorded comparted to the urban residents, with a rate of 4%. Compared to a previous study in AL-Diwaniyah governorate, which recorded the highest infection rate in the rural area 77.07% and lower infection rate in the urban area with percentage of 4e.45% (26). Also, it agrees with results in Tikrit governorate, where the infection with *G. lamblia* in the rural areas (20.87%) and the urban area (11.15%) (29). Furthermore, it agrees with the recorded data in AL-Qadisiyah governorate the infection with *G. lamblia* in the rural areas (24.7%) that is lower than in urban areas (21.11%) (30).

The difference in the incidence of rural was due to several factors, including the lack of clean drinking water and dependence on river water as a direct source of water, as well as the low health and cultural level of the rural population, the raising of parasite-keeping animals and contact with them, and the use of animal and sometimes human waste as organic fertilizer, this was confirmed by [31]

In the same context, the distribution of *G. lamblia* was also investee according to months as shown in Table (6).

Table 6. Number and Percentage of *G.lamblia* according to months by microscopic examination.

Months	No. of sample examine	No. of infected *G.lamblia*	Percentage %
November	50	2	4.0
December	40	1	2.5
January	35	1	2.8
February	40	2	5
March	55	2	3.6
April	60	3	5
May	75	4	5.3
June	95	7	7.4
Total	450	22	4.88

X^2 calculated =13,8, fd 7 , X^2 Tabular = 14,449

* There are no significant differences

It was noted through the results of the current study that the highest infection rate was in the months of June/May/February/ was recording the percentage of (7.4%, 5.3%, 5%) respectively.

This result agrees with the recorded results in AL-Diwaniyah governorate that recorded the highest infection rate in June and May with 10.37% and 8.63%, respectively (26). Also, it is agreement with the recorded date in the months of June and May, which were 10.95% and 6.83%, respectively [32].

The current study recorded that the lowest infection rate was in December and January with a rate of 2.5% which is in agreement with [33], as the lowest percentage of infection was recorded in January with a rate of 1.52%. Also, it agrees with the recorded data in the Tikrit governorate, where the lowest infection recorded in the January month with percentage 6% [29] .This result disagree with the recorded results in Dohuk governorate, where the lower infection rate was recoded in June 28.4% [34].

The high rates of parasite infection in the summer months may be due to the availability of suitable conditions for the growth of the parasite and the presence of vector insects such as flies, cockroaches, which are mechanical carriers of parasite bags [35,36]. Children play in garden filled with cats and go to the swimming pool.

Giardia lamblia diagnosis by PCR

After examining a total of 22 positive stool samples from children infected with the parasite *G. lamblia*, their ages ranged (≤1-12 years old). It was found that 5 positive samples containing genetic materials with percentage (22.8%).

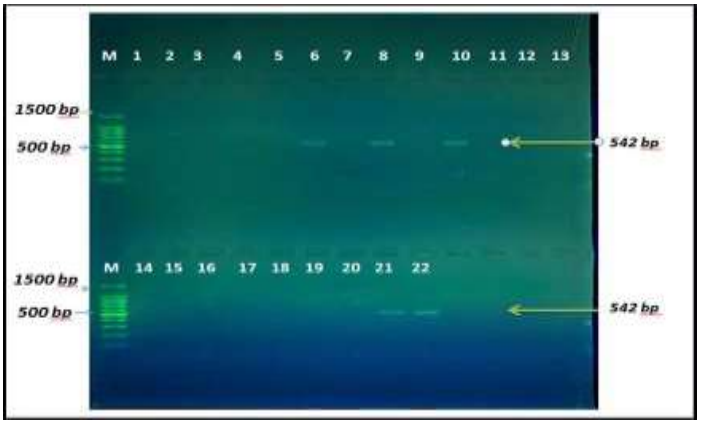

Figure 1. Results of the amplification of *G. lamblia*_18S rRNA gene of stool samples species were fractionated on 1.5% agarose gel electrophoresis M: 100bp ladder marker. Lanes 1,2,4,5 resemble 542bp PCR products.

The difference in the results of the PCR technique may be due to the difference in the methods of extracting DNA from feces samples and the methods of PCR work, and perhaps due to the difference in the number of parasites present in the feces. An error in the microscopic diagnosis of some samples, or the presence of inhibitory substances in some faces may be related to the DNA polymerase enzyme, which inhibits its work and prevents the process of DNA amplification, or it may be due to the time between taking samples

Comparison between microscopic & PCR in the diagnosis of *G. lamblia*

Table7. Distribution *G.lamblia* according to gender by PCR

Gender	No. of examination sample	No. of Positive sample	Percentage %
Male	12	3	25%
female	10	2	20 %
Total	22	5	22.8 %

X^2 calculated = 0,145 fd 1 , X^2 tabular = 3,841
* There are no significant differences

The results of the current study showed that the infection in males is higher than in females by 25% and 20%, respectively, but that there are no significant differences for the sex of the child on the infection with Giardia using PCR technique. This result agreed with the records in AL-Diwaniyah governorate, the higher infection in the male (with percentage of 75%) than in the female (with percentage 70.83%) (26). Also, it agreed with the recorded results in (38), which found that among 70 positive samples by PCR examination, 41 males were infected with a rate of 58.57%, and 29 females were infected with 41.42%. On the other hand , results of Tables (8 & 9) showed a difference in the incidence rate of *G. lamblia* infection among study participant according to age and residence compared the previously obtained result by microscopic examination (39).

Table 8. Number and Percentage of *G.lamblia* according to age group by PCR

Age group / year	No. of sample examination	No. of infected *G.lamblia*	%
< 2 years	5	1	20
2-4	7	3	42.9
4-6	4	1	25
6-8	2	0	0
8-10	3	0	0
10-12	1	0	0
Total	22	5	22.8

X^2 calculated = 5,805 df 5 , X^2 Tabular = 11,070
* There are no significant differences

Table 9. Number and Percentage of G.*lamblia* according to location of residence by PCR

Habitation	No. of sample examination	No. of infected *G.lamblia*	Percentage %
Rural	14	4	28.6
Urban	8	1	12.5
Total	22	5	22.8

X^2 calculated = 0,741 fd 1 , X^2 tabular = 3,841
* There are no significant differences

Because it has a higher sensitivity and specificity than direct microscopy, the Polymerase Chain Reaction (PCR) method is now widely used for giardiasis diagnosis. Furthermore, the PCR method can detect Giardiasis infection in patients with low parasitic cyst counts. Using microscopic diagnostic techniques and PCR at the same time will yield real positive results.

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