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ORIGINAL ARTICLE

## Molecular detection of *Giardia lamblia* genotypes by nested polymerase chain reaction from diarrheic patients in Diyala / Iraq

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### ABSTRACT

**Objective:** Identification of *G. lamblia* genotypes and then determine the role of genotypes in presence of different types of diarrhea.

**Methods:** The populations of this study included 100 patients with different age groups who came to the parasitology laboratory in AL-Batol Hospital, AL-Khalis Hospital, Health Centers in Dali Abbas and Khan Bani Saad in Diyala, suffering from gastrointestinal complaints with acute diarrhea. General fecal samples were collected from them during the period between October 2016 to July 2017.

**Results:** Among 39 fecal samples from patients with giardiasis diagnosed by ELISA, the amplification of these samples showed that 5 (23.80%) contained genotype A and 15 (71.42%) samples contained genotype B, while 1(4.76%) contained mixed A and B genotypes. Regarding to gender, genotypes of *G. lamblia* were more prevalent among males 15/21 (71.42%) than females 6/21 (28.57%), according to age groups differences in distribution of genotypes A and B were found among different age groups of patients. While, according to type of diarrhea *G. lamblia* showed that 12 cases of genotype B isolates were fatty, 3 cases of genotype A were watery/liquid.

**Conclusion:** *G. lamblia* genotype B is the most frequent genotype among patients followed by genotype A, and mix A and B., the highest prevalence of genotype B with male, 2-5 age group and link with watery/liquid diarrhea.

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### INTRODUCTION

*Giardia lamblia* is one of the most important non-viral infections causing diarrheal illness in humans. It is recognized as the most common intestinal protozoan parasite infecting humans in Iraq<sup>1</sup>.

Giardiasis is traditionally considered an epidemic and zoonosis disease between human and animals (farm animals, dogs, cats, birds and rodents)<sup>2</sup>, the infection in humans is usually asymptomatic or mild enough to escape diagnosis, most cases are self-limited, yet

significant acute and chronic infection can occur<sup>3</sup>. Acute infection can produce bloating, crampy abdominal pain and explosive diarrhea, with pale, frothy, steatorrheic stool (foul smelling, greasy stool often mixed with mucus but not blood)<sup>4</sup>. Person-to-person transmission occurs by hand-to-mouth transmit of cysts from the feces of a person infected with *Giardia*. Outbreaks of *Giardia* infections in families and institutions, such as day care centers and nursing homes, especially those

with diapered children, have been associated with fecal-oral route<sup>5</sup>.

Traditionally, the diagnosis of *Giardia* infection is performed through the identification of trophozoites and cysts by microscopy, in fecal samples. However, this parasite presents variable patterns of excretion, which can cause a false-negative outcome<sup>6</sup>. Besides the microscopy, a variety of different diagnostic tests have been reported: immunoassays such as enzyme-linked immunosorbent assay (ELISA), rapid tests (immunochromatographic tests), and the detection of *Giardia* specific genes by conventional polymerase chain reaction (PCR)<sup>7</sup>. *Giardia* isolates are morphologically identical; they vary significantly in their biology, virulence and genetics<sup>8</sup>.

However, molecular methods like PCR are used to classify *G. lamblia* into assemblages and subassemblages. Most studies use tests depend on one or more of genetic loci. However, the use of a various gene, or even a various set of PCR primers, can occasionally assign the same isolate to a different assemblage<sup>9</sup>. It was shown that *G. lamblia* consists of eight assemblages (or genotypes). Only assemblages A and B infect humans. Human infections of assemblage B (~58% of the cases) are more common worldwide compared to assemblage A (~37%)<sup>10</sup>.

The present study was conducted to detection and characterization of *G. lamblia* genotypes and association with gender, age and types of diarrhea.

## MATERIALS AND METHODS

### Patients and samples

The populations of this study included 100 patients with different age groups who came to the parasitology laboratory in AL-Batol Hospital, AL-Khalis Hospital, Health Centers in Dali Abbas and Khan Bani Saad in

Diyala, suffering from gastrointestinal complaints with acute diarrhea. General fecal samples were collected from them during the period between October 2016 to July 2017.

### Diagnosis of stool samples

#### A – Macroscopic examination

Macroscopic examination was depended on consistency, color, odor and presence of blood and mucus in stool samples.

#### B –Microscopic examination

Each fresh stool samples were examined under light microscopic with normal saline and Logules iodine, smears were prepared by add one drop of saline and the other with Logules iodine on clean slides and take small amount from stool by wood sticks from location when found blood and pus, and thoroughly emulsify the stool in saline and logulas iodine, thereafter each slide was covered with a cover slip. Slides were examined fully under the low (10x) and high (40x) powers of microscope.

#### C-Immunological study

The RIDASCREEN® *Giardia* test is any enzyme immunoassay for the qualitative determination of *G.lamblia* in fecal samples performed according to the manufacture's protocol RIDASCREEN® *Giardia* provided by R-Biopharm, Germany.

#### D- Molecular study

##### -DNA extraction from stool samples

The DNA extraction was done by using Accuprep® stool DNA extraction Kit from BioNer with minor modification.

##### -Determination of *G. lamblia* genotypes

Primers: Three sets of primers (Table 1) were used to amplify the fragments of the *G. lamblia* Triose phosphate isomerase (*tpi*) gene.

Table 1: Sequence of primers utilized in the present study

Primary PCR ROUND				
Primer	Sequence	TM°C	GC %	Product size
AL3543	AAA TIA TGC CTG CTC GTC G	55.2	47.4	605bp
AL3546	CAA ACC TTI TCC GCA AAC C	54.9	47.4	
Secondary PCR Round				
Primer	Sequence genotype A			
AssAF	CGC CGT ACA CCT GTC	52.6	66.7	332bp
AssAR	AGC AAT GAC AAC CTC CTT CC	56.8	50.0	
Primer	Sequence genotype B			
AssBF	GTT GTT GTT GCT CCC TCC TTT	58.0	47.6	400bp
AssBR	CCG GCT CAT AGG CAA TTA CA	56.8	50.0	

### PCR programs

A nested PCR was performed to amplify the *tpi* gene, for the primary PCR, a PCR product of 605 bp was amplified by using primer set forward primer AL3543 and reverse primer AL3546 designed by<sup>11</sup>.

PCR amplification mixture was performed in 20 µl final volume with 2 µl of template DNA in PCR PreMix (1 U of Taq polymerase, 250 µM each of deoxynucleoside triphosphate (dNTP), { dATP, dCTP,

dGTP, dTTP}, 10mM Tris-HCl, 30mM KCl, 1.5mM MgCl<sub>2</sub>, stabilizer and tracking dye), 1 µl of each primer, 16 µl distilled water. The thermocycling conditions were as follows:

An initial denaturation step	-95°C for 5 min
35 cycles	-94°C for 45 s -50°C for 45 s -72°C for 60 s
final extension step	-72°C for 10 min

The secondary cycle was performed as separate PCR reactions for all genotype. Undergo further replication using set of separate A and B assemblage specific primers<sup>12</sup>. Found mixed infection was discovered via vision the appearance of band in the agarose, at the 332bp for genotype A multiplied using primer groups forward primer AssAF and reverse primer AssAR and at 400bp for genotype B multiplied using primer groups forward AssBF and reverse product as a template in pre Mix, in total volume 25  $\mu$ l. The thermocycling conditions were as follows:

An initial denaturation step	-94°C for 10 min
35 cycles	-94°C for 45 s -64°C for 45 s - 72°C for 60 s
final extension step	-72°C for 10 min

The amplified products were analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 mg/mL ethidium bromide.

A questionnaire on personal information was prepared.

### Statistical analysis

The Statistical Analysis System- (SAS) was used to influence of different factors in study parameters. The Chi-square-  $\chi^2$  test was used to significant compare between the data. The lower level of accepted statistical significant difference is bellow or equal to ( $p \leq 0.05$ ), and the high significant difference is bellow or equal to ( $P \leq 0.001$ )<sup>13</sup>.

## RESULTS

### Identification of *G. lamblia* genotypes according to the amplification of *tpi* gene

Twenty one samples amplified out of 39 samples of the gene *tpi* (53.84%). However, the amplification of these samples showed that 5 (23.80%) contained genotype A and 15 (71.42%) samples contained genotype B, while

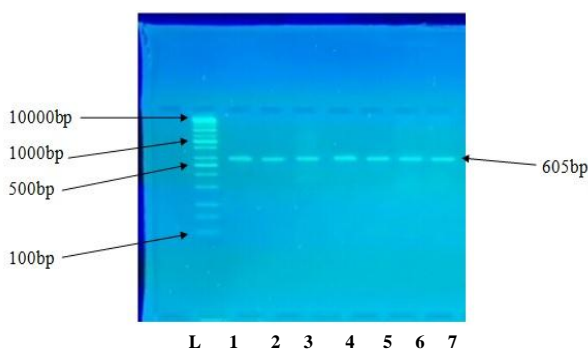


Figure 1: *Tpi* – specific PCR for *G.lamblia* genotyping, first round on 1.5% agarose gel stained with red stain. Lane's L: 100-DNA ladder; and lanes 1-7: PCR products of *G. lamblia* (band 605 bp) from examined samples.

1(4.76%) contained mixed A and B genotypes, the successful amplification of these genotypes was shown in Figure (1, 2 and 3). Statistically, highly significant differences appeared in the distribution of genotypes among giardiasis patients at  $p \leq 0.001$  as shown in Table2.

Table 2: Identification of *G. lamblia* genotypes according to the amplification of *tpi* gene.

Genotypes	No. of samples	%
A	5	23.80
B	15	71.42
A and B	1	4.76
Total	21	100
$\chi^2$ : 22.285		
p-value: < 0001**		
**=Highly significant ( $P \leq 0.001$ ).		

### Distribution of giardiasis genotype groups according to gender and age groups

Regarding to gender, genotypes of *G. lamblia* were more prevalent among males 15/21 (71.42%) than females 6/21 (28.57%); while mixed A and B genotypes were found in male 1/1 (100%).

The present study appeared differences in distribution of genotypes A, B and A&B were found statistically among different age groups of patients. The highest distribution of genotype B was found in 7/15 patients of 2-5 years, then 4/15 of 6-11 years, 3/15 of above 19 year, only 1/15 patient less than 2 years and no successful amplification of this genotype in patients with 12-18 year. Genotype A was found in 2/5 of 2-5 years and only 1 case found for each other patients group and no successful amplification of this genotype in patients with 6-11 year, while mixed A and B genotype infection was found in 2-5 years age group as shown in Table 3.

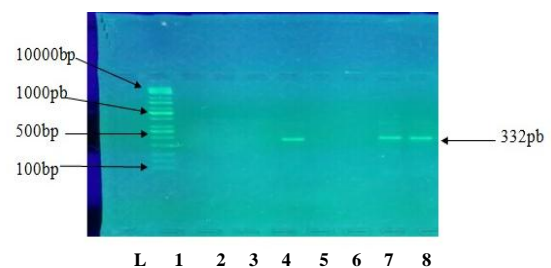


Figure 2: *Tpi* – specific PCR for *G.lamblia* genotyping, second round on 1.5% agarose gel stained with red stain. Lane's L: 100-DNA ladder; and lanes 4, 7,8: PCR products of genotyping A (band 322bp) from examined samples.

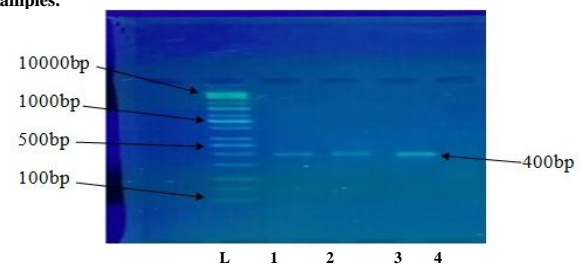


Figure 3: *Tpi* – specific PCR for *G. lamblia* genotyping, third round on 1.5% agarose gel stained with red stain. Lane's L: 100-DNA ladder; and lanes 1-2-3: PCR products of genotyping B (band 400bp) from examined samples.

Table 3: Distribution of giardiasis genotype groups according to gender and age groups of 21 patients.

Gender	Assemblage A	Assemblage B	Mix A and B	Total
No.	5	15	1	21
Male	4 (80%)	10 (66.66%)	1 (100%)	15(71.42%)
Female	1 (20%)	5 (33.33%)	0(0.00%)	6(28.57%)
p-value	0.005 *			
Age groups Stages according to biostatistics				
Less 2 year	1(20%)	1(6.66%)	0(0.00%)	2(9.52%)
2-5 year	2(40%)	7(46.66%)	1(100%)	10(47.61%)
6-11 year	0(0.00%)	4(26.66%)	0(0.00%)	4(19.04%)
12-18 year	1(20%)	0(0.00%)	0(0.00%)	1(4.76%)
Above 19 year	1(20%)	3(20.00%)	0(0.00%)	4(19.04%)
p-value	0.005 *			
*>= significant (P≤ 0.05)				

### Distribution of giardiasis genotypes according to types of diarrhea

The successful amplification of 21 sample of *G. lamblia* showed that 12 cases of genotype B specimens were fatty, 3 cases of genotype A were watery/liquid and only 1 cases of genotype B associated with bloody, while mixed A and B genotypes were fatty (Table 4).

Table 4: Correlation between types of diarrhea and *G. lamblia* genotypes.

Types of diarrhea	Assemblage A	Assemblage B	Mix A+B
Watery/liquid	3 (60.00%)	2 (13.33%)	0 (0.00%)
Fatty	2 (40.00%)	12 (80.00%)	1 (100%)
Bloody	0 (0.00%)	1 (6.67%)	0 (0.00%)
Total	5	15	1

### Discussion

Eight genotypes of *G. lamblia* have been recognized (A-H). Genotyping of a large number of human *G. lamblia* isolates from different parts of the world has demonstrated that humans are almost exclusively infected with assemblage A or B. Among 39 fecal samples from diarrheal patients with giardiasis diagnosed by ELISA, the *tpi* gene was amplified from 21/39 with nested PCR assay, the failures in the amplification of stools samples, may be due to the low quantity of DNA samples, or to their degrading in time, or to the presence of some of PCR inhibitors such as lipid, hemoglobin, cellulose, bile salts, polysaccharides from mucus, bacteria, food degradation product, and handling error<sup>14</sup>.

Data of this study indicated that the amplification percentage of *G. lamblia* genotype B and A for human isolates were 71.42% and 23.80% respectively. The results is in agreement with other studies which showed that an analysis of human *G. lamblia* isolates from different geographical location by PCR amplification of DNA extraction directly from stool samples demonstrates that almost exclusively, only *G. lamblia* assemblage A and B are associated with human infections<sup>15</sup>. The predominance of genotype B in

patients with diarrhea in present study is in agreement with a study done by AL-Barghash, (2007)<sup>16</sup> reported that the prevalence of assemblage A and B found in 17 human *G. lamblia* isolates recorded 29.4% and 70.5% respectively, which give similarity to the results of this study. This may be related to that patients with assemblage B show a greater rate of elimination of cysts, this could have resulted in a higher transmission rate and consequently higher incidence of infection in the population studied, in which assemblage B was predominant<sup>17</sup>. This observation is in accordance with most large-scale studies where the distribution of assemblage B was more commonly identified in developing (58%) than in developed countries (55%) and at a higher prevalence than assemblage A (37% versus 40%)<sup>18</sup>. Assemblage A is also known to most often responsible for the zoonotic transmission with a broad range of animals offered as reservoir hosts, although assemblage B probably transmission from human to human, it has been reported in some animals and may also be an animal potential<sup>19</sup>.

In the present study, *G. lamblia* assemblage A and assemblage B were detected together; whereas this mixture of these assemblages has been reported previously in other studies<sup>20,8</sup>. The causes may be due to several assemblages of *G. lamblia* reflects the complex circulation of the parasite in the environment and the exposure of humans to multiple sources<sup>21</sup>. The reliable detection of cases of mixed infections is influenced by several factors, including the proportion of each assemblage in the specimen and biased amplification efficiencies of one assemblage over the other<sup>22</sup>. This result agreement with study in Al-Qadisiya Governorate, Iraq that found assemblage B (68%), assemblage A (12%) and mix infection (20%)<sup>23</sup>, and in England show assemblage B (64%), assemblage A (33%) and mix infection (3%)<sup>24</sup>. The differences in social and environmental condition might have contributed to the variations in the distribution of *G. lamblia* assemblages<sup>25</sup>. Therefore, the detection these factors may be lead to control on the parasitic infections.



The current study showed that *G. lamblia* of both genotype A and B more prevalent among males than females, males were more exposed to giardiasis due to spending most of their time out the house and may be drink the water that may be contaminated with *Giardia* cysts, therefore they would be more exposed to infection<sup>23</sup>.

Interestingly, the study showed that children age range from 2 to 5 and 6 to 11 years were at higher risk of being infected with genotype B. This finding was consistent with other study that detected this age group as a high risk group for giardiasis<sup>26</sup>. This result was also in agreement with worldwide report suggesting that giardiasis is one of the serious health problems among population of younger age groups<sup>27</sup>. However, this may be related to the fact that children are susceptible to both genotypes with variability in predominance from one region to other. The susceptibility could be related to the practicing inappropriate personal hygiene. Lacking in effective immunity has also been postulated to explain this age specific manner. Interestingly, the study revealed a strong associated between infection with assemblage B and young age group than genotype A, compared to previous studies conducted in different countries, children were found to be at risk of both types of assemblage, with contrast in the pattern over the other from country to country<sup>28</sup>. This may be related to the reality that genotype B differs from genotype A in a diversity of biological important ways. B more infectious, pathogenic and associated with diarrhea than A. The predominance of genotype B is in agreement with other studies<sup>29,30</sup>.

However, the study showed that assemblage B associated with fatty diarrhea (80%), this result agreement with other studies in Egypt and Iraq that found assemblage B high associated with fatty diarrhea<sup>29,31</sup>. While assemblage A associated with watery diarrhea (60%). Finally mix genotypes infection found only in fatty diarrhea. In generally fatty diarrhea found in all *G. lamblia* assemblage, due to poor absorption of fat, fat-soluble and vitamins may happened, when every day losses fat in stool more than 7 gm, case is classified as a steatorrhea<sup>32</sup>. While, one case of assemblage B giardiasis associated with bloody diarrhea, that may be indication of co-infection with *E. histolytica* \ *dispar*, this parasites attacking and destroying the lining of the intestines<sup>33</sup>.

## Conclusions

*G. lamblia* genotype B is the most frequent genotype among patients followed by genotype A, and mix A and B. Also factors of age, gender, and types of diarrhea are genotypes specific.

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