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Molecular Identification of *Giardia lamblia* Genotypes Isolates from Children with Diarrhea

Rawaa A. Hussein[1] *MSc*, **Nada T. Al-Bashier**[2] *PhD*, **Areej A. Mohamed**[3] *CAPB*

[1]Dept. of Microbiology, College of Medicine, University of Diyala, Iraq. [2]Medical Research Unit, [3]Dept. of Pediatrics, College of Medicine, Al-Nahrain University, Baghdad, Iraq.

Abstract

Background Infection with *Giardia lamblia* (*G. lamblia*) parasite regarded as the most important causative agent for diarrhea, and a major public health problem.

Objectives Molecular identification and characterization of *G. lamblia* genotypes and association with gender, age and presence of different clinical signs.

Methods One hundred children with diarrhea were included. Fecal samples were taken from them during the period from May 2014 to February 2015. The age range was 2 months to 18 years. All stool samples were examined by microscopic examination, multiplex real time polymerase chain reaction and nested PCR.

Results Among 42 fecal samples from patients with giardiasis diagnosed by multiplex real time PCR, amplification of triosphosphate isomerase gene of *G. lamblia* was successful among 25/42 (59.52%) samples. However, the amplification of these samples showed that 7 (28%) contained genotype A and 18 (72%) samples contained genotype B; genotype B was more prevalent than A in males 11/18 (61.11%) and females 7/18 (38.89%) respectively. Regarding age, the results showed that no differences in distribution of genotypes were found statistically among different age groups of patients. Regarding to clinical aspects, the rates of abdominal pain, weight loss, anorexia and fever of giardiasis genotype B are higher than the genotype A.

Conclusion *G. lamblia* genotype B is the most frequent genotype among children with diarrhea. Also, the presence of the clinical aspects is genotype specific.

Keywords *Giardia lamblia*, children with diarrhea, and molecular identification.

List of Abbreviations: *Giardia lamblia = G. lamblia,* triosephosphate isomerase = tpi, Polymerase Chain Reaction = PCR and Multiplex Real-Time PCR = Multiplex RT PCR.

Introduction

The protozoan *Giardia* is an intestinal parasite that can infect many species in the animal kingdom including mammalian, avian, reptilian, domesticated animals, and human [(1)]. *Giardia spp*. is unique in their possession of two nuclei that are identical in appearance, DNA content, transcription and time of replication. There are 5 chromosomes per haploid genome. The genome shows little evidence of heterozygosity, supporting that *Giardia* is sexual [(2)].

In human, it can cause gastrointestinal infections ranging from mild to severe as well as chronic disease [(3)]. *Giardia lamblia* (*G. lamblia*) is typically characterized in human by diarrhea, steatorrhea, maldigestion, abdominal cramps, bloating, malabsorption and weight loss. 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 [(4)].

Microscopic diagnosis of this protozoan is neither sensitive nor specific. Studies have found that excretion of trophozoites or cysts in the feces can be intermittent and therefore could lead to missed infections due to the low numbers of the diagnostic stages in the feces [5], other diagnostic methods have been developed such as the Enzyme-linked immunosorbent assay, culture and the Polymerase Chain Reaction (PCR). However, molecular methods like PCR are used to classify *G. lamblia* into assemblages (genotype) and subassemblages (subgenotype). Most studies use tests depend on one or more of genetic loci: Small SubUnit Ribosomal RNA, elongation factor 1 alpha, triosephosphate isomerase (tpi), glutamate dehydrogenase and β-giardin genes. 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 [6].

A large amount of data has shown that *G. lamblia* should be considered a species complex whose types show little variation in their morphology still can be assigned to at least eight distinct assemblages (A to H) depend on genetic analyses [7]. The assemblages A and B are important in human infection; they also can infect other mammals [8]. Interestingly, several reports have suggested a role of the assemblages for the presence and the severity of *G. lamblia* infections [9,10]. However, some studies found a stronger relationship with assemblage B [11,12]. Also, other study showed that assemblage B exhibited more extensive association with persistent symptoms, while assemblage A was found in connection with intermittent diarrhea [13].

The present study was conducted to do molecular identification and characterization of *G. lamblia* genotypes and association with gender, age and presence of different clinical signs.

Methods

Patients and samples: One hundred patients were included in the current study, who were examined and interviewed by pediatric physician and attend to the Parasitology Laboratory in Al-Imamain Al-Kadhimain Medical City in Baghdad, suffering from diarrhea with different gastrointestinal complaints by asked them about different clinical aspects.

General fecal samples were taken during the period from May 2014 to February 2015. The age range was 2months to 18 years.

Stool samples examinations

1-Macroscopical Examination

Samples were noticed in terms of consistency, color, odor and presence of blood and mucus.

2- Microscopical Examination

-Direct Method

From each fecal sample, smears with normal saline and lugol's iodine were prepared. Two direct smears were examined from each fecal sample, by preparing two clean dry microscope slides, one with normal saline and the other with lugol's iodine agents. By using clean wood stick, the fecal specimen was touched in various sites, especially where streaks of blood or pus were noticed, then mixed thoroughly with each drop of normal saline and lugol's iodine agents on the prepared slides, then each slide was covered with a cover slip. The smear was examined thoroughly under the low (x10) and high (x40) powers of the microscope.

3. Molecular study

DNA-extraction

The DNA extraction of *G. lamblia* was performed according to the manufactures protocol of AccuPrep® Stool DNA extraction Kit provided by Bioneer/Korea.

Multiplex Real-Time PCR

Direct, qualitative detection and differentiation of *G. lamblia* was performed according to the manufacture's protocol of RIDA®GENE Parasitic

Stool Panel is a multiplex RT- PCR kit provided by R-Biopharm/Germany**.**

Determination of *G. lamblia* genotypes

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 Sulaiman *et al*., (2003) [(14)].

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}, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$, 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 round was performed as separate PCR reactions for each genotype. Underwent further amplification using a set of separate A [(15)] and B [(16)] assemblage specific primers Presence of mixed infection was detected by visualizing the occurrence of bands in the agarose gel 1.5%, at 332 bp for assemblage A amplified using primer sets forward primer AssAF and reverse primer AssAR and at 400 bp for assemblage B amplified using primer set forward primer AssBF and reverse primer AssBR as shown in Table 1.

PCR amplification mixture was performed in 50 µl final volume with 10 µl of primary PCR product as a template DNA in PCR PreMix (2.5 U of Taq polymerase, 250 µM each of deoxynucleoside triphosphate (dNTP), {dATP, dCTP, dGTP, dTTP}, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye), 4 µl of each primer, 32 µl distilled water.

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.

Table 1. Primers used in the present study for amplification of fragments of the *G. lamblia* tpi gene.

Primary PCR Round		
Primer	**Sequence 5'-3'**	**Product**
AL3543	AAA TIA TGC CTG CTC GTC G	605 bp
AL3546	CAA ACC TTI TCC GCA AAC C	
Secondary PCR Round		
Primer	**Sequence 5'-3'**	**Product**
Genotype A		
AssAF	CGC CGT ACA CCT GTC	332 bp
AssAR	AGC AAT GAC AAC CTC CTT CC	
Genotype B		
AssBF	GTT GTT GTT GCT CCC TCC TTT	400 bp
AssBR	CCG GCT CAT AGG CAA TTA CA	

Questioner performance

A questionnaire was prepared, asking patients (or their parents) about their clinical symptoms and habitation, besides other questions about hygienic habits.

The study was approved by the Ethical committee of the College of Medicine, Al-Nahrain University.

Statistical analysis

The Statistical Analysis System (SAS, 2012) was used to show the influence of different factors in study parameters. The Chi-square- x^2 test was used to compare between percentages. The lower level of accepted statistical significant difference is below or equal to ($p \leq 0.05$), and the high significant difference is below or equal to ($P \leq 0.001$) [(17)].

Results

Identification of *G. lamblia* genotypes

Among 42 fecal samples from patients with giardiasis diagnosed by multiplex RT PCR, amplification of tpi gene of *G. lamblia* was successful among 25/42 (59.52%) samples. However, the amplification of these samples showed that 7 (28%) contained genotype A (Figure 1) and 18 (72%) samples contained genotype B (Figure 2). Statistically, significant differences appeared in the distribution of genotypes among giardiasis patients at p≤ 0.05 as shown in table (2).

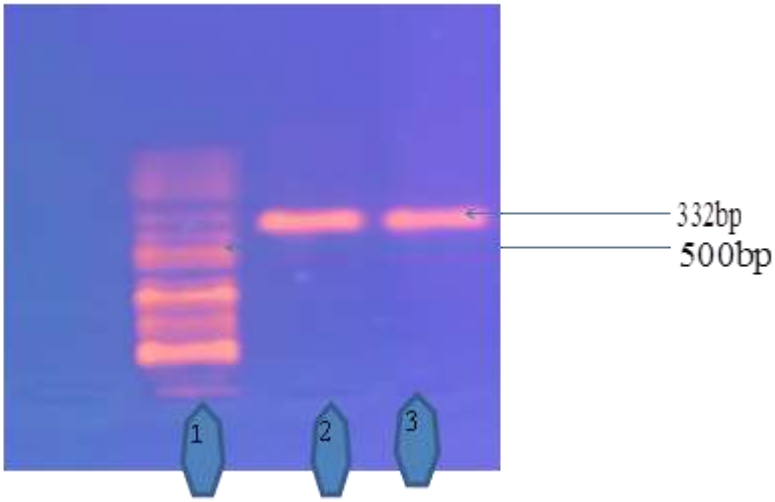

Figure 1. Agarose electrophoresis of PCR amplification for tpi gene (332bp). Lane 1 represents DNA ladder (100bp), Lane 2, 3 represent PCR product of genotype A from examined samples. Fragments were resolved on 1.5% agarose gel and visualized by ethidum bromide staining.

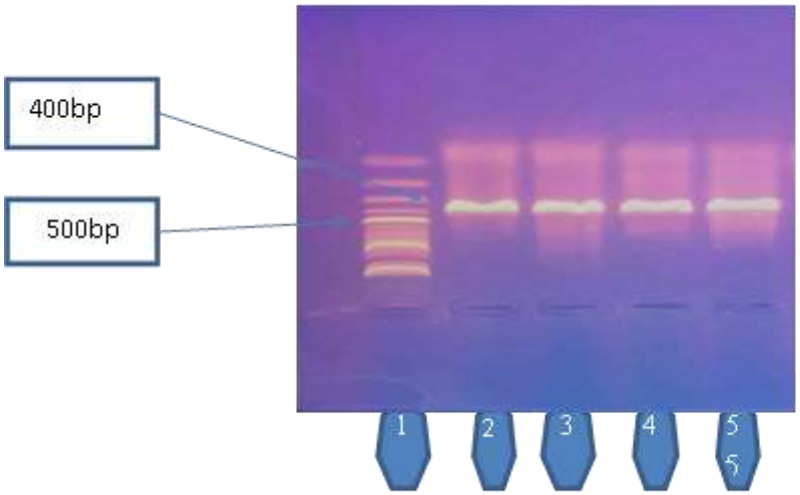

Figure 2. Agarose electrophoresis of PCR amplification for tpi gene (400bp). Lane 1 represents DNA ladder (100bp), Lane 2,3,4,5 represent PCR product of genotype B from examined samples. Fragments were resolved on 1.5% agarose gel and visualized by ethidum bromide staining.

Table 2. Identification of *G. lamblia* genotypes according to the amplification of tpi gene by nested PCR in 25/42 examined samples

Genotype	No. of samples	%
A	7	28
B	18	72
Total	25	100
	***P* = 0.027**	

Characteristics of giardiasis genotypes groups

In regarding to gender, genotypes A and B of giardiasis were more prevalent among males 16/25 (64%) than females 9/25 (36%) as shown in Table (3).

Regarding to age groups, the results showed that no differences in distribution of genotypes were found statically among different age groups of patients. The highest distribution of genotype B was found in 11/18 patients of 2-5 years, 4/18 of 6-11 years, 2/18 of 12-18 years and only 1/18 patient less than 2 years. While genotype A was found in 4/7 of 2-5 years and only 1/7 found in other patients groups, as illustrated in table (4).

Table 3. Distribution of giardiasis genotypes according to gender of 25 patients

Genotype		Male		Female	
		No.	%	No.	%
A	**7/25**	5	74.4	2	28.57
B	**18/25**	11	61.11	7	38.89
		***P* = 0.202**			

Table 4. Distribution of giardiasis genotypes according to age groups of 25 patients

Genotype		2 years		2-5 year		6-11 year		12-18 year	
		No	%	No	%	No	%	No	%
A	**7/25**	1	14.28	4	57.14	1	14.28	1	14.28
B	**18/25**	1	5.55	11	1.11	4	22.22	2	11.11
				***P* = 0.875**					

The clinical aspects among giardiasis patients A and B genotype groups

Regarding clinical aspects, abdominal pain, weight loss, anorexia and fever were statistically of significant differences between the genotype A and B of giardiasis patients, while flatulence, fatigue and vomiting were not significant between genotypes groups as shown in table (5). On the other hand, most the rates of clinical aspects of giardiasis genotype B are higher than the genotype A.

Discussion

Human giardiasis is caused by two distinct genetic groups of *G. lamblia* genotypes A and B. Both assemblages are present associated with human infection globally and have also been detected in different animals. Among 42 fecal samples from diarrheal patients with giardiasis, the tpi gene was amplified from 25/42 (59.52%) with nested PCR assay. The amplification percentages of the tpi gene from stool specimens agree and disagree with studies of several authors. In Egypt, the tpi gene was amplified from 41 (42.3%) fecal samples [13]. Among 26 fecal samples from patients with sporadic giardiasis diagnosed by hospital laboratories, the tpi gene was amplified from 25 (96%) samples [18].

The failures in the amplification of some stool specimens may be derive from the low quantity of specimens DNA, either related to their degrading in time or may be existence of some of PCR inhibitors such as lipids,

hemoglobin, bile salts, polysaccharides from mucus, bacteria, and food degradation product) [19,20].

In the present study, *G. lamblia* assemblage A and assemblage B were never detected together, whereas a mixture of these assemblages has been reported previously in a other studies [21,22].

The predominance of genotype B in patients with diarrhea in present study is agreement with recently study in Baghdad of AL-Obaidi (2014) who showed that (75%) genotype B and (15.62%) genotype A while (9.37%) mixed genotype [23].

However, *Giardia* genotype B was detected to be the only genotype identified in both diarrheal and non-diarrheal children. The significance of this finding is that, children are getting infected via, human-to-human transmission, as the genotype B has been found to be exclusively anthroponotic [24,25]. Among Saudi children, all assemblage B reported was associated with diarrhea [26]. Guinea-Bissau showed predominance of genotype B in diarrheal cases [27].

This may be related to that the most studied genotype B isolates, differs from genotype A isolates in a diversity of biological important ways; host infectivity, metabolism and growth requirements [28]. Beside, in human volunteer studies, B consistently infected normal volunteers, while A was unable to stimulate infections. B was more pathogenic than A in gerbils [29].

Table 5. Correlation between clinical aspects of *G. lamblia* genotype A and genotype B

Clinical aspects	A	%	B	%	*P* value
Abdominal pain	1/7	14.29	15/18	83.33	0.003
Weight loss	1/7	14.29	13/18	72.22	0.008
Flatulence	3/7	42.86	8/18	44.44	0.942
Fatigue	3/7	42.86	10/18	55.56	0.568
Anorexia	1/7	14.29	12/18	66.67	0.018
Fever	2/7	28.57	13/18	72.22	0.045
Vomiting	0/7	0.00	6/18	33.33	0.079

The diversity of *G. lamblia* genotype among the studied populations could be related to different modes of transmission in each area, comprising human to human, foodborne, waterborne or zoonotic transmissions. It has been also found that these variation in the prevalence of genotypes may be attributed to the geographical location, but this finding could indicate a possible risk of waste exposure to the origin of drinking water, and in addition the life style of the studied population who may be in close contact with animal wastes, especially in rural regions [30].

It has been known that genotype A is most often responsible for zoonotic transmission with wide range of animals presenting as reservoir hosts. Although genotype B is most likely transmitted from human to human, it has been reported in some animals and may represent a zoonotic potential as well [31]. However, assemblage B isolates are more infectious [29,32].

Interestingly, identification of different genotypes that contribute to disease enables differentiation in host specificities, transmission methods and sources of infection [33]. Therefore, it may lead to control on the parasitic infections.

The present study showed that *G. lamblia* of both genotypes A and B more prevalent among males than females. Statistically, no significant difference between assemblages' distribution and gender was found. Similar findings were reported by Anthony *et al*. in 2007, which showed no significant difference between genotypes prevalence and gender in

Philippines [34]. On the other hand, Mohammed Mahdy *et al*. in 2009 showed that females were at two fold higher risk of acquiring giardiasis of assemblage B compared to males [35]. Also, 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 Mohammed Mahdy *et al*. (2009) and Sadek *et al.* (2013) that detected, this age group as a high risk group for giardiasis [35,36]. 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 [37]. 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 effective immunity has also been postulated to explain age specific manner.

The significant differences between genotypes and clinical symptoms of gastroenteritis (abdominal pain, weight loss, anorexia and fever). The results correlate genotype B with high rates of symptomatic giardiasis than genotype A, at the same time, the current study found that assemblage B of *G. lamblia* presents with all kinds of clinical features ranging from mild to severe. Similarly, Mohammed Mahdy *et al*. (2009) and Pelayo *et al*. (2008) showed that genotype B was more significantly among symptomatic patients [35,38]. This is due to alternations in the nutrient absorption caused by *G. lamblia,* and the association between *G. lamblia* genotype A and B in the development of clinically overt diarrhea and other gastrointestinal symptoms [39].

In contrast, other studies reported a significant association between assemblage A and the presence of symptoms and genotype B and asymptomatic giardiasis [9,40]. On the other hand, Eligio-Carcia *et al*. (2002) reported there was no correlation between digestive manifestations and genotypes [41]. While other study showed that genotype A and B exhibit no apparent differences in virulence, suggesting that host factors more than type of *G. lamblia* genotypes play a dominant role in determining the clinical symptoms of the infection [42].

Recent advances suggest that variability in *Giardia* strains, host nutritional status, the composition of microbiota, co-infecting enteropathogens, host genetically determined mucosal immune responses, and immune modulation by *Giardia* are all relevant factors influencing disease manifestations after *G. lamblia* infection [43].

This study concluded that G.lamblia genotype B is the most frequent genotype among children with diarrhea. Also, the presence of the clinical aspects is genotype specific.

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Author contributions

Dr. Al-Bashier put the study concept and design; Dr. Mohamed did the physical examination and diagnosis; and Hussein collect stool samples, did the laboratory analyses and preparation of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Corresponding to Rawaa A. Hussein
Email: rawaa.abd1978@gmail.com
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