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Genotype Analysis of *Giardia lamblia* Isolated From Children in Ahvaz, Southwest of Iran

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A B S T R A C T

Background: *Giardia lamblia* is an enteric protozoan parasite, which infects human and a wide range of vertebrate hosts.
Objectives: The aim of this study was to investigate genotypes of *G. lamblia* from children fecal samples in Ahvaz, South West of Iran by PCR-RFLP method.
Materials and Methods: Fecal samples were collected from 58 children who were positive for *G. lamblia*. DNA extractions were performed by QIAamp Stool Mini Kit. DNA were evaluated by semi nested PCR-RFLP assay, targeting the glutamate dehydrogenase (gdh) gene, which was used to distinguish within and between genotypes A and B.
Results: Fifty samples (86%) were confirmed by semi-nested PCR. Genotype analysis among 50 isolates indicated 5 (10%) and 8 (16%) assemblages AII and B, respectively. Mixed Infections with both assemblages AII and B were also detected in 37 (74%) cases.
Conclusions: Current study indicated the molecular characterization of *G. lamblia* in southwest of Iran. Postulated sources of contamination by accidental discharge of sewage effluent and faecal contamination from animals may contribute to this high rate of mixed infection in the current study. Further studies are needed to underestand the source and route of infection better.

Keywords: Giardia lamblia, Glutamate dehydrogenase (gdh), Semi nested PCR-RFLP, Ahvaz, Iran

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▸Implication for health policy/practice/research/medical education:
Results of current study indicated the genotypes of *Giardia lamblia* that would help us to understand the route of infection better.

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1. Background

Giardia lamblia is an intestinal parasite found in a wide range of mammals, including humans, and is considered as a zoonotic agent by the WHO (1, 2). It is one of the most frequent gastrointestinal pathogens in children that may cause malabsorption and weight loss (3). It has a global distribution in developed and developing countries, especially, in poor health conditions and over crowded papulations (4). The rates of infection among humans in different areas of the world vary from 5% to 43% (4). *Giardia*infection rates have been reported 5% - 23% in different parts of Iran and recently 9.1% infection has been reported in rural areas of Khuzestan province, South west of Iran (5, 6).

Based on molecular studies, a variety of genotyping techniques, including PCR-RFLP and sequence analysis of the *gdh, tpi, efla* and *18SrDNA* gene have shown that *G. lamblia* is composed of a range of diverse genotypes A to G (7, 8). Assemblages A and B have been reported in humans and a broad range of other hosts, including livestock, cats, dogs, beavers and wild mammals (8-11). Assemblage A has been further grouped into subtypes I and II. The assemblage B has been classified into subtypes III and IV. Assemblages AII and BIV are considered to be specific for human (8-11). Assemblages C, D, E, F and G have been reported only in domestic animals, livestock and wild animals (10). PCR-RFLP is used as a sensitive, simple,and rapid method, and it has been successfully used by a number of researchers for Giardia genotyping (12-14). In addition, PCR-RFLP is advantageious for its ability to detect and identify the presence of mixed genotypesclearly. The *gdh* gene has been used successfully to genotype isolates of *G. lamblia* from mammals (13-15).

2. Objectives

The present study aimed to determine the genotypes of *G. lamblia* isolates from infected children cases in Ahvaz, southwest of Iran by a PCR-RFLP assay on the organism's glutamate dehydrogenase (*gdh*) gene.

3. Materials and Methods

3.1. Collection and Purification of Cysts

Giardia-positive fecal samples were collected from children less than 15 years old who were referred to Ahvaz health centers clinics for medical examinations, during September 2011 to July 2012. Positive fecal samples were transferred to the Department of Parasitology, School of Medicine, Ahvaz University of Medical Sciences. The positive samples were confirmed by wet smear stained with Lugol's iodine and formalin ether using light microscope at 400 × magnification (16). Fifty eight positive fecal samples for *G. lamblia* cysts were used in the study. The intensity of infection was estimated by average cyst count per high power field (HPF) of light microscope. Samples` scores were divided into three categories: 1-5 (1+), 6-10 (2+) and more than 10 cysts (3+). All samples were stored as whole feces at 4°C without preservatives until used. Cysts were purified from the faeces by sucrose density gradient centrifugation and washed with sterile distilled water and then stored at -20 °C until used (17).

3.2. DNA Extraction

G. lamblia cysts were 10 times freez-thawed on -80°C/+80°C. Subsequentely, DNA was extracted from 200 µl of purified samples by the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. DNA samples were stored at -20°C until used.

3.3. PCR Amplification

The amplification of the *gdh* gene was performed by a semi-nested PCR protocol. In the semi- nested PCR reaction, a fragment of approximately 432 base pairs of the *gdh* gene were amplified with external forward primer GDHeF (TCA ACG TYA AYC GYG GYT TCC GT), internal forward primer GDHiF (CAG TAC ACC TCY GCT CTC GG), and reverse primer GDHiR (GTT RTC CTT GCA CAT CTC C; (15). The primers were tested with positive control. The positive control for *G. lamblia* was received from a colleague in Kerman University of Medical Sciences. Distilled water was used as negative control.

The PCR reaction mixture consisted of 5 µl genomic DNA, 5 µl of 10X buffer (Fermentas, Lithuania), 1.5 µl (50mM) MgCl2, 1 µl (10 mM) dNTP mix , 0.3 µl (5U/ µl) *Taq* DNA polymerase, and 1 µl (12.5 pmol) of each primer. The reactions were carried out in 50 µl volumes. GDHeF and GDHiR were used in the primary PCR reaction. One microliter of the PCR primary reaction product was added to the secondary PCR containing primers GDHiF and GDHiR. PCR was carried out on a thermocycler (iCycler, BioRad) with the following amplification condition: 1 cycle of 94 °C for 3 min, 56 °C for 1 min and 72°C for 2 min, followed by 35 cycles, 94°C for 1min, 56 °C for 20 s and 72°C for 45 s. A final extension of 72 °C for 7 min and a 20°C hold was used (15). The PCR products were electrophoresed on ethidium bromide stained 1% agarose gels (Roche, Germany).

3.4. PCR-RFLP of Region of gdh Gene

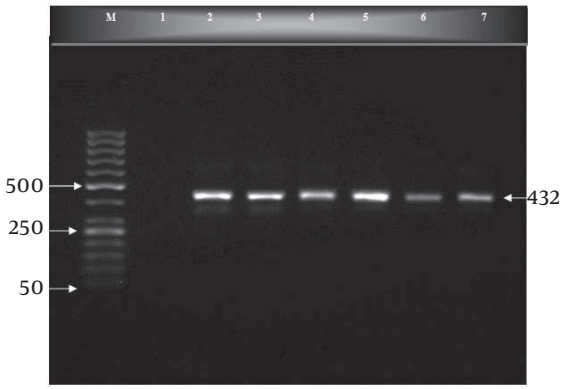
RFLP analysis was carried out by digesting 10 µl of PCR product. It was added to 1 X enzyme buffer, and 2 µl (10 U/ µl) *BspL1* (*NlaIV)* enzyme (Fermentas, Lithuania) or 2 µl (10 U/ µl) *Rsa1*enzyme (Roche, Germany) for 16 h at 37°C. The final volumes of *Rsa1* and *BspL1* enzymes were 25 and

30 µl, respectively. The *BspL1* enzyme digestion was used to distinguish between groups I and II of assemblages A and assemblages B. *Rsa1* enzyme digestion distinguished between subtypes BIII and BIV (15). Restriction fragments were separated in 3% high resolution grade agarose (Roche, Germany) stained with ethidium bromide. A 50bp DNA ladder (Fermentas, Lithuania) was used as size marker.

4. Results

The *gdh* gene was successfully amplified from 50 samples. A 432 bp fragment of *gdh* gene was amplified in the PCR using primers GDHiF and GDHiR (*Figure 1*). PCR products of these samples were digested with *BspL1* and *Rsa1*enzymes.

Figure 1. PCR Products on An Ethidium Bromide-Stained 1% Agarose Gel



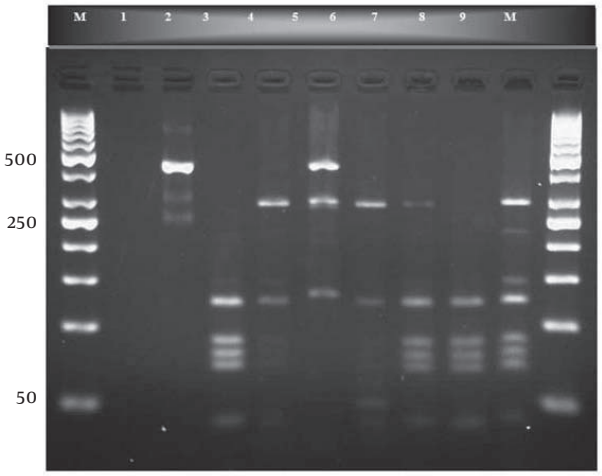
lane M, molecular weight marker (50 bp); lane 1, negative control; lane 2, positive control; lanes 3-7, PCR products from clinical samples.

The predicted restriction fragment sizes are provided in *Table 1* . Thirty seven (74%) cases generated six bands of 40, 70, 80, 90, 120 and 290 bp, which indicated mixed genotypes AII and B. Eight (16%) samples generated two bands of 130 and 300 bp indicating assemblages BIII. Five (10%) samples revealed four bands of 70, 80, 90 and 120 bp, indicating assemblage AII (*Figure 2* and *Table 2*). Assemblages AI and BIV were not detected in the study.

Table 1. Predicted Fragment Sizes (bp) of *G. lamblia* Assemblages After Digesting With BspL1 and Rsa1 Enzyme

Assemblage	Enzyme	Predicted fragment Sizes	Diagnostic genotyping profile
AI	*BspL1*	16, 18, 39, 87, 123, 149	90, 120, 150
AII	*BspL1*	16, 18, 39, 72, 77, 87, 123	40,70, 80, 90, 120
B	*BspL1*	18, 123 291	120, 290
BIII	*Rsa1*	2,133, 297	130, 300
BIV	*Rsa1*	I2, 430	430

Figure 2. Enzyme Digestion of gdh- PCR Products of *G. lamblia* on An Ethidium Bromide-Stained 3% High Resolution Grade Agarose Gel.



Lane M, molecular weight marker (50 bp). Lane 1, negative control. Lane 2 PCR product (432 bp fragment). Lane 3, Bspl1 enzyme digestion of *G. lamblia* standard strain (genotype AII). Lanes 4,6,7,9 mixed genotype AII and B. Lane 5, Assemblage B group BIII (Rsa1 enzyme digestion) and Lane 8, assemblage AII (Bspl1 enzyme digestion).

5. Discussion

G. lamblia , distributed globally, is the most common intestinal parasite of humans in the developed and developing countries (4, 18). This protozoa is recently included in the 'Neglected Diseases Initiative' by the WHO (19). The routine diagnostic method for giardiasis is the microscopic detection of *Giardia* cysts in stools. Molecular analyses such as PCR provided alternative methods to diagnose *G. lamblia* with greater sensitivity than microscopic examination (20-22). The present study indicated that 86% (50/58) microscopic positive samples were identified by seminested PCR. Eigth (14%) samples with low numbers of cysts were negative after seminested PCR. Bertrand *et al.* (23) reported 19.3% false negative result when the *gdh* gene was amplified by nested PCR. In a study conducted by Amar *et al.* (12) the tpi gene was amplified by semi-nested PCR and the study indicated 6% false negative results. Although there is no clear explanation for such false negative results, it could be due to low DNA level, or existence of a robust wall that inhibits release of DNA from the cystes.

the current study results indicated a mixture of assemblages AII and B in 74% (37/50) of samples. Assemblages B and A were detected in 16% (8/50) and 10% (5/50), respectivly. A few studies are done in Iran on *G. lamblia* genotypes, Babaei *etal.* (24) reported 87% assemblages AII and 8.7% assemblage B in Tehran. Another study in Kerman, South east of Iran indicated simillar frequency results in-

cluding 60% assemblage AII, 23.4% assemblage B and 16.6% assemblage AI (25).

reported by Etemadi *et al.* (25) and Hatam-Nahavandi *et al.* (26) where not detected in the current study.

Table 2. All Data About Laboratory Outcomes and Assemblages of *G. lamblia* Isolates Examined by RFLP Analysis at the gdh Gene in Children Cases

Case No.	Stool Microscopy	*Gdh* RFLP	Case No.	Stool Microscopy	*Gdh* RFLP
1	Cyst	AII+B	26	Cyst+Trophozoite	AII+B
2	Cyst	AII+B	27	Cyst	AII+B
3	Cyst	AII+B	28	Cyst	AII+B
4	Cyst	B	29	Cyst	AII+B
5	Cyst	AII+B	30	Cyst	AII+B
6	Cyst	AII+B	31	Cyst	AII+B
7	Cyst	AII+B	32	Cyst	AII+B
8	Cyst	AII+B	33	Cyst+Trophozoite	AII+B
9	Cyst	AII+B	34	Cyst	AII
10	Cyst	AII+B	35	Cyst+Trophozoite	B
11	Cyst	AII	36	Cyst	B
12	Cyst	AII+B	37	Cyst	B
13	Cyst	AII+B	38	Cyst	AII
14	Cyst	AII+B	39	Cyst	B
15	Cyst	AII+B	40	Cyst	AII
16	Cyst+Trophozoite	AII+B	41	Cyst	AII+B
17	Cyst	AII+B	42	Cyst	AII+B
18	Cyst	B	43	Cyst+Trophozoite	AII+B
19	Cyst	AII+B	44	Cyst	AII+B
20	Cyst	AII+B	45	Cyst	AII
21	Cyst	B	46	Cyst	AII+B
22	Cyst	AII+B	47	Cyst	AII
23	Cyst	AII+B	48	Cyst	B
24	Cyst	AII+B	49	Cyst	AII+B
25	Cyst	AII+B	50	Cyst	AII+B

Results of a study conducted in the North west of Iran, indicated 66.7% assemblage B and 33.3% AII (26). Most of researches from different parts of the world have reported the assemblages A and B as the most prevelant *G. lamblia* genotypes in human infection.

In Egypt Helmy *et al.* (27) found 58.5% AI, 17.1% AII and 19.5% genotypes B among 41 patients. In Saudi Arabia, Hamdan (28) reported that in 40 gastrointestinal symptomatic and asymptomatic Saudi children57.5% were assemblages A and 37.5% were assemblage B. On the other hand, results of a study conducted by Singh *et al.* (29) in Nepal indicated that assemblage B (74%) was more prevalent than assemblage A (20%). In Brazil, Kohli *et al.* (30) reported 74.1% and 5% assemblages B and A , respectively among 47 children. Regarding types of assemblages, results of the current research is in agreement with Babaei *et al.* (24). In contrast, assemblages AI and BIV which were

According to the current study literature review, it seems that the rate of mixed infection with genotypes AII and B in the current study report is higher than those of others (1, 29, 31-33). In addition, mixed infection which was predominant in South west of Iran, was only reported by Babaei *et al.* (24) with the lowest prevalence in their report. It has been also reported that these differences in the prevalence of assemblages may be attributed to the geographical location, but this finding could indicate a potential risk of waste exposuer to the source of drinking water, and in addition the life style of the studied population who may be in close contact with animal wastes, specially in rural areas.

In conclusion, this multiple infection may reflect ingestion of contaminated sources by human and animals wastes. Furthermore patients may be repeatedly and cumulatively exposed to the parasite due to poor sanitation in the

studied area. Finally it seems that significance of the predominant mixed infection in the current study is unclear and further studies with a variety of enviromental samples are needed in order to examine, and figure out the dynamics and transmission route of *Girardia* better.

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All authors declare that they have no conflict of interest.

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Authors' Contribution

None declared.

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