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Glutamate dehydrogenase and triose-phosphate-isomerase coding genes for detection and genetic characterization of *Giardia lamblia* in human feces by PCR and PCR-RFLP*

Kareem HATAM NAHAVANDI[1], Esmaeel FALLAH[1], Mohammad ASGHARZADEH[3], Nasrin MIRSAMADI[2], Behroz MAHDAVIPOUR[1]

Aim: The purpose of this study was comparison of the glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) genes for detection and genetic characterization of *Giardia lamblia* (*G. lamblia*) in human stool by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP). Our assay was specific and distinguished between *G. lamblia* assemblages A and B.

Materials and methods: Among the 325 stool samples obtained from patients with acute gastroenteritis hospitalized in the Pediatric Hospital and infected humans referred to the Tabriz Reference Laboratory (TRL), 34 *Giardia*-positive stool samples were identified by conventional techniques. Two assays–PCR and PCR-RFLP–targeting the *tpi* and *gdh* genes were developed to detect and genetically characterize *G. lamblia* isolates in human stool.

Results: The *tpi* gene was amplified from 31 (91.1%) samples by A-PCR and B-PCR assays. Of these samples 13 (41.9%) contained assemblage B, 17 (54.8%) contained assemblage A, 1 (3.2%) contained a mixture of assemblage A and assemblage B, and 3 (8.8%) samples were negative. Only 18 (52.9%) samples were positive when the *gdh* gene was targeted by GDH-PCR. RFLP analysis of the *gdh* gene classified 6 samples (33.33%) in assemblage A subgenotype II, 8 (44.44%) in assemblage B group III, and 4 (22.22%) in assemblage B group IV. Of these, 6 (33.33%) samples contained assemblage A and 12 (66.66%) samples contained assemblage B.

Conclusion: These study results reveal that the PCR technique is sensitive, simple, and specific for *Giardia* detection in fecal samples. In addition, the results demonstrate that the *tpi* gene is well adapted for *G. lamblia* genotyping.

Key words: *Giardia lamblia*, glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), PCR, PCR-RFLP

Introduction

Giardia lamblia (synonym of *G. intestinalis* and *G. duodenalis*) (1,2) is an intestinal protozoan found in a wide range of mammalian hosts (2). In humans giardiasis is a common cause of parasitic gastroenteritis and is a major health concern worldwide (3). The disease is principally acquired by oral ingestion of *G. lamblia* cysts, and the clinical manifestations vary from asymptomatic infection to acute diarrheal illness (4). In immunocompetent individuals giardiasis is usually self-limited, but can develop into persistent and life-threatening diarrhea for both immunodeficient individuals (5) and malnourished children in developing countries (6). The genus of *Giardia* currently comprises

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[1] Department of Parasitology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz - IRAN
[2] Tabriz Reference Laboratory, Tabriz - IRAN
[3] Hematology and Oncology Research Centre, Tabriz University of Medical Sciences, Tabriz - IRAN
Correspondence: *Kareem HATAM NAHAVANDI, Department of Parasitology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz - IRAN*
E-mail: karim.hatam@gmail.com

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6 species which are distinguished on the basis of the morphology and ultra-structure of their trophozoites (7). *G. lamblia* is the only species found in humans, although it is also found in other mammals including pets and livestock (1). A variety of molecular tools, including PCR-RFLP and sequence analysis of housekeeping genes, have shown that *G. lamblia* is a species complex made up of morphologically indistinguishable isolates that are classified into 7 assemblages based on the characterization of the glutamate dehydrogenase (*gdh*), small-subunit (SSU) rRNA, and triosephosphate isomerase (*tpi*) genes (8-12). These assemblages include A and B, which are potentially zoonotic, and C, D, E, F, and G, which appear to be host restricted. Assemblage A consists of isolates that can be divided into 2 distinct clusters, I and II. Assemblage B has been divided into clusters III and IV (1,13). Current methods for the detection of *Giardia* in stool are usually based on visual recognition under light microscopy of stained or unstained *Giardia* cysts or trophozoites (7,14). However, these methods are time consuming, require experienced microscopists, have low sensitivity, and cannot distinguish between genetically distinct *G. lamblia* isolates. Molecular biology provides powerful analytical tools that can be used to develop new and non-subjective tests, which have not yet had widespread application, to the study of the molecular epidemiology of human giardiasis. This study evaluates 2 *tpi* and *gdh* genetic loci by using 2 assays–PCR and PCR-RFLP–concurrently for rapid and specific detection and classification of *G. lamblia* cysts from human stool samples into A and B assemblages.

Materials and methods

Source of stool samples

The 325 stool samples examined in this study were collected from individuals in 2 localities in Tabriz, in the central East Azerbaijan province in the northwest of Iran. The samples came from patients with acute gastroenteritis hospitalized in the Pediatric Hospital and infected humans referred to the Tabriz Reference Laboratory (TRL). These samples were collected from sporadic cases of giardiasis between May and October of 2007. Details about the age, sex, and recent travel history of the patients were obtained from the original request forms.

Stool samples

Stool samples were collected in plastic cups. Approximately 5 g was then transferred to the parasitology laboratory of the Faculty of Medicine at the Tabriz University of Medical Sciences. Stool samples were examined under light microscopy using Lugol's iodine-stained wet mount and formalin-ethyl acetate concentration techniques for parasite diagnosis (15). During this study 34 stool samples containing *G. lamblia* cysts were obtained. Of the 34 patients, 80% were adults between 20 and 60 years of age and 73% were males.

Cyst purification

Cysts were partially purified from stool material through the sucrose density gradient method (16) followed by washing with sterile distilled water. All samples were stored at 4 °C without preservatives for up to 7 weeks (17).

Extraction and purification of DNA

The genomic DNA of *G. lamblia* isolates was extracted through the freeze-thawing technique [10 cycles of freezing (30 min at liquid nitrogen) and thawing (30 min at 65 °C)], followed by the modified proteinase K, SDS, and CTAB methods. This method has been used before (18), but we modified it as follows:

A 300 µL suspension of cysts obtained from the freezing/thawing was suspended in 150 µM of TE buffer (0.1 M Tris-HCl, 0.1 M EDTA) by vortexing. Next 60 µL of 10% SDS and 10 µL of 20 mg/mL proteinase K were added, and the solution was vortexed and incubated overnight at 60 °C. Afterwards 100 µL of 5 M NaCl was added, the solution was vortexed, and 80 µL of CTAB/NaCl solution (1:7) warmed to 65 °C was added. The solution was vortexed until the liquid content became milky and incubated for 10 min at 65 °C. At this stage 700 µL of chloroform/isoamyl alcohol (24:1) was added, and the solution was vortexed for 10 s and centrifuged for 8 min at 11,000 × *g*. Nucleic acid was precipitated by adding 0.6 volume (420 µL) 2-propanol to the aqueous supernatant and incubating the mixture for 30 min at -20 °C, followed by 15 min centrifugation at 12,000 × *g* at room temperature (RT). Then the DNA pellet was

washed by adding 1 mL of cold 70% ethanol, and the solution was centrifuged for another 5 min at 12,000 × *g*. The supernatant was carefully removed, and the pellet was allowed to dry at RT for approximately 15 min. Finally, the pellet was redissolved in 30 µL of deionized water. The DNA was stored at -20 °C.

Primers

Three sets of oligonucleotide primers were used for the analysis of the stool samples. Two sets of primers for detection of *G. lamblia* assemblages A and B were used for the coding region of the *tpi* gene. The primers used to amplify a 148-bp segment of the assemblage A gene (A-PCR) and a 81-bp fragment of the assemblage B gene (B-PCR) have been previously described (17). One set of primers for detection of *G. lamblia* assemblages A and B was used against the coding region of the *gdh* gene. The primer set GDHiF/GDHiR was used for amplification of the *gdh* gene of *G. lamblia* assemblages A and B, respectively. In the GDH-PCR reaction a 432-bp fragment was amplified using the forward primer (GDHiF) 5′-CAG TAC AAC TCY GCT CTC GG-3′and the reverse primer (GDHiR) 5′-GTT RTC CTT GCA CAT CTC C-3′ (10).

PCR amplification

As described above, 2 separate A-PCR and B-PCR amplifications were done with primers A-for/A-rev and B-for/B-rev. Amplification reactions (20 µL) contained 2 µL of template DNA, 1 × PCR reaction buffer corresponding to a final concentration of 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 50 mM KCl, 20 mM Tris-HCl (Cinnagen, Iran), each deoxynucleotide triphosphate (dNTP) at a concentration of 100 µM (Fermentas, Lithuania), 0.5 µM of each primer (F/R), and 2.5 U of *Taq* DNA polymerase (Cinnagen, Iran) for A-PCR and B-PCR. The samples were subjected to an initial denaturation of 94 °C for 10 min; 50 cycles of 94 °C for 35 s, 63 °C for 35 s, and 72 °C for 45 s; and a final extension at 72 °C for 7 min. For B-PCR, cycling parameters were 10 min at 95 °C (initial heat activation step); followed by 50 cycles of 35 s at 94 °C, 30 s at 65 °C, and 40 s at 72 °C; and a final extension of 7 min at 72 °C. Both positive and negative controls (distilled water) were included in A-PCR and B-PCR to validate results. Positive and negative controls were also included in each PCR to validate results. The amplification of the *gdh* gene was performed using a single GDH-PCR protocol. Amplification conditions were as follows: the PCR mixture consisted of 1 × buffer containing 1.5 mM $MgCl_2$ (Cinnagen, Iran), 200 µM of each dNTP (Fermentas, Lithuania), 0.5 µM of each primer (GDHiF/GDHiR), 2.5 U of *Taq* DNA polymerase (Cinnagen, Iran), and 2 µL of template DNA in a final volume of 20 µL. PCR was performed on a Mastercycler gradient thermal cycler (Eppendorf-Germany) with the following amplification conditions: 1 cycle of 94 °C for 10 min (initial heat activation step); 50 cycles of 35 s at 94 °C, 35 s at 61 °C, and 50 s at 72 °C; and a final extension of 7 min at 72 °C. Both positive and negative controls were included to validate results.

RFLP analysis

Restriction digestion was carried out by using *RsaI* and *NlaIV* (Fermentas, Lithuania) restriction enzymes on the GDH-PCR products for differentiation of *G. lamblia* assemblages and subgenotypes (17). The assemblages and subgenotypes were characterized according to restriction patterns that have been previously described (10).

PCR product and restriction fragment detection

PCR (A-PCR, B-PCR, and GDH-PCR) products and restriction fragments were visualized after electrophoresis on 1% and 2% agarose gels, stained in an ethidium bromide solution (0.5 µg/mL), and recovered by UV transillumination (15,17). A 100-bp DNA ladder (Fermentas, Lithuania) was included as a size marker.

Results

Studies were performed with the DNA extracted from purified cysts by using the modified proteinase K, SDS, and CTAB methods. A 148-bp fragment of the assemblage A gene and a 81-bp fragment of the assemblage B gene were amplified along A-PCR and B-PCR, respectively (Figures 1, 2). Among the stool samples in which sporadic cases of giardiasis were identified by conventional techniques (n = 34), the *tpi* gene was amplified from 31 samples (91.1%) using A-PCR and B-PCR (2 separate amplification steps) developed in our laboratory. Of these samples, 13 (41.9%) contained assemblage B, 17 (54.8%) contained assemblage A, 1 (3.2%) contained a mixture of assemblage A and assemblage

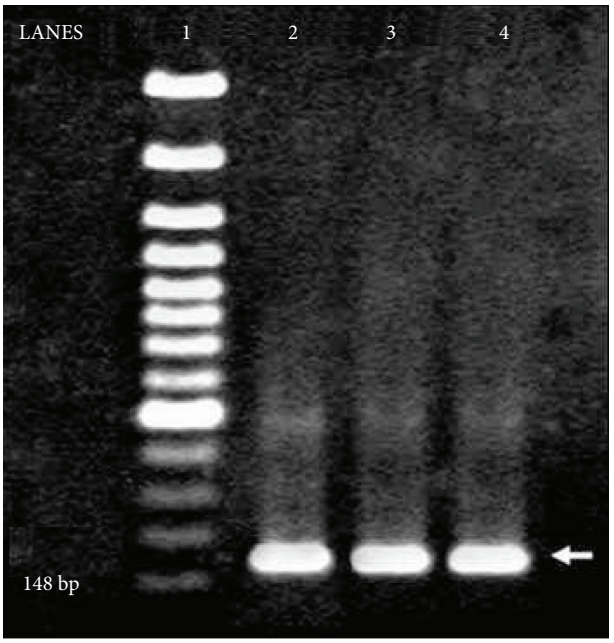

Figure 1. Triose phosphate isomerase gene-based PCR products on an ethidium bromide-stained 2% agarose gel. Lane 1: 100-bp DNA ladder (Cinnagen/Iran). Lanes 2, 3, and 4: *G. lamblia* assemblage A (148-bp fragment).

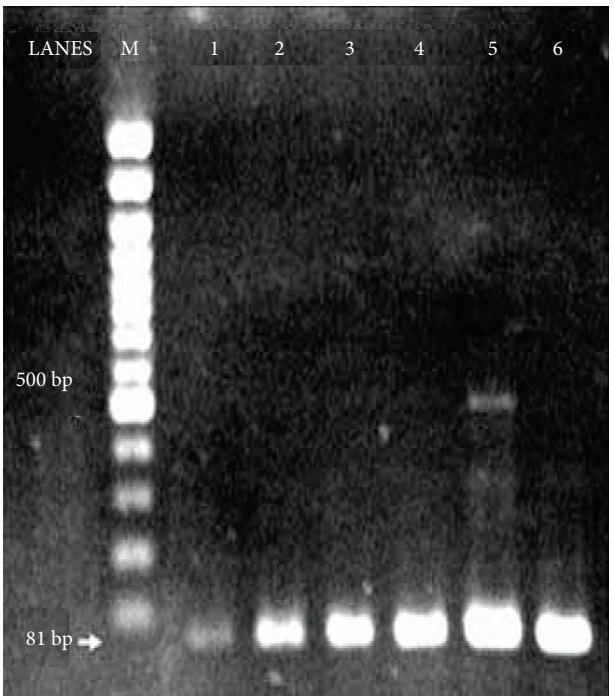

Figure 2. Triose phosphate isomerase gene-based PCR products on an ethidium bromide-stained 2% agarose gel. Lanes 1-6: *G. lamblia* assemblage B (81-bp fragment). Lane M: 100-bp DNA ladder (Cinnagen/Iran).

B, and 3 (8.8%) samples were negative. The *gdh* gene was amplified from 18 samples (52.9%) using PCR developed in our laboratory. A 432-bp fragment of *gdh* locus was amplified in the GDH-PCR using primers GDHiF and GDHiR (Figure 3). An RFLP assay of the 18 specimens recovered from humans revealed *G. lamblia* assemblage A cluster II in 6 (33.33%) specimens, *G. lamblia* assemblage B cluster III in 8 (44.44%) specimens, and *G. lamblia* assemblage B cluster IV in 4 (22.22%). Of these, 6 (33.33%) samples contained assemblage A and 12 (66.66%) samples contained assemblage B (Table). The negative results observed could be explained by the presence of parasites at a very low level (17). Both positive and negative controls (distilled water) were included in A-PCR and B-PCR to validate the results. The *tpi* gene fragment from assemblages A and B could be amplified by using 0.5 and 0.05 pg of DNA per reaction mixture, respectively, equivalent to 50 and 5 copies of the *tpi* gene, respectively, on the basis of a genome size of 1.2×10^7 bp (19). The genotype of *G. lamblia* cysts from 1 stool sample classified in assemblage A with GDH-PCR was reconfirmed by sequencing analysis. This analysis showed a match

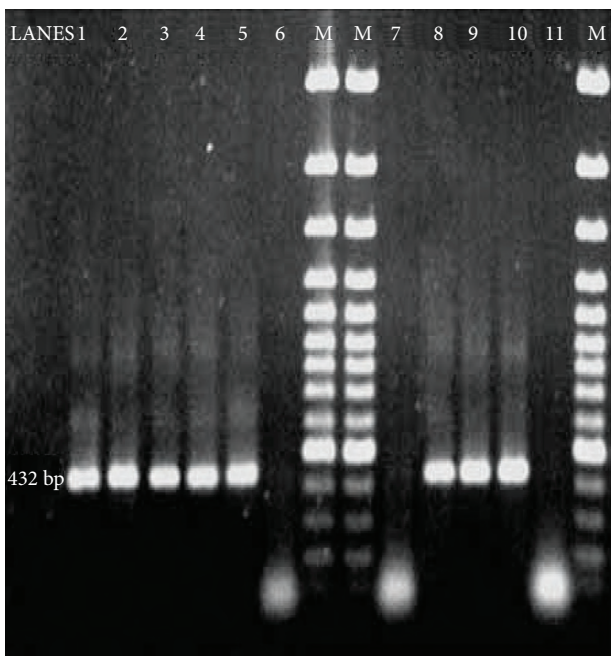

Figure 3. Glutamate dehydrogenase gene-based PCR products on an ethidium bromide-stained 2% agarose gel. Lanes 1-5, 8, 9, and 10: *G. lamblia* (432-bp fragment). Lanes 6, 7, and 11: Negative control. Lane M: 100-bp DNA ladder (Cinnagen/Iran).

Table. Summary of genotyping results obtained with the 2 methods used.

Genotypes (assemblages)	PCR-based methods (No. of samples identified)		
	tpi-based PCR	*gdh*-based PCR-RFLP	Final results
A	17	6	17
Group I			
Group II		6	6
Group not determined			11
B	13	12	13
Group III		8	8
Group IV		4	4
Group not determined			1
A and B	1		1
Total	31	18	31

Total positive samples by microscopic examination = 34.

(95%) between the amplified product obtained with GDH-PCR (432-bp) and the sequence with GenBank accession number L40510, corresponding to *G. lamblia* assemblage A subgroup II (positive control).

Discussion

There is little data about distribution and transmission patterns of the *G. lamblia* genotypes in developing countries. The application of genetic characterization of *G. lamblia* is likely to lead to more rational approaches to disease control. We know this is the first report on the study of *Giardia* genotypes infecting humans performed in the north of Iran.

For the sporadic cases of giardiasis confirmed by analysis with conventional techniques (n = 34), A-PCR/B-PCR resulted in 91.1% positive samples with 2 separate amplification steps, whereas only 52.9% were positive when the *gdh* gene was targeted by GDH-PCR. The majority of sporadic giardiasis isolates were assemblage A genotype (54.8%). The proportion of samples in which the *tpi* and *gdh* genes could not be amplified was higher than that reported by Bertrand et al. (17) using the same primers. In our study this could be explained by the presence of PCR inhibitors in some of the stool samples, which resulted in the prevention of amplification. These failures could stem from the fact that the proportion of DNA in the stool samples was not sufficient to counteract the effect of the inhibitors that would have co-purified with the nucleic acids (20). Surveys in several countries showed a diverse prevalence of genotypes A and B. Studies carried out in Germany, China, Uganda, Italy, New Zealand, Egypt, and Mexico reported a prominence of genotype A (21-25). Results reported in our study correspond with those from studies published in Iran by Babaei et al. (26). They found 33 examples of genotype A II, 3 examples of genotype B, and 2 examples that displayed a mixture of genotypes A and B (n = 38). Similarly, a recent study in Egypt by Helmy et al. (27) found that infection with assemblage A was more prevalent (75.5%) than infection with assemblage B (19.5%). A mixture of assemblage A group II and assemblage B (n = 41) occurred in 2 samples. On the other hand, a UK study that examined 35 human clinical samples found that 64% were assemblage B and 27% were assemblage A. The remaining samples were a mixture of assemblages B and A (15). In Canada Guy et al. (28) found 9 B isolates, 3 genotype A isolates, and 3 that were mixed. In Bangladesh Ng et al. (29) found 32 genotype B, and 3 genotype A isolates. In the study conducted by Singh et al. (30) in Nepal, 26 samples were found to contain assemblage B, 7 contained assemblage A, and a mixture of genotypes A and B were detected in 2 samples. Results from each of these studies are not strictly comparable since amplifications were done on different *G. lamblia* genes. In addition, differences may be attributed to the geographical locations of the populations studied. Differentiation between assemblages A and B genotypes is significant because these genotypes differ in virulence. Assemblage A isolates are usually detected in patients with intermittent diarrheal complaints, while assemblage B isolates are present in patients with persistent

diarrheal complaints (31). RFLP analysis of the *gdh* gene classified 6 samples (33.33%) in the assemblage A subgenotype II, 8 (44.44%) in assemblage B group III, and 4 (22.22%) in assemblage B group IV. In our study *G. lamblia* assemblage A and assemblage B were detected together in 1 sample. A mixture of these assemblages has been reported previously in a few studies (15,25,28). In conclusion, our data provide the first information about the distribution of the 2 major assemblages of *G. lamblia* in sporadic human giardiasis in Northwestern Iran. In addition, the results presented here reinforce the evidence that humans are susceptible only to assemblages A and B, and not to the host-specific assemblages C, D, E, F, and G. Studies carried out with a greater number of samples, both human and animal, from different areas will increase our understanding of the epidemiology of giardiasis in Iran. Our observations show that the *tpi* gene is better adapted for detecting the *G. lamblia* cysts from human stool samples than the *gdh* gene. There is no gold standard for the diagnosis of giardiasis. The initial method of diagnosis is through detection of the trophozoite or cysts of *G. lamblia* in the stool by microscopy. Our results demonstrate, however, that PCR is a reliable diagnostic tool for the rapid and sensitive detection of *G. lamblia* cysts in human stool.

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