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Original Article

Molecular characterization of the Iranian isolates of *Giardia lamblia:* application of the glutamate dehydrogenase gene

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Abstract

Background: This study was conducted to determine of molecular epidemiology of the *Giardia lamblia* by PCR-RFLP method in Tehran, capital of Iran.

Methods: Thirty eight stool samples were randomly selected from 125 patients diagnosed with giardiasis using microscopy in Tehran. DNA extraction of some samples were performed by phenol/chloroform/isoamyl alcohol method and to raise the sensitivity of the PCR assay, the genomic DNA of the others were extracted using glass beads and the QIAamp Stool Mini Kit in order to effectively remove the PCR inhibitors. A single step PCR-RFLP assay, targeting the glutamate dehydrogenase (*gdh*) locus, was used to differentiate within and between assemblages A and B that have been found in humans.

Results: Of the 38 isolates, 33 samples (87%) were found as *G. lamblia* (genotype AII), 3 (7.8%) belonged to assemblage B, genotype BIII, the mixed of genotype AII and B were detected only in two samples (5.2%).

Conclusions: PCR-RFLP is a sensitive and powerful analytical tool that allows effective genotype discrimination within and between assemblages at targeting *gdh* gene, and makes it possible to identify the presence of mixed genotypes. Our data suggest that there is an anthroponotic origin of the infection route, assemblage A group II, in Tehran so it seems that the main reservoir of *Giardia* infection is humans in the area studies.

Keywords: *Genotype,* Giardia, *Glutamate dehydrogenase, Iran*

Introduction

G.lamblia (synonyms: *G.intestinalis*, *G.duodenalis*) is a universal and well-known entric protozoa that is found in the intestines of mammalian hosts, including both domestic and wild animals and humans. *Giardia* is one of the most common gastrointestinal pathogens in children, causing severe intestinal disorder and growth retardation (1). The *G.lamblia* occurrences, identified in the *Giardia* isolates based on morphologic criteria, vary significantly in their biology, host specificity, and genetics. These morphologically indistinguishable isolates can genetically be differentiated into several major assemblages (A-G). Some genotypes appear to be restricted to one host, whereas others have a broad range of host including the humans. The genetic diversity between these groups suggests that separate species names, e.g., *G.simondi*, correspond to assemblage G. Recently Hunter and Thompson proposed some cryptic species as *G.enterica*, *G.canis*, *G.catti* and *G.bovis* correspond to assemblages B, C, F, and E, respectively (2-5).

Although all human-derived *Giardia* isolates belong to assemblages A and B, these assemblages have also been found in isolates from the other domestic and wild animals such as dogs, cats and cattle (6). Some researchers believe that *G. lamblia* presents as a risk of zoonosis from cattle (7), dogs (8-10), wild moose, reindeer (11), farm and wild animals (12).

A variety of genotyping techniques, including PCR-based diagnostic system, cloning, and se-

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quencing with housekeeping genes, have proven to be valuable tools for showing high sensitivity and ability to discriminate between all assemblages and genotypes, and providing powerful analytical tools which can be used to understand molecular epidemiology of human giardiasis (13-17). Genetic studies have revealed that assemblage A comprises genetic group I-VIII (A1-A8), and assemblage B includes subtypes I-VI (B1-B6). However, the AI, AII, BIII, and BIV genotypes have been more reported up to now (2, 14, 18).

The main goal of this study was to determine the genotypes of *G.lamblia* isolates in Tehran, Iran, as our search has been no reported in this regard. We used the PCR-RFLP method that particularly suited for direct typing of the *Giardia* cysts present in fecal or crude samples. This method was used successfully to identify mixed genotypes (3, 14, 16).

Materials and Methods

Samples collection During June 2006 to November 2007, 125 *Giardia* positive-microscopic human fecal samples were collected. The unpreserved specimens were stored at 4º C until more analyses. Positive trophozoite samples were aliquoted in one portion 70% ethanol was added and stored at room temperature, the second aliquot was stored at -20° C for subsequent analysis. Specimens, including cysts, were purified and concentrated by flotation on sucrose with specific gravity of 0.85 M (19), and a harvested axenic cultured *Giardia* that was used as a standard (ATCC® Number: 30888™), were stored at -20° C until further examination.

DNA extraction DNA extraction was randomly performed on 38/125 samples. The trophozoite genomic DNA was extracted by PCI method as following description, approximately 250 µl of each concentrated sample was suspended in a mixture of 300 µl TE buffer (50 mM Tris–HCl, 50 mM EDTA, pH 7.5). After adding SDS 1M (1/20 total volume), and 10 µl of proteinase K (10 mg/ml), the suspension was incubated at 56° C for 1h. The DNA lysate was first treated with phenol/chloroform/isoamyl alcohol (24:24:1), and then by chloroform/isoamyl alcohol (24:1). DNA was precipitated by the addition of 1 ml chilled ethanol. The dried DNA was suspended in 40 µl distilled water (20), and used as a template for PCR.

The cysts wall were mechanically disrupted using glass beads (0.45-0.52 mm diameter), and lysed by vortexing. The DNA of some samples after treating by glass beads were isolated using the QIAamp DNA Stool Mini Kit (QIAgen Company, Germany) as described by manufacture instructions and the DNA of remaining samples were extracted by PCI method as described above. All extracted DNA were stored at -20° C.

PCR amplification Amplification of the *gdh* gene was performed as a single PCR with a forward 5' TCAACGTCAACCGCGGCTTCCGT 3', and reverse 5' GTTGTCCTTGCACATCTCC 3' primer as described before (3) expect some slight modifications by replacing degenerated nucleotide. The primers were tested by standard *Giardia* DNA. The PCR reaction mixture consisted of 1-10 µl of template DNA according to the DNA concentration, 10 µl of 10X PCR buffer (Roche), 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 1U of Taq polymerase (CinnaGen Tehran, Iran), and 50 pmol of each primer.

DNA was amplified using Primus, MWG-BIOTECH, Thermal Cycler under the following conditions: 8 min at 94° C as a initial hot start step, followed by 35 cycles, of 1 min at 94° C, 90 sec at 60.5° C, 2 min at 72° C, and a final extension step 5 min at 72° C. Distilled water used as a negative control. The PCR products were electrophoresized on ethidium bromide-staining 1% (W/V) agarose gel.

PCR-RFLP at the gdh locus To distinct groups I and II of assemblages A and assemblage B, 15 µl of PCR product was digested by 0.5 unit of *BspLI* (*NlaIV*, Fermentase) and for distinction between subtypes BIII and BIV 0.5 U of *RsaI* (Fermentase) was added. Restriction fragments were separated in 3% agarose/ethidium bromide gels, or were visualized on 8% polyacrylamide gel by PAGE electrophoresis.

Results

On 38 samples, *gdh* gene was amplified. By glass beads and PCI method, only 12 of the samples (30%) were amplified but because of using glass beads and QIAgen kit in all remaining 26 isolates the 458 bp expected size were amplified (Fig. 1). Therefore, this study showed that the latter method was more effective for extraction of cyst DNA.

The expected fragments after digesting by specific restriction *BspLI* and *RsaI* enzymes are shown in Table 1.

Table 1: The RFLP profiles of *G. lamblia* assemblages after digesting with *BspLI* and *RsaI*

Assemblage	Enzyme	Expected fragment sizes	Diagnostic genotyping fragments
AI	*BspL*I	16,39,47,87,123,146	87,123,146
AII	*BspL*I	16,39,47,69,77,87,123	69,77,87,123
BIII	*BspL*I	47,123,288	47,123,288
BIV	*BspL*I	47,123,288	47,123,288
BIII	*Rsa*I	30,131,298	131,298
BIV	*Rsa*I	30,428	428

Digested the 458 bp amplified fragment by restriction enzymes revealed that the majority of the isolates had a characteristic four-band pattern of assemblage AII. The standard sample showed pattern AI so had a molecular weight band of 146 bp and lacked the 69- and 77-bp bands. For discrimination between the subtypes of assemblage B using the specific restriction enzyme, *RsaI*, it was necessary to observe 2 different profiles since digesting with *BspLI* enzyme in the B-group isolates showed a same pattern (Table 1).

According to our result, 33(87%) of the 38 specimens were typed as assemblage AII, 3(7.8%) assemblage BIII, and in 2(5.2%) isolates a mixture of assemblages AII and B were detected (Fig. 2 and 3). Interestingly, assemblages AI and BIV were not detected in our samples. The genotyping results are summarized in Table 2.

Table 2: Assemblages and genotypes of *G.lamblia* determined by PCR-RFLP of *gdh* locus

Isolate code	Stool Examination	*Genotype*	Isolate code	Stool Exsamination	Genotype
TIG1	Cyst+Trophozoite	BIII	TIG20	Cyst	AII
TIG2	Cyst Cyst+Trophozoite	AII	TIG21	Cyst	AII
TIG3	Cyst	AII	TIG22	Cyst	AII
TIG4	Cyst	AII	TIG23	Cyst+Trophozoite	AII
TIG5	Cyst	AII	TIG24	Cyst	AII
TIG6	Cyst	AII	TIG25	Trophozoite	BIII
TIG7	Cyst	AII+B	TIG26	Cyst	AII
TIG8	Cyst	AII	TIG27	Cyst	AII+B
TIG9	Cyst	AII	TIG28	Cyst	AII
TIG10	Cyst	AII	TIG29	Trophozoite	AII
TIG11	Cyst	AII	TIG30	Trophozoite+Cyst	AII
TIG12	Cyst	BIII	TIG31	Cyst	AII
TIG13	Trophozoite	AII	TIG32	Cyst	AII
TIG14	Cyst	AII	TIG33	Cyst	AII
TIG15	Cyst	AII	TIG34	Cyst	AII
TIG16	Cyst	AII	TIG35	Cyst	AII
TIG17	Cyst	AII	TIG36	Cyst	AII
TIG18	Cyst	AII	TIG37	Cyst	AII
TIG19		AII	TIG38	Cyst	AII

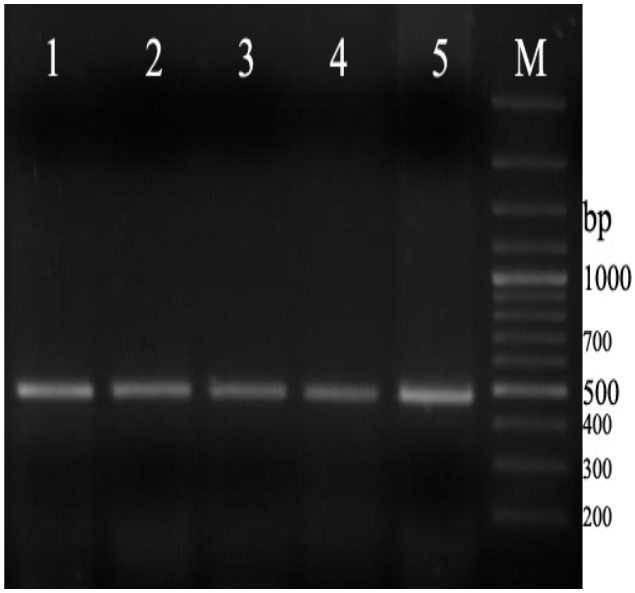

Fig. 1: Electrophretic separation of PCR product from DNA amplified at the *gdh* locus of *G. lamblia*, lanes 1-5 PCR products from clinical samples, lane M, 100 bp ladder

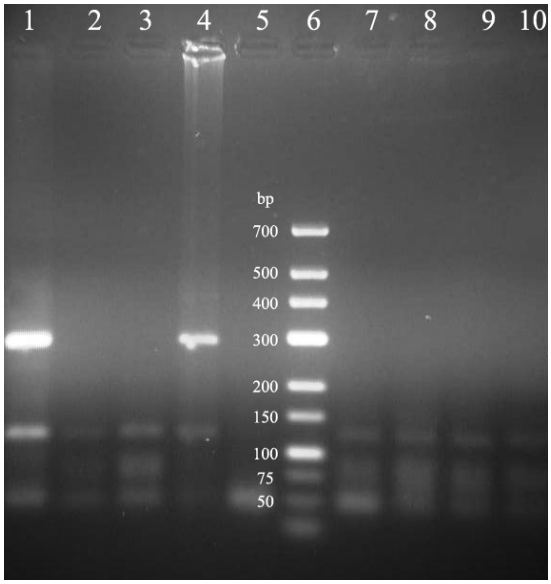

Fig. 2: *BspLI* digestion of single PCR product of *G. lamblia* on an ethidium bromide-stained 3 % gel. Lanes 1 and 4, *G.lamblia* assemblage B, lanes 2, 3 and 7-10 genotype AII and lane 6, (O'Gene Ruler DNA Ladder , Low Range, Fermentas)

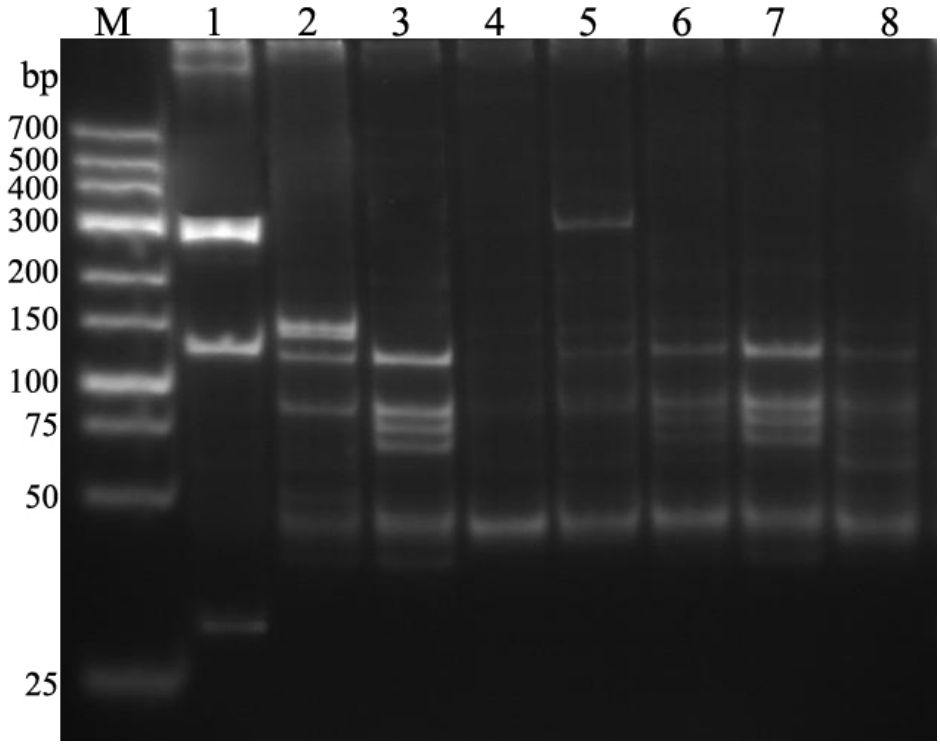

Fig. 3: Enzyme digestion of PCR product of *G lamblia* on an ethidium bromide-stained 8% polyacrylamide gel. Lane M (O'Gene Ruler DNA Ladder, Low Range, Fermentas), lane 1 *G. lamblia* assemblage B group BIII (*RsaI* digestion), lanes 2, *G.lamblia* assemblage A genotype AI, lanes 3, 4, and 6-8 genotype AII and lane 5 mixed genotypes AII and B. (lanes 2-8: profiles *BspLI* digestion of PCR product of *G.lamblia* the standard strain (genotype AI) and *G.lamblia* isolates, respectively)

Discussion

G.lamblia is considered as one of the most important human intestinal parasites in terms of morbidity in several countries (14). This protozoa was recently taken into the World Health Organization's (WHO's) "Neglected Diseases Initiative" (21). Moreover, the heterogeneity in the results of molecular analyses has revealed that *G.lamblia* is a complex species, comprised of a range of diverse genotypes (22, 23). For this reason, to use advanced tools for molecular epidemiology determination of this diverse and interesting parasite in the world is critical.

PCR-RFLP is a sensitive and powerful analytical tool that is capable of providing the level genotyping discrimination between and within assemblages by targeting some loci such as *gdh* and *tpi*, making it possible to identify the presence of mixed genotypes (3,6,13-15,23,24). It is important to note that all loci enable successful grouping at level assemblage of *G.lamblia* isolates, and could characterize the sub-assemblages AI and AII, whereas only a few loci allow sub-assemblages differentiation within the B assemblage. Such markers like the SSU-rRNA, the elongation factor 1α gene can only be used to discriminate major assemblages, whereas the glutamate dehydrogenase gene, the triose phosphate isomerase, and β Giardin allow us to distinguish between the subgroups of the assemblages A and B (25).

Moreover, direct amplification of cysts DNA from feces help to solve important questions such as: presence of mixed genotypes, correlation between genotypes and host (pathogenicity), and selection for irrelevant genotypes during cultivation (15, 16, 24, 26). But using directly stool for DNA amplification cause to decrease the yield of DNA extracted that can be improved by apply a more effective approach. In addition, there are many PCR inhibitors (e.g. lipids, hemoglobin, bile salts, polysaccaharides from mucus, bacteria and food degradation product) which can affect the result of amplification. For this reason, some extraction and amplification methods have been improved to develop more sensitive assays to identify gene. In some studies, specific DNA was detected at all target concentrations, demonstrating that QIAamp DNA kit extraction method could effectively remove PCR inhibitory substances. (27-29).

Unsuccessful amplification of some samples in the initial of our study, especially in whole stool, suggested the presence of impurities in the extracted DNA that inhibit PCR amplification, or existence of a robust wall of cyst that inhibit release of the DNA from the cysts. To solve these problems, we used a modified protocol in which using glass beads for rupturing the cyst wall and QIAamp DNA Stool Mini Kit in order to remove the inhibitors, after which all samples were amplified.

For the first time, our study reports the distribution of the genotypes of *G.lamblia* from humans with sporadic giardiasis in Iran. In this work, we studied 38 human samples by PCR-RFLP analysis at the *gdh* gene that showed presence assemblages A and B to be associated with human infections. According to the results of previous studies, it appears that genotype AI and assemblage B (especially BIII) have a more zoonotic potential than subgroup AII, and have a more host range (30) however subgroup BIV appears to be human-specific (22).

The differences in the prevalence of assemblages A and B may be attributed to the spatial locations of the populations studied (17, 31).

Our results revealed that genotype A is widely distributed in Tehran. The higher rate of assemblage A in Tehran consistent with previous reports. A study conducted in South Korea had also shown that all seven isolates from humans belonged to assemblage A. Similarly, all 26 human isolates in Mexico were from assemblage A. The predominance of assemblage A in wastewater and humans in Italy also has been reported (15, 23, 32). In the present study, the majority (87%) of the samples (33/38) belonged to *G.lamblia* assemblage AII consistent with an anthroponotic origin of infection, and corresponded to other reports. Caccìo et al. described that assemblage AII predominated in the examined stool samples by PCR-RFLP at targeting

β-giardin gene (15). Moreover, an unusual prevalence of the *G.intestinalis* subtype AII among isolates from humans and domestic animals in Mexico has been reported (33). Our work suggested that genotype AII of assemblages A was the most prevalent assemblage/genotype in the study region.

Interestingly, in the present study, mixed infection with genotype AII and assemblage B were detected in our samples. Some before study also reported mixed infection with some genotypes. Amar et al, observed a mixture of assemblage A genotype II and assemblage B in 9% of 35 samples, whereas multiple infections of assemblage A genotype I and assemblage B have also been reported (4, 14, 34). These multiple infections may reflect ingestion of sources contaminated by heterogeneous mixtures of parasites (14).

In conclusion, determination of the genetic grouping of *G.lamblia* is a useful way to understand the infection route, to prevent infection effectively, to reveal the critical issues in the molecular epidemiology of this parasite, and finally to address important questions related to human health in Iran. In the base of our results, an anthroponotic origin of the infection route is suggested and underscored the fact that for human infection, other human are the main reservoir while the zoonotic source plays a minor role. Because of the possibility of zoonotic transmission and the potential of domestic animals for hosting the parasite suggested by some researcher (1), further studies with a variety of humans and animals samples is recommended. Besides, more studies in other region for finding the pattern of distribution of this parasite in Iran is required.

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