Original Article

Genotyping of *G. duodenalis* in the People Referred to Health Centers of Semnan City

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ABSTRACT

Giardia duodenalis (G. duodenalis), is one of the major causes of gastrointestinal disorders worldwide, infecting the small intestine of humans and animals. Based on the genetic characteristics of the parasite, eight genotypes (A to H) have been identified in clinical samples. The main purpose of the present study was to find the genetic diversity of *Giardia* in people referred to health centers in Semnan, Iran, using PCR. Totally, 300 stool samples were collected from people referred to health centers in Semnan. The stool samples were first examined using the microscopic method (direct method and Lugol staining), and the samples were checked with trichrome staining. After DNA extraction, the GDH gene of positive samples was amplified by the semi-nested PCR method. The genotype of positive samples was determined by the sequencing method. Out of 300 samples, only 20 (6.66%) samples were found to be positive in the microscopic examination of the stool. In the PCR test, only 13 (4.33%) of the samples were positive. According to the multiple alignment results, it was found that the isolates belonged to AII, BIII, and BIV genotypes. Most of which are related to people without clinical symptoms of diarrhea. Identification of AII, BIV, and BIII genotypes indicates the anthroponotic and anthropozoonotic transmission cycle of *Giardia* infection in Semnan.

Keywords: *G. duodenalis*, Glutamate Dehydrogenase Gene, Genotype, Iran

1. Introduction

Giardia duodenalis (G. duodenalis) is a flagellated protozoan parasite that belongs to the Hexamitidae Family and lives in the human digestive tract. *Giardia* is one of the most common intestinal parasites in humans, and more than two hundred million people in Asia, Africa, and Latin America show infections with clinical symptoms (1). This parasite is pathogenic for a wide range of vertebrates, including humans and domestic animals such as dogs and cats, livestock, and wild animals (2). It has a direct life cycle that includes a non-invasive trophozoite stage with rapid multiplication on the intestinal mucosal surface. Small infectious cysts are extremely resistant to adverse environmental conditions, and humans and animals become infected after swallowing them. One of the most common ways of transmitting this cyst is through drinking water and contaminated food. Other risk factors of cyst transmission are poverty, living in crowded places, poor environmental hygiene, unhygienic personal habits, lack of safe water supply, and low economic and social levels (1). The pooled prevalence of *G. lamblia* infection among the healthy population lived in different parts of Iran was estimated at 10.6% (3).

G. duodenalis contains a group of several genotypes, based on their genetic characteristics, at least eight genetic groups (A to H) have been known. The most important of these subgroups in humans are genotypes A and B, which are divided into subspecies, such as BIV, BIII, AIII, AII, and AI, some of which are common between humans and animals (1). To indicate the anthroponotic and anthropozoonotic transmission cycle of *Giardia* infection, the differentiation of human and animal genotypes of *Giardia* human isolates is important. Additionally, to properly understand the pathogenicity of this parasite, the subjects such as the role of farm and domestic animals in the transmission of giardiasis, the infectivity of *Giardia* cysts, the ability of *Giardia* cysts to infect water sources and drinking water, biological differences of different genotypes and subtypes, their clinical manifestations and distribution is essential. In this regard, various studies have been conducted in different regions of the world by some researchers, such as in Italy, Netherlands, Japan, Ethiopia, Brazil, Argentina, and Iran, and so on (2-9).

Many of these studies indicated that humans are often infected with genotypes A or B, or group B, as group B predominates in humans (1), and there is a difference in virulence between the A and B subspecies (10). Genotype A has three subgenotypes, namely AI, AII, and AIII. The AI is found in humans and dogs, AII is found only in humans, and AIII is found only in animals. Genotype B has two subgenotypes of BIII and BIV, and the BIII genotype is common among humans, dogs, goats, rabbits, etc., while the BIV genotype has been reported only in humans. In addition, other genotypes (e.g., C and D genotypes) have rarely been seen in humans (1).

In Iran, a number of genotypic studies have been carried out in different parts of the country in the last decade, such as East Azerbaijan (11), Tehran (12, 13), Khorramabad (14), south of Iran (15), Fars province(16), Shahrekord (17), Kerman (18), Urmia (19), and Isfahan (20).

Considering that no such study has been conducted in Semnan province so far, the present study aimed to investigate the rate of *Giardia* infection and to determine the common *Giardia* genotypes in the *Giardia*-infected residents of Semnan.

2. Materials and Methods

2.1. Type of the Study

The present descriptive-cross-sectional study was carried out on 300 stool samples of people referred to Semnan medical centers during 2022(figure 1).

2.2. Location of the Study

Semnan is located in the south of the Alborz mountain range and the north of the desert plain on the way from Tehran to Khorasan. This city is located between the three cities of Damghan, Garmsar, and Mahdishahr at a geographic longitude of 53 degrees and 23 minutes and a geographic latitude of 35

degrees and 34 minutes, and its average height is 1130 meters above sea level. Its climate is dry and moderate. According to the results of the census of 2015, the population of Semnan was 185,129 people. This city has 13 health-medical centers, of which only one health center has a laboratory and two private laboratories and a clinic were also selected for sample collection.

2.3. Stool Microscopic Examination

Fresh stool samples were collected from people referred to health-medical centers and stored separately in containers containing polyvinyl alcohol, 75% alcohol, and 5% formalin. The number of samples was calculated based on the sample determination formula considering the infection rate of the previous reported studies (21). Direct, formalin-ether, and trichrome staining techniques were used for microscopic detection of *Giardia*.

2.4. Molecular Study

The primers were used for amplifying *GDH* gene (fragment ~432 bp) by Semi-nested PCR including GDHeF: TCAACGTYAAYCGYGGYTTCCGT; GDHiF: CAGTACAACTCYGCTCTCGG; and GDHiR: GTTRTCCTTGCACATCTCC (25). The primary PCR reaction was carried out in a volume of 15 µLs, including 1 µL sterile distilled water, 5.5 µL of parasite DNA, 7.5 µL of master mix, and 1 µL of each of the forward and reverse primers with a concentration of 10 picomoles. The secondary PCR reaction volume was the same as the primary PCR. The thermal cycle conditions for the primary PCR were as follows: initial denaturation at 95C for 5 min, then 35 cycles with denaturation at 95C for 30 sec, annealing at 55C for 30 sec, extension at 72C for 30 sec, and final extension at 72C for 10 min. The thermal cycling conditions in the secondary PCR were the same as the first step except for the annealing temperature. The annealing temperature in the secondary PCR was 54ºC.

In this study, the Giardia parasite sample available in the Parasitology Department of Tarbiat Modares University was used as a positive control, and sterile distilled water was used as a negative control. Finally, the PCR product was stained with Safe DNA stain on a 1% agarose gel. After the secondary PCR of the *GDH* gene, the fragment ~432 base pairs was loaded on a 1.5% gel, and the desired band was observed using a transilluminator.

2.5. Sequencing and Phylogenetic Analysis

Positive DNA bands were excised from the gel and purified using GeneAll Expin™ Combo GP kit (GeneAll, South Korea), according to the manufacturer's protocol. The final purified bands were eluted in 50 µL of pre-warmed sterile distilled water and sent for sequencing purposes. The obtained sequences were analyzed by Sequencher (version 4.1.4). Multiple alignments were done to evaluate the genetic diversity between isolates in the current study and those registered in the GenBank. Ultimately, the respective phylogenetic tree was drawn using the neighbor-joining method in MEGA 7.0 bioinformatics software.

2.6. Statistical Analysis

The SPSS software (version 16) was used for the statistical analysis of the variables. All data were compared using Chi-square test with a 95% confidence level, and a *P*-value less than or equal to 0.05 was considered statistically significant.

3. Results

Of the 300 stool samples collected, 16(6.66%) samples belonged to men, and 4(6.66%) samples belonged to women (Table 1). In terms of stool consistency, 16 samples were non-diarrheal, and four samples were watery and diarrheal. Out of the total samples, 20(6.66%) samples were positive for *Giardia* cyst infection with the trichrome staining method and microscopic observation, and 13(4.33%) samples with the PCR method (Table 2).

After the secondary amplification of the *GDH* gene in PCR assay, the desired band was observed in the fragment ~432 base pairs on the gel (Figure 2).

After blasting the data, the obtained results had 99-100% homology with the isolates registered as *G.*

Table 1. Number and percentage of positive stool samples collected from men and women in Semnan

Gender	Number of samples	Positive samples	
		Number	%
Male	240	16	6.66
Female	60	4	6.66
Total	300	20	6.66

Table 2. Distribution of absolute and relative frequency (number and percentage) of *Giardia* infection by staining method, microscopic observation, and PCR of 300 residents of Semnan

Clinical symptoms (diarrhea)	Number of samples	Staining method and microscopic observation		PCR method	
		Positive sample (%)	Negative sample (%)	Positive sample (%)	Negative sample (%)
Yes	260	16(%6.15)	244(%93.85)	9(%3.46)	251(%96.54)
No	40	4(%10)	36(%90)	4(%10)	36(%90)
Total	300	20(%6.66)	)280 %93.33)	13(%4.33)	287(%95.66)

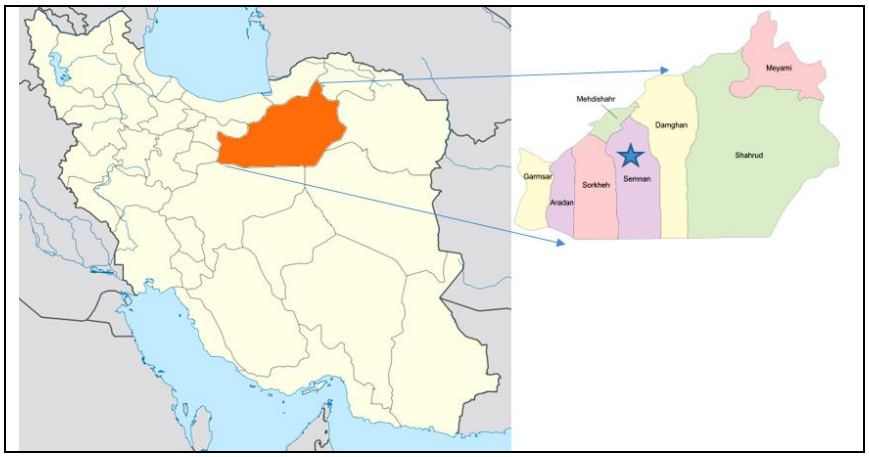

Figure 1. Semnan province map

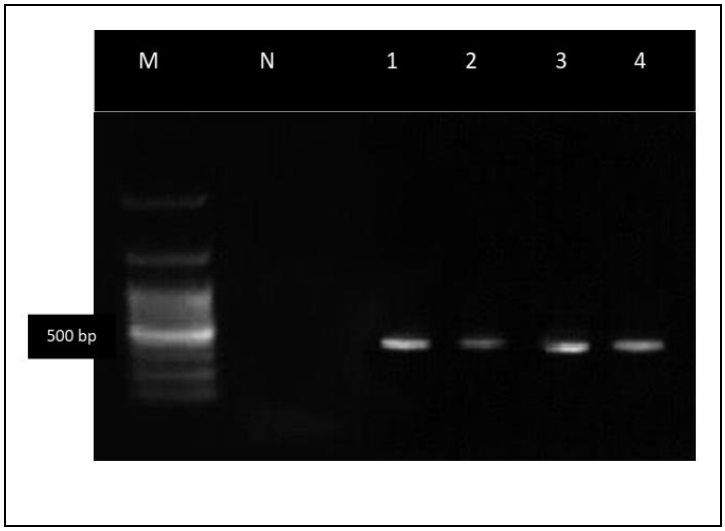

Figure 2. Bands of positive samples related to *Giardia GDH* gene on 1.5% gel electrophoresis. From left to right, M: Molecular marker 100 base pairs, N: Negative control, 1 to 4: Positive sample of 432 bp

duodenalis species in the Genbank. Sequencing results showed that two isolates belonged to genotype B, and two isolates belonged to genotype A. Additionally, the phylogenetic tree of the identified isolates was drawn in Figure 3. Based on the CLUSTAL OMEGA multiple alignment results of the expand.

European Bioinformatics Institute (EBI) site, it was determined that the isolates numbered 3 and 12 belonged to subgroup AII, isolate number 11 belonged to subgroup BIII, and isolate number 8 belonged to subgroup BIV (Fig. 3).

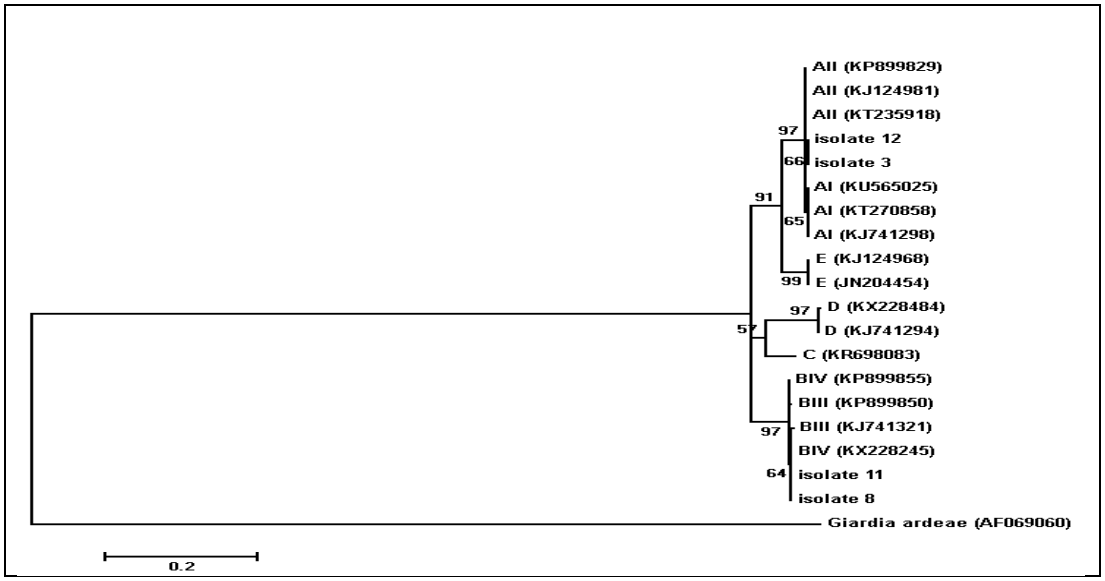

Figure 3. Tree of phylogenic relationships of GDH genotypes of *G. duodenalis* isolates inferred by Maximum Likelihood algorithm. The genotypes of this study are identified with isolates 3, 12, 11, and 8

4. Discussion

The current study was conducted to investigate *Giardia* infection and sequences analysis of G. *duodenalis* assemblage from specimens recovered from infected residents of Semnan. Results of *GDH* gene sequences analysis of specimens revealed assemblage AII and B of *G. duodenalis*. On the other hand, the identification of AII, BIV, and BIII assemblage, respectively, indicated the anthroponotic and anthropozoonotic transmission cycle of *Giardia* infection in this city.

Compared to other studies carried out in different parts of the world, in Italy assemblages A and B were identified among human isolates using the beta giardin gene locus. In addition, the host-specific assemblages C and D were found in most dog isolates; F in the single feline isolate and assemblages A, B, and E among calf isolates have been identified. Five subgenotypes, A1, A2, A3, A4, and B3, were found to be associated with infections of humans, dogs, and calves. These results supported the role of these animals as a source of infection for humans (2). In another study conducted in the Netherlands, on the basis of PCR assays specific for the assemblages A and B and the DNA sequences of 18S ribosomal RNA and the *GDH* genes. Genotyping results showed that *G. duodenalis* isolates originating from Dutch human patients belonged to assemblage A (35%) and in 65% of the cases to assemblage B (4). The genotypes of *G. intestinalis* using the *GDH* gene from the dog, cat, wild monkey, and cow samples in Japan indicated the assemblages A, C, D or A/D for dog isolates, assemblage F for cat, A or E for calf and assemblage B for monkey isolates (4). In a genetic analysis of human isolates of *G. duodenalis* collected in Ethiopia, a fragment of the beta-guardian gene was amplified by nested PCR and analyzed by restriction and sequence analyses. Of the 59 isolates examined, 31(52%) were typed as assemblage A and 13 (22%) as assemblage B and assemblage F. A strong correlation between the presence of symptoms and infection with assemblage B was observed (6).

In a study conducted in the state of São Paulo, Brazil, sequences analysis of specimens recovered from humans revealed *G. duodenalis* assemblage AII and B, and samples from cats, assemblage F and AI. Additionally, C and D were detected in samples from dogs and AI from cattle (7). In another study in Argentina, the triosephosphate isomerase gene was amplified from 60 human fecal samples. Among these, 6.98% were genotype AII, and 93.02% were genotype

B. Genotype B was also found in polysymptomatic people, many of whom presented diarrhea (9).

Compared to other studies conducted in Iran, a study was conducted to determine the genotype of *G. lamblia* isolates in Tehran using PCR-RFLP and glutamate dehydrogenase gene. Of the 38 isolates, 87% were found as genotype AII, 7.8% belonged to assemblage B genotype BIII. In 5.2% of the isolates, a mixture of assemblages AII and B were detected (12). In a study conducted to identify the genotype of *G. lamblia* in the south of Iran, using *GDH* gene by PCR-RFLP method, 74.41% of the samples were typed as assemblage AII, 17.44% assemblage BIII, 3.49% assemblage BIV and in 4.66% isolates, mixed assemblages AII and BIV were detected (15). A study conducted in Khorramabad, based on GDH sequences, indicated the presence of only one genotype assemblage A of *G. lamblia* (14).

In another study performed in Kerman using the PCR-RFLP method for analyzing the genotype sequence of the *GDH* gene of *G. duodenalis*, 16.6% of the samples were found to be from AI subgroup, and 60% and 23.4% belonged to AII subgroup and BIII subgroup, respectively. A significant correlation was observed between the genotype of the parasite and the clinical symptoms of the patients, such as nausea, diarrhea, and abdominal pain (18).

In a study conducted at Motahhari Hospital in Urmia, the *Giardia* samples were genotyped using the glutamate dehydrogenase gene by PCR-RFLP method, BIV (6.7%) and BIII genotypes (93.3%) were identified (19). In a study conducted in Karaj using PCR on the triose phosphate isomerase gene, the dominant genotype was A (23). In another study in Karaj, BIV, BIII, and AII genotypes were detected, some of which belonged to asymptomatic children (24). In a study in Baharestan (southwest of Tehran province), by amplification of the beta-giardin gene, genotypes AII, BIII, and BIV have been detected (13). There are different reports about the relationship between genotype and diarrhea symptoms. In a study in Ethiopia, a strong relationship between genotype B and asymptomatic infection was reported (25). In a study conducted in Bangladesh (26), it has been shown that although genotype B is more common, but it does not play a role in causing diarrhea, on the contrary, genotype A causes diarrhea. Another review study clearly reported a strong relationship between infection with clinical symptoms and AII genotype (27). In the present study, positive samples were observed in people with diarrhea and those without diarrhea. In a study in Karaj (Iran), a direct relationship between diarrheal samples and genotype A was also reported (23). Nevertheless, the results of some studies in Isfahan (20), Kerman (18), and Tehran (8) showed no statistical correlation between asymptomatic infections and genotype B, as well as diarrhea and genotype A (17). These results confirm the findings of the present study that at least these two genotypes do not differ from each other in causing clinical symptoms. One of the limitations of the present study was the lack of easy and sufficient access to clinical samples.

In the present study, *Giardia* cysts were detected in 6.66% of microscopic stained samples and 4.33% of the PCR assay. Out of 4 *G. duodenalis* isolates, one BIV isolate, one BIII isolate, and two AII isolates were detected, some of which are related to people without clinical symptoms of diarrhea. The identification of genotypes AII, BIV, and BIII, respectively, indicate the transmission cycle of both anthroponosis and anthropozoonosis cycles of *Giardia* infection in Semnan.

Authors' Contribution

RO (first author), methodologist/principal researcher ;AD (second author), supervisor, manuscript writer/methodologist/principal researcher/statistical analyst/discussion writer; MP (third author), advisor and methodologist/principal researcher.

Ethic

This study was confirmed by the Medical Ethics Committee of the Faculty of Medical Sciences of Tarbiat Modares University with code No. IR.MODARES.REC.1400.019.

Conflict of Interest

The authors do not have any conflict of interest.

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