ORIGINAL RESEARCH ARTICLE

Molecular Detection of Giardia Duodenalis Sub-Assemblages among Human Diarrheal Patients in Northwestern Iran

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Abstract

Background Giardia duodenalis is a prevalent intestinal protozoan parasite worldwide and consequently, in Iran. The diversity of Giardia genotypes infecting humans in Iran is poorly documented. In the current study, the frequency and diversity of G. duodenalis assemblages and sub-assemblages circulating among human patients with diarrheal symptoms were investigated.

Methods Fecal specimens were collected from 135 patients with diarrhea at health centers in Urmia, Iran, between 2014 and 2015. A total of 45 microscopically Giardia cyst-positive samples were used for DNA extraction, genotyping by PCR amplification, and sequence analysis of the Glutamate Dehydrogenase (gdh) and Triose-Phosphate Isomerase (tpi) loci.

Results Both gdh and tpi markers successfully genotyped in 76.3% (29/38) and 67.6% (25/37) isolates as assemblage A, respectively. All of the assemblage A strains belong to sub-assemblage AII with 100% similarity. Assemblage B isolates were categorized into sub-assemblages BIII (18.9%/7/12/37) and BIV (13.5%/5/12/37) with the tpi marker. The frequency of sub-assemblages BIII and BIV was 7.9% (3/9/29) and 15.8% (6/9/29) when using the gdh marker. Six isolates (13.95%) were genotyped as BIII by the tpi marker and as BIV by the gdh marker.

Conclusion It could be concluded that sub-assemblage AII is the prevalent sub-assemblage of G. duodenalis circulating among human diarrheal patients in Urmia, Iran. The sequence of gdh was more polymorphic than tpi in our analyzed samples.

Keywords Assemblages, Genotyping, Giardia duodenalis, Giardiasis, Iran, Urmia

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1 Introduction

Giardia duodenalis, also known as Giardia intestinalis and Giardia lamblia, is an enteric protozoan parasite of mammals.[1] Giardiasis is still an ubiquitous parasitic disease in the world and joined the WHO neglected disease initiative in 2004.[2]

Giardiasis, caused by G. duodenalis, affects up to 300 million people globally each year, particularly children in low-resource regions, with prevalence varying widely and asymptomatic infections contributing to its ongoing public health challenge.[3–5] The genetic differences in the species may be the reason for the high incidence of this disease.[6] There are eight different assemblages of G. duodenalis (A–H), using Glutamate Dehydrogenase (gdh), Triose-Phosphate Isomerase (tpi), Beta giardin and small subunit ribosomal ribonucleic acid (ssu-rRNA), sequence analysis, and genotypes A and B are often pathogenic to humans.[7] Assemblage A is classified into smaller genetic sub-assemblages, including AI, AII, and AIII. Assemblage AI has been isolated from both humans and animals, while assemblage AII has only been isolated from humans.[8] Assemblage B sub-assemblages include BIII and BIV; BIII appears to be zoonotic, while BIV is mainly anthroponotic.[9] However, a recent update to the review presents sequence and host data for the most commonly detected Giardia species and proposes a taxonomic revision by synonymizing contemporary genotypes with existing species descriptions, based on prior recommendations and host associations supported by extensive sequence datasets.[10]

Despite various distributions of giardiasis in Iran and West Azerbaijan province,[11–13] the current epidemiological scenario of human giardiasis in Iran is very poorly described.[14] The current study was designed to determine and confirm the prevalence of G. duodenalis assemblages and sub-assemblages among patients with Giardia attending health centers in Urmia using sequence analysis of gdh and tpi genes. It will provide more information on the molecular epidemiology of giardiasis in Iran.

2 Methods

Stool Sample Specimen collection & microscopic examination

Giardia duodenalis positive human stool samples were collected from Imam Khomeini (25.92%, 35/135) (37°33’4”N, 45°3’10”E) and Imam Reza (11.12%, 15/135) (37°31’24”N, 45°3’42”E) hospitals, and Velayat health center (62.96%, 85/135) (37°53’88”N,45°07’05”E) in Urmia, Iran, during 2014-2015 (Figure 1).

They were transferred to the Parasitology Laboratory of the Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran. A microscopic examination was performed on all the stool samples, and those with multiple G. duodenalis cysts (10-15 Giardia cysts in a field of view at 40× magnification) were included in the study. The initial diagnosis of the parasite was performed in the stool samples using physiologic serum and Lugol’s iodine under the microscope.

Figure 1 A map showing the sample collection area of G. lamblia isolates in Urmia, West Azerbaijan province, Iran

Cyst collection and concentration

The Giardia positive samples were then concentrated using a 0.85M Sucrose Gradient method.[15] The samples containing Giardia cysts were stored at -20°C for later DNA extraction and PCR amplification, approximately 10 days after sample collection.

DNA extraction

DNA was extracted from condensed samples using the Yekta Tajhiz Azma stool DNA extraction Kit (YTA, Tehran, Iran). The DNA extraction protocol was followed according to the manufacturer’s instructions, with minor modifications. To break the Giardia cyst wall, it was frozen in liquid nitrogen and then thawed in boiling water ten times before using the kit.

Primer designing and PCR amplification

Previously published papers on Giardia genotyping in Iran usually used a 400 bp fragment.[16-18] In the current study, GDHUF (5’AGG TTA TCT TCG AGC GCG3´) as a forward primer with two reverse primers, GDHUR2 (5’TTC TGA GTG GCG CAA GGC3´) and GDHDeg (5’ATC TGG TAG TTC TTG GSG TG3´), were designed using GeneRunner software version 5.1.06 beta to amplify 874 bp and 1145 bp fragments of the gdh gene based on the GenBank ID: M84604. The TPIUF (5’-ATG CCT GCT CGT CGY CCC3´) and TPIUR (5’-ACT GGC CAA GCT TCT CGC3´) primers were designed to amplify a 683 bp fragment of the tpi gene based on the GenBank IDs: L02116 and L02120.

The PCR reactions were performed at a volume of 25 µL.

The annealing temperature was separately optimized for each primer. The gdh gene amplification was performed over 5 minutes at 95 °C in 35 cycles, including 1 minute at 94 °C, 1 minute and 30 seconds at 64 °C, 1 minute and 55 seconds at 72 °C, and finally 10 minutes at 72 °C. The PCR conditions for tpi amplification were as follows: 5 minutes at 95 °C, 1 minute at 95 °C, 1 minute at 62 °C, and 1 minute at 72 °C in 30 cycles, followed by a final extension of 10 minutes at 72 °C. The PCR product was electrophoresed on 1 % agarose gel stained with ethidium bromide under safe conditions. A total of 37 and 38 samples from gdh and tpi genes, respectively, were selected directly for sequencing in both (forward and reverse) directions on an ABI 377 automatic sequencer with the same primers by Takapouzist Company (Tehran, Iran).

Sequence analysis

The quality of the obtained chromatograms was checked by visual inspection, and the presence of ambiguous (double) peak positions was actively searched for to ascertain the potential occurrence of mixed infections involving two assemblages or sub-assemblages of the parasite. Bioinformatics analysis was done using Chromas [19], Basic Local Alignment Search Tool (BLAST)[20], and Clustal Omega.[21] Molecular and Evolution Genetic Analysis software version 6 (MEGA 6) was used for sequence alignment.[22] These sequences were deposited in GenBank and are available in the National Center for Biotechnology Information (NCBI) through accession numbers MH310965 to MH311039.

3 Results

The current study examined 135 stool specimens collected from two hospitals and a health center in Urmia, Iran. Among those, 45 isolates (33%) were concentrated for G. duodenalis. The age of people infected with Giardia ranged from 4 to 60 years old. The highest level of infection was observed in the 10- to 40-year-old age group.

PCR amplification

A 682 bp fragment of the tpi gene and an 874 bp fragment of gdh were amplified in 33.3%/ 45/135 and 18.5%/25/135 samples, using the specific primers, respectively. The 1145 bp fragment of gdh was amplified in 14.8%/20/135 samples using the degenerative primer (Table 1) (Figure 2).

Sequences analysis

- tpi

Comparing the sequences obtained from the current study (n = 37) with GenBank IDs L02120.1 and U57897.1, they were classified into assemblage AII (68%, 25/37) and assemblage B (32%, 12/37). The comparison of assemblage B sequences with BIII and BIV sequences with the GenBank IDs: AY228628.1 and L02116.1 showed that the frequency of both assemblages BIII and BIV was 18.91%/7/12/37 and 13.51%/5/12/37 in Urmia, respectively (Table 2).

Assemblages AI with the GenBank ID: L02120.1 and AII with the GenBank ID: U57897.1 showed 99.26% similarity, while the similarity between assemblages BIII and BIV with the GenBank IDs: AY228628.1 and L02116.1 was 98.68%. The comparison of the sequences obtained in this study with those in GenBank showed 99.25%-99.26% similarity with assemblage BIV and 99.25%-99.62 % with assemblage BIII (alignments were not shown) (Figure 3).

- gdh

A comparison of 38 obtained sequences with sequences submitted from Afghanistan (No. XM77 3614.1) and Canada (No. AY178737.1) revealed that 76.32% of them showed 100% similarity with the AII genotype, while 7.9% matched BIII, 7.9% matched BIV, and 7.9% were identified as recombinant assemblages (Table 1). The sequence similarity between AI and AII assemblages (No. XM 773614.1 and AY 178737.1) was 98.51%. They have mismatches in 11 nucleotides, including transitions, transversions, and deletions. The sequence similarity within BIII and BIV assemblages was 97.64%-99.37% and 97.7-99.50%, respectively, while the similarity

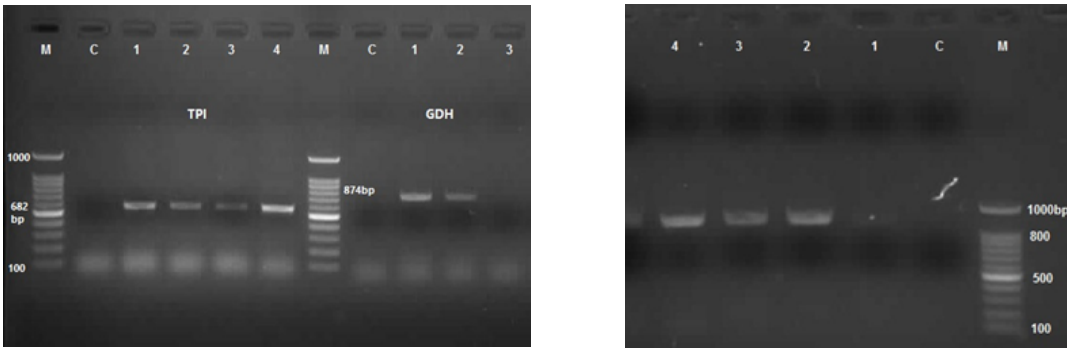

Figure 2 Representative PCR amplification of tpi (A) and gdh (A and B) fragments by using designed primers in G. lamblia isolates collected from Urmia, West Azerbaijan province, Iran

Table 1 Details of genotyping results of Giardia parasites in Urmia based on gdh and tpi sequences

Locus	Assemblage	Sub-assemblage	No. of isolates (%)	Reference sequence	GenBank Submission	SNP
gdh	A	AII	29 (76.3)	AY178737	MH311002-MH311009, MH311012, MH311014-MH311016, MH311018-MH311023, MH31125-MH311030, MH311031, MH311034, MH311035, MH311037-MH311039	None
	B	BIII	3 (7.9)	AY178756	MH311010, MH311011, MH311017	T116C, Y206T, Y254T, T326C, T344C, T416C, G509A, C596T, C620T, G704T, Y764C, G818A, T860C, T974C, T1004C
		BIV	3 (7.9)	AY178756/ EF507665	MH311013, MH311032, MH311036	C116T, T206Y, T254Y, C326T, C344T, C416T, A509G, T596C, T620C, T704G, C764Y, A818G, C860T, C974T, C1004T
		BIII/BIV	3 (7.9)	EF507665	MH311033, MH311034, MH311037	None
tpi	A	AII	25 (67.6)	U57897	MH310965-MH310968, MH310974, MH310976-MH310978, MH310980-MH310985, MH310987-MH310993, MH310995-MH310997, MH310999, MH311000	None
	B	BIII	7 (18.9)	AY228628	MH310971-MH310973, MH310994, MH310998, MH311001	G39A, G27T, G91T, C165T, C168T, C210A, C534T
		BIV	5 (13.5)	L02116	MH310969, MH310970, MH310975, MH310979, MH310986	A39G, T27G, T91C, T165C, T168C, A210C, T534C

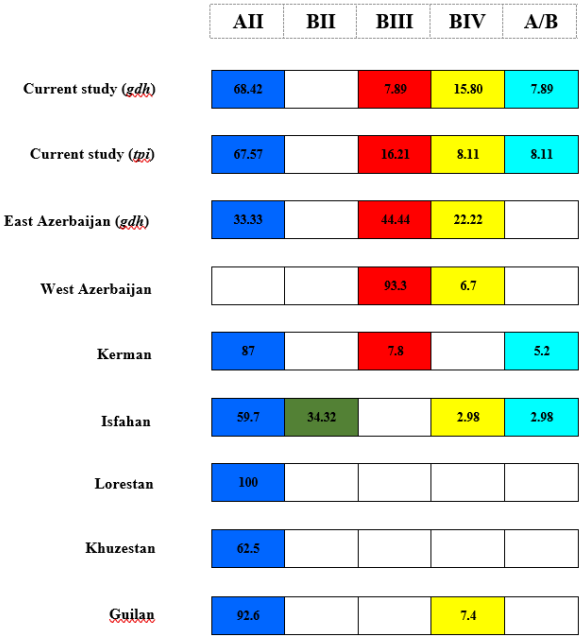

Figure 3 Representative G. lamblia genotypes and their prevalence (%) in different provinces of Iran, based on gdh and tpi genes

between them was 98.5% (alignments were not shown) (Figure 3).

4 Discussion

Giardia duodenalis has a worldwide distribution with higher infection rates in developing countries and children. More than 71% of the 280 million cases of diarrhea occur annually in Asia, Africa, and Latin America.[23] Assemblages A and B are zoonotic and often associated with pathogenicity in humans and mammals.[24,25] Babaei et al. used the gdh gene for genotyping 38 samples and found 33 (87%) AII genotype samples, three (7.8%) BIII genotype samples, and two samples with both genotypes in Tehran, Iran.[28] In Tabriz, Iran, 54.8% of samples were identified as assemblage A, and 41.9% were classified as assemblage B using RFLP of the tpi gene.[17] However, Fallah et al. in East Azerbaijan province, Iran, reported that 67% of the samples were genotype B and 33% genotype AII.[26] Different studies in Iran from 2008–2019 using polymerase chain reaction-random amplification length polymorphism (PCR-

RFLP) reported the presence of AII, BII, BIII, BIV, and A/B from Tehran, Kerman, Isfahan, East Azerbaijan, Khuzistan, Fars, and Lorestan provinces of Iran (Table 2).[16–18,28–30]

However, in Khuzestan, 37.5% of sequences were reported as untypable.[28] However, Hazrati Tappeh et al. reported 93.3% infection with sub-assemblage BIII and 6.7% with sub-assemblage BIV using PCR-RFLP on the gdh gene.[12] In this study, the frequency of assemblage A was 67%, suggesting that humans are the primary source of infection in the region. It could be concluded that the differences in the prevalence of the A and B genotypes in different geographical areas are due to the diverse target populations.[24,40,41]

1). However, the current study reports the presence of AII, BII, BIV, and recombinant assemblages in Urmia (Table 2). This discrepancy could be attributed to the fact that they sequenced the 432 bp fragment of gdh, whereas we used 847 bp and 1145 bp fragments of gdh, as well as 692 bp of tpi fragments, for sequence analysis. It could be suggested that using a large fragment of gdh is essential for detecting Giardia BIV sub-assemblages. The current study reports the presence of G. duodenalis AII, BIII, and BIV sub-assemblages in diarrheal humans for the first time in Urmia based on the sequencing of the gdh and tpi locus.

A high degree of polymorphism in assemblage B has also been reported.[41,42] In this study, no mixed genotypes

Table 2 Summarizing the main findings in studies related to human giardiasis in Iran during 2000 to 2020, in comparison with the current study results

Location	Period of study	No. of collected samples	No. of genotyped samples	Genotyping Marker	Genotyping Results	Reference
Fars	2020	15	15	SSU-rDNA	A, B	[31]
Khorramabad	2015 - 2016	490	70	TPI	AI, BII, BIV	[32]
Hamadan	2014 - 2015	4066	23	GDH & TPI	AII, B	[33]
Andimeshk	2015 - 2016	3580	40	GDH & β-giardin	AII, B	[34]
Kerman	2016 - 2017	199	11	GDH &TPI	AI, AII, mixed (AI + B)	[35]
Kashan	2015	3653	44	GDH	AII, BII, BIV	[36]
West Azerbaijan	2011-2012	720	34	GDH/RFLP	BII, BIV	[18]
Guilan	2016-2017	8356	41	GDH	AII, BIV	[37]
Ahvaz	2017–2018	1073	82	SSU-rDNA, GDH , β-giardin	AII AIII, BII, BIV	[38]
Behbahan	2015 - 2016	450	12	TPI& SSU-rDNA	AII	[28]
Fars	2010	205	172	GDH/RFLP	AII	[39]
West Azerbaijan	**2014-2015**	**135**	**38**	**GDH & TPI**	**AII, BII, BIV, Recombinant**	**Current study**

The fragment for PCR amplification and sequencing in the current study was larger (1145 bp) than the fragment used in the RFLP studies (432 bp). Interestingly, sequence analysis showed that the RFLP fragment had low heterogeneity. Recently, an investigation on the occurrence of G. duodenalis infections through the sequence analysis of the gdh locus in humans residing in the Guilan province of Iran revealed the presence of AII and BIV sub-assemblages.[36] Out of four recognized G. duodenalis sub-assemblages in Iran, only BII was not detected in Urmia. On the other hand, the BIII sub-assemblage was not detected in Guilan,[36] while the prevalence of the BIV sub-assemblage was 7.9% and 13.5% using gdh and tpi sequences, respectively (Table

were observed. Still, gene exchange can occur when there are two types of Giardia assemblage in the same host, causing genetic diversity, especially in assemblage B.[40]

The current study was based on a master's student project with limited budget. Strengthening this study with additional samples from human and animal populations in the region will be beneficial in enhancing our understanding of the current epidemiological scenario of human giardiasis in the area and the country.

Conclusion

Genotyping Giardia is preferred with tpi gene sequencing

rather than gdh gene sequencing. Therefore, the tpi gene is recommended over gdh in the genotyping of Giardia parasites. The dominant assemblage in Urmia, North-Western Iran, was assemblage A, which suggests that humans are the primary source of infection. However, further studies are required to study the role of domestic animals and water reservoirs as potential sources of Giardia infection in Iran.

Declarations

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Authors' Contributions
Conceived and designed the experiments: KHT, SG, and FN. Performed the experiments: FN, KHT, SK, SM, and SG. Analyzed the data: SG, SHAA, and FN. Contributed reagents/materials/analysis tools: KHT and FN. Wrote the paper: SG, FN, KHT, SM, SHAA, EAS, and SK. All authors read and approved the final manuscript.

Availability of Data and Materials
Data supporting this article are included within the article and supplementary file.

Conflict of Interest
The authors declare that they have no competing interests.

Consent for Publication
All authors have read and approved the final manuscript and have provided their consent for publication.

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Ethical Considerations
Human stool samples and the extraction of DNA from these samples were approved by the Ethics Committee of Urmia University of Medical Sciences under the Code of Ethics IR.UMSU.REC.1394.27. The samples were obtained from patients as part of a routine examination for the diagnosis of parasitic infections. They were referred to the healthcare laboratories at Urmia University of Medical Sciences, Urmia, Iran. The Research Ethics Committee waived the requirement for informed consent.

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