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Original Article

Molecular Identification of *Giardia duodenalis* Isolates from Fars Province, Iran

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Abstract

Background: *Giardia duodenalis* is one of the most common human intestinal protozoan parasites worldwide and is endemic throughout the world with a vast range of mammalian hosts. The present study aimed to identify the prevalence of *G. duodenalis* isolates and determine the most common of its assemblages in the patients referring to health centers and hospitals in Fars province, Iran that will be subjected to further molecular investigation.

Methods: We collected 1000 human fecal samples from health centers and hospitals in Shiraz, Iran in a one year period from September 2009 to August 2010. Microscopic examination for the presence of *G. duodenalis* cysts and trophozoites was performed by direct wet mount before and after the concentration techniques. Extraction of DNA was performed by Phenol-Chloroform-Isoamylalcohol (PCI). *G. duodenalis*-positive specimens were analyzed by PCR. A fragment of the SSU-rDNA (292 bp) gene was amplified by PCR using the forward primer RH11 and the reverse primer RH4. Genotyping was performed using sequence analysis of *G. duodenalis* glutamate dehydrogenase gene using primers GDHeF, GDHiF, and GDHiR.

Results: The prevalence of *Giardia* infection was 10.7% (107/1000) examined based on microscopic examination. PCR identified 80% (40/50) of the samples as positive for *G. duodenalis* based on SSU-rDNA amplification on sucrose gradient samples. Besides, genotyping results indicated 32 isolates (80%) as assemblage AII and 8 isolates (20%) as assemblage BIII and BIV based on the DNA sequence analysis of the glutamate dehydrogenase locus of *G. duodenalis*.

Conclusion: The findings of this study emphasize that Iran (Fars Province) is a favorable area for giardiasis with an anthroponotic infection route.

Introduction

Giardia duodenalis (synonymous *G. lamblia* or *G. intestinalis*) is one of the most common intestinal parasites in humans worldwide and is endemic throughout the world with a vast range of mammalian hosts (1). The genus *Giardia* is categorized as phylum Sarcomastigophora, class Zoomastigophorea, and a member of the order Diplomonadida (2). Although the specific name *duodenalis* is as zoological nomenclature, the names *intestinalis* and *lamblia* are often used, particularly for the isolates of human origin (3). The prevalence of this infection varies between 2-5% in industrialized countries up to 20–30% in less developed countries (4). About 200 million people have symptomatic giardiasis in Asia, Africa, and Latin America with some 500,000 new cases reported each year (5, 6). Giardiasis is among the top ten human parasitic diseases that is a widespread intestinal disease, responsible for 2.5 million diarrhea and nutritional deficiencies in children in developing countries (7). *Giardia* grows in the small intestine and multiplies by asexual binary fission on the surface of the mucosa. Trophozoite and cyst are two distinct forms of *Giardia*. Ingestion of tetra nucleated agent is responsible for transmission from one host to another (8, 3). Giardiasis has variable clinical symptoms from the absence of symptoms to acute or chronic diarrhea, dehydration, abdominal pain, nausea, vomiting, and weight loss. The interplay between the virulence of the parasite and the developmental, nutritional, and immunological status of the host determines the severity of the disease (9-11).

Parasitological techniques have been applied for detecting *Giardia* using direct wet-mount, concentration, and staining method (12, 13). Moreover, techniques of molecular detection based on PCR have been developed to detect *G. duodenalis* cysts in feces from isolates of human and animal origin worldwide. In addition, these molecular methods allow the genotyping of *G. duodenalis* cysts. Molecular genetics techniques have successfully provided new powerful tools to characterize *Giardia* isolates and *G. duodenalis* specific. The majority of molecular studies of *G. intestinalis* have relied on the analysis of the small subunit ribosomal RNA (SSU-rRNA), β-giardin (*bg*), glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef-1*), triose phosphate isomerise (*tpi*), and variant surface protein (*vsp*) genes (14). These tools are widely used for identification of *G. duodenalis* genotypes in clinical specimens as well as differentiation of *Giardia* at the species/assemblage and genotype levels. The utility of molecular diagnostic tools is frequently determined by the SSU-rRNA, *gdh*, *tpi*, and *bg* genes targeted. The usage of these loci for the genotyping or subtyping of *G. duodenalis*, their restriction fragment length poly-morphism analysis, sequence characteristics and phylogenetic tree is useful to understanding of the population genetics, epidemiology, and taxonomy of giardiasis in humans and domesticated animals (8). Isolates of *G. duodenalis* are classified into seven assemblages based on the characterization of the glutamate dehydrogenase (*gdh*), subunit ribosomal RNA (SSU-rRNA), and triose phosphate isomerase (*tpi*) genes. Two assemblages (A and B) are found in both humans and animals, whereas the remaining five (C-G) are animal host-specific (15). Besides, DNA sequence studies on *G. duodenalis* have shown the existence of two subgroups in assemblage A (AI and AII) and assemblage B (BIII and BIV). Genotype AI is generally found in animals, whereas genotype AII has mainly been identified in humans. Assemblage B comprises a genetically more diverse group of predominantly human isolates although some animal genotypes have been included, as well (16,17). Information on the genotype of *G. duodenalis* from individual patients over time will assist understanding the course of infection and the rate of reinfection following

treatment. It will also be possible to identify those genotypes of *Giardia* that are more persistent and associated with infections of long-term duration (18). Giardiasis has been one of the most common intestinal infections in Iran. In recent years the Iranian researchers have contributed greatly to increase their knowledge about giardiasis in human and animals (19, 20). Reports of giardiasis prevalence ranged from 1.4% to 59.6% among different socioeconomic and geographical situation in the community. The incidence of *G. duodenalis* in the patients who had referred to hospitals and health care centers was reported as 25.8%, 10.1%, and 21.3% by Arani et al. (12), Haghighi et al. (21), and Taherkhani et al. (22), respectively. Also, another study indicated that the prevalence of giardiasis varied in different parts of Iran (5%-23%) (22). The findings of the studies emphasize that *Giardia* infection is a major public health problem. Thus, the present study aims were to determine the prevalence of *G. duodenalis* and assemblages or sub-assemblage in the patients referring to health centers and hospitals in Fars province based on microscopic and molecular examination that presents a base situation of giardiasis in the understudy region.

Materials and Methods

Sample collection

In a one year period from September 2009, one thousand human fecal samples were collected from the patients with gastrointestinal discomfort, such as diarrhea, dehydration, abdominal pain, nausea, vomiting, referring to the health centers and hospitals in Shiraz, south of Iran. For further examination, the samples were sent to the Research Laboratory of Intestinal Parasites in the Department of Parasitology and Mycology in Faculty of Medicine, Shiraz University of Medical Sciences.

Parasitological examination

Microscopic confirmation for the presence of *G. duodenalis* cysts and trophozoites was performed by examination on direct wet mount before and after the concentration techniques. Direct microscopy was done on all formed and liquid samples. Sedimentation of ethyl acetate was performed in order to maximize the number of detectable organisms which may be too limited to be seen by direct microscopy alone. The fluid on the side of the tube was allowed to drain onto the deposit. Aliquots of the concentrated fecal samples were stored at -20 ºC. Monolayer sucrose gradient with specific gravity at 0.85 M was done on positive stool samples (to purify fecal samples and concentrate of cysts). Fresh stool samples were homogenized 1:10 (v/v) in distilled water or physiologic saline solution, filtered through two layer gauze to discard larger detritus, and washed two times by spin at 400 × g for 5 min in 50 ml falcon tube. Then, 5 ml of the sample suspension was gently added to 5 ml of 0.85 M sucrose solution that made two separate layers in a 15-ml glass tube. After spin at 1700 × g for 10 min in a 4°C centrifuge, the cysts formed a thin ring liquid layer between the sucrose solution and the suspension that was carefully transferred into a 15-ml clean glass tube. The resulting cysts ring solution was diluted in distilled water for twice (first time 1:10 (v/v) and second time 1:5 (v/v) and centrifuged at 400 × g for 5 min. The sediment material was then placed in a clean 1.5 ml ependorf tube. Aliquots of purified fecal samples were stored at 4°C and -20 ºC.

Molecular techniques

DNA extraction

DNA was extracted by Phenol-Chloroform-Isoamylalcohol (PCI) on purified and concentrated fecal by sucrose gradient and sedimentation of ethyl acetate samples as follows. Moreover, glass beads or freeze-thaw was used for disruption of the cyst wall on some samples. First, 200 µl of sucrose gradient or sediment concentrated cysts sample and 200 µl 3%Triton X100 were mixed and incubated in a water bath at 75 ºC for 1 hour. Then, 200

µl of lysis buffer and 10 µl of proteinase K were added to 200 µl of homogenate and incubated at 37 ºC overnight. Phenol-chloroform extraction method (PCI) was used for extraction of genomic DNA from the cysts. PCI was then added to the solution and centrifuged at 15000 rpm at 25 ºC for 10 min. The supernatant was precipitated with ethanol absolute (2 or 4 equal) in a new tube at -20 ºC for 24 hours. The solution was centrifuged at 15000 rpm at 4 ºC for 10 min. After air dry sediment, 100 DDW or TE (100 mMTris, and 25 mM EDTA, pH= 8.0) was added to the sediment in the tube and stored at -20°C until PCR analysis.

Molecular identification

PCR Amplification of SSU-rDNA

A fragment of the SSU-rDNA (292 bp) gene was amplified by PCR using the forward primer RH11 (5'-CATCCGGTCGATCCTGCC-3') and the reverse primer RH4 (5'-AGTCGAAC CCTGATTCTCCGCCCAGG-3') as previously described (23, 24) with some slight modification. The PCR mixture in the 25 µL reaction volume contained 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM for each forward and reverse primer, and 2 U Taq. The PCR was performed under the following conditions: An initial hot start at 96 ºC for 2 min followed by 30 cycles each including 96 ºC for 45 s (denaturing), 58 ºC for 30 s (annealing), 72 ºC for 45 s (extension), and a final extension step at 72 ºC for 4 min using (Corbett Research Thermal Cycler, Australia). The primers were checked by a positive and a negative control. The PCR products were electrophoresized on a 1.5% agarose gel with 5 µl of the reaction solution and visualized by staining the gel with ethidium bromide.

PCR Amplification and sequencing of the glutamate dehydrogenase gene (gdh)

A semi-nested PCR was performed using the primers GDHeF, GDHiF, and GDHiR to amplify a 432 bp fragment of *Giardia* glutamate dehydrogenase gene (*gdh*) (25) with some slight modifications. Genotyping was performed using sequence analysis on 40 PCR products of *G. duodenalis* based on *gdh* amplification in both directions. DNA sequencing reactions were performed by First BASE Laboratories in Malaysia (http://www.base-asia.com) to sequencing commercially in both forward and reverse directions using primers GDHiF and GDHiR for GDH gene fragment.

Results

In this study, microscopy and molecular technique were performed directly on fecal samples. The results indicated that 107/1000 (10.7%) samples were positive for *G. duodenalis* based on microscopy. Parasite load for microscopically positive *G. duodenalis* samples were different in the quantity of cysts from low (less than 2 cysts), moderate (2 to 10 cysts) and high (more than 10 cysts) per 40X field of view. One step monolayer sucrose gradient successfully was done on positive stool samples to purify fecal samples and concentrate *G. duodenalis* cysts (Fig.1 A-B).

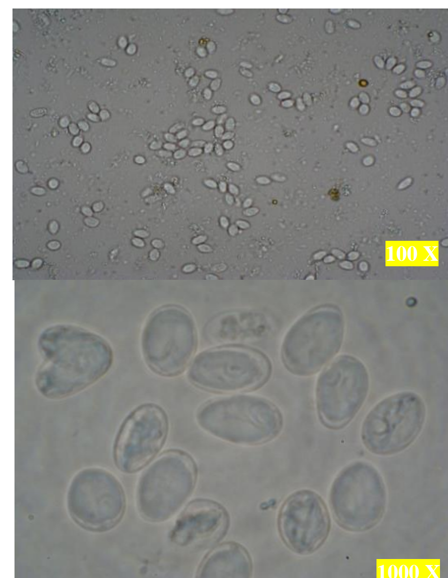

Fig. 1: A and B- Microscopic slides of concentrated and purified *G. duodenalis* cysts observing after 1 step sucrose gradient in the stool samples. (Original picture 100 X, 1000 X)

Pretreatment to disrupt the cysts wall was successfully performed before extraction of DNA using 3% Triton X100 (Fig. 2 A-B).

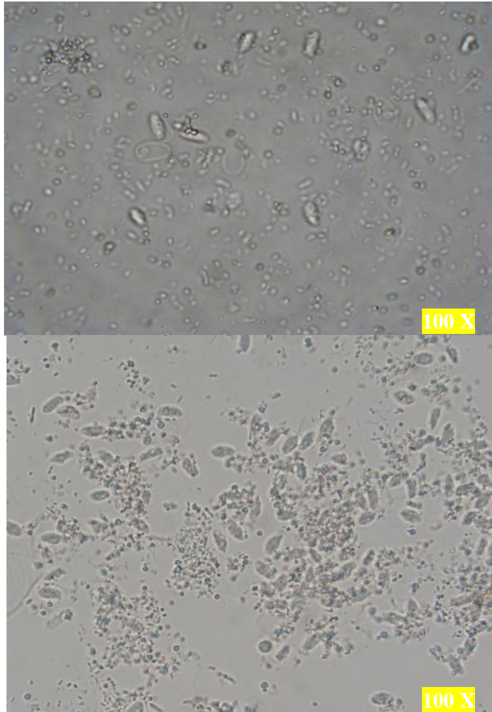

Fig. 2: A and B: Cyst wall disruption after using 3% TritonX100 on fresh sucrose gradient samples. (Original picture 100 X)

Fifty microscopically positive *G. duodenalis* were analyzed by PCR. In molecular identification, PCR identified 40/50 (80%) samples as positive based on SSU-rDNA amplification on sucrose gradient samples. The specific PCR product (292 bp) amplified with primers RH11 and RH4 was observed after pretreatment by incubating in 3% TritonX100 and DNA extraction method by phenol/chloroform/isoamylalcohol extraction method (Fig. 3). Sequence analysis was successfully performed on 40 PCR products of *G. duodenalis* based on *gdh* amplification in both directions. The genotyping results indicated 32 isolates (80%) as assemblage AII and 8 isolates (20%) as assemblage BIII and BIV based on the DNA sequence analysis of the glutamate dehydrogenase locus of *G. duodenalis.*

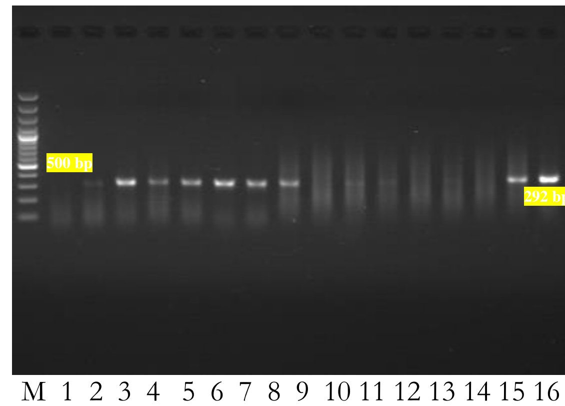

Fig. 3: Electrophoresis of PCR product with SSU-rDNA (292 bp) primers/Lane 2-15=Samples from the patients/Lane 1=Negative control (primers + buffer)/ Lane 16=Positive control (292 bp)/ Lane M=100 bp DNA marker (Fermentas, Canada)

Discussion

In this study, we applied microscopic and molecular detection in order to identify *G. duodenalis* from fecal samples. In medical laboratories *Giardia* diagnosis is based on preparation of wet mount of patient stool and visual recognition by light microscopy. Performing of these methods requires experienced microscopists and medical laboratory technician. In the other hand, these techniques are unable to differentiate between the genetic assemblages of *G. duodenalis* isolates and might not be sensitive enough to detect low numbers of excreted *Giardia* cysts in fecal samples (16, 26, 27). Thus, techniques of molecular detection based on PCR have been developed to detect *G. duodenalis* cysts in feces. These molecular methods allow the genotyping of *G. duodenalis* cysts.

For molecular detection in this study, Polymerase Chain Reaction (PCR) was used to amplify the genes encoding the SSU-rRNA on the microscopically positive *Giardia* samples. The SSU-rRNA locus has been proven to be highly sensitive as a screening tool for *Giardia* directly from faces and is the traditional gene sequence used for identification and phylogenetic analyses (28, 29). The SSU-rRNA sequence is more conserved compared to the

other regions that were used as templates. This could explain why the primers amplifying the SSU-rRNA gene worked better (26). The conserved SSU-rRNA gene is a commonly used marker for the species and assemblage differentiation (mostly genotyping) of *Giardia* (8). For molecular characterization, we used the glutamate dehydrogenase (*gdh*) marker that allows distinguishing between the subgroups of the assemblages A and B. The variable *gdh* locus has been used for successfully genotyping and subtyping the isolates of *G. duodenalis* from a range of vertebrate hosts (30, 31, 32). An effective DNA extraction method is needed to overcome the problems of inadequate yield of extracted DNA and improve molecular detection apart from the sensitive amplification systems for gene identification.

We had chosen the Phenol-Chloroform protocol for DNA extraction of *G. duodenalis* from fecal samples with pretreatment using Triton X100. Our results showed that this method was the most efficient DNA extracting method for the samples was purified by sucrose gradient. Also, all steps of DNA extraction and amplification by PCR were performed in a short time. A high percentage of false-negative results may occur in the PCR assay in clinical specimens, foods, and environmental samples (27). In our experiment, the false negative PCR reaction could have occurred due to some reasons: 1- Parasite load: the low intensity of infection in naturally infected host samples is the problem in using PCR related to DNA extraction from fecal samples (33). 2- Storage of stool specimen: long storage of stool specimens could have caused a false negative PCR reaction (34).

The prevalence of *Giardia* infection was 10.7% of 1000 patients examined in the area under study. Islamic Republic of Iran is a favorite area for giardiasis. The prevalence of giardiasis varies in different parts of Iran (5%-23%) (22). In a national survey of the prevalence of intestinal parasitic infections in the community, *G. duodenalis* was the most common infection (10.9%) (35), which is highly consistent with the results of other similar study (10.2%) (36). The incidence of *G. duodenalis* in the patients referring to hospitals and health centers were reported as 25.8%, 10.1%, and 21.3% by Arani et al. (12), Haghighi et al. (21), and Taherkhani et al. (22), respectively. In all these studies, confirmation of the infection was based on microscopic detection and parasitological techniques. In our study, the prevalence of *G. duodenalis* among the patients was 10.7% (107/1000) which is in agreement with other researchers' findings in Iran. Overall, health promotion, public education, improving sanitation conditions, and improving clean drinking water and food are important strategies which can be used for control and prevention of the giardiasis infection. Studies performed on DNA extracted directly from fecal samples from various countries confirm that only *Giardia* assemblages A and B are associated with human infections. The prevalence of each assemblage varies from country to country and sometimes even within the same country. The reasons behind the geographic variations in the distribution of the *G. duodenalis* assemblages are still unclear. In our study, DNA sequence showed that the assemblage A was the most prevalent (80%) genotype and 20% of the positive samples belonged to assemblage B in humans living in the area. The predominance of assemblage A in this study is similar to the previous studies in Brazil (37), Iran (38), Italy (39), New Zealand (40), and Thailand (41). However, it was in contrast to some other studies showing the predominance of assemblage B from Australia (6), Bangladesh (42), Belgium (43), England (44), Nicaragua (45), and Philippine (46). In this study, subtyping analysis showed that all the 32 assemblage A isolates were identical to sub-genotype AII that has also been shown in Australia (6), Brazil (37), Iran (38), Nicaragua (45) and Peru (47).

Although assemblage AII is most likely transmitted from human to human, assemblage AI is most often responsible for zoonotic transmission with a wide range of animals

acting as the reservoir hosts. Moreover assemblage B appears to be largely human specific, it has been reported in some animals and may represent a zoonotic potential. The predominance of assemblage AII and lack of assemblage AI in our study may be due to the geographical differences among the countries regarding the prevalence of infection that might be influenced by the regional epidemic or local dynamics of the transmission related to host fauna, infection sources, and socioeconomic factors. Despite, the findings of this study suggested anthroponotic transmission from human to human, it is difficult to compare human and animal isolates in this context simply because there was no investigation or study conducted in this area to genotype assemblage A and B from animals isolates thus setting as one of the limitation of the study. Therefore, current result was not enough to demonstrate the role of anthroponotic and zoonotic of giardiasis infections. Of course, further molecular genotypes and epidemiological information about the distribution and prevalence of *G. duodenalis* assemblages and subtypes within human and animal populations will be attended in future studies. We hope to include the prevalence of assemblage A or B in domestic and pet animals that may serve as a potential reservoir for human in zoonotic giardiasis. Also a thorough more molecular characterization at multiple loci shall be performed in future on *G. duodenalis* isolates ranges from humans, animals and the environment.

Conclusion

G. duodenalis is a flagellated protozoan parasite found in the small intestine of many different mammalian species including man and animals. The clinical manifestation of *G. duodenalis* infection varies from asymptomatic to acute diarrhea, flatulence, abdominal pain, fatigue, and anorexia or chronic disease. *G. duodenalis* is one of the most common intestinal parasites in Iran. Important factors, such as socioeconomic, geographical, sanitary and hygienic, cultural, and nutritional factors, contribute to the prevalence of the parasite. The present study showed that *G. duodenalis* subassemblage AII was the predominant assemblage in Fars Province, which indicates an anthroponotic transmission in this population.

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