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The assemblage B of *Giardia duodenalis* is the most common assemblage in isolates from southeast of Iran

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ABSTRACT

Objective: To identify genetic assemblages of *Giardia duodenalis* in human isolates from health centers in the city of Zahedan, Iran.
Methods: The present study included 54 fecal specimens infected with *Giardia* cysts from symptomatic and asymptomatic patients with giardiasis. The fecal specimens were used to identify the parasite genotypes. Genomic DNA was isolated by using QIAamp DNA Stool Mini Kit. Genotyping of *Giardia duodenalis* samples was performed by PCR assay by amplification of P21 locus of *Giardia*.
Results: Our result indicated that genotype B (75.9%) was more prevalent than genotype A (24.1%) as defined by the presence of amplicons at 249 bp and 303 bp for genotypes B and A, respectively. No mixed genotype of A + B was detected in tested samples. Additionally, we observed that the assemblage A was more prevalent in symptomatic patients than in asymptomatic ones.
Conclusions: Our findings revealed that assemblage B was the most common assemblage in isolates examined in Zahedan demonstrating that there is a major non-zoonotic transmission cycle in the area.

1. Introduction

Giardia duodenalis (*G. duodenalis*) (also known as *Giardia intestinalis* or *Giardia lamblia*) is an intestinal protozoan pathogen that infects human and a wide variety of mammalian hosts. It is the most common intestinal protozoan in humans[1]. The incidence of giardiasis is 2%–5% in developed countries and up to 20%–30% in developing countries[2]. Poor sanitary environments, poor water quality control and overcrowding are among factors that contribute to high prevalence of *G. duodenalis* in developing countries. Giardiasis is associated with 2.5 million diarrhea-associated deaths as well as nutritional deficiencies in children in developing countries[3]. In Asia, Africa and Latin America, about 200 million people have symptomatic giardiasis[4]. In Iran, the prevalence rate of giardiasis ranges from 1.4% to 59.6% under diverse geographical or socioeconomic conditions in the region[5,6].

Giardia genus includes six species *e.g. G. duodenalis*, *Giardia agilis*, *Giardia psittaci*, *Giardia muris*, *Giardia microti*, and *Giardia ardeae*[1,7]. Mammalians including human are the major hosts for *G. duodenalis* which is transmitted via fecal-oral route by direct contact or through ingestion of cysts from filthy food or water[8]. The clinical characteristics of giardiasis vary from asymptomatic infection to severe diarrhea, abdominal cramps and weight loss[9,10].

Giardia infection is multifactorial, and several risk factors are associated with its infection, among which the disparities in the virulence of *Giardia* isolates, the host immune response, or a combination of both have been proposed. Although *Giardia* cysts have identical morphology, they are composed of genetically different genotypes, and microscopic examination is incapable of detecting the identity or the infective potential of waterborne cysts in humans[11]. By application of molecular techniques, *G. duodenalis* can be genetically classified into eight major assemblages: A to H. Assemblages A and B infect humans and a variety of other animals (dogs, cats, and live-stock); whereas the remaining six assemblages (C–H) are more common in domestic or

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The study protocol was performed according to the Helsinki Declaration and approved by Ethical Committee of Zahedan University of Medical Sciences, Zahedan, Iran. Informed written consent was obtained from all patients.

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wild animals, livestock, and marine vertebrates[9]. Moreover, DNA sequencing reports on *G. duodenalis* have demonstrated the presence of two clusters in assemblage A (AI and AII) and assemblage B (BIII and BIV). Cluster AI which involves closely linked animal and human isolates is the most frequent in human isolates. Assemblage B is more common in human isolates, but there are some animals as the hosts for this genotype[1]. The information of patients for the genotype of *G. duodenalis* may improve our understanding of the infection course as well as the rate of re-infection after treatment. Determining the most common genotypes of *Giardia* associated with its long-term infection could improve our understanding of disease treatment[5].

In Iran, a few studies assessed the prevalence of giardiasis and its molecular examination, but the results have been inconsistent and conflicting. Therefore, the current study was conducted to determine the prevalence of *G. duodenalis* and its assemblages in the patients from Zahedan by using both microscopic and molecular examinations to demonstrate the general condition of giardiasis in this region. To our knowledge, this is the first study to examine *Giardia* genotypes in Zahedan, Southeast Iran.

2. Materials and methods

2.1. Microscopic examination

In the present study, 90 stool samples were collected from patients that were referred to the internal medicine clinics at various hospitals in Zahedan, southeast of Iran, in the period between March 2014 to July 2015. Examination of fecal samples was done by wet smear stained with Lugol's iodine and using a light microscope. All positive samples (showing presence of cysts and/or trophozoites) were stored in 70% ethanol (1:3, v/v) for the molecular analyses. The examination indicated that 54 samples were confirmed as *G. duodenalis* positive [at least five cysts in each high power field (40×) of light microscope]. Sucrose density gradient centrifugation was applied for isolation of cysts from the feces and then the cysts were washed using sterile distilled water and eventually stored at –20 ˚C until further use[12].

2.2. DNA extraction

Prior to DNA extraction, cysts were freeze-thawed at –80 ˚C and 80 ˚C for 10 times. The commercial QIAamp DNA Stool Mini Kit (Qiagen, Germany) was used for extracting DNA from purified cyst samples according to the manufacturer's guidelines. The isolated DNA was stored at –20 ˚C until further use.

2.3. PCR amplification

The assemblage-specific PCR method was applied for identification of *G. duodenalis* genotypes by using primers designed by Vanni *et al.*[13]. The sequences of primer pairs as well as the amplicons band length were shown in Table 1. Each PCR tube contained 10 μL of master mix (Genetbio, South Korea), 10 pmol/L of each primer, 11 μL distilled deionized water and 200 ng of DNA in a final volume of 25 μL. PCR reactions were initiated with denaturation at 94 ˚C for 5 min and 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 60 ˚C (for assemblage A) and 62 ˚C (for assemblage B) for 30 s and extension at 72 ˚C for 30 s. A final extension was performed at 72 ˚C for 7 min. PCR amplicons were separated on 1.5% gel electrophoresis and were stained with ethidium bromide. We used 100 bp DNA marker as size standard in gel electrophoresis.

Table 1
List of primers for the assemblage-specific PCRs and sizes of the amplicons.

P21 gene	Primer	PCR product (bp)
Assemblage A	F: ATGCTAGCCGTAGTTAATAAGG R: ACCGGCCTTATCTACCAGC	303
Assemblage B	F: TTAATAGAAATGCTTTCGACACG R: TTGCTACAGCAGAAAGGTGC	249

2.4. Study participants

In the present study, 45 males and 9 females with positive samples were recruited, and the participants were divided into four separate groups based on their age: 22 cases in Group 1 (0–12 years), 19 in Group 2 (12–26 years), 7 in Group 3 (27–37 years) and the remaining 6 in Group 4 (above 37 years). All patients were asked to fill in a questionnaire to obtain data on their demographic information and clinical symptoms like age, sex, abdominal cramps, diarrhea, fatty diarrhea, constipation and medication intake. The study protocol was performed according to the Helsinki Declaration and approved by Ethical Committee of Zahedan University of Medical Sciences, Zahedan, Iran. Informed written consent was obtained from all patients.

2.5. Statistical analysis

SPSS software version 16 was used for data analysis, and the *Chi*-square test was used for evaluating the association between variables. Besides, Mann-Whitney *U* test was applied to examine the relationship between genotypes and clinical symptoms. *P* value below 0.05 was considered as statistically significant.

3. Results

The PCR analysis indicated that out of a total of 54 samples, 13 (24.1%) had assemblage A defined by the presence of DNA bands at 303 bp and 41 (75.9%) had assemblage B determined by the presence of DNA bands at 249 bp. Assemblage A + B was not observed in the tested samples. Data analysis showed that there was no significant association between distribution of assemblages A or B and age ($P > 0.05$) and sex ($P = 0.670$) of subjects as shown in Table 2.

Among the 54 participants, 37 cases had diarrhea. The PCR indicated that among subjects with assemblage A, 76.9% suffered

from diarrhea. In contrast, among subjects with assemblage B, 65.9% had diarrhea. In spite of the difference, the statistical analysis showed lack of any significant association between prevalence of genotypes and diarrhea (P = 0.517). With respect to abdominal cramps, among subjects with assemblage A, 92.3% suffered from abdominal cramps, and 72.5% of subjects with assemblage B had abdominal cramps (P = 0.253). Similarly, no statistically significant relationship between distribution of genotypes A or B and fatty diarrhea (P = 0.740) as well as constipation (P = 0.241) was observed.

Table 2
Relationship between distribution of genotypes A or B and subjects' characteristics and symptoms [*n* (%)].

Characteristics and symptoms		Genotype A	Genotype B	*P* value
Subjects		13 (24.1)	41 (75.9)	-
Sex	Male	10 (76.9)	35 (85.4)	0.670
	Female	3 (23.1)	6 (14.6)	
Age (year)	1–12	7 (53.8)	15 (36.6)	
	13–26	2 (15.4)	17 (41.5)	0.140
	27–37	3 (23.1)	4 (9.8)	0.665
	> 37	1 (7.7)	5 (12.2)	0.639
Diarrhea	Yes	10 (76.9)	27 (65.9)	0.517
	No	3 (23.1)	14 (34.1)	
Abdominal cramps	Yes	12 (92.3)	29 (72.5)	0.253
	No	1 (7.7)	11 (27.5)	
Fatty diarrhea	Yes	5 (38.5)	13 (31.7)	0.740
	No	8 (61.5)	13 (31.7)	
Constipation	Yes	2 (15.4)	2 (4.9)	0.241
	No	11(84.6)	39 (95.1)	

4. Discussion

Giardiasis is a common intestinal infection in Iran. A number of factors including geographical, socioeconomic, sanitary and hygienic, and nutritional factors contribute to the spread of this disease[7]. The incidence of giardiasis varies among different cities of Iran. For instance, the occurrence rate is 10.1% in Zahedan[14], 10.7% in Isfahan[5] and 28.7% in South Khorasan[15]. According to these reports, it can be understood that *Giardia* infection is a main health issue in Iran.

Human giardiasis results from two separate genetic groups of *G. duodenalis* recognized as assemblages A and B, which are present in different species. Both assemblages have been connected to worldwide human infection, and also observed in a variety of animals[16]. The PCR analysis in this study demonstrated that assemblage B (75.9%) was more common in studied subjects with giardiasis compared to assemblage A (24.1%). The high prevalence of assemblage B in our study is in agreement with the previous reports conducted in India (n = 44 *vs.* n = 6)[17] and (82% *vs.* 9%)[18], United Kingdom (27% for assemblage A, 64% for assemblage B, 9% for assemblage A + B)[19] and Nipal (20% for assemblage A, 74% for assemblage B, 6% for assemblage A + B)[20]. In contrast, assemblage A was found to be the more prevalent genotype detected in the studies performed in Australia (50% *vs.* 39%)[21] and Brazil (78.4% for assemblage A, 21.6% for assemblage B)[22]. Furthermore, several Iranian researchers have determined *Giardia* genotypes from samples in different cities of Iran. For example, Pestehchian *et al*.[6] examined 67 isolates from Isfahan using PCR-RFLP assay, and observed the AII genotype in 40 isolates (59.7%), BIII genotype in 23 (34.32%) samples and BIV genotype in 2 (2.98%) isolates. Besides, two samples (2.98%) were positive for both AII and B genotypes. The study of Sarkari *et al*.[23] on 172 positive samples from Fars Province (Southern Iran) indicated the presence of different genotypes including AII in 128 (74.41%), BIII in 30 (17.44%), BIV in 6 (3.49%) and the mixed AII and BIV genotypes in 8 (4.66%) isolates. Besides, Rayani *et al*.[5] reported that 32 samples (80%) had genotype AII and 8 samples (20%) were positive for genotypes BIII and BIV.

Microscopic detection of *Giardia* (cysts and/or trophozoites) in fecal samples is an old-fashioned method for diagnosis of giardiasis. This method needs trained microscopists, takes a lot of time, and is unable to discriminate between genetically different *G. duodenalis* isolates[24]. In recent years different molecular methods have been used to discriminate these genotypes chiefly by nested PCR followed by DNA sequencing, RFLP or real-time PCR[25]. Most of molecular assays are dependent upon the amplification of a gene section using primers that bind to a common DNA sequence that is preserved in the two assemblages (or preserved in all *G. duodenalis* assemblages or in *Giardia* species). The conventional PCR assay performed in our study is able to identify and discriminate *G. duodenalis* assemblages A and B by using the P21 specific primers[13]. By binding to unique DNA sequence for each assemblage, specific primers yielded products of visible size for each assemblage at each locus (249 bp and 303 bp).

Furthermore, we found no association between the assemblages and clinical symptoms including diarrhea, abdominal cramps, fatty diarrhea as well as constipation. Our results support the findings of a study by Sarkari *et al*.[23], which was performed in Fars Province of Iran (southern region). They reported that the prevalence of assemblages A and B was not related to clinical symptoms of subjects. Similarly, there was no relation between genotypes of *G. duodenalis* and clinical presentation in Brazilian children[26]. However, the study of Etemadi *et al*. in Kerman, a province in the central south region of Iran, indicated that assemblage B was more significantly common in symptomatic patients[27]. They found that assemblage B isolates were more common in patients with persistent diarrheal complaints. Rafiei *et al*. also reported that intensity rate of cysts was higher in symptomatic cases compared with asymptomatic ones, which demonstrated the increased parasite activity in symptomatic cases[12]. In Saudi Arabia, Homan and Mank showed that assemblage B was more prevalent in symptomatic patients with continuous diarrheal complaints[28].

In conclusion, our study demonstrated that *G. duodenalis* assemblage B was the predominant assemblage in Zahedan, which indicates that there is a major non-zoonotic transmission cycle in the area. This study on giardiasis by applying the PCR molecular assay is the first report from Zahedan. However, one limitation of this study was the lack of data regarding sub-assemblages due to limited financial resources. Additional studies on human using more genes are needed to better characterize the molecular epidemiology of giardiasis at the level of assemblage and sub-assemblage in all areas of our country.

Conflict of interest statement

We declare that we have no conflict of interest.

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