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Full Length Research Paper

Molecular identification of *Giardia lamblia* isolates from adult human cases in southwest of Iran

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Giardia lamblia **is a flagellated protozoa that infects the intestinal tract of a wide range of mammalian hosts, including both wild and domestic animals as well as humans. Two genotypes A and B are commonly reported among humans. The purpose of this study was to investigate the genotypes of *G. lamblia* among adult infected cases in Ahvaz, southwest of Iran. Fecal samples were collected from 50 patients who had been tested positive to *G. lamblia*. The samples were analyzed by semi-nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) using the gdh gene. Our results indicate 38 and 16% assemblages of BIII and AII, respectively. Mixed infections with both assemblages AII and BIII were also detected in 46% of the positive samples. This higher rate of mixed infection in the region may be explained by the common occurrence of repeated and cumulative infection by the parasite in the study area or may reflect environmental contamination of water resources. Therefore, it seems further studies are needed to clarify the route of infection in the study area.**

Key words: *Giardia lamblia*, glutamate dehydrogenase (gdh), semi-nested polymerase chain reaction (PCR), PCR-RFLP, Ahvaz, Iran.

INTRODUCTION

Giardia lamblia has been considered as the most commonly identified intestinal parasite that infects human and other mammals (Adam, 2001; Garcia, 2005; Feng and Xiao, 2011). Its prevalence has been estimated to be 5 to 43% in different parts of the world and 5 to 23% in Iran (Garcia, 2005; Thaherkhani et al., 2009). It seems that this parasite is still a public health problem in rural areas of Khozestan with prevalence rate of 9.1% (Mowlavi et al., 2009). Person-to-person, zoonotic, foodborne and waterborne transmissions may occur through the fecal-oral route after contact with the infective stage of the parasite (Caccio et al., 2005; Hunter and Thompson, 2005; Dawson, 2005; Karanis et al., 2007; Smith et al., 2007; Carmena, 2010). Water and food are recognized as the most important vehicles for the transmission of *G. lamblia* (Smith et al., 2007; Carmena, 2010). This parasite causes a wide range of clinical symptoms from asymptomatic infection to diarrheal illness with or without malabsorption and weight loss (Thompson and Monis, 2004). Isoenzyme and DNA analyses revealed that *G. lamblia* is classified into eight major assemblages: A to H (Monis and Thompson, 2003;

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Abbreviations: PCR, Polymerase chain reaction; **RFLP,** restriction fragment length polymorphism.

Thompson, 2004; Lasek-Nesselquist et al., 2010). Assemblages A and B have been detected in the feces of humans and a broad range of other hosts, including livestock, cats, dogs and beavers (Monis et al., 1999; Thompson, 2004; Xiao and Fayer, 2008; Feng and Xiao, 2011). The assemblage A comprises two subgroups, I and II. Assemblage AI is mainly zoonotic and assemblage AII is commonly anthroponotic (Monis et al., 1999; Thompson et al., 2000; Hunter and Thompson, 2005; Xiao and Fayer, 2008). The assemblages B have been grouped into subgroups III and IV. Assemblage BIV appears to be human-specific (Hunter and Thompson, 2005). Genetic assemblages C, D, E, F and G appear to be host restricted to domestic animals, livestock and wild animals (Monis and Thompson, 2003; Xiao and Fayer, 2008; Feng and Xiao, 2011). Recently, assemblage H has been identified in marine vertebrates (Lasek-Nesselquist et al., 2010). Current method for Giardia infection is the microscopic detection of Giardia cysts or trophozoites in stool (Isaac-Renton, 1991; Adam, 2001), but this method cannot distinguish between isolates of *G. lamblia*. Molecular analytical tools such as polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) provide sensitive and specific diagnostic methods to distinguish between genetically distinct *G. lamblia* isolates (Caccio et al., 2002; McGlade et al., 2003; Caccio and Ryan, 2008). In this study, we aimed to identify the genotypes of *G. lamblia* isolates from adult human cases in Ahvaz, southwest of Iran by PCR-RFLP assay using glutamate dehydrogenase (gdh) gene.

MATERIALS AND METHODS

Giardia-positive fecal samples were collected from symptomatic and asymptomatic human populations who were referred to Ahvaz health centers clinics for medical examinations, during September 2011 to July 2012. Positive fecal samples were transferred to the Department of Parasitology, School of Medicine, Ahvaz University of Medical Sciences. The positive samples were confirmed by wet smear stained with Lugol's iodine and formalin ether using light microscope at 400× magnification. Fifty (50) positive human (16 to 65 years old; 42 males, eight females) fecal samples for *G. lamblia* cysts were used in the study. All samples were stored as whole faeces at 4°C without preservatives until used. The cysts were purified and concentrated from the faeces by sucrose density gradient centrifugation and washed with sterile distilled water and then stored at -20°C until genomic DNA extraction was performed within one month.

DNA extraction

The cysts were disrupted by 10 freeze-thaw cycles (-80°C, +80°C). The genomic DNA of *G. lamblia* was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. DNA samples were preserved at -20°C until used.

PCR amplification

The amplification of the gdh gene (432 bp) was performed as a semi-nested PCR with an external forward primer GDHeF (TCA ACG TYA AYC GYG GYT TCC GT), internal forward primer GDHiF (CAG TAC ACC TCY GCT CTC GG) and reverse primer GDHiR (GTT RTC CTT GCA CAT CTC C) as described by Read et al. (2004). The primers were tested with positive DNA control. The positive control was generously donated by Kerman University of Medical Sciences. The positive control was isolated from human sources. Distilled water was used as negative control. The PCR reaction mixture comprised 5 µl genomic DNA, 5 µl of 10X buffer (Fermentas, Lithuania), 1.5 µl (50 mM) $MgCl_2$, 1 µl (10 mM) dNTP mix, 0.3 µl (5U/ µl) Taq DNA polymerase (Fermentas, Lithuania) and 1 µl (12.5) pmol of each primer. The reactions were performed in 50 µl volumes. GDHeF and GDHiR were used in the primary PCR reaction. One microlitre of PCR product from the primary reaction was added to the secondary PCR containing primers GDHiF and GDHiR. The DNA was amplified using iCycler, BioRad Thermal Cycler under the following condition: 1 cycle of 94°C for 3 min, 56°C for 1 min and 72°C for 2 min, followed by 35 cycles, 94°C for 1 min, 56°C for 20 s and 72°C for 45 s. A final extension of 72°C for 7 min and a 20°C hold was used. The PCR products were visualized on 1% agarose gels (Roche, Germany) stained with ethidium bromide stain.

PCR-RFLP of region of gdh gene

RFLP analysis was carried out by digesting 10 µl of PCR product. It was added to 1 X enzyme buffer, and 2 µl (10 U/ µl) BspL1 (NIaIV) (Fermentas, Lithuania) or 2 µl (10 U/ µl) Rsa1 (Roche, Germany) for 16 h at 37°C. The final volumes of Rsa1 and BspL1 were 25 and 30 µl, respectively. The BspL1 digestion was used for the distinction between groups I and II of assemblages A and assemblages B. Rsa1 digestion was distinguished between subtypes BIII and BIV. Restriction fragments were visualized on 3% high resolution grade agarose (Roche, Germany) stained with ethidium bromide. A 50 bp DNA ladder (Fermentas, Lithuania) was used as a size marker.

RESULTS

All the 50 human fecal samples which were positively identified by microscopy were successfully amplified in semi-nested PCR using primers GDHiF and GDHiR (Figure 1). Therefore, the semi-nested gdh PCR showed 100% correlation with the microscopic examination. Among these fecal samples, 23 (46%) cases were identified with mixed genotypes AII and BIII, defined by the presence of DNA bands at 40, 70, 80, 90, 120 and 290 bp. 19 (38%) samples revealed assemblages BIII, defined by the presence of DNA bands at 130 and 300 bp and 8 (16%) samples were assemblage AII, determined by the presence of DNA bands at 70, 80, 90 and 120 bp (Figures 2, 3, 4 and Table 1).

DISCUSSION

G. lamblia is the most common intestinal parasite found throughout the world; it has different major assemblages (Garcia, 2005; Ivanov, 2011), which can only be distinguished by molecular techniques such as PCR-RFLP (Monis et al., 1999; Thompson, 2004; Caccio and Ryan, 2008). This is a sensitive, simple, rapid and powerful method that is capable of detecting mixed

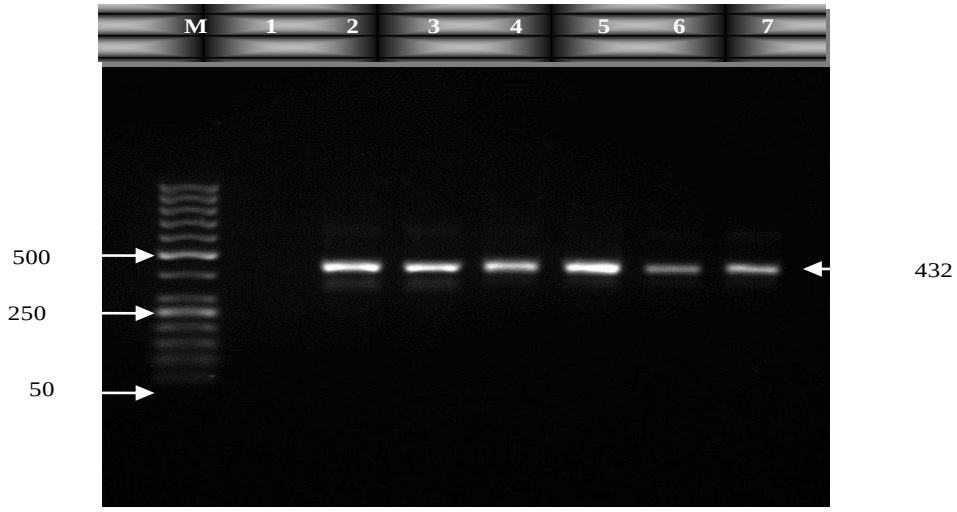

Figure 1. Glutamate dehydrogenase gene-based PCR products on an ethidium bromide-stained 1% agarose gel. Lane M, molecular weight marker (50 bp); lane 1, negative control; lane 2, positive control; lanes 3 to 7, PCR products from clinical samples.

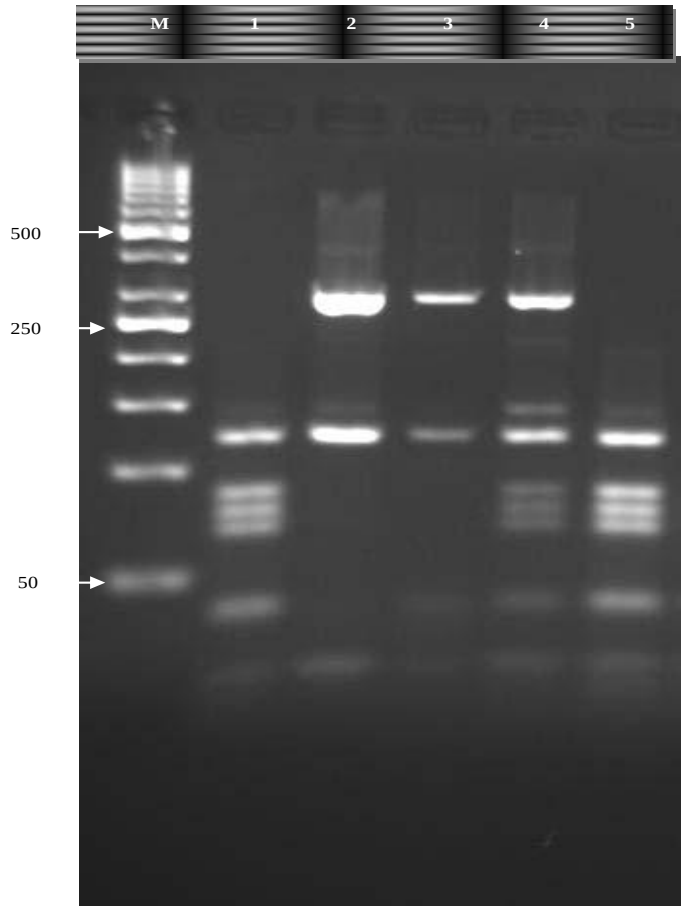

Figure 2. *Bspl1* digestion of *gdh*-PCR products on an ethidium bromide-stained with 3% high resolution grade agarose gel. Lane M, molecular weight marker (50 bp); lane 1, *G. lamblia* positive control (genotype AII), lanes 2 and 3, genotype B; lane 4, mixed genotype AII and B; lane 5, genotype AII.

genotypes (Homan et al., 1998; Amar et al., 2002; Read et al., 2004). For the first time, the present study provides information on the distribution of the genotypes of *G. lamblia* from humans in Ahvaz, southwest of Iran. All 50 fecal samples that were identified as positive with microscopic examination were also successfully amplified in semi-nested PCR. Some previous studies had false negative results using PCR (Amar et al., 2002; Bertrand et al., 2005; Hatam-Nahavandi et al., 2011). These failures may be as a result of: initial misidentification, low DNA levels, the existence of a robust wall that inhibits the release of DNA from the cysts, the presence of PCR inhibitors in some of the fecal samples or degradation of parasite material during storage. In the current study, assemblages BIII and AII were detected in 38 and 16% of the samples, respectively. A higher prevalence rate of assemblage B in our study is in agreement with the results of Hatam-Nahavandi et al. (2011). Their study indicated that 66.7% of human clinical samples contained assemblage B (44.4% BIII and 22.2% BIV) in the northwest of Iran, while other Iranian studies showed a higher rate of assemblage AII (Babaei et al., 2008; Etemadi et al., 2011). In agreement with our results in other countries, Tungtrongchitr et al. (2010) in Thailand proved that assemblage B (51%) was more common than assemblage A (8%). Similarly, Breathnach et al. (2010) observed 73% of assemblage B and 24% assemblage A in 199 human fecal samples in the southwest of London. Singh et al. (2009) in Nepal, indicated that infection with assemblage B (74%) was more prevalent than assemblage A (20%) in patients older than 12 years of age. Mohammed et al. (2009) found that assemblage B was significantly more common among 42 fecal samples in Malaysia. Sahagun et al. (2008) observed 53.8% of assemblage B and 41.5% assemblage A in 65 patients

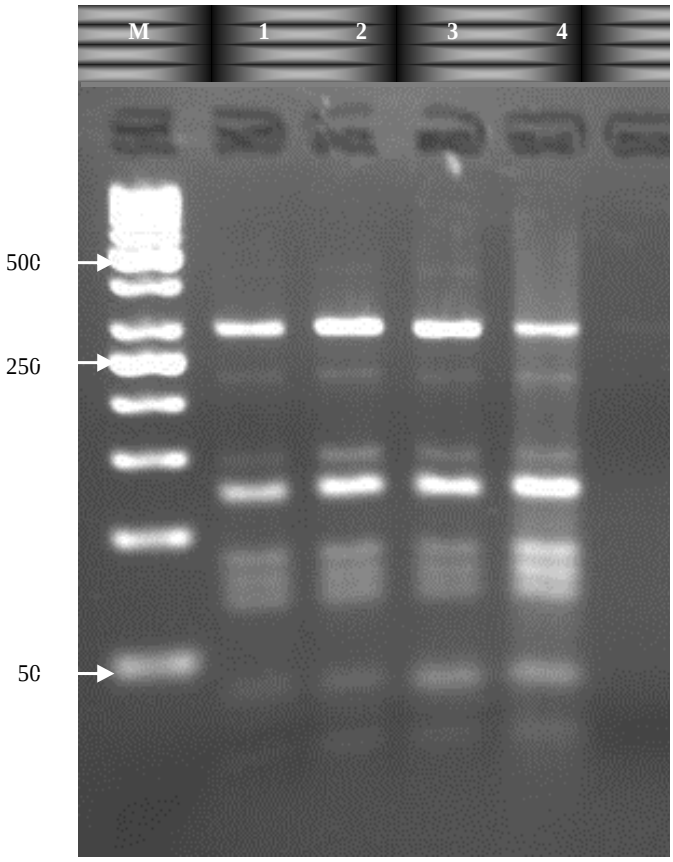

Figure 3. *Bspl1* digestion of *gdh*-PCR products on an ethidium bromide-stained with 3% high resolution grade agarose gel. Lane M, molecular weight marker (50 bp); lanes 1 to 4, mixed genotypes AII and B.

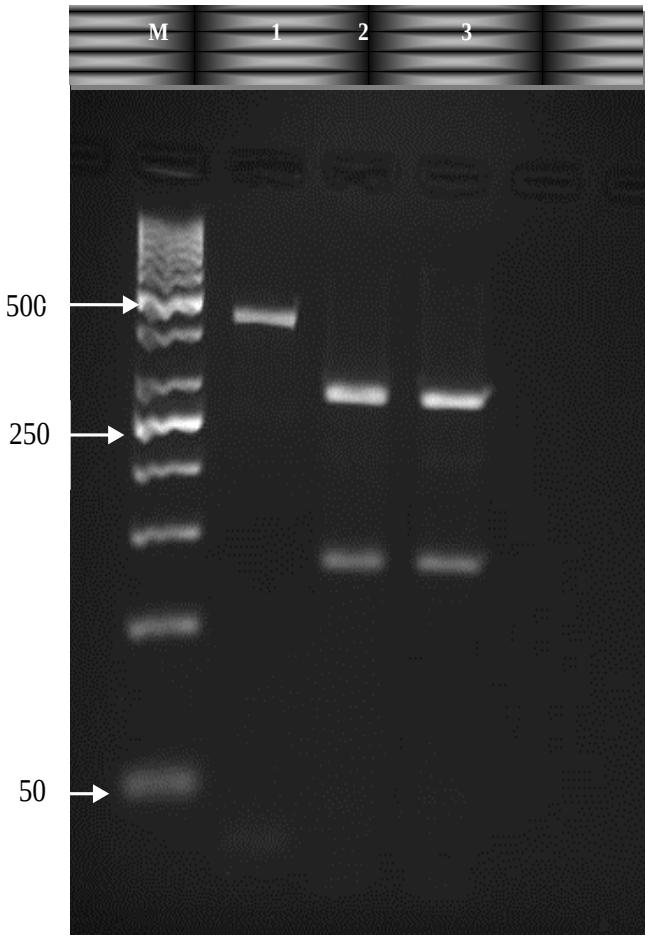

Figure 4. *Rsa1* digestion of *gdh*-PCR products on an ethidium bromide-stained with 3% high resolution grade agarose gel. Lane M, molecular weight marker (50 bp); lane 1, PCR products (432 bp fragment); lanes 2 and 3, genotype BIII.

from 5 to 72 years of age in Spain. In Egypt, Foronda et al. (2008) found that assemblage B was the most prevalent (80%) genotype among 97 fecal samples. In the study conducted by Guy et al. (2004), the majority of clinical fecal samples were assemblage BIII. In India, the proportion of assemblages B and A in giardiasis patients was 61 and 39%, respectively (Traub et al., 2004). Amar et al. (2002) found that 64% of human clinical samples were assemblage B in the UK. In such studies, some of the researchers mentioned that, aside from human to human transmission of giardiasis in their area, zoonotic transmission also plays a role.

In contrast, in Palestine, Hussein et al. (2009) observed that assemblage AII was more prevalent in eight fecal samples. In Egypt, Helmy et al. (2009) reported 75% assemblage A and 19.5% assemblage B among 41 patients. Souza et al. (2007) indicated that infection with assemblage AII (78.4%) was more prevalent in Brazil. Homan and Mank (2001) observed that assemblage A was more prevalent in patients between 8 to 60 years of age in the Netherlands. These researchers concluded that humans are probably the most important reservoirs in the studied regions. Differences in the prevalence of assemblages A and B may be due to the geographical locations of the populations studied, the role of animals in the transmission of the disease and the levels of hygiene conditions. In the present study, mixed infection of *G. lamblia* assemblages group AII and BIII were detected in 46% of the samples, which is in agreement with some previous studies, but it seems that the rate of mixed infection with genotypes AII and BIII, in our report is higher than the others (Amar et al., 2002; Guy et al., 2004; Yason and Rivera, 2007; Sahagun et al., 2008; Singh et al., 2009; Tungtrongchitr et al. 2010). We do not have a clear explanation for this higher rate of mixed infection in the region, but the multiple infections of *G. lamblia* may be explained by the common occurrence of repeated and cumulative infection by the parasite in the study area. Finally, it seems that the significance of the predominant mixed infection in our study is unclear and further study with human and animal fecal samples, as well as environmental water resources, are needed in order to better understand the route of Giardia

Table 1. Predicted fragment sizes (bp) of *G. lamblia* assemblages after digesting with *BspL1* and *Rsa1.*

Assemblage	Enzyme	Predicted fragment size	Diagnostic genotyping profile
AI	*BspL1*	16, 18, 39, 87, 123, 149	90, 120, 150
AII	*BspL1*	16, 18, 39, 72, 77, 87, 123	40,70, 80, 90, 120
B	*BspL1*	18, 39, 123, 291	120, 290
BIII	*RsaI*	2,133, 297	130, 300
BIV	*RsaI*	12, 430	430

transmission.

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