Tropical Biomedicine 29(3): 366–371 (2012)

Genotyping of *Giardia lamblia* isolates from human in southern Iran

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Received 24 October 2011; received in revised form 31 March 2012; accepted 25 May 2012

Abstract. *Giardia lamblia* cysts isolated from human faeces in South of Iran were analyzed with PCR-restriction fragment length polymorphism (RFLP) assay, based on the detection of glutamate dehydrogenase (gdh) genes. Among 205 faecal samples from microscopically diagnosed giardiasis patients, the gdh gene was amplified from 172 cases with a semi nested PCR assay and typed by RFLP analysis. Of the 172 positive samples, 128 (74.41%) were typed as assemblage AII, 30 (17.44%) assemblage BIII, 6 (3.49%) assemblage BIV and in 8 (4.66%) isolates, mixed assemblages AII and BIV were detected. Clinical features were available for 52 successfully typed cases and the possible correlation of *Giardia* assemblages and clinical symptoms was evaluated. Both assemblages caused similar illness, but assemblage AII was significantly more frequently associated with abdominal pain, nausea and vomiting. Since these isolates, A and B, are of human origin, anthroponotic transmission of *Giardia* can be suggested for the route of infection in this region.

INTRODUCTION

Giardia lamblia is a ubiquitous parasite involved in human parasitic infection all over the world. Transmission of *G. lamblia* cyst to human occurs mostly following drinking of contaminated water. *Giardia* is a common infection in Iran especially in preschool and school age children and the rates of infection among these groups in different regions vary from 1 to more than 10 percent (Sayyari *et al.*, 2005; Arani *et al.*, 2008; Kia *et al.*, 2008; Haghighi *et al.*, 2009; Nasiri *et al.*, 2009). Clinical manifestations of symptomatic giardiasis include fatty stools, flatulence, diarrhoea and abdominal cramps, although a majority of cases are asymptomatic. While spontaneous clearance of the infection is not uncommon, reinfection is frequent in hyper-endemic areas. In one study in Iran, more than 34% of children were reinfected three months after treatment (Saffar *et al.*, 2005).

Although the *Giardia* isolates are morphologically identical, they vary significantly in their biology, virulence and genetics. Isolates of *G. lamblia* are classified into seven assemblages, based on the characterization of the glutamate dehydrogenase (gdh), small-subunit (SSU) rRNA, and triose phosphate isomerase (tpi) genes (Cacciò & Ryan, 2008). These assemblages can infect different hosts. The analysis of human isolates from different geographical locations, examined by PCR amplification of DNA extracted from stool samples, demonstrated that in almost all cases only *G. lamblia* assemblages A and B are associated with human infections (Cacciò & Ryan, 2008). The assemblage A isolates have been further grouped into subgroups I and II. The assemblage B isolates have been divided into subgroups III and IV. These assemblages also infect a wide range

of other hosts, including livestock, cats, dogs, and wild mammals.

Molecular characterization of *Giardia* isolates have been done in some parts of Iran including Tehran (Babaei *et al.*, 2008). However, no such study has been performed in south of the country where the rate of *Giardia* infection is substantial (Sayyari *et al.*, 2005). Considering the molecular differences and the diversity of the prevalence of *Giardia* assemblages in different geographical regions of the world, and in view of probable correlation between *Giardia* assemblages and clinical symptoms, this study was conducted to assess the genetic diversity of *Giardia* isolated from patients with giardiasis in Fars province, south of Iran. The study also aimed to evaluate any possible relationship between *Giardia* assemblages and clinical features of the disease.

MATERIALS AND METHODS

Sample collection

Human stool samples microscopically positive for *G. lamblia* cysts (n=205) were collected at clinical laboratories in Fars province in South of Iran, from April to December 2010. Samples were collected from both females and males and from different age groups, ranging from 6 months to 65 year of age. Epidemiological data such as age, gender and clinical signs and symptoms of some of cases were also collected using a predesigned questionnaire. Sucrose gradient was used to concentrate the cysts from each specimen.

DNA extraction

DNA was extracted from concentrated cysts by a manual procedure, using Triton X100. Briefly, 100 µl of cyst suspension (in distilled water) was mixed with 100 µl of 2% Triton X100 and heated for 30 min at 72ºC. Sample was equally mixed with 100 µl of lysis buffer and then 10 µl of proteinase K was added and incubated overnight at 37ºC. Afterward 100 µl of phenol:chloroform:isoamyl alcohol was added and centrifuged at 4000 g for 10 minutes. Aqueous top phase was removed and absolute alcohol was used to precipitate the DNA.

PCR amplification

Amplification of the glutamate dehydrogenase (gdh) gene was performed using a two-step semi nested PCR as originally described by Read *et al.* (2004). The primers which were used are: GDHeF (5'TAC ACG TYA AYC); GDHiR (5'GTT RTC CTT GCA CAT CTC C3'); and GDHiF (5'CAG TAC AAC TCY GCT CTC GG). The first-round of PCR amplification was performed using mixtures of 5 U of *Taq* polymerase with 2.5 µl of 10X PCR buffer, 16.3 µl deionized distilled water, 25 mM $MgCl_2$, 10 mM of dNTP, 10 pmol of each primer (GDHeF and GDHiR), and 2 µl of DNA template, in a total volume of 25 µl. The thermal cycling conditions were as follows: 94ºC for 2 minutes and then 56ºC for 1 minute, 72ºC for 2 minute (one cycle) and then 94ºC for 30 seconds, 56ºC for 20 seconds, 72ºC for 45 seconds (31 cycles) and finally 72ºC for 7 minutes.

The second-round PCR was performed using mixtures of 5 U of *Taq* polymerase with 5 µl of 10X PCR buffer, 35.6µl of deionized distilled water, 25 mM $MgCl_2$, 10 mM of dNTP, 10 pmol of each primer (GDHiF and GDHiR), and 1 µl of DNA template, in a total volume of 50 µl. The thermal cycling conditions were as follows: 94ºC for 2 minutes and then 56ºC for 1 minute, 72ºC for 2 minute (one cycle) and then 95ºC for 60 seconds (one cycle), followed by 94ºC for 35 seconds, 61ºC for 35 seconds, 72 ºC for 50 seconds (9 cycles) and finally 72ºC for 7 minutes. PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Restriction fragment length polymorphism (RFLP)

RFLP analysis was performed by digesting 10 µl of semi nested gdh PCR products using 5 U of NIa IV (Fermentase) in a total volume of 20 µl, for 16 hours at 37ºC. Restriction fragments were separated on 4% agarose gel in TBE (Tris Base/Boric Acid/EDTA) buffer. For those isolates which showed the genotyping profile of assemblage B, Rsa I

(Fermentase) restriction enzyme was used to differentiate between BIII and BIV assemblages.

RESULTS

Out of 205 samples, DNA was successfully extracted from 174 samples and *Giardia* assemblage was determined in 172 samples. After the first-round of PCR amplification, using GDHiR and GDHeF primers, a 457 bp band was amplified. In the second-round of PCR amplification, using GDHiF and GDHiR primers, a 432 bp band was amplified (Fig. 1). Products of the second round of semi nested PCR were used for RFLP analysis. The 432 bp amplified fragment was first digested with NlaIV (Bsp L1) and the results showed the pattern of AII or AII and B assemblages (Fig. 2). RsaI digestion was used for discrimination between the subtypes of assemblage B, BIII and BIV (Fig. 3). According to our findings, 128 (74.41%) of the 172 specimens were typed as assemblage AII, 30 (17.44%) assemblage BIII, 6 (3.49%) assemblage BIV. The remaining 8 (4.66%) isolates showed patterns that were compatible with the presence of mixed infections, including assemblages A and B.

Moreover, the possible correlation of *Giardia* genetic variability and clinical symptoms of patients was evaluated in this study. In our study, clinical features were available for 52 successfully typed cases. Both assemblages caused similar illness, while a significant association was found between AII assemblage and symptoms such as abdominal pain, nausea and vomiting.

DISCUSSION

The molecular genotyping of human isolates of *Giardia* from different regions of the world demonstrates that in almost all cases only *G. lamblia* assemblages A and B are related to human infections (Caccio & Ryan, 2008). Assemblages C–G are likely to be host-specific, as assemblages C and D have been identified in dogs, cats, coyotes and wolves, assemblage E in cattle, sheep, goats, pigs, water buffaloes and assemblages F and G in cats and rats, respectively (Caccio & Ryan, 2008).

In our study, the main assemblage of the isolates was AII followed by BII and BIV. This was consistent with Babaei *et al.* (2008) study in the region of Tehran, where they reported AII as the most common assemblage in human isolates (Babaei *et al.*, 2008). Assemblage BIII was also the second most frequent assemblage in our study and this is again consistent with those reported by Babaei *et al.* (2008).

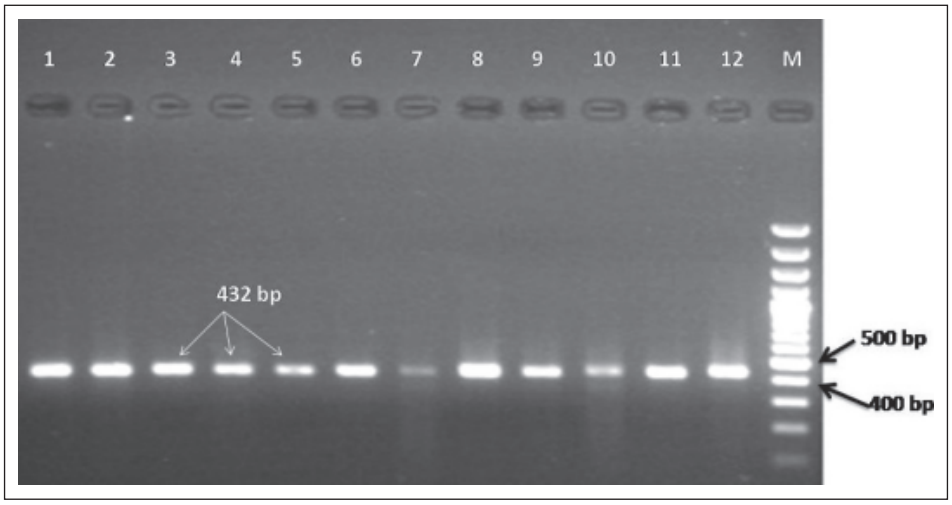

Figure 1. Electrophretic separation of semi nested PCR product from DNA amplified at the *gdh* locus of *G. lamblia*. Lane 1-12: Positive samples (432 bp); M: 100 bp DNA marker

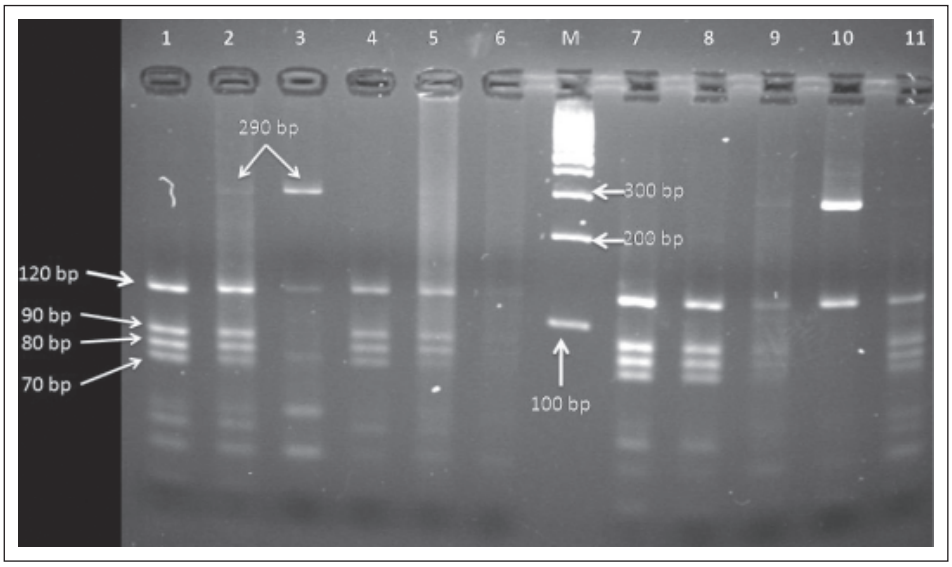

Figure 2. Nla IV (Bsp L1) digestion of semi nested PCR product of gdh of *Giardia lamblia* and identification of genotype AII and B by RFLP analysis. Lane 1,4,5,6,7,8: Assemblage AII. Lane 2,3,9,10,11: Assemblage AII and B (Mix); M: 100 bp DNA marker

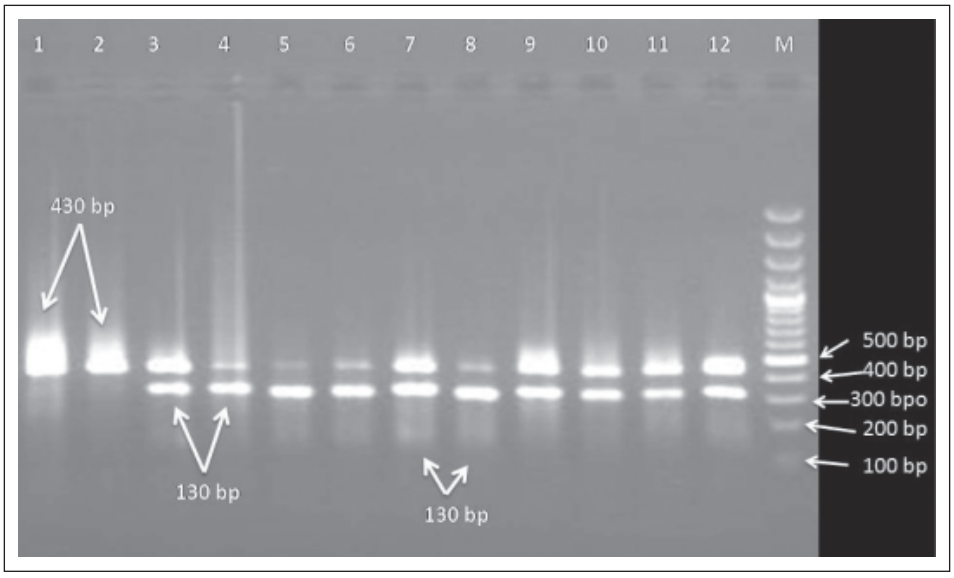

Figure 3. Rsa I digestion of semi nested PCR product of gdh of *Giardia lamblia* and identification of genotype BIII and BIV by RFLP analysis. Lane 1, 2: *Giardia* Assemblage BIV; Lane 3-12: *Giardia* Assemblage BIII; M: 100 bp DNA marker

Genotype A, especially AII, and B are the frequent genotypes in human isolates in various areas of the world. Genotyping of *G. lamblia* isolates among residents of slum area in Manila, Philippines, revealed that from 130 samples, fifty (37.59%) samples contained assemblage A (mainly AII), while 115 samples (86.47%) contained assemblage B (Yason & Rivera, 2007). Fallah *et al.* (2008) evaluated the genotyping of *Giardia* in

Tabriz, northwest of Iran. They reported assemblages of AII, BIII and BIV of *Giardia* in human patients.

In another study, the genotypes of *Giardia* in Kerman, in the central south of Iran was evaluated. In this study, from 130 isolates, 18 samples (60%) were found as assemblage All, 5 (16.7%) belonged to assemblage Al and 7 samples (23.3%) were reported as BIII assemblage (Etamadi *et al*., 2011).

Clinical picture of giardiasis were very variable in our study, ranging from asymptomatic infection to the existence of moderate symptoms, with diarrhoea being the most common. Host factors and strain divergence of the parasite are both likely to be involved in the diversity of the symptoms. The correlation between clinical symptoms of *Giardia* and assemblages is controversial. A few studies have found a connection between symptomatic infection with assemblage B and asymptomatic infection with assemblage A (Paintlia *et al*., 1999; Read *et al*., 2004; Aydin *et al*., 2004; Mohammed Mahdy *et al*., 2009) while some reports describe severe, actual persistent diarrhea with assemblage B and a strong correlation of the mild, intermittent type of diarrhea with *Giardia* assemblage A (Homan & Mank, 2001).

On the other hand, in a case-control study in Bangladesh (Haque *et al*., 2005), diarrhoea was more associated with assemblage A than with assemblage B. In another study in Thailand, all subjects with AI assemblage were symptomatic while only 50% of the subjects with BIII assemblage were symptomatic (Tungtrongchitr *et al*., 2010).

In our study, clinical features were available for 52 successfully typed cases. Both assemblages caused similar illness, but assemblage AII was significantly more frequently associated with abdominal pain, nausea and vomiting.

Our results are in keeping with those reported by Breathnach *et al*. (2010) which described cases of giardiasis in Southwest London where both assemblages, A and B, caused similar illness. Higher rate of cyst shedding in children with assemblage B in comparison with assemblage A have been reported from Brazil, although no significant difference in diarrheal symptoms between A and B assemblages was confirmed (Kohli *et al*., 2008).

Since genotypes A and B are mainly human origin, anthroponotic transmission of *Giardia* can be assumed for the infection in this region of Iran and control measurement should be implemented accordingly.

Acknowledgments. We are grateful to Mr M. Kalantari for his technical support. This study was financially supported by the office of Vice-chancellor for research of Shiraz University of Medical Sciences.

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