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Genetic Characterization of *Giardia intestinalis* Strains from Patients Having Sporadic Giardiasis by Using PCR Assay

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In this study, 325 stool samples from sporadic cases giardiasis were examined by conventional techniques for parasite diagnosis. A simple and rapid procedure for the extraction of DNA from fecal samples was developed. Triose phosphate isomerase (*tim*) based PCR assay was applied for definitive identification and genetic characterization of *Giardia intestinalis* strains collected from Tabriz Reference Laboratory and pediatric Hospital in Tabriz. Among 34 DNA samples extracted, the *tim* gene was amplified from 31 (91.1%). Of these, 13 (41.9%) samples contained assemblage B, 17 (54.8%) contained assemblage A and one (3.2%) contained a mixture of assemblage A and assemblage B. Of these, three samples (8.8%) were negative. The results indicated that PCR technique provides an applicable and feasible method for detection and identification of Giardia cysts in stool samples. The results of furthermore, demonstrated that *Giardia intestinalis* assemblage A and B exist in East Azerbaijan province of Iran.

Key words: *Giardia intestinalis*, Giardiasis, Triosephosphate isomerase (*tim*), PCR

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INTRODUCTION

Giardia intestinalis [synonym of *G. lamblia*, *G. duodenalis* (Haque *et al*., 2007; Di Matteo *et al*., 2008; Sokmen *et al*., 2008; Ponce-Macotela *et al*., 2008)] is a common cause of parasitic gastroenteritis and is a major health concern worldwide (Wolfe, 1992). Giardiasis is believed to be responsible for 2.5 million diarrhoea-associated deaths and nutritional deficiencies in children in developing countries (WHO, 1998). This organism is endemic throughout the world, with the highest prevalence occurring in the tropics and subtropics (Jones, 1998). Worldwide incidence is believed to range from 20 to 60% (Tripathi *et al*., 1999). The incidence rate is 2-7% in industrialized nations (Upcroft and Upcroft, 2001). The prevalence of giardia in Iran has been reported to 10.9% (Sayyari *et al*., 2005). This infection is diversely dispersed throughout allover Iran, such as East Azerbaijan province. The incidence rate in this province is variable among 15.2% (Tabriz City) to 43.8% (country of Naghadeh) (Saebi, 2005). This protozoan produces robust cysts, which are voided in the feces and transmitted directly through faecal/oral contact, or by ingesting the contaminated water or food (Gelanew *et al*., 2007). Although morphologically identical isolates of *G. intestinalis* have been shown to be phenotypically and genotypically heterogeneous (Thompson *et al*., 2000), the majority of *G. intestinalis* isolates from humans and some species of domestic animals can be grouped into two distinct genetic assemblages, A and B (Homan *et al*., 1992; Thompson *et al*., 2000). The assemblage A isolates have been further grouped into two distinct clusters AI and AII. Cluster A-I consists of a mixture of closely related animal and human isolates that appear to have undergone a recent global dispersion. Much of the focus regarding the potential for zoonotic transmission of *Giardia* has centered on this group and to a lesser extent, genotypes in Assemblage B. The assemblage B isolates have been separated into cluster BIII and BIV. In contrast, Cluster AII consists entirely of human isolates (Thompson *et al*., 2000). Isolates of *G. intestinalis* have been shown to differ in their pathogenicity, virulence and other biological characteristics (Read *et al*., 2002). In the present study, we used tim-based PCR technique to characterize and determine distribution of human Giardiasis assemblages A and B in fecal samples collected from humans from the Tabriz city, Iran.

MATERIALS AND METHODS

Collection and purification of cysts: Three hundred and twenty five faecal samples were collected from individuals in two localities at the Tabriz (Center of East Azerbaijan province at the Northwest of Iran), including: patients with acute gastroenteritis hospitalized in the Paediatric Hospital and infected humans referred to the Tabriz Reference Laboratory (TRL). These samples were collected from sporadic cases giardiasis between May and October 2007. The most of faecal samples of the collected from TRL were related to workers that they were coming from suburb to center place of Tabriz. The children's samples of collected from pediatric hospital related to children belonging to households which they were settlement of areas that characterized by a high number of children per household, poor sanitation and personal hygiene and lack of health education. Stool samples were collected in plastic cups; approximately 5 g were then transferred to the Parasitology Laboratory of the Faculty of Medicine of the Tabriz Medical University. Fecal samples were examined using light microscopic examination by Lugol's iodine-stained wet mounts and formalin-ethyl acetate concentration technique for parasite diagnosis (Amar *et al*., 2002). Thirty-four fecal samples (10.46%) containing *G. intestinalis* cysts were obtained during this study. Of the 34 patients, 80% were adults aged between 20 to 60 years and 73% were males. Cysts were partially purified from faecal material by sucrose density gradient method (Barazesh *et al*., 2006) and washing with sterile distilled water and then all samples were stored at 4°C without preservatives for up to 7 weeks (Bertrand *et al*., 2005).

Genomic DNA extraction: Genomic DNA of *G. intestinalis* isolates were extracted by freeze-thawing technique and then by method of modified proteinase K, SDS and CTAB. This method was done before (Van Soolingen *et al*., 1994), but we did set up the modified method as follow:

Suspend 300 μL of suspension of cysts obtained from the feces in 150 μm of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by vortexing. Then, 60 μL of 10% SDS and 10 μL of 20 mg mL^{-1} proteinase K added and vortex and incubate (overnight) at 60°C. After than, add 100 μL of 5 M NaCl and vortex. Which followed by added 80 μL of CTAB/NaCl solution (1:7) (was warmed in 65°C), vortex until the liquid content becomes milky and incubate for 10 min at 65°C. In this stage add 700 μL of Chloroform/Isoamyl Alcohol (24:1), vortex for 10 sec and centrifuge for 8 min at 11,000 g and precipitate the nucleic acid by adding 0.6 volume (420 μL) of 2-propanol to the aqueous supernatant and keep the mixture for 30 min at -20°C. Which followed by 15 min centrifugation at 12,000 g at room temperature (RT). Then, wash the DNA pellet by adding 1 mL of cold 70% ethanol and centrifuge for another 5 min at 12,000 g. Carefully remove the

supernatant and permit the pellet to dry at room temperature for approximately 15 min. Finally redissolve the pellet in 30 µL of deionized water. The DNA is stored at -20°C.

Primers: Primers sets A-for/A-rev (A-PCR) and B-for/B-rev (B-PCR) were used for amplification of the *G. intestinalis* Triose phosphate isomerase (*tim*) gene, as previously (Bertrand *et al*., 2005). The primers used for assemblage A amplification were: A-for 5'-GGAGACCGACGAGCAAAGC-3' (positions 839 to 857 on the WB sequence, GenBank no. L02120) and A-rev 5'-CTTGCCAAGCGCCTCAA-3' (positions 970 to 986 on the WB sequence). The primers used for assemblage B amplification were: B-for 5'-AATAGCAGCACARA ACGTGTATCTG-3' (positions 126 to 150 on the BAH-12 sequence) and B-rev 5'-CCCATGTCCAGCAG CATCT-3' (positions 188 to 206 on the BAH-12 sequence) (Cinnagen, Iran). A 148 bp fragment of the assemblage A gene with primers A-for and A-rev (A-PCR) and a 81 bp fragment of assemblage B gene with primers B-for and B-rev (B-PCR) were amplified.

PCR amplification for identification of *G. intestinalis* assemblages A and B: PCR amplification of the Triose phosphate isoerase (*tim*) gene was performed by using conventional thermocycler (model Eppendrof-Germany). Two separated PCR amplification were done with primers A-for/A-rev and B-for/B-rev as described above. Amplification reactions (20 µL) contained 2 µL of template DNA, 1×PCR reaction buffer corresponding to a final concentration of 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 50 mM KCl, 20 mM Tris-HCl (Cinnagen, Iran), each deoxynucleotide triphosphate at a concentration of 100 µM (Fermentas, Lithuania), 0.5 µM of each primer (F/R) and 2.5 U of Taq DNA polymerase (Cinnagen, Iran) For A-PCR and B-PCR. Samples were subjected to an initial denaturation of 94°C for 10 min, 50 cycles of 94°C for 35 sec, 63°C for 35 sec and 72°C for 45 sec and a final extension at 72°C for 7 min. For B-PCR, cycling parameters were 10 min at 95°C (initial heat activation step), followed by 50 cycles of 35 sec at 94°C, 30 sec at 65°C and 40 sec at 72°C, with a final extension of 7 min at 72°C. Both positive and negative controls (distilled water) were included in A-PCR and B-PCR to validate results. For positive control, first the genotype of *Giardia intestinalis* cysts from one fecal sample classified in assemblage A with GDHiF/GDHiR (GDH-PCR) (Read *et al*., 2004) was confirmed by sequencing analysis (Unpublished data). This analysis showed 100% matches between the amplified product obtained with GDH-PCR (432 bp) and the sequence of *G. intestinalis* assemblage A.

Gel electrophoresis: PCR products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) and visualized under a UV transilluminator. A 100 bp, plus DNA ladder (Fermentas, Lithuania) was included as a size marker.

RESULTS AND DISCUSSION

Studies were performed with DNA extracted from purified cysts by using method of modified proteinase K, SDS and CTAB. A 148 bp fragment of the assemblage A gene and a 81 bp fragment of assemblage B gene were amplified along A-PCR and B-PCR, respectively (Fig. 1). Among the one hundred fecal samples collected from children hospitalized in pediatric hospital, no cyst was observed and all of the 34 positive samples were related to patients referred to the Tabriz Reference Laboratory. Among fecal samples of sporadic cases of giardiasis identified by conventional techniques (n = 34), the *tim* gene was amplified from 31 samples (91.1%) with A-PCR and B-PCR (two separated amplification step) developed in our laboratory. Of these, 13 (41.9%) samples contained assemblage B, 17 (54.8%) contained assemblage A, one

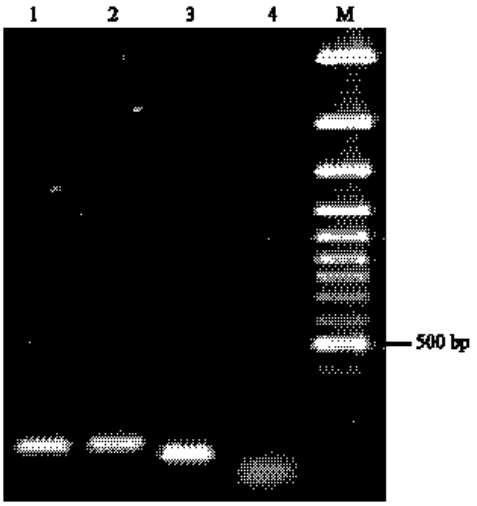

Fig. 1: Differentiation of *G. intestinalis* assemblages A and B with the *tim*-based on PCR technique with the primer pairs A-for/A-rev and B-for/B-rev in Tabriz city. The figure shows a 2% low-melting-point ethidium bromide-stained agarose gel of A-PCR and B-PCR products (148 and 81 bp). Lanes 1, *Giardia intestinalis* assemblage A group II (positive control); lanes 2, assemblage A; lane 3; assemblage B of *G. intestinalis* specimens from patients with giardiasis; lane 4; Negative control and Lane M, 100 bp DNA ladder (Cinnagen/Iran) (the 500 bp fragments are indicated)

Table 1: Results of PCR analysis of the *G. intestinalis tim* gene amplified from DNA extracted from feces

Sample and type of DNA extracted from feces (No. of samples)	No. and type of samples which DNA was extracted from feces					
	Positive samples by conventional techniques	Positive PCR samples	A-PCR Assemblage A	B-PCR Assemblage B	Assemblage A and B	None[b]
Pediatric hospital, Tabriz (n = 100)[a]	-	-	-	-	-	-
Tabriz Reference Laboratory (225)[a]	34 (15.1%)	31 (13.7%)	17 (54.8%)	13 (41.9%)	1 (3.2%)	3 (8.8%)

[a]Total No. = 325, [b]Not amplified

(3.2%) contained a mixture of assemblage A and assemblage B and three (8.8%) samples were negative (Table 1). The negative results observed could be explained by the presence of parasites at a very low level or degradation of parasite DNA during transport of the samples to our laboratory (Bertrand *et al*., 2005). The *tim* gene fragment from assemblages A and B could be amplified by using 0.5 and 0.05 pg of DNA per reaction mixture, respectively, equivalent to 50 and 5 copies of the *tim* gene, respectively, based on a genome size of 1.2×10^7 bp (Adam, 2000). The mixture of these assemblages has been reported previously in a few studies (Amar *et al*., 2002; Guy *et al*., 2004; Lalle *et al*., 2005). Amar *et al*. (2002) observed a mixture of assemblage A and assemblage B in 9% of 35 samples, The observation that the majority of sporadic giardiasis case isolates were assemblage A genotype (54.8%) in contrast with the findings of several studies conducted in France [61.5%, n = 26 (Bertrand *et al*., 2005)], India [100%, n = 10 (Sulaiman *et al*., 2003)], Peru [76%, n = 25 (Sulaiman *et al*., 2003)], United States [80%, n = 15 (Weber *et al*., 1992)] and United Kingdom [64%, n = 35 (Amar *et al*., 2002)]. However, an Italian study reported 80% assemblage A in 30 stool samples examined by sequencing or PCR-RFLP analysis of the β-giardin gene (Caccio *et al*., 2002). These differences in the prevalence of assemblages A and B may be attributed to the geographical locations of the populations studied. The predominance of assemblage B in samples collected in sewage treatment facilities was shown in one study (Guy *et al*., 2003). With respect to different pathogenicity (Read *et al*., 2002; Aydin *et al*., 2004) and zoonotic aspects (Thompson *et al*., 2000) of assemblages A and B, this study provides, for the first time, information on the distribution of the genotypes of *G. intestinalis* from humans with sporadic giardiasis in East Azerbaijan province of Iran. Present findings agree with the findings of other investigators which have identified two very distinct genotypes of *G. intestinalis* each of which appear to be distributed throughout the world (Van Keulen *et al*., 1995) according to Polish and Belgian (Homan *et al*., 1992) and groups ½ and 3 (Nash, 1995), although, assemblage B isolates appear to be less widespread and restricted to localized endemic foci (Meloni *et al*., 1995). Higher frequency of assemblage A was shown in this study. From a public health importance view, among the *G. intestinalis* strains, assemblage A subgroups AI and assemblage B subgroups BIII and BIV have zoonotic importance. Thus, further studies by application of appropriate genotyping tools in endemic foci where predictive assessments can be made about transmission patterns are needed to characterise the full genetic nature of parasites from human and animal to identify the source of contamination and human-infective potential of species. In summary, we developed a highly sensitive PCR technique to detect and distinguish *G. intestinalis* assemblages A and B. This study provides the first information about the distribution of the two major assemblages of *G. intestinalis* in sporadic human giardiasis in Iran. However, further studies with a larger series of fecal or environmental samples could lead to better knowledge of the distribution of these assemblages in humans as well as the role of domestic animals and livestock as a potential source of infection for humans. The results of this study might help public health care systems in management of Giardiasis.

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