

The comparison of “*tim*” gene of *Giardia lamblia* in laboratory animals and human and the importance of cross transmission probability in Iran

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Abstract:

BACKGROUND: *Giardia* has the ability to infect many mammals including dogs, cats, deer, mice, ground squirrels, chinchillas, swine, rabbits, pocket mice, oxen, guinea pigs, and humans. *Giardia lamblia* (also *Giardia duodenalis*, *G.intestinalis*) isolates have been variably divided into two or three genotypes by different investigators, and each group can be divided into subgroups. **OBJECTIVES:** We have compared the triosephosphate isomerase (*tpi*) sequences of these genotypes by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) to determine *G.lamblia* genotype in Iran for the first time. **METHODS:** In this study, 4 sets of primers were used in which 2 sets were designed by other investigator, and 2 sets were designed by the authors of the present study to confirm the results of the first two primers and also to differentiate the subgroups. **RESULTS:** Among *Giardia* isolates, 2/10 and 1/19 of PCR-RFLP of rabbit and mouse respectively amplified with primer PM290. **CONCLUSIONS:** There is evidence that suggests that direct transmission from companion animals to human does occur. Zoonosis is controversial regarding *Giardia*; however, most researchers believe that its zoonotic potential merits adequate precaution when working with feces of animals that may be infected.

Introduction

Giardia intestinalis, the causative agent of the diarrheal disease giardiasis, is the most common gastrointestinal protozoan parasite detected in humans worldwide (Kappus et al., 1994). The etiology of the disease is poorly understood and re-infection, especially in poorer communities, is common (Gilman et al., 1988; Farthing, 1994). The spectrum of

clinical manifestations seen in human giardiasis include temporary, mild, and transient intestinal complaints that resolve completely, and a rather characteristic complex of symptoms consisting of an acute onset of diarrhea, abdominal cramps, bloating, and flatulence that are often accompanied by nausea and weight loss that last for up to 7 weeks. In particular, in the undernourished host and in children, the infection can occasionally become

chronic with profound diarrhea, weight loss, malabsorption, and failure to thrive (Farthing, 1994). The Factors determining the variability in clinical outcome in giardiasis are still poorly understood (Buret, 1994). Host factors, such as immune status, nutritional status, and age, as well as differences in virulence and pathogenicity of *Giardia* strains are recognized as important determinants for the severity of infection (Thompson et al., 1993; Buret, 1994). However, no, or only limited, differences in phenotypic traits such as parasite growth, the parasite influence on host growth, and pathogenicity have been found between asymptomatic and symptomatic isolates of *G. duodenalis* in vivo and in vitro studies (cedilla-Rivera et al., 1989).

Giardia exists in two forms, the trophozoite and the cyst. *Giardia duodenalis*, originally regarded as a commensal organism, is the etiologic agent of giardiasis, a gastrointestinal disease of humans and animals. Transmission is by the fecal-oral route. Both humans and animals may become infected either by direct fecal ingestion or by the ingestion of contaminated water or food. Freshly passed cysts are immediately infective. The ingestion of a mere ten or fewer *Giardia* cysts is enough to cause infection. The most common sign is chronic or intermittent foul-smelling bowel diarrhea. Diarrhea is usually lightly colored, greasy, and mixed with mucus. Diarrhea is not usually watery and does not generally contain blood.

Defined previously as *G. duodenalis* (Syn *G. lamblia*) on the basis of shared morphological characteristics (Filice, 1952), the species includes organisms that have the ability to infect many mammals including dogs, cats, deer mice, ground squirrels, chinchillas, swine, rabbits, pocket mice, oxen, guinea pigs, and humans (Pantchev et al., 2014). However, this morphological similarity masks significant genetic differences.

Giardiasis causes major public and veterinary health concerns worldwide. Due to its

invariant morphology, investigation on aspects such as host specificity and transmission patterns requires a direct genetic characterization of cysts/ trophozoites from host samples. A number of molecular assays have been developed to help unravel the complex epidemiology of this infection, indicating genetic characterization of *G. duodenalis* isolates has revealed the existence of seven genetic groups (or assemblages), two of which (A and B) (Mayrhofer et al., 1995) are found in both humans and animals; however, the role of animals in the epidemiology of human infection is still unclear. Despite the fact that the zoonotic potential of *Giardia* was recognized by the WHO some 30 years ago, the remaining five (C-G) are host-specific. Sequence-based surveys have identified a number of genotypes within assemblages A and B in animal species, some of which may have zoonotic potential. Recently, however, molecular approaches have been complicated by the recognition of intra-isolate sequence heterogeneity (i.e., “mixed templates” that affect identification of subtypes within each assemblage) and by the unreliable assignment of isolates to *G. duodenalis* assemblages generated by different genetic markers. Assemblage “A” and assemblage “B” include all isolates from humans, and they correspond respectively to group I plus II and groups III plus IV of Andrews et al. (1989), to the “Polish” and “Belgian” genotype of Homan et al. (1992), and to groups 1 plus 2 and group 3 of Nash et al. (1992).

This raises concerns about previous interpretation of genotyping data, especially when single genetic markers have been used. (Cacciò SM, 2008).

Although the genetic distance between both assemblages is larger than that detected between species in other protozoan parasites (Mayrhofer et al., 1995), there are no phenotypic characteristics supporting differentiation into two species (Thompson et al., 2000). However, in most of these studies the geno-

type characterization was performed on cultured isolates, a method that heavily selects for isolates from one of the two assemblages (Andrew et al., 1992). Also, most described molecular methods use DNA retrieved from trophozoites established in culture (Nash et al., 1985; Mayrhofer et al., 1992; Morgan et al., 1993; Safaris and Issac-Renton, 1993). Few reported studies have attempted to biotype parasites using DNA from cysts obtained directly from original specimen (Weiss et al., 1992; Paintlia et al., 1998).

The aim of the present study was to biotype groups of *G.lamblia* in laboratory animals that were used in research and the possibility of cross transmission between human and these animals and the importance of this transmission in research in Iran.

Materials and Methods

Animal - source isolates were obtained from unpreserved fecal specimens from rabbit and mice with laboratory- confirmed- giardiasis. Cysts were purified using sucrose gradient density centrifugation (Thompson et al., 1976).

After treating purified cysts with 0.1N HCl (1 ml of 1:10 cyst suspension and 9ml HCl pH=2) and 3 times washing with sterile distilled water, pellets of cysts were inoculated into borosilicate glass culture tube, previously filled with modified TYIS- 33 medium (Keister, 1983). The culture tubes were incubated at 37°C on slant and examined daily with an inverted microscope until trophozoite formed a compact monolayer on the inner wall of the tubes.

DNA extraction: In this study, cysts were frozen and thawed for several times before DNA extraction. DNA extraction was performed using phenol-chloroform method (Sambrook et al., 1989). Briefly, equal volumes of STE (0.1M NaCl and 1M Tris and 1mM EDTA pH=8), 1% SDS and proteinase K (20mg/ml) were added to each tube. The

tubes were briefly vortexed. The samples were incubated for half an hour at 50°C. After boiling, equal volumes of tris- saturated phenol (pH= 8) were added to each tube. The tubes were shaken for 5 to 15 min and centrifuged at 10000rpm for 5min. The aqueous layers were immediately transferred to clean tubes with equal volumes of chloroform and centrifuged at 12000 rpm for 5min. Each aqueous layer was transferred to a clean tube with an equal volume of sodium acetate (3M sodium acetate pH=5.2). Following the addition of the isopropyl alcohol, the tubes were incubated at -20°C overnight. Samples were centrifuged at 12000 rpm for 10min, and pellets were then rinsed in of 70% ethanol, centrifuged at 12000 rpm for 5min. The pellets were allowed to dry, and then sterile distilled water was added to each tube.

PCR parameters: Using a system that distinguishes sequence differences in the metabolic enzyme, triosephosphate isomerase (tpi), DNA collected from cyst was amplified using 2 sets of primers (PM290-F/PM290-R) and (PM924-F/PM924-R) was designed to distinguish group 2 and subgroups of it.

After optimization of PCRs with primer sets PM924 and PM290 for MgCl₂ concentration, and annealing temperature, the following conditions were found to be optimal for both PCRs: 0.5U Taq DNA Polymerase (Cinagen, Tehran, Iran), 100 mol of each of deoxynucleotide triphosphates (dNTP), 0.5 M of forward and reverse primers, 1.5 mM MgCl₂ for PM 924 and 3 mM MgCl₂ for PM 290 in a 50 µl reaction mixture. Amplifications were performed on a Curbet Gradient thermal cycler (C.R. Australia) under the following conditions for the PCR: 94°C for 5 min, then 35 cycles comprising 94°C for 30 sec at 58.5°C for PM924 and 52.5°C for PM290 and 30 sec at 72°C; and a final extension of 5 min at 72°C. The sequences of primers for “tpi” gene amplification for group 2 and its subgroups are as follows:

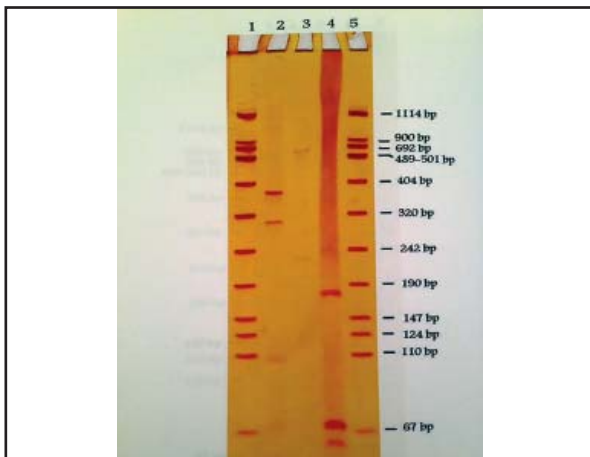


Figure 1. PCR- RFLP result of Human cyst on silver stained 8% PAGE. Lanes 1 and 5: DNA Molecular weight marker. VIII (Roche), Lane 2:RFLP of 924 bp with *RsaI* (Roche), Lane 3: :RFLP of 924 bp with *AvaI* (Roche) Lanes 4:RFLP of 290 bps with with *RsaI*.

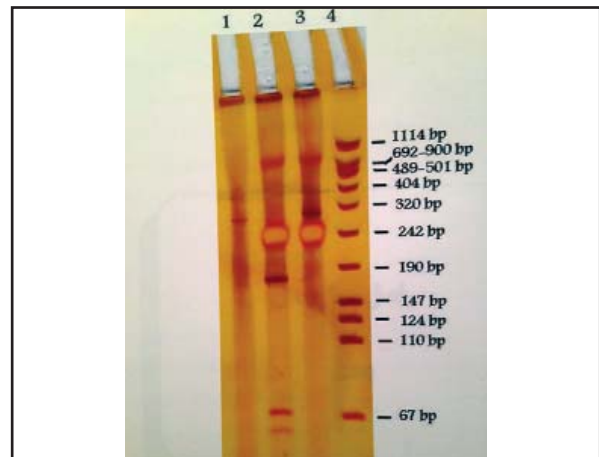


Figure 2. PCR-RFLP result of PM290-F/PM290-R PCR product (290 bp) of Rabbit cyst on silver stained 8% PAGE. Lane 1: 290 bp fragment, Lane 2; RFLP of 290 bp with *RsaI* (Roche), Lane3 : RFLP of 290 bp with *AvaI* (Roche), Lane 4: DNA molecular weight marker. VIII (Roche) (The blobs in all wells that include RFLP samples are caused by the enzyme in silver stained).



Figure 3. PCR-RFLP result of PM290-F/PM290-R PCR product (290 bp) of Mouse cyst on silver stained 8% PAGE. Lane 1: DNA molecular weight marker. VIII (Roche), Lane 2: 290 bp fragment, Lane 3; RFLP of 290 bp with *RsaI* (Roche), Lane 4 : RFLP of 290 bp with *AvaI* (Roche). (The blobs in all wells that include RFLP samples are caused by the enzyme in silver stained).

PM290-F(5'-GCCATTGCTGCCCA-CAAGAT - 3')

PM290-R (5'-GTCATCCCCTTTTCTA-GAGC - 3')

PM 924-F (5'-TCATGCACCGTGATTG-GAC- 3')

PM 924-R(5'-AGTTGCTTCCATTGGC-CGAT-3')

For detection of amplified products, PCR products were electrophoresed on ethidium

bromide 2% agarose gels and visualized on UVP translaminator (UVI Tec England).

Using DNAsis software program, the restriction enzymes *RsaI* and *AvaI* were selected to digest the 924bp and the 290 bp amplification products. Digestion of 924 bp PCR products of PM924-F/PM 924-R Primers with *RsaI* and *AvaI* enzyme mefor isolates AC#U57897 and AC# L02120 are as follows: *RsaI*: 65, 97,101,297,364 and 65,97,297,465 and for *AvaI*: 119,213,592 and 119,805 respectively.

Digestion of 290 bp PCR Products of PM240-F/PM 290-R Primers with *RsaI* enzyme for isolates AC# 069558, AC#069559, AC#L02120 or AC# U57897 (together) are as follows: 123,167 and 58,65,69,98 and 58,65,167 respectively.

Ten microliter aliquots of PCR products were digested at 37°C for 2.5hr, using 4U restriction endonuclease (Fermentas) in a 25 µl final volume of the appropriate digestion reaction mixture.

Digestion products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE) and the bands were visualized by silver- nitrate staining.

Results

Two sets of primers were designed to differentiate between these *Giardia* isolates. The PM924 primer set was used to differentiate between AC#L0210 (WB) and AC#U57897 (JH) isolates, and PM290 primer set was used to differentiate between C#AF069559 and AC#AF069558. DNA from 10 isolates with rabbit origin and 19 isolates with mice (souri) origin were used for PCR. Only 2 samples from rabbit and 1 sample from souri were successfully amplified by PCR.

The findings indicate that none of the animal isolates cultured in axenic culture media TYIS-33. Human isolates from axenic culture (trophozoites) and from cysts amplified with either PM924 and PM290 or only PM290 (Fig.1). 2/10 rabbit (Fig. 2) and 1/19 souri (Fig.3). origin cysts amplified only with primer PM290.

Discussion

This study constitutes the first description of biotyping of *Giardia lamblia* in Iran.

The TPI- based genotype tool is also useful in epidemiologic investigations of giardiasis in humans (Thompson, 2000; Irshad M.Sulaiman, 2003).

Triosphosphate isomerase is a glycolytic enzyme whose catalytic mechanism has been thoroughly studied. The “tpi” sequences of many species, including several parasites have been reported (Lodi et al., 1994; Lu et al., 2002). Using a variety of genetic-based methods; *G.lamblia* isolates have been divided into three genotypes, called groups 1, 2, and 3. (Nash, 1992). Their representative isolates are WB, JH, and GS, respectively, and they are registered in the GenBank. The accession numbers are L02120 (WB), U57897 (JH), and L02116 (GS). The tim (tpi) sequence of the three genotypes were compared by Lu et al. (1998) who showed that groups 1 and 2 were

very similar, while group 3 was markedly different from the other two groups.

A recent study of sporad cases of human giardiasis used a tpi- based PCR- RFLP genotyping tool of the 33 tpi-PCR-positive infected patients, 21 (64%) were infected with assemblage B, 9 (27%) with assemblage A, and 3 (9%) samples were mixed infections of assemblages A and B (Amar CFL et al., 2002).

A study in Australia also showed that 30% of children were infected with isolates of *G.duodenalis* that were genotyped as assemblage A, and 70% had isolates that were genotyped as assemblage B. Statistical analysis revealed that children infected with *Giardia* isolates from assemblage A were 26 times more likely to have diarrhea than those with assemblage B organism. (Thompson, 2002).

Our results from *Giardia* infection in Iran are in contrast with those of these investigators.

Is *Giardia* a zoonotic concern? There is evidence that suggests that direct transmission from companion animals to humans does occur. Zoonosis is controversial regarding *Giardia*; however, most researchers believe that its zoonotic potential merits adequate precaution when working with feces of animals that may be infected. In this study, partial sequence similarity was observed between human “tpi” gene and the animals tested.

Giardia is a potential health concern for both man and animals. Correct measures should always be employed in order to properly diagnose, control, and treat giardiasis. Much work has been done in the area of *Giardia* research; nevertheless, there is still much to be done. Preventing and controlling giardiasis will require the joint efforts of both human veterinary medical professionals. The zoonotic potential of both assemblages A and B is evident when studied at the level of assemblages, sub-assemblages, and even at each single locus. Although the zoonotic potential of *G. duodenalis* is evident, evidence on the contribution and

frequency is (still) lacking. This newly developed molecular database has the potential to tackle intricate epidemiological questions concerning protozoan diseases (Sprong, 2009; van Keulen et al., 2002).

The importance of zoonotic transmission of *Giardia* spp. and similarities between human and laboratory animals such as rabbit and souri mouse is important in researches that can interfere with the culture of other organism or may infect other laboratory animals in animal house or may infect the person who works with them.

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