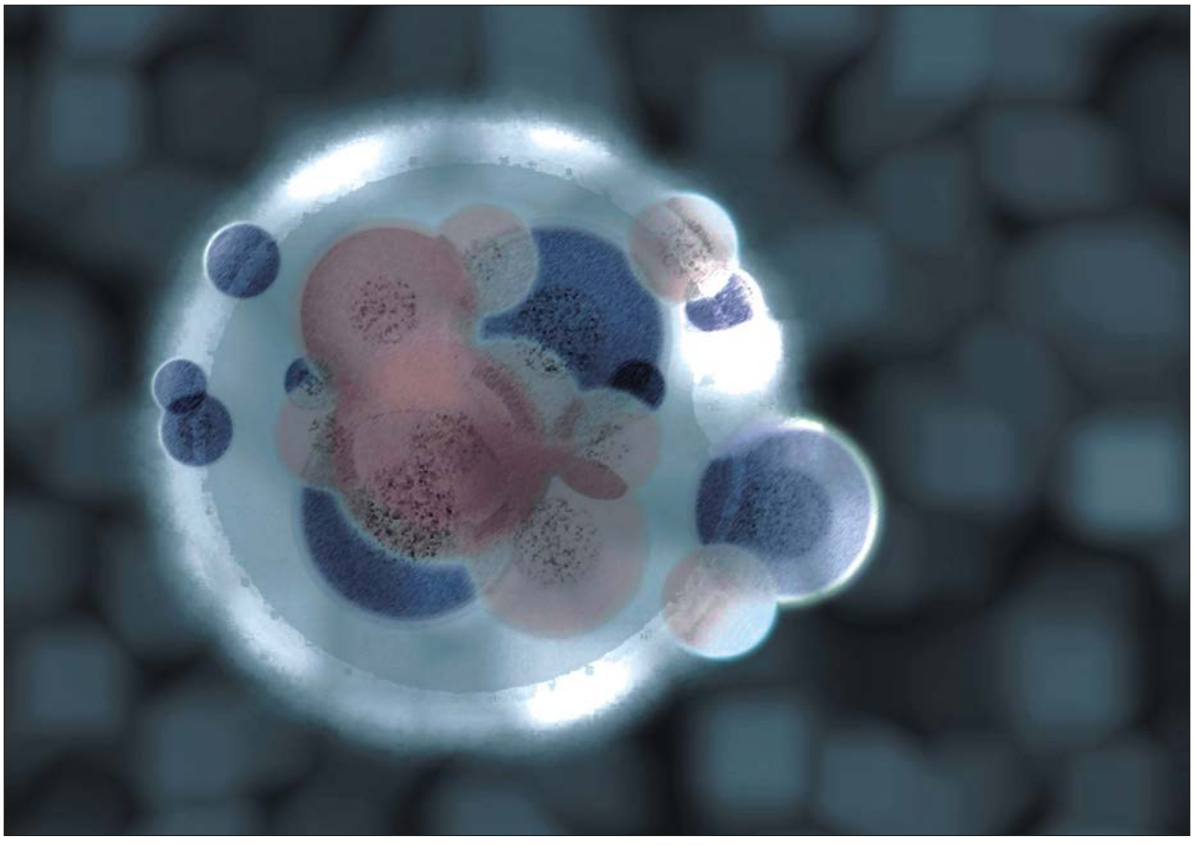

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (5): 896-901, 2008
ISSN 1727-3048

Molecular Identification of *Giardia duodenalis* Isolates from Human and Animal Reservoirs by PCR-RFLP

[1]Esmaeel Fallah, [1]Kareem Hatam Nahavandi, [1]Rasul Jamali,
[1]Behroz Mahdavi Poor and [2]Mohammad Asgharzadeh
[1]Department of Parasitology, Faculty of Medicine,
[2]Haematology and Onchology Research Center,
Tabriz University of Medical Sciences, Tabriz, Iran

Abstract: The purpose of this study was to investigate the genotypes of *Giardia duodenalis* from human and animal feces and their epidemiological in Iran. Molecular characterization of cysts of human and animal origin represents an objective means to approve or reject this hypothesis. In this study, cysts of *Giardia duodenalis* were collected from feces of naturally infected cats (n = 2), human (n = 34), dog (n = 2) and cattle (n = 3). PCR-RFLP analysis of the 34 specimens recovered from humans revealed 6 *G. duodenalis* assemblage AII, 8 *G. duodenalis* assemblage BIII and 4 *G. duodenalis* assemblage BIV. Among samples from cats, 1 was classified into assemblage AI. Genetic subgenotypes identified from human reveals that genetic diversity of this protozoan in East Azerbaijan, Iran, is similar to that of *Giardia* from other parts of the world. The present study represents the first contribution to the knowledge of *G. duodenalis* genotypes in Iran.

Key words: *Giardia duodenalis*, humans, domestic animals, glutamate dehydrogenase (*gdh*), PCR-RFLP

INTRODUCTION

Giardia is a genus of intestinal flagellates that infect a wide range of vertebrate hosts. The genus currently comprises six species, namely: *Giardia agilis*, *Giardia ardeae*, *Giardia duodenalis*, *Giardia microti*, *Giardia muris* and *Giardia psittaci*, which are distinguished on the basis of the morphology and ultrastructure of their trophozoites (Adam, 2001). *G. duodenalis* (syn. *G. intestinalis*, *G. lamblia*) is the only species found in humans, although it is also found in other mammals, including pets and livestock (Thompson *et al*., 2000). In humans, giardiasis is a common cause of parasitic gastroenteritis and is a major health concern worldwide (Wolfe, 1992). A variety of molecular tools including isoenzyme analysis, PCR-RFLP and sequence analysis of housekeeping genes have shown that *G. duodenalis* is a species complex, made up of morphologically indistinguishable isolates that are genetically and phenotypically distinct (Meloni *et al*., 1988; Hopkins *et al*., 1997; Homan *et al*., 1998; Monis *et al*., 1999). Assemblages A and B infect humans and a broad range of other hosts, including livestock, cats, dogs and wild mammals. The assemblage A isolates have been further grouped into subgenotypes I and II. The assemblage B isolates have been separated into subgenotypes III and IV (Thompson, 2000). Genetic assemblages C, D, E, F and G appear to be host restricted to domestic animals, livestock and wild animals (Monis *et al*., 2003). The assemblages C and D are considered to be specific for dogs (Hopkins *et al*., 1997; Monis *et al*., 1998; Thompson *et al*., 2000), while assemblage E seems to be specific for livestock (Ey *et al*., 1997), assemblage F for cats and G for rats (Monis *et al*., 1999). All human isolates characterized to date have been grouped into either assemblage A or B, whereas, cats, dogs and livestock are susceptible to infection with isolates from either their own host specific assemblage or the more widely distributed potentially zoonotic assemblages A and B (Read *et al*., 2004). It has been suggested that for humans, other humans are the main reservoir of infection, with zoonotic sources constituting a minor reservoir (Thompson, 1998). The finding that similar genotypes are dispersed in different species of host is not by itself conclusive evidence that zoonotic transmission is taking place (Monis and Thompson, 2003). This requires the application of appropriate genotyping tools in endemic foci where predictive assessments can be made for the transmission patterns (Monis and Thompson, 2003). The *gdh* gene is proven useful for the genotyping of *Giardia* isolates from mammals (Read *et al*., 2004). PCR-RFLP has successfully been used

Corresponding Author: Kareem Hatam Nahavandi, Department of Parasitology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran Tel: 00989124574331

by a number of researchers to genotype *Giardia* from human (Amar *et al*., 2002) and animals (Leonhard *et al*., 2007). The East Azerbayjan Province is located in the North West of Iran, its estimated population is 3,500,000 of which about two-fifth are inhabitants of Tabriz, the capital city of the province. Iran is an endemic foci for giardiasis and the prevalence has been reported to be 10.9% (Sayyari *et al*., 2005). This infection is diversely dispersed throughout all over Iran, such as East Azerbaijan Province. Incidence in this province is variable from 15.2% (Tabriz City) to 43.8% (district of Naghadeh) (Saebi, 2005). The aims of this study were to determine the genetic diversity of *G. duodenalis* isolates in East Azerbaijan Province, Iran and to detect the potential zoonotic reservoir of transmission of the infection in this area.

MATERIALS AND METHODS

Collection and purification of cysts: Human *Giardia*-positive fecal samples (n = 34) were collected from individuals in two localities at the Tabriz, including: patients with acute gastroenteritis hospitalized in the Pediatric Hospital and infected humans referred to the Tabriz Reference Laboratory (TRL), between May and October 2007. Dog *Giardia*-positive faecal samples (n = 2) were collected from dogs visiting veterinary clinics (kept individually) and from refuges located in the urban areas of Tabriz. The cats were sampled (n = 2) in catteries and refuges located in the urban areas of Tabriz. The samples from cattle (n = 3) were from the slaughterhouse and the calves from dairy herds located in the municipalities of Tabriz. Positive cyst samples had been detected by conventional techniques. Cysts were partially purified from fecal material by sucrose density gradient centrifugation (Luchtel *et al*., 1980) 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. duodenalis* isolates were extracted by freeze-thawing technique and then by method of proteinase K, SDS and CTAB (Fallah *et al*., 2008).

PCR amplification and sequencing of the *gdh*: The amplification of the *gdh* gene was performed using a single PCR protocol. In the PCR reaction, a 432 bp fragment was amplified using the forward primer (GDHiF) 5'-CAG TAC AAC TCY GCT CTC GG-3' and the reverse primer (GDHiR) 5'-GTT RTC CTT GCA CAT CTC C-3' (Read *et al*., 2004). Amplification conditions were modified as follows-the PCR mix consisted of 1X buffer containing 1.5 mM $MgCl_2$ (Cinnagen, Iran), 200 μM of each dNTP (Fermentas, Lithuania), 0.5 μM of each primer (F/R), 2.5 U of Taq DNA polymerase (Cinnagen, Iran) and 2 μL of template DNA in a final volume of 20 μL. PCR was performed on a Mastercycler gradient (Eppendrof-Germany) thermal cycler with the following amplification conditions: 1 cycle of 94°C for 10 min (initial heat activation step), 50 cycles of 35s at 94°C, 35 sec at 61°C and 50 sec at 72°C, with a final extension of 7 min at 72°C. Both positive and negative controls were included to validate results. PCR products were visualised on ethidium bromide (0.5 μg mL^{-1}) stained 1% agarose gel. Also, six PCR products of the samples were sequenced to validate results. These samples were purified using the DNA extraction kit (PureExtreme, Fermentas, Lithuania), according to manufacturer's instructions. DNA sequencing reactions were performed by Fermentas Company. The amplicons were sequenced in both directions using. Multiple alignments of the nucleotide sequences were performed using Clustal W (Thompson *et al*., 1994).

RFLP analysis: RFLP analysis was performed by digesting 10 μL of PCR product with 2 U of RsaI (Fermentas, Lithuania) or 2 U of NlaIV (Fermentas, Lithuania) in 1X enzyme buffer in a final volume of 20 μL for 3 h at 37°C. The assemblage-specific restriction enzyme profiles patterns were obtained by Nla IV and Rsa 1 digestion of PCR products. The predicted restriction fragment sizes are described previously (Read *et al*., 2004). The NlaIV digestion was used for the distinction between all of the major assemblages and assemblage A subgenotype 1, assemblage A subgenotype 2 after amplification. RsaI digestion distinguished between assemblage B subgenotype 3 and assemblage B group 4. Restriction fragments were separated by horizontal electrophoresis in 2% agarose gels, with ethidium bromide staining. A 100 bp DNA ladder (Fermentas, Lithuania) was included as a size marker. Restriction fragments were recorded by UV transillumination.

RESULTS AND DISCUSSION

PCR and sequencing of *gdh*: Among human fecal samples of sporadic cases of giardiasis identified by convectional techniques (e.g., formalin-ethyl acetate) (n = 34), the *gdh* gene was successfully amplified from 18 samples (52.9%) with PCR developed in this laboratory. A 432 bp fragment of *gdh* locus was amplified in the PCR using primers GDHiF and GDHiR. Among animal faecal samples containing cysts of *Giardia*, the gdh gene was amplified

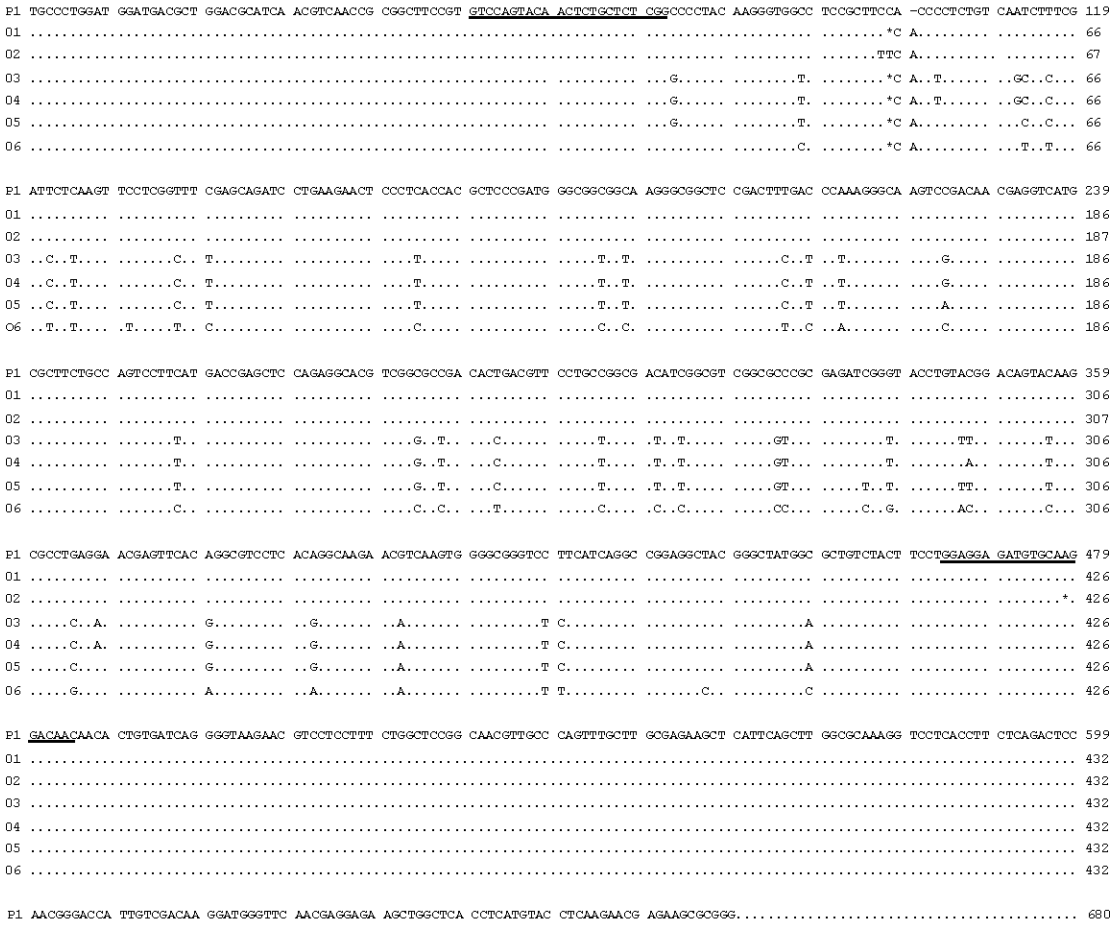

Fig. 1: Multiple alignment, determined with the Clastal W program, of partial *gdh* nucleotide sequences (432 bp fragment) of six *Giardia duodenalis* Assemblages in the present study. P1, the Portland-1 reference strain (GenBank accession number L40510); 01, 02, 03, 04 and 05, human *Giardia duodenalis* isolates and 06, cat *Giardia duodenalis* isolate that were comprised with GeneBank reference sequence. The underscores show the positions of the primers in the sequences. The asterisks indicate base deletions and dots indicate nucleotide identity

from only one faecal isolate from cat. No product was amplified with DNA extracted from purified *G. duodenalis* obtained from cattle and dog. The negative remaining observed could not be amplified because there was insufficient DNA. Analysis of the *gdh* sequences for these samples revealed a 100% match with GenBank reference sequences from each assemblage with the exception of 43 point mutations in the five human isolates and 39 point mutations in the cat isolate that were sequenced (Fig. 1). These mutations did not affect the RFLP profile.

RFLP analysis: RFLP assay of the 18 specimens recovered from humans revealed 6 (33.33%) *G. duodenalis* assemblage A cluster II (Fig. 2), 8 (44.44%) *G. duodenalis* assemblage B cluster III and IV (22.22%) *G. duodenalis* assemblage B cluster IV (Fig. 3). Of these, 6 (33.33%) samples contained assemblage A and 12 (66.66%) samples contained assemblage B. Among samples from cat, one was classified into assemblage A cluster I (Table 1).

Infection with *G. duodenalis* is widespread both in humans and animals and multiple transmission routes exist, with water and food playing an increasingly recognized role worldwide (Thompson, 2000). To understand the epidemiology of the infection and to implement control measures, it is important to determine whether *G. duodenalis* can infect humans through a zoonotic route and, if so, to identify the animals that can act as a source of infection. The risk posed by animals can be better understood by the molecular characterization of

Table 1: Genotypic characterization of human and animal *Giardia* isolates at the *gdh*

Sample	Assemblage A		Assemblage B			
(No. of samples)	AI	AII	BIII	BIV	Assemblages C, D, E	Total
Human (n = 34)	0	6 (33.33%)	8 (44.44%)	4 (22.22%)	0	18 (52.94%)
Cat (n = 2)	1	0	0	0	0	1 (50%)
Dog (n = 2)	0	0	0	0	0	0
Cattle (n = 3)	0	0	0	0	0	0
Total (n = 41)	1	6	8	4	0	19 (46.34%)

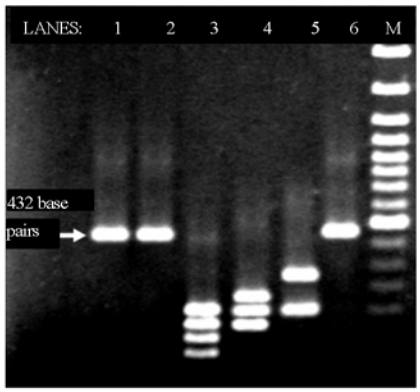

Fig. 2: PCR products and Nla IV digestion of PCR products on an ethidium bromide-stained 2% high resolution agarose gel. Lanes 1, 2 and 6, PCR products (432 bp fragment); lane 3, *G. duodenalis* assemblage A group II (120, 90, 80 and 70 bp); lane 4, *G. duodenalis* assemblage A group I (Cat isolate) (150, 120 and 90 bp); lane 5, *G. duodenalis* assemblage B (290 and 120 bp); lane M, 100 bp, Plus marker (Fermentas, Lithuania) (the 432 bp fragments is indicated)

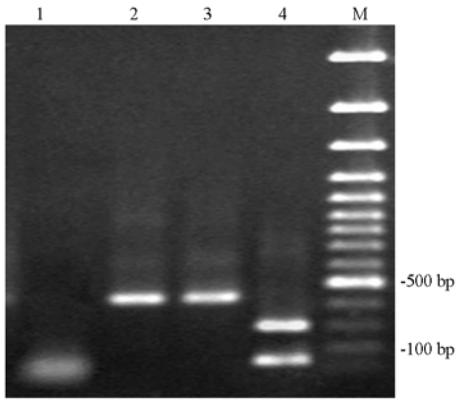

Fig. 3: Rsa I digestion of gdh-PCR products on an ethidium bromide-stained 2% agarose gel. Lane 1, negative control; lane 2 and 3, *G. duodenalis* assemblage B subgenotype IV (430 bp); lane 4, *G. duodenalis* assemblage B subgenotype III (300 and 130 bp); lane M, 100 bp, Plus molecular weight marker (Fermentas, Lithuania)

cysts, as molecular data represent an objective means of determining the source of infection both in outbreaks and sporadic cases (Isaac-Renton *et al*., 1993; Hopkins *et al*., 1997; McIntyre *et al*., 2000). The *gdh*-based PCR assay of *G. duodenalis* has proven to be highly effective in detecting the sources of infection and in providing data for improving the control programs of giardiasis. In this study, we used PCR-RFLP typing to investigate active giardiasis transmission in East Azerbaijan Province of Iran. In this work, we studied 18 human and 1 animal isolates by RFLP analysis of PCR products of the *gdh* locus. As expected, only assemblage A and B were found to be associated with human infections. All *G. duodenalis* assemblage A strains obtained from human were identified as assemblage A group 2, corresponds to the findings of a UK study that examined 35 human clinical samples and found 27% assemblage AII and 64% assemblage B (Amar *et al*., 2002) and different from a Brazilian study that found of the *G. duodenalis* detected in humans (78.4%) to be assemblage AII (Souza *et al*., 2007). Read *et al*. (2002) and Traub *et al*. (2004) also found assemblage B to occur more frequently than assemblage A in humans. The present study provides, for the first time, information on the distribution of the genotypes of *G. duodenalis* from humans with sporadic giardiasis in East Azerbaijan Province of Iran. The results presented here reinforce the evidence that humans are susceptible only to assemblages A and B but not to the host-specific assemblages C, D, E and F. In addition, the results corroborate that assemblage AII is highly human specific. One isolate from cat was classified into assemblage AI. If the assemblage AI of *G. duodenalis* represents risk to human population, it could be hypothesized that the public health risk is related to the cats in the municipality of Tabriz. The high frequency of occurrence of zoonotic assemblages in cats from urban areas were already described in Italy, where ten cats were found to be infected with *Giardia* and harbouring cysts belonging to the zoonotic assemblage A (Papini *et al*., 2007). In the study of the Souza *et al*. (2007) in Brasil, among 19 samples from cats, 11 were classified into assemblage F (Cat genotype) and 8 into assemblage AI (Souza *et al*., 2007). The general picture of the *gdh* polymorphism of samples of *G. duodenalis* in the Province of East

Azerbaijan, Iran, like to those of other parts of the world. In this study, neither the zoonotic assemblage AI of *G. duodenalis* was found in humans, nor the zoonotic assemblage B was found in pets. This does not necessarily indicate that the participation of such hosts in urban zoonotic cycle of giardiasis is minimal in the urban areas of the East Azerbaijan. In fact, any conclusion about zoonotic transmission should be drawn with caution, because finding similar or different genotypes dispersed in different hosts is not by itself conclusive that zoonotic transmission is or is not taking place (Thompson, 2002). Although molecular characterization of *Giardia* from different hosts is imperative for the epidemiology of giardiasis in animals and humans, further studies contemplating the classical risk factors and cross-sectional surveys are needed in order to better understand the transmission dynamics of *Giardia* spp. in endemic foci and to evaluate the level of participation or each host as source of infection for susceptible individuals and populations.

ACKNOWLEDGMENT

We thank colleagues in Tabriz Reference Laboratory and Tabriz Paediatric Hospital Laboratory for providing stool samples.

REFERENCES

Adam, R.D., 2001. Biology of *Giardia lamblia*. Clin. Microbiol. Rev., 14 (3): 447-475.

Amar, C.F., P.H. Dear, S. Pedraza-Diaz, N. Looker, E. Linnane and J. McLauchlin, 2002. Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. Clin. Microbiol. J., 40 (2): 446-452.

Bertrand, I., L. Albertini and J. Schwartzbrod, 2005. Comparison of two target genes for detection and genotyping of *Giardia lamblia* in human feces by PCR and PCR-Restriction fragment length polymorphism. J. Clin. Microbiol., 43 (12): 5940-5944.

Ey, P.L., M. Mansouri., J. Kulda, E. Nohynkova., P.T. Monis., R.H. Andrews and G. Mayrhofer, 1997. Genetic analysis of *Giardia* from hoofed farm animals reveals artiodactyls-specific and potentially zoonotic genotypes. J. Euk. Microbiol., 44: 626-635.

Fallah, E., K. H. nahavandi., R. Jamali., B. Mahdavi and M. Asgharzadeh, 2008. Genetic characterization of *Giardia intestinalis* strains from patients having sporadic giardiasis by using PCR assay. J. Med. Sci. (In Press).

Homan W.L., M. Gilsing, H. Bentala, L. Limper and F. van Knapen, 1998. Characterization of *Giardia duodenalis* by polymerase-chain-reaction fingerprinting. Parasitol. Res., 84 (9): 707-714.

Hopkins, R.M., B.P. Meloni, D.M. Groth, J.D. Wetherall, J.A. Reynoldson and R.C.A. Thompson, 1997. Ribosomal RNA sequencing reveals differences between genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. J. Parasitol., 83 (1): 44-51.

Isaac-Renton, J.L., C. Cordeiro, K. Sarafis and H. Shahriari, 1993. Characterization of *Giardia duodenalis* isolates from a waterborne outbreak. J. Infect. Dis., 167 (2): 431-440.

Leonhard, S., K. Pfister, P. Beelitz, C. Wielinga and R.C.A. Thompson, 2007. The molecular characterisation of *Giardia* from dogs in Southern Germany. Vet. Parasitol., 150 (1-2): 33-38.

Luchtel, D.L., W.P. Lawrence and F.B. Dewalle, 1980. Electron microscopy of *Giardia lamblia* cysts. Applied Env. Microbiol., 40 (4): 821-832.

McIntyre, L., L. Hoang, C.S. Ong, P. Lee and J.I. Isaac-Renton, 2000. Evaluation of molecular techniques to biotype *Giardia duodenalis* collected during an outbreak. J. Parasitol., 86 (1): 172-177.

Meloni, B.P., A.J. Lymbery and R.C.A. Thompson, 1988. Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and felines. Am. J. Trop. Med. Hyg., 38 (1): 65-73.

Monis, P.T., R.H. Andrews, G. Mayrhofer, J. Kulda, J.H. Isaac-Renton and P.L. Ey, 1998. Novel lineages of *Giardia intestinalis* identified by genetic analysis of organisms isolated from dogs in Australia. Parasitol., 116 (1): 7-19.

Monis, P.T., R.H. Andrews, G. Mayrhofer and P.L. Ey, 1999. Molecular systematics of the parasitic protozoan *Giardia intestinalis*. Mol. Biol. Evol., 16 (9): 1135-1144.

Monis, P.T. and R.C.A. Thompson, 2003. *Cryptosporidium* and *Giardia*-zoonoses: Fact or fiction? Infect. Genet. Evol., 3 (4): 233-244.

Monis, P.T., R.H. Andrews, G. Mayrhofer and P.L. Ey, 2003. Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect. Genet. Evol., 3 (1): 29-38.

Papini, R., G. Cardini, B. Paoletti and A. Giangaspero, 2007. Detection of *Giardia* assemblage A in cats in Florence Italy. Parasitol. Res., 100 (3): 653-656.

Read, C., J. Walters, I.D. Robertson and R.C.A. Thompson, 2002. Correlation between genotype of *Giardia duodenalis* and diarrhoea. Int. J. Parasitol., 32 (2): 229-231.

Read, C.M., P.T. Monis and R.C.A. Thompson, 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol., 4 (2): 125-130.

Saebi, E., 2005. Protozoal Diseases in Iran. Textbook of Clinical Parasitology. 4th Edn. Ayexh, Tehran, pp: 79-97.

Sayyari, A.A., F. Imanzadeh, S.A. Bagheri Yazdi, H. Karami and M. Yaghoobi, 2005. Prevalence of intestinal parasitic infections in the Islamic Republic of Iran. East. Mediterr. Health. J., 11 (3): 377-383.

Souza, S.L.P., S.M. Gennari, L.J. Richtzenhain, H.F.J. Pena, M.R. Funada, A. Cortez, F. Gregori and R.M. Soares, 2007. Molecular identification of *Giardia duodenalis* isolates from humans, dogs, cats and cattle from the state of Sao Paulo, Brazil, by sequence analysis of fragments of glutamate dehydrogenase (gdh) coding gene. Vet. Parasitol., 149 (3-4): 258-264.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22 (22): 4673-4680.

Thompson, R.C.A., 1998. *Giardia* Infections. Zoonoses: Biology. In: Clinical Practice and Public Health Control, Palmer, S.R., E.J.L. Soulsby and D.I.H. Simpson (Eds.). Oxford University Press, Oxford, United Kingdom, pp: 545-561.

Thompson, R.C.A., 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. Int. J. Parasitol., 30 (12):1259-1267.

Thompson, R.C.A., R.M. Hopkins and W.L. Homan, 2000. Nomenclature and genetic groupings of *Giardia* infecting mammals. Parasitol. Today, 16 (5): 210-213.

Traub, R.J., P.T. Monis, I. Robertson, P. Irwin, N. Mencke and R.C.A. Thompson, 2004. Epidemiological and molecular evidence supports the zoonotic transmission of Giardia among humans and dogs living in the same community. Parasitology, 128 (3): 258-262.

Wolfe, M.S., 1992. Giardiasis. Clin. Microbiol. Rev., 5 (1): 93-100.