4

Genotyping of *Giardia intestinalis* Isolates from Dogs by Analysis of *gdh*, *tpi*, and *bg* Genes

Enedina Jiménez-Cardoso, Leticia Eligio-García,
Adrian Cortés-Campos and Apolinar Cano-Estrada
Laboratorio de Investigación en Parasitología
Hospital Infantil de México Federico Gómez, México, D.F.
Mexico

1. Introduction

Giardia intestinalis, a flagellated protozoan parasite, is the most prevalent human intestinal protozoan worldwide (Adam, 2001). About 200 million people in the world are infected with giardiasis and each individual eliminates up to 900 million cysts per day (Minivielle , 2008). Higher prevalence is found in tropical and subtropical areas, where *Giardia* affects up to 30% of the population. In epidemiological studies carried out in Mexico and other sudamerican countries, prevalence between of 10-16% has been found in urban areas and 34% in shantytowns (Gamboa "et al", 2003; Giraldo-Gomez, 2005; Sulaiman, 2004). *G. intestinalis* is a cosmopolitan pathogen with a very wide host range, including humans, domestic animals, and wild animal species (Caccio, 2008; Thompson, et al 1993). The most common cause of infection with *Giardia* is the consumption of contaminated food or water (Ortega, 1997), although zoonotic transmission is also possible. Once a person is infected, the parasite lives in the intestines and is passed in the stool of the infected person. Animals such as cats, dogs and cattle can also be infected and spread the disease to humans.

Infections may be asymptomatic or include symptoms of chronic diarrhea, weight loss, and malabsorption. When children infected with *Giardia* have no symptoms of giardiasis, the parasite is present in their feces and they can pass the infection to others. Other symptoms of chronic giardiasis include: Loose, soft, greasy stools, discomfort in the abdomen, general feeling of discomfort or illness, weakness and fatigue.

The parasite has two interchangeable forms that guarantee a simple and efficient life cycle. The cyst that contaminate the environment and the trophozoite, which attach to the intestinal villi via a specialized microtubule structure, the ventral disc.

There are at least seven major genotypes referred to as assemblages (A-G) including 2 (A and B) known to infect humans (Mcpherson, 2005; Monis, 2009).

Assemblages A and B, of clinical significance to humans, differ from each other by as much as 20% at the DNA sequence level (Caccio, 2008). There is also evidence that genetic exchange has resulted in hybrids, or mixed types, based on assemblage-specific PCR of Giardia isolates from cases of human infection (Monis, 1999).

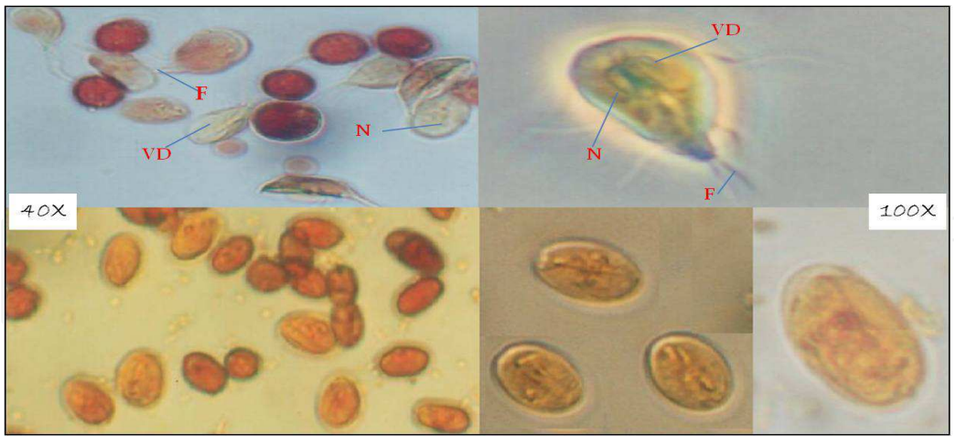


Fig. 1. Trophozoite (top) and Cyst (bottom) of *Giardia intestinalis* lugol´s stained. Ventral disc (VD), Flagella (F) and Nuclei (N). Olympus Bh2 microscopy. Laboratorio de Investigacion en Parasitologia. Hospital Infantil de Mexico.

Clinically, *G. intestinalis* assemblage A appears to be less prevalent than assemblage B and other mixed types worldwide (Tungtrongchitr, 2010; Singh A, 2009). However, infections with assemblage A appear to be more symptomatic. Although assemblages A and B are the only ones that infect humans, they also can infect other mammals (Volotao, et al. 2007). Interestingly, assemblage A is more frequently associated with animal hosts that may serve as reservoirs of infection for humans (Ballweber, 2010).

Although *Giardia* isolated from dogs typically belong to assemblages C or D, assemblages A and B have been identified in dogs in regions of high endemicity (Cooper, 2010). Genotypes E, F, and G have been isolated from pigs and other farm mammals (Jerlström-Hultqvist J, 2010), cats (Ballweber, 2010), and rodents (Monis, 1999), respectively.

The ability to genetically characterize *Giardia* strains isolated from clinical and animal samples should contribute to the understanding of the epidemiology and pathogenesis of *Giardia* infection and the relative contributions of distinct genotypes to the severity of clinical infection and zoonotic potential. Here we report our attempts to develop assays for the characterization of *Giardia* genomic DNA extracted from dog stool samples and from *Giardia* trophozoites Portland I, based on the Polymerase Chain Reaction amplification of multiple loci. We targeted the *G. intestinalis tpi*, *gdh*, and *bg* genes that encode the enzyme *triose-phosphate isomerase*, the enzyme *glutamate dehydrogenase*, and *β-giardin*, respectively.

2. Methodology

2.1 Biological samples

The 9 fecal specimens from dogs included gala1, gala2, gala3, croquetilla1, croquetilla2, croquetilla3, mila1, mila2, and mila3. One *Giardia* containing human fecal specimen was named 454LP. The Portland1 strain of *Giardia* served as a control representing the sub-genotype assemblage AI.

2.2 Coproparasitoscopic analysis

Fecal specimens were stained with Lugol's iodine (Faust, 1938) and examined by microscopy to find cysts of *Giardia intestinalis*. Cysts were concentrated from dog feces by repeated washing in distilled water and stored at 4 ºC until use.

2.3 DNA extraction

DNA was extracted from the fecal samples by use of the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. All DNA concentrations were determined by using an Epoch spectrophotometer (Biotek, Winooski, VT).

2.4 PCR amplification

The *gdh* gene encoding *glutamate dehydrogenase*, the *tpi* gene encoding *triose phosphate isomerase*, and the *bg* gene encoding *β-giardin* were each amplified using the Polymerase Chain Reaction (PCR) as follows.

2.5 *gdh* gene amplification

The *gdh* gene was amplified by using 0.8 μM of each primer (578: 5´-GAGAGGATCCTTGARCCNGAGCGCGTNATC-3´ and 579:5´-CCGCGNTTGTADCGNCCNAAGATCTTCCA-3´) in 50 μL reactions containing 10 mM Tris-HCl, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM each dNTP, 1 U Taq DNA Polymerase and 200 ng of genomic DNA. Samples were subjected to 30 cycles of [94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min], with an initial denaturation step at 94 °C for 4 min, and a final extension step at 72 °C for 6 min (Monis, 1996).

2.6 *bg* gene amplification

The *bg* gene was amplified in two steps via nested-PCR. The first round of PCR was conducted in a 25-μL reaction containing 200 pmol each primer (G7: 5'-AAGCCCGACGACCTCACCCGCACTGC-3' and G759: 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'), 10 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U Taq DNA Polymerase and 200 ng of genomic DNA. This was amplified for 45 cycles of [95 °C 30 s, 65 °C 30 s and 72 °C 1 min]. The second round of PCR, using the product of the first reaction as template, was performed in a 50-μL reaction with 200 pmol each primer (F: 5´-GAACGAGATCGAGGTCCG-3´; R: 5'-CTCGACGAGCTTCGTT-3'), 10 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of Taq DNA Polymerase and 3 μL of template. Amplification was for 35 cycles of [94°C 30 s, 53°C 30 s and 72°C 1 min] (Lalle, 2005).

2.7 *tpi* gene amplification

The tpi gene was amplified by nested-PCR in which the first round was a duplex reaction to amplify two fragments corresponding to genotypes A and B simultaneously using four primers (TPIAF 5´-CGAGACAAGTGTTGAGATGC-3´, TPIAR 5´-GGTCAAGAGCTTACAACACG-3´ and TPIBF 5´-GTTGCTCCCTCCTTTGTGC-3´, TPIBR

5´-CTCTGCTCATTGGTCTCGC-3´). PCR amplification was performed in a volume of 50-µL with 500 ng of DNA in 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM of dNTP and 1 U of Taq DNA Polymerase. Amplification was achieved with 25 cycles of [94 ºC for 20 s, 50 ºC for 30 s and 72 ºC for 1 min]. The second round of PCR comprised two separate hemi-nested PCRs to amplify internal fragments of 476 bp and 140 bp corresponding to the A and B genotypes respectively.

To amplify genotype A, primers TPIAIF: 5´-CCAAGAAGGCTAAGCGTGC-3´ and TPIAR were used using 3 µL of the first round amplicon as template in a 50-µL volume reaction. The amplification step used 33 cycles of [94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min]. Alternatively, the 140 bp fragment corresponding to genotype B was amplified with primers TPIBIF: 5´-GCACAGAACGTGTATCTGG-3´ and TPIBR. Amplification was performed under the same conditions used for A except that the $MgCl_2$ concentration in the PCR mixture was 1.5 mM. (Amar, 2003. Molina, 2005)

2.8 Restriction analysis

The amplicons generated by PCR were digested with restriction enzymes for the purpose of subtyping. The *tpi* gene amplicons were digested with restriction enzyme *RsaI*, the *bg* gene amplicons were digested with *HaeIII*, and the *gdh* amplicons were digested with *BspHI*. The products of restriction enzyme digestion were separated by 2% agarose gel electrophoresis, using 100bp DNA ladder (Promega, Madison,Wi. USA) as a size standard, and visualized by staining with ethidium bromide.

3. Results

We developed a molecular method to test stool samples for the presence of *G. intestinalis* genotypes that are of clinical significance to human infection possibly by zoonotic transmission from dogs. *Giardia* infection of dog stool samples was confirmed by coproparasitoscópico analysis (data not shown). *Giardia* cysts isolated from feces was genotyped by a combination of multi-locus (*gdh, bg, tpi*) PCR followed by restriction analysis of the PCR amplicons. We analyzed nine samples of dog stool, one sample of human stool, and *G. intestinalis* cysts from the Portland-1 control strain. The Portland-1 standard is a control for the A-I assemblage.

3.1 Figure 2

Illustrates genotyping based on amplification and subsequent *BspHI* enzyme digestion of the *gdh* locus encoding *glutamate dehydrogenase*. All of the dog and human samples tested in this way yielded the same 1200 bp fragment as the Portland-1 control and after digestion they yielded two fragments of 900 and 300 bp respectively indicative of assemblage A.

3.2 Figure 3

Illustrates genotyping based on amplification and *HaeIII* enzyme digestion of the *bg* locus encoding *β-giardin*. Three fragments ranging from 100 to 200 bp are indicative of assemblage A-I. All 9 dog samples and 1 human sample were classified as assemblage A-I, the same as the Portland-1 control.

3.3 Figures 4

Illustrate genotyping based on amplification and *RsaI* enzyme digestion of the *tpi* locus encoding *triose-phosphate isomerase.* Again, all samples yielded products consistent with their identification as belonging to *G. intestinalis* genotype AI.

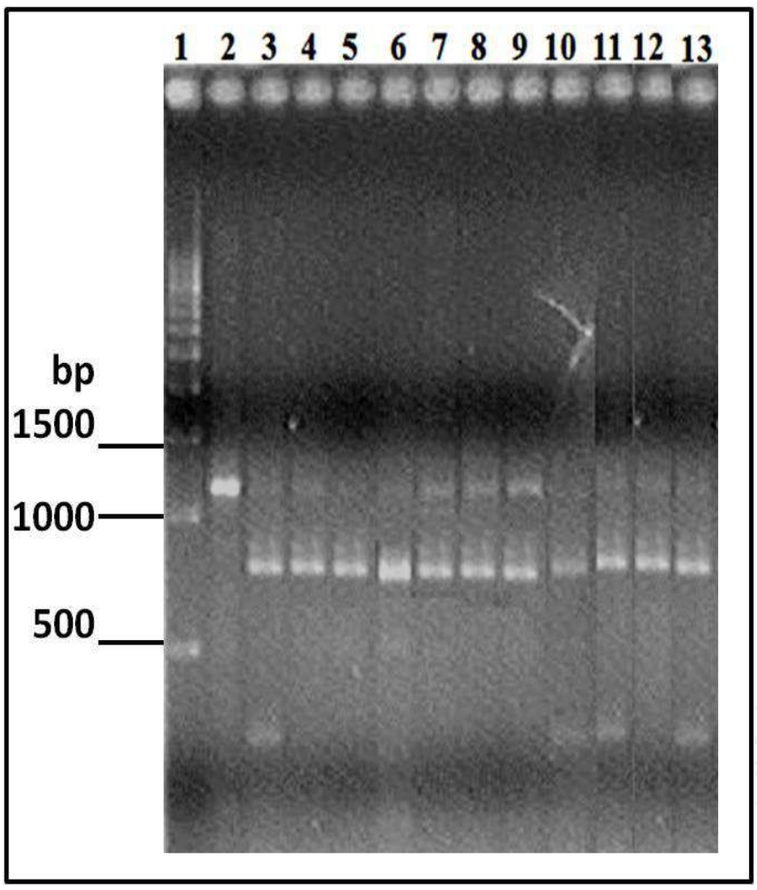


Fig. 2. Genotyping by *gdh*-specific PCR and restricted analyzed by agarose gel electrophoresis. The 900 bp band corresponds to the *BspHI*-digested *gdh*-specific PCR product. 1) Molecular weight marker (500 bp ladder), 2) Portland-1 control without digestion, 3) Portland-1 control restricted, 4) gala1, 5)gala2, 6)gala3, 7)croquetilla1, 8)croquetilla2, 9)croquetilla3, 10)mila1, 11) mila2, 12) mila3, 13) 454 LP.

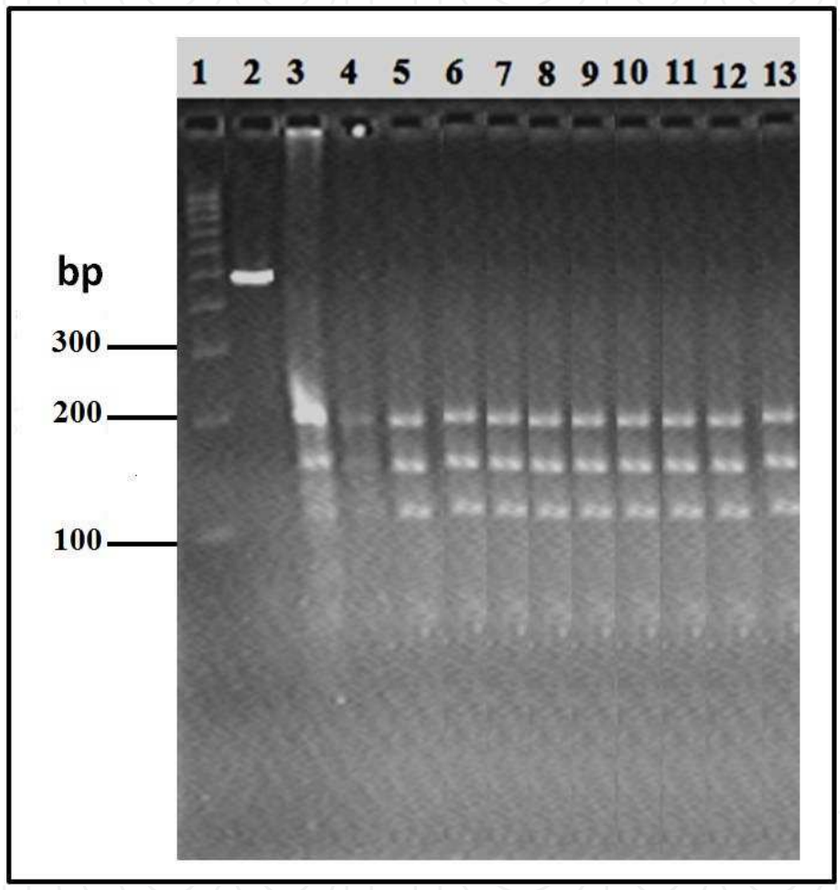


Fig. 3. Genotyping by *bg*-specific PCR and restriction analyzed by agarose gel electrophoresis. The fragments ranging from 100 to 200bp corresponding to the *HaeIII*-digested bg-specific nested-PCR product. 1) Molecular weight marker (500 bp ladder), 2) Portland-1 control without digestion, 3) Portland-1 control restricted, 4) gala1, 5)gala2, 6)gala3, 7)croquetilla1, 8)croquetilla2, 9)croquetilla3, 10)mila1, 11) mila2, 12) mila3, 13)454 LP.

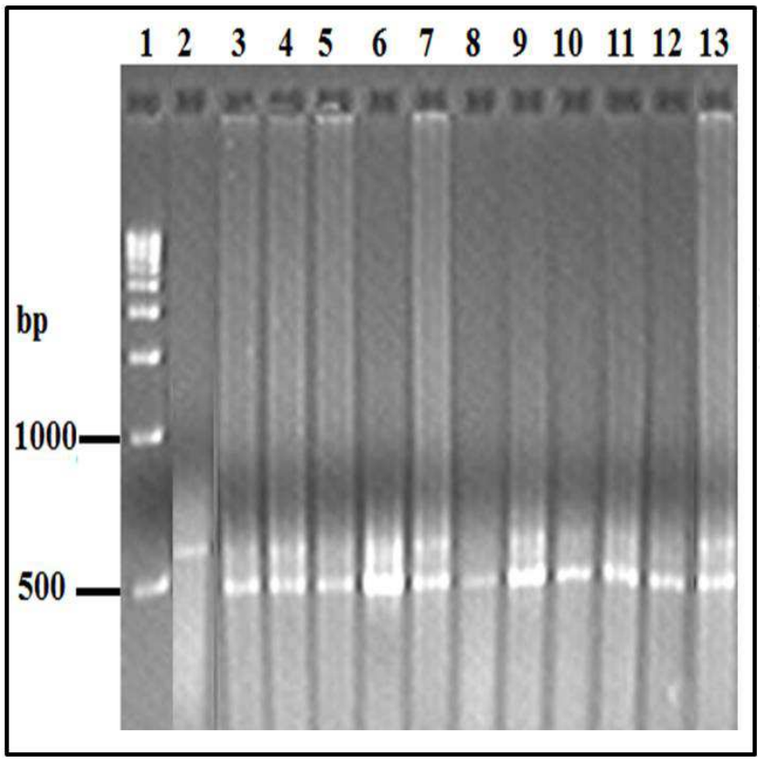


Fig. 4. Genotyping by *tpi*-specific PCR and restriction analyzed by agarose gel electrophoresis. The 437 bp band corresponds to the *RsaI*-digested amplicon representative of assemblage A-I. 1) Molecular weight marker (500 bp ladder), 2) Portland-1 control without digestion, 3) Portland-1 control restricted, 4) gala1, 5)gala2, 6)gala3, 7)croquetilla1, 8)croquetilla2, 9)croquetilla3, 10)mila1, 11) mila2, 12) mila3 13) 454 LP.

4. Discussion

Human giardiasis is caused by two genetically distinct assemblages (A and B) of *G. intestinalis*. A number of molecular assays have been developed for their specific detection in stool and environmental samples (Caccio, 2008).

We have developed a method to detect *Giardia* based on the PCR amplification of three genes (*gdh, bg, tpi*) used in prior genotyping studies.

Although DNA-based methods reported in the literature have been used with success to genotype *Giardia*, we did not have observed differences among the analysis of different genes in studied samples. Some researchers had found frequent mismatches, intra-assemblage discordances and mixed positions, in *tpi* and in *bg* sequences, especially in assemblage B (Bonhomme, 2011).

All of the fecal samples analyzed in this report (9 from dogs, 1 from humans) were determined to belong to the sub-genotype A-I assemblage. This predominance of assemblage A-I probably reflects the mechanism that led to infection of the animals from which the fecal samples came. The dogs might have drunk from water that had been

contaminated by livestock rather than by humans. This has important epidemiological ramifications.

5. References

Adam RD. (2001). Biology of *Giardia lamblia*. *Clinical Microbiology Review*. ; 14 (3), pp.447-75.

Adam RD, Nigam A, Seshadri V, Martens CA, Farneth GA, Morrison HG, Nash TE, Porcella SF, Patel R. (2010). The *Giardia lamblia* vsp gene repertoire: characteristics, genomic organization, and evolution. *BMC Genomics*, 11 (9), pp. 424.

Almeida A, Pozio E, Cacciò SM. (2010). Genotyping of *Giardia duodenalis* cysts by new Real-Time PCR assays for detection of mixed infections in human samples. *Applied and Environmental Microbiology*, 76(6), pp. 1895–1901.

Almeida A, Moreira MJ, Soares S, Delgado ML, Figueiredo J, Silva Magalhães E, Castro A, Da Costa AV, Correia da Costa JM. (2010). Biological and genetic characterization of *Cryptosporidium spp*. and *Giardia duodenalis* isolates from five hydrographical basins in northern Portugal. *Korean Journal of Parasitology*, 48(2) pp. 105–111.

Ballweber LR, Xiao L, Bowman DD, Kahn G, Cama VA. (2010). Giardiasis in dogs and cats: update on epidemiology and public health significance. *Trends in Parasitology*, 26(4), pp. 180-9.

Bonhomme J, Le Goff L, Lemée V, Gargala G, Ballet JJ, Favennec L. (2011). Limitations of tpi and bg genes sub-genotyping for characterization of human *Giardia duodenalis* isolates. *Parasitology International*, 60(3), pp. 327-30.

Cacciò SM, Ryan U. (2008). Molecular epidemiology of giardiasis. *Molecular and Biochemical Parasitology*, 160(2), pp.75-80.

Cooper MA, Sterling CR, Gilman RH, Cama V, Ortega Y, Adam RD. (2010). Molecular analysis of household transmission of *Giardia lamblia* in a region of high endemicity in Peru. *The Journal of Infectious Diseases*, 202(11), pp. 1713-21.

Amar CF, Dear PH, McLauchlin J. (2003). Detection and genotyping by real-time PCR/RFLP analyses of *Giardia duodenalis* from human feces. *Journal of Medical Microbiology*, 52, pp. 681–683.

De Boer RF, Ott A, Kesztyüs B, Kooistra-Smid AMD. (2010). Improved Detection of Five Major Gastrointestinal Pathogens by Use of a Molecular Screening Approach. *Journal of Clinical Microbiology*, 48(11), pp. 4140–4146.

Faust EC, D´antonio JS, Odom V, Miller MJ, Peres C, Sawitz W, Thomen LF, Tobie J, Walker JH . (1938). A critical study of clinical laboratory techniques for the diagnosis of the protozoan cysts and helminthes egg in feces. *The American Journal of Tropical Medicine and Hygiene*, 18, pp. 169-83.

Jerlström-Hultqvist J, Franzén O, Ankarklev J, Xu F, Nohýnková E, Andersson JO, Svärd SG, Andersson B. (2010). Genome analysis and comparative genomics of a *Giardia intestinalis* assemblage E isolate. *BMC Genomics*, 11, pp.543.

Lalle M, Jimenez-Cardosa E, Cacciò SM, Pozio E. (2005). Genotyping of *Giardia duodenalis* from humans and dogs from Mexico using a beta-giardin nested polymerase chain reaction assay. *The Journal of Parasitology*, 91(1), pp. 203-5.

Macpherson CN. (2005). Human behaviour and the epidemiology of parasitic zoonoses. *International Journal of Parasitology*, 35(11-12), pp.1319-31.

Minvielle MC, Molina NB, Polverino D, Basualdo JA. (2008). First genotyping of *Giardia lamblia* from human and animal feces in Argentina, South America. Memorias do Instituto Oswaldo Cruz, 103(1), pp. 98-103.

Molina N, Polverino D, Minvielle M, Basualdo J. (2007). PCR amplification of triosephosphate isomerase gene of *Giardia lamblia* in formalin-fixed feces. *Revista Latinoamericana de Microbiología*, 49(1-2), pp. 6-11.

Monis PT, Mayrhofer G, Andrews RH, Homan WL, Limper L, Ey PL. (1996). Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. *Parasitology*, 112 (Pt 1), pp. 1-12.

Monis PT, Andrews RH, Mayrhofer G, Ey PL. (1999). Molecular systematics of the parasitic protozoan *Giardia intestinalis. Molecular Biology and Evolution*, 16(9), pp. 1135-44.

Ortega YR, Adam RD. (1997). Giardia: overview and update. *Clinical of Infectious Diseases*, 25, pp. 545-550.

Plutzer J, Ongerth J, Karanis P. (2010). *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. *International Journal of Hygiene and Environmental Health,* 15(5), pp. 321-33.

Singh A, Janaki L, Petri WA Jr, Houpt ER. (2009). *Giardia intestinalis* assemblages A and B infections in Nepal. *The American Journal of Tropical Medicine and Hygiene,* 81(3), pp. 538-9.

Sulaiman IM, Jiang J, Singh A, Xiao L. (2004). Distribution of *Giardia duodenalis* genotypes and subgenotypes in raw Urban Wastewater in Milwaukee, Wisconsin. *Applied Environmental Microbiology,* 70(6), pp. 3776–3780.

Ten Hove RJ, van Esbroeck M, Vervoort T, van den Ende J, van Lieshout L, Verweij JJ. (2009) Molecular diagnostics of intestinal parasites in returning travelers. *European Journal of Clinical Microbiology and Infectious Diseases*, 28(9), pp. 1045–1053.

Thompson RC, Reynoldson JA, Mendis AH. (1993). *Giardia* and giardiasis. *Advances in Parasitology*, 32, pp. 71-160.

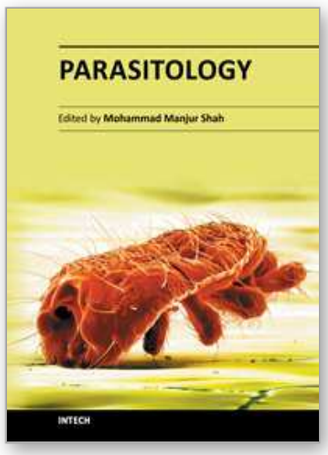
Tungtrongchitr A, Sookrung N, Indrawattana N, Kwangsi S, Ongrotchanakun J, Chaicumpa W. (2010). *Giardia intestinalis* in Thailand: identification of genotypes. *Journal of Health, Population and Nutrition*, 28(1), pp. 42-52.

van Keulen H, Macechko PT, Wade S, Schaaf S, Wallis PM, Erlandsen SL.(2002). Presence of human Giardia in domestic, farm and wild animals, and environmental samples suggests a zoonotic potential for giardiasis. *Veterinary Parasitology*, 108(2), pp.97-107.

Volotão AC, Costa-Macedo LM, Haddad FS, Brandão A, Peralta JM, Fernandes O. (2007). Genotyping of *Giardia duodenalis* from human and animal samples from Brazil using beta-giardin gene: a phylogenetic analysis. *Acta Tropica*, 102(1), pp.10-9.

Gamboa M, Basualdo J, Córdoba M, Pezzani B, Minvielle M, Lahitte H. (2003). Distribution of intestinal parasitoses in relation to environmental and sociocultural parameters in La Plata, Argentina. Journal of Helminthology, 77, pp. 15-20.

Giraldo-GómezI J; LoraII F; Henao LH; Mejía S, Gómez-Marín J. (2005) Prevalence of giardiasis and intestinal parasites in pre-school children from homes being attended as part of a state programme in Armenia, Colombia. *Revista de Salud Pública*, 7(3), pp. 327-338.



Parasitology

Edited by Dr. Mohammad Manjur Shah

ISBN 978-953-51-0149-9
Hard cover, 206 pages
Publisher InTech
Published online 14, March, 2012
Published in print edition March, 2012

Parasitology is an established discipline that covers a wide area of subjects, ranging from the basics (study of life cycle, ecology, epidemiology, taxonomy, biodiversity, etc) to the advanced and applied aspects (human and animal related, although control aspect remains the most important task). There is a great scarcity in the amount of available literature that is freely accessible to anyone interested in the subject. This book was conceptualized with this in mind. The entire book is based on the findings of various studies performed by different authors, comprising reviews and original scientific papers. I hope this book will be helpful to diverse audiences like biologists, zoologists, nematologists, parasitologists, microbiologists, medical doctors, pathologists as well as the molecular biologists, by providing them with a better understanding of the subject.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen