Genotyping and Phylogenetic Analysis of *Giardia duodenalis* Isolates from Turkish Children

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1 **Gulden Sonmez Tamer**
DEF 2 **Murat Kasap**
BF 3 **Doganhan Kadir Er**

1 Department of Medical Microbiology, Kocaeli University, Umuttepe Campus, Kocaeli, Turkey
2 Department of Medical Biology, Kocaeli University, Umuttepe Campus, Kocaeli, Turkey
3 Department of Medical Microbiology, Institute of Health Sciences, Kocaeli University, Umuttepe Campus, Kocaeli, Turkey

Corresponding Author: Gulden Sonmez Tamer, e-mail: guldensonmez@hotmail.com
Source of support: Departmental sources

Background: Giardiasis is caused by the intestinal protozoan parasite *Giardia duodenalis* (synonyms: *G. lamblia*, *G. intestinalis*), which is one of the most frequent parasites that infect Turkish children. However, molecular characterization of *G. duodenalis* in Turkey is relatively scarce. The present work aimed at genotyping *G. duodenalis* isolates from Turkey using molecular techniques.

Material/Methods: In the present study, 145 fecal samples from children were collected to search for the presence of *Giardia* by microscopy and PCR screening. PCR generated a 384 bp fragment for *β-giardin*. The PCR products were sequenced and the sequences were subjected to phylogenetic analysis by using PHYLIP.

Results: Based on the phylogenetic analysis of the sequences, assemblage A, B, and mixed subtypes were determined. Of 22 isolates, 11 were identified as assemblage A (50%), 7 were assemblage B (31.8%), and 4 were assemblage AB (18.2%). Association between *G. duodenalis* assemblages and the epidemiological data was analyzed. No correlation was found between symptoms and infection with specific assemblages (*P*>0.05), but we found statistically significant association between age and the assemblage AB (*P*=0.001).

Conclusions: The association between *G. duodenalis* and the epidemiologic data were analyzed. Since assemblage A is the more prevalent subgroup compared with assemblage B, this subgroup might be responsible for common Giardia infections in Turkey. This is the first study that included a detailed phylogenetic analysis of *Giardia* strains from Turkey.

MeSH Keywords: **Giardia • Molecular Epidemiology • Phylogeny • Polymerase Chain Reaction**

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/892318

📄 2038   📊 2   📈 2   📚 31

Background

Giardia duodenalis (syn. *G. intestinalis* and *G. lamblia*) is a flagellate protozoan that is the cause of giardiasis. The protozoan infects humans and animals worldwide, and has been considered as a neglected disease by the World Health Organization since 2004 [1]. Giardiasis is especially common in children in developing countries and about 200 million people have symptomatic infection [2,3]. The main route of infection is fecal-oral transmission via contaminated food and water. The infection has a broad spectrum, ranging from asymptomatic infections to chronic diarrhea [4]. *G. duodenalis* is an important intestinal protozoan in Turkey, with infection rates of children ranging from 17.3% to 33.3% [5].

Based on genotyping studies, *G. duodenalis* isolates are grouped into 8 assemblages, A-H [3]. Different symptoms are linked to different assemblages in different populations [6] Therefore, several molecular studies have divided this into various assemblages or genotypes, which not only demonstrate host specificity patterns but also differ in a range of other phenotypic aspects. As a result, several *loci* have been described for determining these assemblages: triose phosphate isomerase (*tpi*), *β-giardin*, small subunit ribosomal RNA (*SSU rRNA*), glutamate dehydrogenase (*gdh*), and elongation factor genes [4]. Assemblages A and B are zoonotic, infecting humans and animals and the prevalence varies in different geographic areas [7]. Assemblage B is seen more frequently in humans [8]. Assemblages C-H seem to be host-specific [3]. The protozoal or host mechanisms responsible for this assemblage distribution are important to define, given that they influence a variety of *Giardia* prevention measures, from transmission to vaccine development. Since assemblage A is the more prevalent subgroup compared with assemblage B, this subgroup might be responsible for common *Giardia* infections in Turkey. The present study aimed to establish an association between *Giardia duodenalis* and the epidemiologic data. We determined the subtypes of human *G. duodenalis* isolates by using PCR-based sequence analysis and BLAST search and also determined the phylogenetic relationships among these isolates.

Material and Methods

Sample collection

The samples were collected from June 2013 to March 2014 from children (aged 1–13 years) who were referred to the parasitology laboratory of Kocaeli University Hospital, Turkey. A questionnaire to obtain epidemiological and clinical data was completed by the parents or caregivers of the patients. These surveillance data included information about some epidemiological variables (sex, age, and place of residence) and clinical symptoms (fever, flatulence, nausea, vomiting, headache, anorexia, fatigue, and weight loss). Permission for the present work was granted by the local ethics committee.

Microscopy

Stool samples from 145 symptomatic and asymptomatic children were examined for intestinal parasites by a wet smear staining with Lugol's iodine, followed by formalin ethyl acetate concentration technique [9]. All stool samples stained with modified acid-fast stain for *Cryptosporidium spp.*, *Cyclospora*, and *Cystoisospora* [10], and were tested for common bacterial pathogens using standard culture methods [11]. Samples positive for *G. duodenalis* and lacking bacterial and other parasitic pathogens were used in this study.

DNA extraction and PCR amplification

A collection of 34 samples was used for DNA isolation. DNA was extracted from 200-mg stool samples using QIAmp DNA Stool Mini Kit (Qiagen, GmbH. Germany) following the manufacturer's instructions. Elution was accomplished by adding 30 µl elution buffer (Qiagen, GmbH. Germany). Both positive (DNA isolated from the Portland-1 strain (ATCC 30888D™ LGC Promochem) and negative controls (no template added) were included in each series of PCR reactions. A 384-bp fragment of the *β-giardin* gene was amplified using the forward primer G376 (5'-CATAACGACGCCATCGCGGCTCTCAGGAA-3') and the reverse primer G759 (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3') [12]. A ready-to-use PCR mixture, FastMix/Frenche i-Taq (iNtRON Biotechnology, Korea) was used to set up PCR reactions; the reaction was contained 1× reaction buffer, 250 µM of dNTP solution, 2 mM of $MgCl_2$, 0.5 µM of each primer, 2.5 units of DNA polymerase, and 1 µl of template in a final volume of 20 µl. Reactions were heated to 95°C in an automated thermal cycler (iCycler; BioRad, USA) for 5 min to initial denaturation. This was followed by 35 cycles of denaturation (94°C, 30 s); annealing (65°C, 30 s), extension (72°C, 1 min), and a final extension (72°C, 5 min). Samples were analyzed in 2% agarose gels stained with ethidium bromide to confirm the amplification of expected product size. PCR samples that gave a 384-bp band on agarose gel (Figure 1) were purified by using a PCR purification kit (Qiagen, GmbH. Germany) and sequenced from both strands (Iontek Inc., Turkey).

DNA sequencing and phylogenetic analyses

Bands were excised from agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, GmbH. Germany) according to the manufacturer's instructions. DNA sequencing was conducted in both directions using the PCR primers (Iontek Inc., Turkey). The sequences (23 of them) were contig assembled in vector NTI (Life Tech, USA), edited in BIOEDIT, and used in
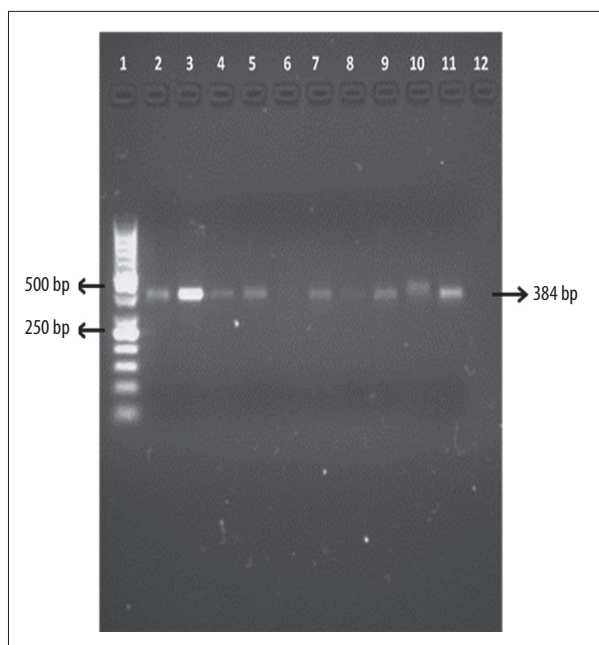
Figure 1. A representative 2% agarose gel to visualize PCR-amplified fragment of the *β-giardin* gene. Lane 1, 50 bp marker; lane 2, positive control; lane 12, mixture control; other lines, clinical samples.

BLAST search for identification of assemblages [13]. The results revealed that all sequences belonged to *β-giardin*.

The sequences were then aligned and examined. Examination of the alignment revealed the presence of partial sequences and the sequences that generated dubious quality. These sequences or sequence parts were systematically eliminated. The edited sequences were then aligned with Clustal W using default parameters and were subjected to phylogenetic analysis using the freely available PHYLIP package [14]. In brief, the sequence data were bootstrapped 1000 times by randomly choosing columns from the original alignment by using the SEQBOOT program. The input order of the sequences was randomized with a jumble number of 10. Then NJ (NEIGHBORJOINING) trees were built by using the generated bootstrapped data. The majority rule consensus trees were created by using CONSENSUS and the tree was drawn with DRAWTREE and edited in Adobe Illustrator 10.

Statistical analysis

All statistical analyses were performed using IBM SPSS for Windows version 20.0 (SPSS, Chicago, IL, USA). Kolmogorov-Smirnov tests were used to test the normality of data distribution. Continuous variables are expressed as mean ± standard deviation, and median and categorical variables are expressed as percentages. Comparisons of categorical and continuous variables between the groups were performed using the one-way ANOVA and Tukey post hoc test and Monte Carlo chi-square test. A 2-sided *P* value ≤0.05 was considered statistically significant.

Results

A total of 22 isolates (15%) were identified for the presence of *G. duodenalis* DNA by PCR. PCR analysis of *β-giardin* produced the expected targeted amplicons in 22 samples, which were successfully sequenced (Table 1).

Sequence comparison with *G. duodenalis* sequences available in the GenBank database revealed that 50% (11/22) were assemblage A, 31.8% (7/22) were assemblage B, and 18.2% (4/22) were assemblage AB. The epidemiological data and the association with infections by *G. duodenalis* assemblages are shown in Table 2. We found no significant association between assemblages of *Giardia* and the distinct types of enrollees of children or with sex and residence (urban or rural) (*P*>0.05). No correlation was found between symptoms (fever, flatulence, nausea, vomiting, headache, anorexia, fatigue, and weight loss) and infection with specific assemblages (*P*>0.05). However, we found a statistically significant association between age and the assemblage AB (*P*=0.001).

When the phylogenetic tree was mid-pointed, 2 main clusters were detected (Figure 2). While cluster I contained the assemblage A subtype, cluster II contained the assemblage B and the assemblage AB subtypes. The bootstrap value for cluster I was 74%, indicating that assemblage A sequences did not diverge much in time, forming a relatively tight cluster. In contrast, assemblage B was separated into 2 distinct branches. One of the branches was formed by 95% confidence rate, while the other branch displayed a low confidence rate, as indicated by the low bootstrap value. This group was a sister cluster to the assemblage AB group (11, 20) and the other assemblage B group was a sister cluster to the other assemblage AB group (18, 21). This indicates that group B is genotypically more similar to AB and might have emerged from the group AB assemblage subtype.

Discussion

The purpose of this study was to establish a link between clinical symptoms and genotyping of *G. duodenalis* from Turkish isolates. We also provided the first phylogenetic data on Turkish isolates. *G. duodenalis* infection rate is high in children [5]. In the present work, 145 fecal samples from children ages 1–13 years were analyzed. The observed infection rate obtained with microscopic examination was high – 13.8% (20/145) – and was even higher taking into account the results obtained

Table 1. Blast search results of the isolated *β-giardin* sequences.

Sample number	Assemblages	% coverage of the query	E-value	% Identity
9	B	%96	4e-175	%99
2	B	%96	1e-180	%99
3	B	%96	3e-177	%98
29	A	%100	1e-176	%99
22	A	%100	1e-169	%99
7	A	%100	1e-188	%99
33	A	%100	1e-180	%99
38	A	%100	1e-103	%99
45	A	%99	1e-103	%99
42	A	%100	1e-175	%99
10	A	%100	1e-175	%99
13	A	%99	1e-180	%99
16	A	%99	1e-175	%99
14	A	%99	1e-176	%99
18	AB	%99	1e-150	%99
21	AB	%100	1e-152	%99
19	B	%97	1e-162	%98
11	AB	%55	8e-103	%100
20	AB	%55	8e-103	%100
36	B	%97	1e-180	%98
5	B	%100	1e-175	%99
40	B	%97	1e-165	%99

Table 2. Association between *G. duodenalis* assemblages and the epidemiological data analyzed in studied children.

Characteristic	Assemblage A (n=11)		Assemblage B (n=7)		Mixed assemblages A+B (n=4)		P
	n	%	n	%	n	%	
Symptomatic group	8	72.7	2	28.6	3	75	0.163*
Asemyptomatic group	3	27.3	5	71.4	1	25	
Sex							
Male	7	63.6	5	71.4	2	50	0.854*
Female	4	36.4	2	28.6	2	50	
Residing area							
Urban	3	27.3	3	42.9	2	50	0.602*
Rural	8	72.7	4	57.1	2	50	
Age							
Mean value (±SD)	3.82 (±1.72)		8.14 (±2.73)		6.5 (±1.91)		
Median	3		7		7		0.002**
Interval	2–7		5–13		4–8		

* Monte Carlo Chi-Square test; ** One-way ANOVA.
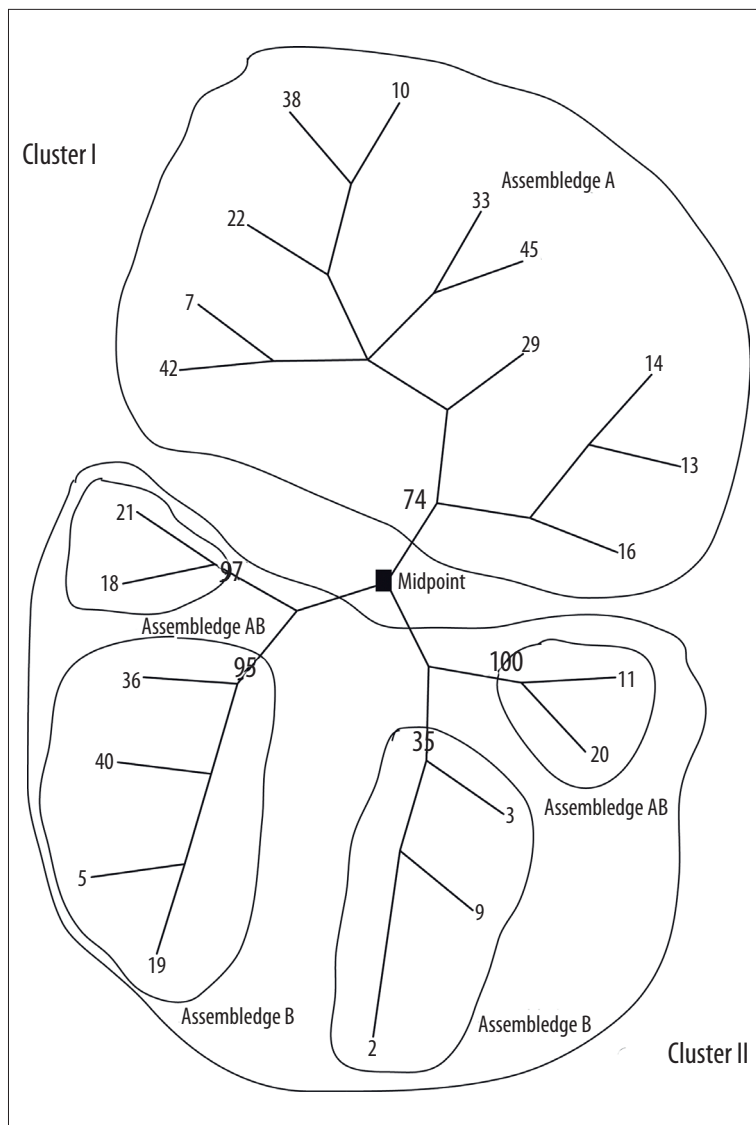
Figure 2. Phylogenetic analysis of *G. duodenalis* assemblages.

through the detection of *Giardia*-specific amplicons using molecular methods (PCR). DNA from all *Giardia*-positive samples identified by microscopy was efficiently extracted and detected by PCR. Additionally, DNA from another 2 samples, previously identified as negative for microscopy, were amplified by PCR. Assemblages A, B, and AB were found in this study, as indicated by sequence homology analysis and phylogenetic inference results.

Our results show a clear predominance of assemblage A, corresponding to 50% of analyzed DNA sequences. A recent study reported that children infected with assemblage A are less associated with greater cyst shedding than children infected with assemblage B, which may promote its spread. The present study was able to discriminate among assemblages A, B, and AB, showing again that all 3 groups exist in Kocaeli and confirming the presence of natural *G. intestinalis* variations in

Turkish hosts, as stated previously by Aydin et al. (2004) and Degerli et al. (2012) [15,16]. Our findings agree with the findings of Degerli et al. (2012), but contradict with the findings of Aydın et al. (2004) [15,16].

Distribution of assemblages among human-associated *Giardia* isolates show variability in different parts of the world. For example, in the Americas, there are pockets of areas with differing predominant assemblages. The frequencies of assemblage A are higher in Mexico, Brazil, and Colombia, while in Nicaragua and Argentina assemblage B is predominant [17,18]. As in our study, the occurrence of mixed infections has been reported in surveys performed in Australia, the United Kingdom, India, Italy, and Peru. Interestingly, the percentages of mixed infections range from 2.0% to 21.0% [19–22]. Because there are genetic and phenotypic differences among the assemblages, there should be a correlation between clinical and epidemiological

differences. However, we did not find significant differences in the epidemiological aspects that we evaluated. It is important to note that in some studies, correlations between the assemblages and symptoms were reported [15,23]. For instance, there seems to be a significant association of assemblage A with diarrhea [24, 25] and in assemblage B several studies reported a correlation with symptoms [26,27]. The discrepancies in the clinical manifestations of *Giardia* assemblages in different reports could be due to the variation in the virulence of the different genotypes, host factors, or the combination of both [28]. In our study, clinical features were available for 22 successfully typed cases. All assemblages caused similar illness, but there was no correlation among the symptoms and the assemblages. Our results agree with those of Breathnach et al. (2010) who described cases of giardiasis in Southwest London where both assemblages, A and B, caused similar illness [29]. However, higher rates of cyst shedding in children with assemblage B in comparison with assemblage A have been reported from Brazil [30]. Similar studies were performed in the Arabian Peninsula. A study carried out in Saudi Arabia showed that assemblage A was more prevalent than B (57% *vs.* 37.5%, respectively) [23]. In Egypt, a community-based study reported that the proportion of *Giardia* attributable to assemblage B was 80% compared to 5% for assemblage A [31]. However, these studies also reported no conclusive correlations among the assemblages and the clinical symptoms.

Conclusions

There is a need for the evaluation of genetic variability within assemblages because it will help to clarify *G. duodenalis* epidemiology, including the role of animals in human infection and the sources of infection [8]. This work was a pilot study to demonstrate *G. duodenalis* genotyping in Kocaeli, Turkey. Our results show that *Giardia* assemblages A and B are prevalent in children in Kocaeli, with a predominance of assemblage A. There is need for a study focused on the source of these infections in Turkish children. Our findings are important in understanding of distribution of assemblages and their phylogenetic relationships in the Kocaeli region of Turkey. The findings demonstrated that determining the distribution of assemblages is important in understanding the lineages of these subtypes and should be performed with more subjects to be conclusive.

References:

1. Savioli L, Smith H, Thompson A: *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol, 2006; 22: 203–8

2. Lima AA, Moore SR, Barboza MS et al: Persistent diarrhea signals a critical period of increased diarrhea burdens and nutritional shortfalls: a prospective cohort study among children in northeastern Brazil. J Infect Dis, 2000; 181: 1643–51

3. Feng Y, Xiao L: Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin Microbiol Rev, 2011; 24: 110–40

4. Tsourdi E, Heidrich FM, Winzer M et al: An exotic cause of exudative enteropathy. Am J Case Rep, 2014; 15: 226–29

5. Tamer SG, Erdogan S, Willke A: The frequency of the presence of intestinal parasites in students of Arslanbey Primary School. Turkish Society for Parasitology, 2008; 32: 130–33

6. Robertson LJ, Hanevik K, Escobedo AA et al: Giardiasis –why do the symptoms sometimes never stop? Trends in Parasitology, 2010; 26: 75–82

7. Monis PT, Thompson RC: *Cryptosporidium* and *Giardia*-zoonoses: fact or fiction? Infect Genet Evol, 2003; 3: 233-44

8. Caccio SM, Beck R, Lalle M et al: Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int J Parasitol, 2008; 38: 1523–31

9. Ritchie LS: An ether sedimentation technique for routine stool examination. Bull US Army Med Dep, 1948; 8: 326–32

10. Garcia LS: Laboratory method for diagnosis of parasitic infections. In: Barron EJ and Finegold SM (eds.) Bailey and Scott's diagnostic microbiology. St Louis: Mosby, 1990; 776–61

11. Murray P, Baron E: Manual of clinical microbiology, 9th edn. ASM, 20007, Washington

12. Caccio SM, De Giacomo M, Pozio E: Sequence analysis of the *β-giardin* gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol, 2002; 32: 1023–30

13. Altschul SF, Madden TL, Schäffer AA et al: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res, 1997; 25: 3389–402

14. Felsenstein J: PHYLIP. Phylogenetic inference package, Version 3.5c. Seattle: Department of genetics, 1993 University of Washington

15. Aydin AF, Besirbellioglu BA, Avci IY et al: Classification of *Giardia duodenalis* parasites in Turkey into Groups A and B using restriction fragment length polymorphism. Diagn Microbiol Infect Dis 2004; 50: 147–51

16. Degerli S, Degerli N, Celiksoz A et al: Genotyping of *Giardia intestinalis* isolated from people living in Sivas, Turkey. Turk J Med Sci, 2012; 42: 1268–72

17. Eligio-Garcia L, Cortes-Campos A, Cota-Guajardo S et al: Frequency of *Giardia intestinalis* assemblages isolated from dogs and humans in a community from Culiacan, Sinaloa, Mexico using *β-giardin* restriction gene. Vet Parasitol, 2008; 156: 205–9

18. Lebbad M, Ankarklev J, Tellez A et al: Dominance of *Giardia* assemblage B in Leon, Nicaragua. Acta Trop, 2008; 106: 44–53

19. Hopkins RM, Meloni BP, Groth DM et al: Ribosomal RNA sequencing reveals differences between the assemblages of *Giardia* isolates recovered from humans and dogs living in the same locality. J Parasitol, 1997; 83: 44–51

20. Amar CFL, Dear PH, Pedraza-Diaz S et al: Sensitive PCR restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. J Clin Microbial, 2002; 40: 446–52

21. Traub RJ, Monis P, Robertson I et al: Epidemiological and molecular evidence support the zoonotic transmission of *Giardia* among humans and dogs living in the same community. Parasitology, 2004; 128L 253–62

22. Lalle M, Pozio E, Capelli G et al: Genetic heterogeneity at the *β-giardin locus* among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic sub-assemblages. Int J Parasitol, 2005; 35: 207–13

23. Al-Mohammed HI: Genotypes of *Giardia intestinalis* clinical isolates of gastrointestinal symptomatic and asymptomatic Saudi children. Parasitol Res, 2011; 108: 1375–81

24. Haque R, Roy S, Kabir M et al: *Giardia* assemblage A infection and diarrhea in Bangladesh. J Infect Dis, 2005; 192: 2171–73

25. Sahagun J, Clavel A, Goni P et al: Correlation between the presence of symptoms and the *Giardia duodenalis* genotype. Eur J Clin Microbiol Infect Dis, 2008; 27: 81–83

26. Homan W, Mank T: Human giardiasis: genotype linked differences in clinical symptomatology. Int J Parasitol, 2001; 31: 822–26

27. Mahdy MAK, Surin J, Wan KL et al: *Giardia intestinalis* genotypes: risk factors and correlation with clinical symptoms. Acta Trop, 2009; 112: 67–70

28. Thompson R, Hopkins R, Homan W: Nomenclature and genetic groupings of *Giardia infecting* mammals. Parasitol Today, 2000; 16: 210–13

29. Breathnach AS, McHugh TD, Butcher PD: Prevalence and clinical correlations of genetic subtypes of *Giardia lamblia* in an urban setting. Epidemiol Infect, 2010; 138: 1459–67

30. Kohli A, Bushen OY, Pinkerton RC et al: Giardia duodenalis assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg, 2008; 102: 718–25

31. Foronda P, Bargues MD, Abreu-Costa N et al: Identification of genotypes *Giardia intestinalis* of human isolates in Egypt. Parasitol Res, 2008; 103: 1177–81