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

**Background:** Giardiasis is caused by the intestinal protozoan parasite *Giardia duodenalis* (synonyms: *G. lamblia*, *G. intesti-nalis*), 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

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**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 30888™ 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’-CATACCGACCCATGGGCTCTCGAGAA-3’) and the reverse primer G759 (5’-GAGGGCCCTTGCATCCTGAGACG-3’) [12]. A ready-to-use PCR mixture, FastMix/Frenchi 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 MgCl₂, 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 (lontek 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 (lontek Inc., Turkey). The sequences (23 of them) were contig assembled in vector NTI (Life Tech, USA), edited in BIOEDIT, and used in
Continuous variables between the groups were performed using expressed as percentages. Comparisons of categorical and continuous variables are expressed as mean ± standard deviation, and median and categorical variables are 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 by using different methods of analysis.
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-163  | %99        |
| 7             | A           | %100                    | 1e-188  | %99        |
| 33            | A           | %100                    | 1e-180  | %99        |
| 38            | A           | %100                    | 1e-163  | %99        |
| 45            | A           | %99                     | 1e-103  | %99        |
| 42            | A           | %100                    | 1e-175  | %99        |
| 10            | A           | %100                    | 1e-176  | %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-176  | %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*       |       |
| Asemptomatic 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.
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 Aydin 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

Figure 2. Phylogenetic analysis of *G. duodenalis* assemblages.
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.

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