

1-1-2012

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SERPİL DEĞERLİ

NACİ DEĞERLİ

ALİ ÇELİKSÖZ

SEMRA ÖZÇELİK

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DEĞERLİ, SERPİL; DEĞERLİ, NACİ; ÇELİKSÖZ, ALİ; and ÖZÇELİK, SEMRA (2012) "Genotyping of *Giardia intestinalis* isolated from people living in Sivas, Turkey," *Turkish Journal of Medical Sciences*: Vol. 42: No. 7, Article 18. <https://doi.org/10.3906/sag-1104-32>

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Genotyping of *Giardia intestinalis* isolated from people living in Sivas, Turkey

Serpil DEĞERLİ¹, Naci DEĞERLİ², Ali ÇELİKSÖZ¹, Semra ÖZÇELİK¹

Aim: The technique of polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) genotyping was used to characterise morphologically identical isolates of *Giardia intestinalis* from human stool samples.

Materials and methods: In this study a total of 17 trophozoite samples, obtained either directly from stool samples or after excystation, or by duodenal aspiration, were used. A set of primers was chosen to amplify the different regions of *triose phosphate isomerase (tpi)* and a segment of the *glutamate dehydrogenase (gdh)* genes. A single-stranded conformational polymorphism technique was also used in an attempt to discriminate among some subgroups.

Results: Only primers of the 683-bp segment of the *tpi* gene from the trophozoite samples were suitable for obtaining a PCR product. In the total of 17 trophozoite DNAs where the *tpi* gene segment was amplified, 9 belonged to assemblage A (53%) and 4 to assemblage B (23.5%). It was not possible to identify assemblages for the remaining 4 samples (23.5%).

Conclusion: PCR–RFLP *tpi* gene application was able to discriminate between *G. intestinalis* assemblage A and B, but not the other subgroups. Since assemblage A is the more prevalent subgroup compared with assemblage B, this subgroup can be said to be responsible for common *Giardia* infections in Turkey.

Key words: *Giardia intestinalis*, *gdh*, *tpi*, RFLP, genotyping, subgroups

Introduction

Giardia intestinalis is an intestinal protozoan parasite found in a wide range of mammals (1–3). Giardiasis is considered as one of the most common waterborne diseases and, along with *Cryptosporidium*, is a major health concern in water installations, both in developing and developed countries (4). *Giardia* are flagellated parasites but are accepted as amitochondriate organisms, belonging to the class of Zoomastigophorea (4,5).

Molecular techniques provide powerful analytical tools for the epidemiology of human and animal giardiasis. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis showed that *G. intestinalis* is a complex organism, made up of morphologically indistinguishable isolates

that are genetically and phenotypically distinct (4,6,7). Using the appropriate genotyping tools in endemic foci, predictive assessments could be made about transmission patterns (8). For this purpose different gene loci were used to predict transmission events in different hosts groups. Gene segments such as 18S rDNA, *glutamate dehydrogenase (gdh)*, *triose phosphate isomerase (tpi)*, *topoisomerase*, *elongation factor alpha*, and *trophozoite surface antigen (tsa)* are commonly used to discriminate between different assemblages (7,9). The majority of *G. intestinalis* isolates from humans and some domestic animals species can be grouped into 1 of 2 distinct genetic assemblages: A and B (8–11). The assemblages A and B are accepted as potentially zoonotic and were recovered from humans and a wide variety of animals,

Received: 13.04.2011 – Accepted: 04.04.2012

¹ Department of Parasitology, Faculty of Medicine, Cumhuriyet University, 58140 Sivas - TURKEY

² Department of Molecular Biology and Genetics, Faculty of Sciences, Cumhuriyet University, 58140 Sivas - TURKEY

Correspondence: Serpil DEĞERLİ, Department of Parasitology, Faculty of Medicine, Cumhuriyet University, 58140 Sivas - TURKEY

E-mail: sdegerli@cumhuriyet.edu.tr

while C, D, E, F, and G appear to be restricted to specific hosts (7,12).

Molecular techniques using the sequence analysis of the *gdh* locus were able to differentiate among subgroups such as AI, AII, BIII, and BIV (7). The PCR-RFLP technique was successfully used to determine and identify the presence of mixed groups (7,13,14).

Giardia specimens obtained from stool or duodenal aspirate samples have been studied worldwide. There are 2 major groups of *G. intestinalis* recognised as infecting humans worldwide, including in Turkey. Karanis and Ey (15) found that the genotype of *Giardia* isolates provided from Turkey belonged to assemblage B. In a further genotyping study conducted by Aydin et al., both assemblages (A and B) were commonly seen in Turkey (16). The aim of this study was to attempt molecular differentiation and determine the general distribution of *G. intestinalis* isolates from humans in a different province, Sivas, using the PCR-RFLP technique. The genetic diversity of the parasite's *tpi* gene was characterised on the basis of nucleotide sequence due to the high genetic heterogeneity displayed by *Giardia* at this locus (2,4,9,13).

Materials and methods

Sample collection

Giardia DNA was obtained from samples containing cysts or trophozoites collected during the period from May 2003 until September 2005. Most of the human stool samples were collected during school surveys, while some samples were obtained from patients who visited hospitals in different districts of Sivas. Stool samples were subjected to *Giardia* isolation to obtain cysts or trophozoites and were stored at -20°C until DNA isolation. In total 17 trophozoite samples were obtained: 10 from stool samples, 3 after excystation, and 4 after duodenal aspiration. DNA for PCR amplification was obtained from samples sent to the Parasitology Laboratory of Cumhuriyet University for routine stool examination as well as from samples taken in the Internal Medicine Services during endoscopic aspirations.

Giardia cyst purification

The specimens were examined under a light microscope (100–400 \times magnification) for the presence of cysts and trophozoites. *Giardia* cysts were

isolated using the technique of Buchel et al. (17). The ultimate precipitant consisted of *Giardia* cysts, which were dissolved in 200 μL of 100 mM sodium tris hydroxyethylenediaminetetraacetic acid (STE) buffer (pH 8.00) and stored at -20°C until use.

Obtaining trophozoites through excystation

The method for inducing excystation of trophozoites in vitro was used according to Bingham and Meyer (18). Isolated cysts (0.5 mL) were treated with 5 mL of pepsin–acid solution at 37°C for 1 h, then transferred to a trypsin Tyrode solution (0.5%) (pH 8.00) filtered with filter paper and centrifuged at $600 \times g$ for 15 min. Excystated trophozoites were transferred to a trypticase yeast extract iron serum (TYI-S-33) medium for culture.

DNA purification

For DNA purification from cysts and trophozoites, first genomic DNA was extracted using a conventional DNA isolation method involving SDS and proteinase K digestion (20 mg/mL), and then phenol–chloroform–isoamyl alcohol (PCI) extraction and precipitation with decreasing ethanol gradients was carried out, with some modification (19). Finally, a small amount (approximately 10 mg) of lysozyme powder was added to the already digested cyst suspension, which was then incubated for 2 h and precipitated by PCI/ethanol treatment. After every step, extracted DNA was resuspended in 20 μL of 1X TE buffer (pH 8.0) and checked in the 0.8% agarose gel, and then stored at -20°C until further analysis.

PCR amplification

To amplify *G. intestinalis* cyst and trophozoite DNA, 2 different primer sets, i.e. 1 for the *tpi* gene and 1 for the *gdh* gene, were used (17,18). Initially, the *tpi* gene segment was amplified according to the method of Amar et al. (13). Due to the lack of a reference standard DNA (we were unable to obtain any *Giardia* isolates from the American Type Culture Collection), it was not possible to decide whether or not the DNA in the gel media originated from *Giardia*.

In a further attempt to obtain *Giardia* cyst DNA, a segment of the *gdh* gene was amplified as described by Monis et al. (14). Finally, an attempt was made to amplify cyst genomic DNA from *G. intestinalis* using a shorter segment of the *tpi* gene. The primers were used as previously described by

Aydin et al. (16) with a slight modification (forward 5'-TGGACTGGCGAGACAAG-3' and reverse 5'-TCCGGCTTGAGGGAAGC-3'). In order to improve PCR amplification, 5% acetonitrile and 1X ammonium sulphate (AS) Taq polymerase buffer was used, but no PCR amplification was obtained. However, trophozoite DNA was successfully amplified with this primer set.

Since limited amounts of trophozoite template DNA were present, subsequent experiments were conducted with the latter oligonucleotide primer set in order to amplify the 683-bp segment of the *tpi* gene (16,20,21). Taq buffer with AS was used and included in 5% acetonitrile. A single-stranded conformational polymorphism (SSCP) technique was also used in an attempt to discriminate between other possible subgroups.

Polyvinylpyrrolidone and cetyltrimethylammonium bromide treatment

Due to failure of the PCR amplification, isolated *Giardia* DNA samples were subjected to polyvinylpyrrolidone (PVP) (2,20) or cetyltrimethylammonium bromide (CTAB) (22).

PCR product and RFLP with *Xho I*

PCR products and restriction fragments were separated by horizontal electrophoresis in 0.8% and 2.5% agarose gels, respectively, with ethidium bromide staining, and were recorded by UV box. RFLP analysis was performed by digesting 5 µL of the *tpi* PCR product with 20 U of *Xho I* in 1X enzyme buffer in a final volume of 20 µL for 1 h at 37 °C. To assess the size of the PCR and RFLP products, 50- and 100-bp DNA ladders were used.

Results

In order to determine the distribution of *G. intestinalis* subgroups in Sivas and reveal the molecular substructure of the *Giardia*, a bp fragment of the *tpi* gene was amplified from 17 different trophozoite DNA samples isolated from humans. Before the PCR amplification, a large number of stool samples were surveyed for *Giardia* cysts and over 150 cyst-positive samples were obtained from humans. All attempts to amplify *Giardia* cyst DNA were unsuccessful.

At the end of digestion, 442 and 241 bp were obtained for group B. A putative 40-bp fragment was also obtained but fragments of less than 50 bp were not included. Overall, 9 samples were identified as *Giardia* assemblage A (53%) and 4 as assemblage B (23.5%), and 4 samples remained unidentified (23.5%). Due to the existence of unclear band patterns in the RFLP gel, the SSCP technique was additionally used. However, because of the numerous artefacts existing in the background, these samples remained unidentified. Since no reference isolates were used for the discrimination, the putative *tpi* gene segment was recognised based on molecular mass marker (using Lambda DNA *EcoRI*+*HindIII* and a 50-bp GeneRuler molecular marker) and PCR-RFLP digested fragment size.

Discussion

Molecular tools have been used recently to characterise the epidemiology of human giardiasis. Although isolates of *G. duodenalis* from humans and various animals are morphologically similar, distinct host-adapted genotypes have been demonstrated within *G. duodenalis* (23,24). There are 2 major groups of *G. duodenalis* recognised as infecting humans worldwide, but there are some differences in the naming of these groups, as evidenced by the following categorisations: Polish and Belgian genotypes (10) and assemblages A and B (11).

Currently there are limited PCR-based methods that allow amplification and characterisation of *Giardia* cyst DNA directly from faeces (7,21,23). The number of cysts observed by microscopy may not be relative to the quantity of intact template DNA, because the substance of cysts may be diminished prior to extraction of DNA from stool samples (13).

In our study, the conventional proteinase K/PCI extraction method was used for the DNA isolation from trophozoites, but this technique was not suitable for digestion of cysts. All attempts to obtain a template for DNA amplification failed and none of the primers isolated from *Giardia* cysts were suitable for amplifying the DNA in the stool samples. For this reason, in order to remove potential inhibitors of the PCR, some isolated DNA samples were treated with PVP to remove potential inhibitors. The purified

cyst DNA was also treated with CTAB, in order to eliminate possible remnants; however, no clear PCR product was obtained.

The *tpi* gene was specially chosen for this study because of the high genetic heterogeneity displayed by *Giardia* spp. at this locus, as depicted by others (4). The predicted restriction enzyme profiles revealed that one enzyme, *Xho I*, was capable of discriminating between assemblages A and B (7,14,21). This *tpi*-based genotyping tool is also useful in epidemiologic investigations of giardiasis in humans (11,24). The distribution or frequency of assemblage A subgroups is predominantly higher than subgroup B and also higher than the remaining unidentified possible subgroups.

A recent study in the United Kingdom of sporadic 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%) were infected with assemblage A, and 3 (9%) samples were mixed infections of assemblages A and B (13). Similar results were obtained with samples from a nursery outbreak, in which 21 (88%) of 24 samples were shown to be *G. duodenalis* assemblage B parasites; the rest were assemblage A parasites (25). The intragenotypic variations of *tpi* in assemblage B identified in the depicted study should be useful in subtyping outbreak isolates (26). According to the results of Sulaiman et al. (24), the *tpi* gene is a good phylogenetic marker for analysis of the molecular evolutionary and taxonomic relationship of *G. duodenalis* parasites. The genetic relationship shown by phylogenetic analysis of the *tpi* gene is largely in agreement with that obtained at other genetic loci. Results of the molecular analyses support the conclusion that *G. duodenalis* is a species complex, a finding that should be useful in the revision of *Giardia* taxonomy and standardisation of *Giardia* nomenclatures.

There were 2 genotype groups recognised, which were in accordance with the assemblages A and B described by Aydin et al. (16) and Sousa et al. (21). The present study was able to discriminate between isolates from assemblages A and B, showing again that groups A and B exist in Sivas and confirming the presence of natural *G. intestinalis* variations in human hosts as stated previously by Aydin et al. (16), who also showed that in Turkey the group A subset of *G.*

intestinalis is more prevalent than group B. However, most of the samples enrolled in the study of Aydin et al. (16) were obtained from 1 locality, i.e. from 1 hospital in 1 province, and specifically from patients admitted to the hospital due to gastrointestinal and other clinical complaints. In the mentioned study, predominance of the group A subset was associated with cases of patients with gastrointestinal symptoms. This is in contradiction with the findings of Singh et al. (27), who reported a high endemicity of group B subset present in patients with gastrointestinal symptoms. In the present study no B subgroups were observed in any of the 4 endoscopic aspirates, while the subgroup A (3 of 4) appears to be related with observed diarrhoeal symptoms, which is in contrast with the findings of Aydin et al. (16).

In order to determine the general distribution of these common subgroups throughout the country, most of the trophozoite samples used in our study were randomly collected from localities in Sivas regardless of any clinical symptoms. In addition, most of the DNA used in the present study was obtained directly from trophozoite samples and a few samples were obtained after excystation, i.e. not all samples were obtained by excystation of cysts or passage of the isolates through an animal model such as gerbils or suckling mice. Since the determined subgroups were obtained after very extensive stool screening, it can be said that assemblage A is the most prevalent subgroup and responsible for common *Giardia* infections in Sivas.

DNA obtained from trophozoites rather than cysts can be amplified successfully and used in the molecular diagnosis of *Giardia*, as demonstrated in the present study. The reason for the failure of observed cyst DNA amplification for the relatively longer or shorter DNA segment is unclear. One possibility could be the need to use harsh methods for the release of DNA from cysts. It could be not determined whether the DNA bands observed in the 0.8% agarose gel were of *Giardia* origin or contamination.

Acknowledgement

We would like to thank Dr Kosta Y. Mumcuoğlu, Dr Jacqueline Miller, and Dr Avi Keysary for their critical review of the manuscript. This work was supported by the Cumhuriyet University Research Fund under Contract No. T - 210.

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