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RESEARCH ARTICLE

Genotyping of *Giardia lamblia* in a Cohort of Turkish Patients: A Search for a Relationship between Symptoms and Genotypes

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Summary

Recent surveys investigating the molecular biology of *Giardia lamblia* revealed two distinct assemblages with different clinical outcomes. However, there is not a universal compromise about the clinical effects of each assemblage, warranting further studies. Here, we report the results of the first analyses of the assemblages of *G. lamblia* in Manisa province located in western Turkey, together with their relationships with the symptoms and DNA sequence analyses of the PCR products. DNA samples were isolated from the stools of 63 patients infected with *G. lamblia* and 54 DNA samples, amplified successfully with PCR, were digested with the enzyme *Xho* I for RFLP. Thirty-eight of 54 samples (70.4%) were found to be in Assemblage A, while the remaining 16 samples (29.6%) were found to be in Assemblage B. The number of female patients was found significantly higher in Assemblage B (P=0.18). There was a statistically significant relationship between the occurrence of both abdominal pain and diarrhea and Assemblage B (*chi-square*, 10.52; P<0.05). No other statistically significant relationship was detected between the assemblages and neither with the symptoms nor with the age groups of the patients. The comparison of the DNA sequences of the PCR products from two assemblage B (one subtype B1 and one B) and one assemblage A samples both with each other and with other DNA sequences in the NCBI website by multialignment analyses, revealed specific regions for assemblages B (B1-B) and A on *tpi* gene region. Further studies with more patients are required to assess these initial results. Now, our aim is to design a probe for *tpi* gene region to set up a real-time PCR assay that is easier to conduct and requiring shorter time for the analyses.

Keywords: *Giardia lamblia, Diagnosis, PCR, Genotyping, Symptom*

Bir Türk Hasta Kohortunda *Giardia lamblia* Genotiplendirilmesi: Semptomlar ile Genotipler Arası İlişkinin Araştırılması

Özet

Giardia lamblia ile yapılan moleküler düzeydeki çalışmalar iki farklı genotipin farklı klinik belirtilere sahip olduğunu ortaya çıkarmıştır. Bununla birlikte, hangi genotipin ne tür klinik belirtilere neden olduğuna dair henüz kesin bulgular elde edilmiş değildir. Bu araştırmada Türkiye'nin Manisa yöresinden elde edilen *G. lamblia* izolatları polimeraz zincir reaksiyonu (PZR) ve sonrasında DNA dizi analizi yöntemleriyle araştırılmış ve saptanan genotipler ile hastalardaki klinik semptomlar arasındaki ilişkiler incelenmiştir. Toplam 63 hastadan DNA örnekleri izole edilmiş ve bunların 54'üne *Xho* I restriksiyon enzimi kullanılarak RFLP analizi uygulanmıştır. Sonuçlar incelendiğinde, 54 örneğin 38'inin (%70.37) A, 16'sının ise (%29.63) B genotipine ait olduğu belirlenmiştir. Kadın hasta sayısının B genotipinde anlamlı düzeyde daha fazla olduğu (p=0.18), ayrıca hastalarda birlikte bulunan karın ağrısı ve ishal yakınması ile *G. lamblia* B genotipi arasında istatistiksel olarak anlamlı bir ilişki olduğu tespit edilmiştir (*ki-kare testi*, 10.52; P<0.05). Hastaların yaşı ya da yakınmaları ile *G. lamblia* genotipleri arasında başka anlamlı bir ilişkiye rastlanmamıştır. A genotipine ait bir ve B genotipine ait iki ayrı (B1 alt tipine ait bir ve B genotipine ait bir olmak üzere) örneğin PZR ürünlerinin DNA dizileri, çoklu dizi analizi yöntemiyle birbirleriyle ve NCBI veb sitesindeki ilgili DNA dizileri ile karşılaştırılmıştır. Sonuç olarak *G. lamblia*'ya ait *tpi* gen bölgesi üzerinde A ve B (B1 ve B) genotiplerine özgü bölgeler tespit edilmiştir. Daha fazla hasta örneği kullanılarak yapılacak ileri çalışmalarla elde edilen sonuçların değerlendirilmesi gerekmektedir. Sonraki çalışmamızda, *tpi* gen bölgesine özgü bir prob tasarlamak suretiyle daha hızlı ve güvenilir analizler yapılmasında kullanacağımız bir gerçek zamanlı PZR (GZ-PZR) testi geliştirmeyi amaçlamaktayız.

Anahtar sözcükler: *Giardia lamblia, Tanı, PZR, Semptom*

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INTRODUCTION

Giardia is a genus of intestinal flagellate protozoa, including six species that infect several vertebrates [1]. *Giardia lamblia (syn. G. intestinalis, G. duodenalis)* is currently the only known species found in humans, while it is also found in other mammals like pets and livestock. *G. lamblia* is a common cause of endemic and epidemic diarrhoea worldwide. Infection with *G. lamblia* includes asymptomatic cyst passage, acute self-limited diarrhoea, and a chronic syndrome including diarrhoea, malabsorption and weight loss. Most cases are asymptomatic, but symptomatic infections involving recurrent intestinal symptoms may interrupt the normal growth and development in affected children [2,3]. In addition to its wide spectrum of clinical symptomatology, it also has varied routes of transmission, including anthroponotic, zoonotic, foodborne and waterborne [1-4]. The conventional diagnostic method for *G. lamblia* in stool specimens is microscopy, including the examination with wet mount, formalin ethyl acetate concentration and trichrome staining [3].

The morphological differences among the isolates of *G. lamblia* are quite little; however, latest molecular biological studies have demonstrated seven distinct assemblages (A-G) belonging to *G. lamblia*, which is not possible to discriminate with routine microscopic methods [3,5]. Among these seven assemblages, only A and B have yet been associated with human infections [1,4-8], whereas C and D have been identified in dogs, cats, coyotes and wolves, E in cattle, sheep, goats and pigs, and F and G in cats and rats, respectively [1]. Molecular studies have revealed longer genetic distance between these assemblages compared to that used to delineate other protozoal species [9].

There are no extensive studies in the literature that aim to reveal the ratio of the *Giardia* genotypes causing symptomatic infections in humans [1]. Therefore, it is not known why some patients go asymptomatic while some suffer from clinical symptoms. Host factors as well as differences in the genotypes of *Giardia* isolates are probably involved in the pathogenesis of the infection [4,10]. There are conflicting reports about the correlation between the genotypes and clinical manifestations of giardiasis; some of them reported an association between mild or intermittent diarrhoea and assemblage A and between severe or persistent diarrhoea and assemblage B [11,12], while others reported that assemblage B was more prevalent in asymptomatic children [13-15]. In a recent study with Brazilian children, it was reported that children with assemblage B shed more cysts than children with assemblage A [16].

The aim of the present study was to reveal, for the first time, the genotypic differences of *Giardia* isolates in patients from Manisa province located in western Anatolia with a population of 1.4 million, and assess the relationships between the characteristics of patients and the genotypes.

MATERIAL and METHODS

Samples

A total of 63 DNA samples of *G. lamblia*-infected patients diagnosed during the routine microscopic examination with wet mount, formalin ethyl acetate concentration and trichrome staining in the laboratory of Celal Bayar University School of Medicine Department of Parasitology, were included in the analysis. Personal characteristics were noted for each individual during registration. Stool samples were kept at -20°C until DNA isolation.

DNA Isolation

The isolation of DNA was done with "Genomic DNA Purification Kit" (Fermentas®#K0512), according to the instructions of the producer. DNA samples were then kept at -20°C until the day of PCR application.

PCR

PCR was done according to the protocol [13,17] with minor modifications. The primer set of "forward; 5-TGGA

TGGACCGGCGAGACAAGCGTCGAGATGCTGCTGGACATGGGGCTGAGCCATGTAATAATAGGACA
CTCTGAAAGACGTAGAATCATGGGCGAGACCAATGAGCAGAGTGCTAAGAAGGCGAAGCGTGCTC
TGGACAAAGGTATGACTGTTATCTTCTGCACCGGAGAGACCCTGGATGAACGCAAGGCCAATAACA
CTATGGAGGTGAATATTGCTCAGC^TCGAGGCTCTTAAGAAGGAGATTGGAGAATCAAAGAAGTTA
TGGGAGAACGTTGTAATGCCTATGAGCCGGTGTGGTCTATCGGCACGGGTGTGGTGGCCACACCG
GAGCAGGCAGAGGAAGTCCATGTGGGACTCCGCAAATGGTTTGCGGAAAAGGTTTGCGCAGAAGG
TGCGCAGCACATCCGCATCATCTATGGAGGGTCTGCCAATGGGAGTAACTGCGAGAAGCTTGGCCA
GTGCCCGAATATCGACGGATTCCTCGTCGGAGGTGCTTCCCTCAAGCCGGA

Fig 1. GenBank: L02116.1 *Giardia lamblia* (GS/M) triosephosphate isomerase gene, complete cds (220/290=510 bp). The primers were indicated with green, and the restriction site with red

Şekil 1. *Giardia lamblia* triozfosfataz isomeraz geni. Genbankası L02116.1 Primerler yeşille, restriksiyon bölgesi ise yeşille gösterilmiştir

CTGGCGAGACAAG-3 and reverse 5-TCCGGCTTGAGGG AAGC-3" encoding tpi gene was used *(Fig. 1)*. The reaction was performed in a 50 µl volume with 3 µl DNA in 10 X PCR buffer, 2 mM $MgCl_2$, 100 nM of each dNTP, 1 µl of each primer and 2.5 U *Taq* DNA polymerase (all reagents from Fermentas®). Samples were then subjected to an initial denaturation of 95°C for 3 min than followed by 35 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The amplified samples (10 µl) were run horizontally in 1% of agarose gel, stained with ethidium bromide and finally examined by UV trans-illumination.

RFLP

RFLP analysis was conducted by digesting 5 ml PCR product with 20 U *XhoI* enzyme in 2X enzyme buffer (Buffer R^+, MBI Fermentas®, Cat. no. #BR5, Hannover, Germany) in a final volume of 20 ml for 1 h at 37°C. Restriction fragments were then separated in 2% of agarose gel by horizontal electrophoresis and examined under UV transillumination.

Sequence Analysis

DNA sequence analysis of the PCR products from two assemblage B and one assemblage A samples were performed by a commercial company (RefGen, http://www.refgen.com) and examined by BLAST using the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These TPI sequences were compared by multi-alignment (ClustalW2, http://www.ebi.ac.uk/Tools/clustalw2/index.html) with each other and also other DNA sequences in the NCBI website. The comparison is presented in the Results section.

Statistical Analyses

The statistical analyses of the study were done with SPSS 11.5® using chi-square and Fischer's exact tests, and *P* values of 0.05 or lower were regarded as significant.

RESULTS

Fifty four of 63 genomic DNA samples were amplified successfully with PCR and analyzed for the genotypic differences of *G. lamblia*. The mean age of the patients is 14.0 years (±12.4; range 3-67), but more than half of them (51.90%) were below 10 years and female (1:1.3) *(Table 1)*.

The results of the RFLP analysis demonstrated that 38 of 54 samples (70.37%) were belonging to assemblage A, while the remaining 16 samples (29.63%) to assemblage B. Both sexes were equal among the patients in assemblage A while the number of women were significantly higher in Assemblage B (P=0.18) *(Table 2)*.

Many patients (71.20%) complained of one or more symptoms related to giardiasis and abdominal pain was found to be the leading symptom followed by diarrhea *(Table 2)*. A statistically significant relationship was detected between Assemblage B and the occurrence of abdominal pain and diarrhea together (Chi-square, 10.52; P<0.05) *(Table 3)*.

Table 1. *Characteristics of 54 patients with Giardia lamblia*
Tablo 1. *Giardia lamblia'lı 54 hastanın özellikleri*

Characteristics		Assemblage of *G. lamblia* *				Total	%
		A	%	B	%		
Age Groups	0-9	18	64.3	10	35.7	28	100
	10-19	11	64.7	6	35.3	17	100
	20-29	1	100	0	0	1	100
	30-39	5	100	0	0	5	100
	40-49	2	100	0	0	2	100
	≥ 50	1	100	0	0	1	100
	Total	**38**	**70.37**	**16**	**29.63**	**54**	100
Sex	Female	19	65.5	10	34.5	29	100
	Male	19	76.0	6	24.0	25	100
	Total	**38**	**70.37**	**16**	**29.63**	**54**	100

** Percentages represent the total values on the lines*

Table 2. *Distribution of symptoms reported by patients*
Tablo 2. *Hastalar tarafından bildirilen semptomların dağılımları*

Semptom	Number	%
None	15	28.80
Abdominal Pain	11	21.20
Diarrhoea	7	13.0
Abdominal Pain and Diarrhea	5	9.60
Growth Retardation	5	9.60
Anorexia	3	5.80
Allergic Reactions	3	5.80
Flatulence	3	5.80
Enuresis Nocturna	2	3.70
Total	**54**	**100.0**

The PCR revealed a product of *G. lamblia* with 510 bp *(Fig. 2)*. The RFLP analyses of this product demonstrated either a single band of 510 bp (Assemblage A) or formation of two bands of 290 bp and 220 bp (Assemblage B) *(Fig. 3)*.

Table 3. *Distribution of symptoms according to assemblages*
Tablo 3. *Genotiplere göre semptomların dağılımları*

Symptoms	*Giardia* Assemblage				Total	
	A		B			
	n	%	n	%	n	%
Abdominal Pain	9	81.80	2	18.20	11	100
Diarrhoea	4	57.10	3	42.90	7	100
Abdominal Pain and Diarrhoea	0	0	5	100	5	100
Growth Retardation	4	80.0	1	20.0	5	100
Flatulence	3	100	0	0	3	100
Allergic Reactions	3	100	0	0	3	100
Enuresis Nocturna	2	100	0	0	2	100
Anorexia	2	66.67	1	33.33	3	100
Unknown	5	83.33	1	16.67	6	100
None	7	87.50	1	12.50	8	100

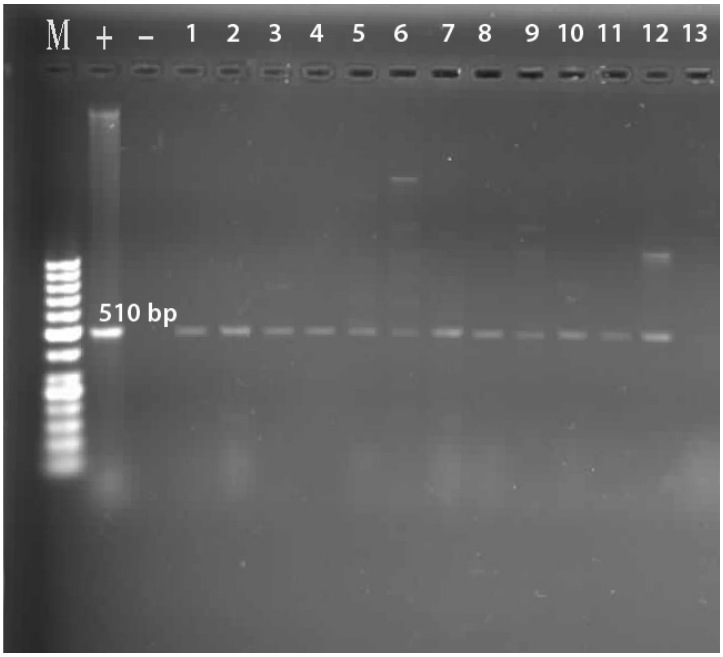

Fig 2. Images of the PCR product (510bp) of *G. lamblia* (M: Marker 50 bp)

Şekil 2. *G. lamblia*'nın PZR görüntüsü

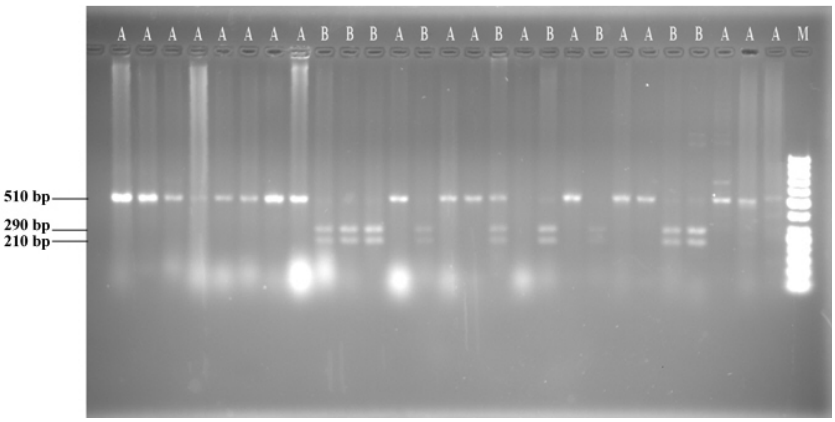

Fig 3. The images of the PCR products restricted with *Xho*I, stained with ethidium bromide and run in 2% of agarose gel: The formation of two bands of 290 and 220 bp represent Assemblage B, while the single band of 510 bp represent Assemblage A. (M: Marker 50 bp)

Şekil 3. *Xho*I ile sınırlandırılan ve etidyım bromid ile boyanarak %2 agaroz jelde yürütülen PZR ürününün görüntüsü. B tipinde 290 ve 220 bp'de iki adet bant oluşurken, A tipinde sadece bir adet 510bp'de bant oluşmuştur

According to the results of sequence analyses, 477 bp were compared with other sequences obtained from NCBI website of the BLAST program with accession numbers as follows: L02116.1, EU014511.1, EU014503.1, EU014515.1, EU014504.1 (Assemblage B), EF688021.2, EF688020.2, EF688019.2, EF688018.2, EF688022.1 (Assemblage A). Sequencing results of *tpi* gene region for the selected samples were also multialigned with each other. The sequence results of two samples from assemblage B (B, B1) and one from assemblage A were shown in *Fig. 4*. The rate

```
B1    -CCGGTACTGTGGCTGTGGCTGAGCCTGTAATA-TAGGACACTCTGAA-GACGTAGAATC 57
B     CGCCGTGGTGTTGATGTGGCTGAGCCTGTAATAATAGGACACTCTGAAAGACGTAGAATC 60
A     -TCTGTAGTACGACTGCGTTTGAGCATGTGATAGTAGGGCACTCTGAA-GACGCAGAATC 58
        * ** *   ** * ***** *** *** **** ********* **** ******

B1    ATGGGCGAGACCAATGAGCAGAGTGCTAAGAAGGCGAAGCGTGCTCTGGACAAAGGTATG 117
B     ATGGGCGAGACCAATGAGCAGAGTGCTAAGAAGGCGAAACGTGCTCTGGACAAAGGTATG 120
A     ATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCTAAGCGTGCCCTGGAAAAGGGGATG 118
        ***** ****** * ***** ** ** ******** ** ***** ***** ** ** ***

B1    ACTGTTATCTTCTGCACCGGAGAGACCCTGGATGAACGCAAGGCCAATAACACTATGGAG 177
B     ACTGTTATCTTCTGCACCGGAGAGACCCTGGATGAACGCAAGGCCAATAACACTATGGAG 180
A     ACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGCGCAAGGCCAACCGCACCATGGAG 178
        ** ** *********  ********** ******* ***********  *** ******

B1    GTGAATATTGCTCAGCTCGAGGCTCTTAAGAAGGAGATTGGAGAATCAAAGAAGTTATGG 237
B     GTGAATATTGCTCAGCTCGAGGCTCTTAAGAAGGAGATTGGAGAATCAAAGAAGTTATGG 240
A     GTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAGCTCGGAGAGTCCAAGATGCTCTGG 238
        ***** ** ** ***** ***** ***  ****** * ***** ** **** * * ***

B1    GAGAACGTTGTAATTGCCTATGAGCCGGTGTGGTCTATCGGCACGGGTGTGGTGGCCACA 297
B     GAGAACGTTGTAATTGCCTATGAGCCGGTGTGGTCTATCGGCACGGGTGTGGTGGCCACA 300
A     AAGGAGGTTGTCATTGCTTACGAGCCCGTGTGGTCCATTGGCACGGGCGTGGTGGCCACG 298
        ** * ***** ***** ** ***** ******** ** ******** ***********

B1    CCGGAACAGGCAGAGGAAGTCCATGTGGGACTCCGCAAATGGTTTGCGGAAAAGGTTTGC 357
B     CCGGAACAGGCAGAGGAAGTCCATGTGGGACTCCGCAAATGGTTTGCGGAAAAGGTTTGC 360
A     CCCGAGCAGGCAGAGGAGGTACATGTGGGGCTCCGAAAGTGGTTTGCGGAGAAGGTTTGT 358
        ** ** *********** ** ******** ***** ** *********** ********

B1    GCAGAAGGTGCGCAGCACATCCGTATCATCTATGGAGGGTCTGCCAATGGGAGTAACTGC 417
B     GCAGAAGGTGCGCAGCACATCCGCATCATCTATGGAGGGTCTGCCAATGGGAGTAACTGC 420
A     GCCGAGGGCGCACAGCATATCCGTATCATTTACGGGGGATCGGCCAATGGAAGCAACTGC 418
        ** ** ** ** ***** ***** ***** ** ** ** ** ******** ** *******

B1    GAGAAACTTGGCCAGTGCCCGAATATCGACGGATTCCTCGTCGGAGGTGCTTCCTTTCAA 477
B     GAGAAGCTTGGCCAATGCCCGAATATCGACGGATTCCTCGTCGGAGGTGCTTCCCT-CAA 479
A     GAGAAGCTCGGCCAGTGTCCGAATATTGACGGCTTCCTTGTCGGTGGCGCTTCCTT-CAA 477
        ***** ** ***** ** ******** ***** ***** ***** ** ******* * ***
```

Fig 4. The results of the multialignment analyses of the assemblages B, B1 and A

Şekil 4. B, B1 ve A genotiplerinde çoklu dizi analizlerinin sonuçları

of correlation between two assemblage B samples were found to be 97%, while the rates of correlation between the assemblage B, B1 and assemblage A were 79% and 81%, respectively. Specific regions were determined for assemblages B (B1-B) and A on *tpi* gene region by multi-alignment analysis.

DISCUSSION

This is the first report of *Giardia* assemblages analyzed with PCR and RFLP in patients living in Manisa province in western Turkey. The results are concordant with the recent findings in Rio de Janeiro - Brazil [2], Mexico [18], Italy [19] and Portugal [4], but contrary to the findings in Fortaleza - Brazil [16], Bangladesh [20], and Nicaragua [21].

Giardiasis is a common parasitic disease in Manisa; our previous studies with different children cohorts from Manisa province revealed prevalence rates of 13.50% and 32.0% [22,23]. It was reported that the prevalence of *G. lamblia* in stool specimens ranged between 2-5% and 20-60% in industrialized and developing countries, respectively [24]. Symptoms related to gastrointestinal tract are manifest in symptomatic patients and many studies have been conducted in the recent years to assess the possible relationships between the symptoms and the genotype of the parasite. We detected symptoms related to giardiasis in 72.20% of all patients in our study group, and the number of symptomatic patients is doubled in assemblage A compared to assemblage B, but the only statistically significant relationship was detected between the combination of abdominal pain and diarrhea and assemblage B (*Chi-square*, 10.52; $P<0.05$). However, this comparison needs to be confirmed in larger-scale studies with more patients. Detection of no other significant relationship between the symptoms and assemblages is similar to the findings of Haque et al.[20] in Bangladesh. However, in the previous study conducted in Turkey, assemblage A was found to be associated with diarrhoeal symptoms while assemblage B was detected in asymptomatic infections [13]. This may be due to the geographical variations of *Giardia* parasites within Turkey, but it clearly indicates the need for further studies with more samples from all parts of Turkey to clarify the association between the symptoms and the genotype of the parasite.

Zoonotic potential of *Giardia sp.* has been well-known by World Health Organization for more than 30 years; however, the role of the animals in the epidemiology of human infection is not clear despite detailed molecular analyses that showed the presence of Assemblages A and B in humans and many animals [25]. In a study from Japan, Giardia genotypes were assessed in fecal samples of some wild and domestic animals [26]. The identified genotypes were assemblages A, C, D or A/D in dogs, assemblage F in cats, assemblages A and E in calves and assemblage B in monkeys. Minvielle et al.[27] investigated the zoonotic potentials of *Giardia* genotypes in a cohort of individuals, dogs and cows from a region. The results showed that Assemblage B was predominant (93.02%) among the patients and was identified in a dog's sample, whereas Assemblage A was not found in any animal sample. The difficulty of obtaining stool samples from animals is the main drawback in such studies, which keep the number of samples low and results with limited reliability.

Initial molecular biological studies investigating the genotypes of *G. lamblia* isolates relied on culturing the organism, which may affect the identification process as some isolates may grow better than others in culture, creating a bias in favor of some genotypes [13]. This drawback is now over as the assemblages of *Giardia* could be determined directly in stool or aspirates of the patient in recent studies [2,12,13]. We also genotyped our *G. lamblia* isolates specifically after extracting their DNA from feces; thus our results exhibit the real, unbiased assemblages of the parasite.

One interesting finding of our study is the association between assemblage B and female sex (7 out of 9 individuals in assemblage B were female). Since the sample size is small and the number of females is slightly higher than males (29 vs. 25) in our study group, more data are required to confirm this association.

Fifty-four of 63 DNA samples were amplified successfully with PCR in the present study. The failure of the amplification of 9 samples may be due to late isolation of DNA after long storage time of the samples at -20°C or insufficiency in overcoming the hard cystic structure of *Giardia* during DNA extraction. DNA isolation with fresh stool samples may lower the number of amplification failures in future studies.

PCR was conducted with the same protocol and primers were selected as previously described by other researchers [13]. A PCR product of 540 bp was obtained, whereas we identified a PCR product of 510 bp for *G. lamblia*, shown in the genebank with accession number L02116.1 *(Fig. 1)*. Comparison of the primer sequences revealed one-base difference between our forward primer and L02116.1 sequence.

Although genotyping is expensive and labour-intensive method requiring specific laboratory equipment for DNA extraction, restriction enzyme digestion, and electrophoretic discrimination of the products, it is out of scope for a routine parasitology laboratory, especially in developing countries. However, identification of non-pathogenic as well as pathogenic *Giardia* assemblages with this sensitive method will surely prevent unnecessary medical treatments, help patients avoid the toxic effects of the drugs, and improve the cost-effectiveness of the treatment. It will also add valuable information to the

biological processes of *Giardia* infection.

The results of the multialignment analysis of three selected samples indicated specific regions for assemblages B (B1-B) and A on *tpi* gene region. Now, our aim is to design a new probe for *tpi* gene region with LightCycler Probe Design Software 2.0 program to differentiate the assemblages and thus to set up a real-time PCR assay which will help analyzing the results in one step and shorten the test time significantly.

In conclusion, this is the first report of *G. lamblia* genotypes from Manisa. Despite the number of patients were relatively low, the statistically-significant correlations identified in the present study surely worth further large-scale assessments in similar patient groups to clarify the correlation between the patient symptoms and *Giardia* genotypes.

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