Polymerase Chain Reaction Based Subtyping of *Blastocystis* spp. Isolates from Symptomatic Patients in Turkey

Türkiye'deki Semptomatik Hastalardan Elde Edilen *Blastocystis* spp. İzolatlarının Polimeraz Zincir Reaksiyonu ile Alt Tiplendirilmesi

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ABSTRACT Objective: *Blastocystis spp.* is a common parasite found in human intestinal tract; however its pathogenic role and factors involved is still under investigation. There are 13 different subtypes of Blastocystis spp. based on genotype analysis. In this study, subtypes of *Blastocystis* spp. were analyzed in 29 symptomatic patients who were positive for *Blastocystis* spp. in Erciyes University Hospital. **Material and Methods:** Stool samples from patients were confirmed to be positive for *Blastocystis* spp. by light microscopy. Genomic DNA was isolated from stool samples of the patients, and *Blastocystis* spp. isolates were classified into subtypes using polymerase chain reaction using 7 subtype-specific primers. Subtypes, symptoms and the presence of additional parasites were comparatively analyzed for each patient. **Results:** Among symptomatic patients, subtypes 3, 1, 2 and 4 were found in 55.2, 37.9, 13.8 and 6.8% of the patients, respectively. There were mixed infections of different subtypes such as 1 and 2 in one patient, 1 and 3 in another patient, and 3 and 4 in two patients. Most commonly encountered symptoms were abdominal pain and diarrhea followed by dermatitis. Additional parasites such as one of the flagellate or amoeba species were observed in 45.5% (5 out of 11 patients), 25% (1 out of 4 patients), 25% (4 out of 16 patients) and 50% (1 out of 2 patients) of patients having subtypes 1, 2, 3 and 4, respectively. **Conclusion:** Our results demonstrate the relative abundance of each *Blastocystis* spp. subtype in symptomatic patients in Kayseri and surrounding cities. Observed subtypes are aligned as 3, 1, 2 and 4 according to frequency of occurrence.

Key Words: Blastocystis; polymerase chain reaction; genotype; signs and symptoms; Turkey

ÖZET Amaç: *Blastocystis* spp. insan bağırsak sisteminde bulunan yaygın bir parazittir, bununla birlikte hastalıktaki rolü ve buna yol açan etmenler araştırılmaktadır. *Blastocystis* spp. genotip analizleri sonucunda 13 alt tipe ayrılmıştır. Bu çalışmada, Erciyes Üniversitesi Hastanesi'nde bulunan ve *Blastocystis* spp. taşıdığı belirlenen 29 semptomatik hastanın *Blastocystis* spp. izolatlarının alt tipleri belirlenmiştir. **Gereç ve Yöntemler:** Hastalardan alınan dışkı örnekleri ışık mikroskobuyla incelenerek *Blastocystis* spp. varlığı tespit edilmiştir. Dışkı örneklerinden DNA izolasyonu yapılmış ve polimeraz zincir rekasiyonu yöntemiyle 7 farklı alt tip spesifik primer kullanılarak herbir dışkıdaki *Blastocystis* spp. parazitleri tiplendirilmiştir. Herbir hasta için alt tip, semptom ve tespit edilen diğer parazitler analiz edilmiştir. **Bulgular:** Semptomu olan hasta grubunda *Blastocystis* spp. alt tip 3, 1, 2 ve 4 sırasıyla hastaların %55,2, %37,9, %13,8 ve %6,8'inde tespit edilmiştir. Ayrıca bir hastada 1. ve 2., bir hastada 1. ve 3., ve iki hastada ise 3. ve 4. alt tipler aynı anda tespit edilmiştir. Hastalarda en yaygın olarak gözlenen semptomlar sırasıyla karın ağrısı, ishal ve dermatittir. Ayrıca, 1., 2., 3. ve 4. alt tiplerin pozitif bulunduğu hastaların sırasıyla %45Ayrıca, 1., 2., 3. ve 4. alt tiplerin pozitif bulunduğu hastaların sırasıyla %45Ayrıca, 1., 2., 3. ve 4. alt tiplerin pozitif bulunduğu hastaların sırasıyla %45. **Sonuç:** Bu çalışma, Kayseri ve çevresindeki semptomatik hastalarda *Blastocystis* spp. alt tiplerinin bulunma sıklığını göstermektedir. Gözlemlenen alt tipler sıklık sırasına göre 3, 1, 2 ve 4'tür.

Anahtar Kelimeler: Blastokist; polimeraz zincir reaksiyonu; genotip; belirti ve bulgular; Türkiye

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Blastocystis spp. is a common parasite found in human intestinal tract.[1, 2] It's one of the most common eukaryotic single-cell organism found in human stool samples.[3] Developed countries have a lower prevalence compared to developing countries. There are different subtypes of *Blastocystis* spp. based on genotyping data. Subtype 3 seems to be the most dominant subtype found in humans.[1-3] Although its pathogenicity is still controversial, infection with *Blastocystis* spp. is associated with intestinal disorders.[4-7] These disorders include diarrhea, abdominal pain and flatulence.[8-11] On the other hand, there are many asymptomatic cases with *Blastocystis* spp. and several reports suggest that *Blastocystis* spp. is not associated with pathogenicity.[12-13]

There are different subtypes of *Blastocystis* spp. based on genotyping data. Small-subunit (SSU) rRNA gene has been used to classify *Blastocystis* spp. into clades. In general, *Blastocystis* spp. can be grouped as seven distinct genotypes.[14-16] However recently, 13 subtypes of *Blastocystis* spp. of which subtypes 1-9 have been detected in human samples, were identified based on SSU rDNA genomic sequence.[17,18] Specific diagnostic primers can be used to discriminate *Blastocystis* spp. subtypes using a polmerase chain reaction (PCR)-based approach. Seven diagnostic primer pairs were designed to classify *Blastocystis* spp. isolates into seven subtypes.[19-22] Subtype 3 is the most dominant subtype found in humans and Subtypes 1, 2 and 4 are subsequent common subtypes.[3]

It has been proposed that some subtypes of *Blastocystis* spp. may have a greater pathogenic potential than others. In a study with asymptomatic individuals and symptomatic patients, *Blastocystis* spp. subtypes 1, 2 and 4 seemed to be associated with gastrointestinal symptoms.[23]

In contrast, a molecular epidemiology study of *Blastocystis* spp. isolates from 87 individuals (69 symptomatic and 18 asymptomatic) in Turkey has stateed that subtype 3 is by far the most common subtype with 75.9% prevalence, however, the authors did not find any association of symptoms with any subtype.[24]

In this study, we aimed to determine different subtypes of *Blastocystis* spp. from symptomatic patients that have been confirmed to have *Blastocystis* spp. on light microscopy.

MATERIAL AND METHODS

COLLECTION OF SAMPLES

This study was approved by Clinical Research Ethics Committee of Erciyes University, and informed consent forms were signed by patients. Stool samples were collected from routine laboratory samples of symptomatic patients in between March and June 2012, following the guidelines of Ethics Committee of the respective institute. Presence of *Blastocystis* spp. was confirmed by light microscopy in 29 patients in Department of Parasitology, Erciyes University, Kayseri, Turkey. Age, gender and symptom data of the patients were recorded. The stool samples were kept at -20 °C until processing.

DNA ISOLATION

DNA isolation was done by using QIAamp stool kit (QIAGEN, Baltimore, USA) from the stool samples according to manufacturer's procedure. Isolated DNA was kept at -20 °C.

PCR-BASED SUBTYPING

In order to determine the subtype of *Blastocystis* spp. in each specimen, genomic DNA isolated from each sample was used in PCR with seven different subtype specific primers, as described by Yoshikawa et al. (Table 1).[25] We used classification nomenclature based on phylogenetic classification by small-subunit ribosomal DNA analysis, proposed by Stensvold et al.[17] PCR reactions (total volume of 25 µl) included 5 µl of master mix (FirePol-Solis Biodyne, Tartu, Estonia), 2 µl genomic DNA and 1 µl of each primer. PCR conditions were as following: Hot start at 94 °C for 5 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min, and at the end, elongation at 72 °C for 5 min.

RESULT

Each specimen was classified into one or more subtypes according to the results of PCR. Two repre-

TABLE 1: Sequenced tag site primers used in this study (Yoshikawa et al. 2004).

Subtypes	STS Primer sets	Sequences of Forward (F) and reverse (R) primers (5′–3′)	Product size (bp)	GenBank accession no.
1	SB83	F: GAAGGACTCTCTGACGATGA R: GTCCAAATGAAAGGCAGC	351	AF166086
2	SB340	F: TGTTCTTGTGTCTTCTCAGCTC R: TTCTTTCACACTCCCGTCAT	704	AY048752
3	SB227	F: TAGGATTTGGTGTTTGGAGA R: TTAGAAGTGAAGGAGATGGAAG	526	AF166088
4	SB337	F: GTCTTTCCCTGTCTATTCTTGCA R: AATTCGGTCTGCTTCTTCTG	487	AY048750
5	SB336	F: GTGGGTAGAGGAAGGAAAACA R: AGAACAAGTCGATGAAGTGAGAT	317	AY048751
6	SB332	F: GCATCCAGACTACTATCAACATT R: CCATTTTCAGACAACCACTTA	338	AF166091
7	SB155	F: ATCAGCCTACAATCTCCTC R: ATCGCCACTTCTCCAAT	650	AF166087

sentative samples were shown belonging to subtype 2 and 3 respectively. Genomic DNA from both samples was used to run PCR with seven subtype-specific primers simultaneously, and PCR products specific for subtype 2 and 3 were obtained respectively (Figure 1A and 1B). A total of 29 patients were studied. *Blastocystis* spp. subtypes 1, 2, 3, and 4 were found in 11 (37.9%), 4 (13.8%), 16 (55.2%) and 2 patients (6.8%), respectively. Percentages for each subtype included mixed infections; therefore total percentage exceeds 100%. There were mixed infections of different subtypes such as 1 and 2 in one patient, 1 and 3 in another patient, and 3 and 4 in two patients. A detailed description of each patient, the symptoms and the corresponding *Blastocystis* spp. subtypes are summarized in Table 2. Presence of additional parasites along with *Blastocystis* spp. infection was monitored and occasionally other symptomatic or asymptomatic parasites were detected (Table 2). An additional parasite was found in 45.5% of patients with *Blastocystis* spp. subtype 1 (5 out of 11), 25% of patients with subtype 2 (1 out of 4), 50% of patients with subtype 4 (1 out of 2) and 25% of patients with subtype 3 (4 out of 16).

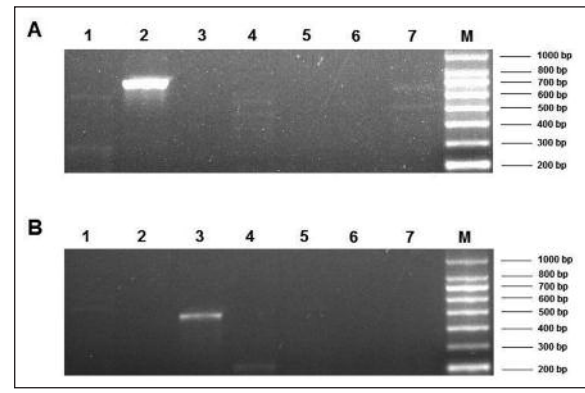

FIGURE 1: Polymerase chain reaction (PCR) analysis of Blastocystis spp. subtypes in two symptomatic patients. Genomic DNA isolated from stools of patients was amplified with seven distinct primer pairs designed for the identification of seven subtypes of Blastocystis spp. PCR products were visualized in agarose gel electrophoresis along with the DNA marker. Numbers 1 to 7 represent each PCR reaction with a subtype specific primer pair. A. A PCR product of 704 bp that corresponds to subtype 2 was amplified. B. A PCR product of 526 bp that corresponds to subtype 3 was amplified.

DISCUSSION

Blastocystis spp. is frequently found in humans; however its presence does not always cause disease symptoms[3]. In Turkey, *Blastocystis* spp.-positive patients without any other parasites have been reported to have frequent intestinal symptoms and abdominal pain.[7] It's been proposed that *Blastocystis* spp. can be transmitted to humans from their pets and tap water.[26] Dogruman-Al et al. reported that subtype 3 was the most common one in both symptomatic and asymptomatic patients; however they have found a correlation between subtype 2

TABLE 2: Age, gender, subtypes of Blastocystis spp. isolates and observed symptoms in symptomatic patients from Erciyes University Hospital.

Patient	Age/Gender	Subtypes	Symptoms	Additional Parasite
1	8/F	1	Abdominal Pain	
2	40/M	1	Dermatitis	Retortamonas intestinalis
3	64/F	1	Abdominal Pain	Entamoeba hartmanni
4	70/M	1	Diarrhea	
5	26/F	1	Diarrhea	
6	13/M	1	Abdominal Pain	
7	43/F	1 and 3	Abdominal Pain	Endolimax nana
8	54/M	1 and 2	Abdominal Pain	Entamoeba coli
9	55/F	1	Diarrhea	
10	5/M	1	Dermatitis	
11	7/F	1	Growth Retardation	Entamoeba histolytica/dispar
12	11/F	2	Dermatitis	
13	29/F	2	Abdominal Pain	
14	15/M	2	Growth Retardation	
15	30/M	3	Diarrhea	
16	46/F	3 and 4	Diarrhea, Abdominal Pain	Giardia intestinalis
17	11/F	3	Constipation	Entamoeba coli
18	13/F	3	Growth Retardation	
19	46/F	3	Diarrhea	
20	55/M	3 and 4	Diarrhea, Abdominal Pain	
21	68/M	3	Abdominal Pain	
22	55/M	3	Abdominal Pain	Endolimax nana
23	52/M	3	Diarrhea	
24	13/M	3	Diarrhea	
25	13/M	3	Abdominal Pain	
26	39/M	3	Dermatitis	
27	42/M	3	Abdominal Pain	
28	16/F	3	Abdominal Pain	
29	73/F	3	Diarrhea	

and asymptomatic groups, but they did not find a correlation of any subtype with symptomatic patients.[27] In another study from Turkey, subtype 3 has been found to be by far the most common in both symptomatic and asymptomatic groups, but any subtype was not found to be associated with pathogenicity.[24] In contrast, others reported that subtype 1 was associated with symptomatic patients and pathogenicity.[28, 29]

In this study, subtypes of *Blastocystis* spp. in a symptomatic patient group in Erciyes University Hospital were analyzed. *Blastocystis* spp. subtype 3 was observed in 55.2% of patients, followed by *Blastocystis* spp. subtype 1 observed in 37.9% of patients, *Blastocystis* spp. subtype 2 observed in 13.8% of patients and *Blastocystis* spp. subtype 4 observed in 6.8% of patients. There were mixed infections of different subtypes such as 1 and 2, 1 and 3, and 3 and 4. The most common symptoms were abdominal pain and diarrhea followed by dermatitis. Presence of additional parasites was analyzed, and parasites such as *Giardia intestinalis* or one of the amoeba species were detected. Previously, simultaneous presence of *Iodomoeba butschlii* and *Blastocystis* spp. was reported.[30] Interestingly, an additional parasite was detected in 45.5%, 25%, 25% and 50% of patients with *Blastocystis* spp. subtype 1, subtype 2, subtype 3 and subtype 4, respec-

tively. This is an interesting observation because subtype 1 was reported to be associated with symptoms, and the observation of additional parasites with subtype 1 more frequently may be related to its reported pathogenicity.[28,29] Subtype 1 was detected only in symptomatic patients. In a study from Turkey, all 20 of symptomatic patients were found to have subtype 1.[29] Although subtype 3 is the most common subtype in humans, it has been detected in both symptomatic and asymptomatic patients.

CONCLUSION

Our results demonstrated the relative abundance of each *Blastocystis* spp. subtype in symptomatic patients in Kayseri and its surrounding cities. Observed subtypes are aligned as 3, 1, 2 and 4 according to the frequency of occurrence.

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REFERENCES

1. Stenzel DJ, Boreham PF. Blastocystis hominis revisited. Clin Microbiol Rev 1996;9(4):563-84.
2. Stensvold CR, Nielsen HV, Mølbak K, Smith HV. Pursuing the clinical significance of Blastocystis--diagnostic limitations. Trends Parasitol 2009;25(1):23-9.
3. Tan KS. New insights on classification, identification, and clinical relevance of Blastocystis spp. Clin Microbiol Rev 2008;21(4):639-65.
4. Brites C, Barberino MG, Bastos MA, Sampaio Sá M, Silva N. Blastocystis hominis as a Potential Cause of Diarrhea in AIDS Patients: a Report of Six Cases in Bahia, Brazil. Braz J Infect Dis 1997;1(2):91-94.
5. Cirioni O, Giacometti A, Drenaggi D, Ancarani F, Scalise G. Prevalence and clinical relevance of Blastocystis hominis in diverse patient cohorts. Eur J Epidemiol 1999;15(4):389-93.
6. Graczyk TK, Shiff CK, Tamang L, Munsaka F, Beitin AM, Moss WJ. The association of Blastocystis hominis and Endolimax nana with diarrheal stools in Zambian school-age children. Parasitol Res 2005;98(1):38-43.
7. Kaya S, Sesli Çetin E, Cicioğlu Arıdoğan B, Arıkan S, Demirci M. Pathogenicity of Blastocystis hominis, a clinical reevaluation. Turkiye Parazitol Derg 2007;31(3):184–7.
8. Kuştimur S, Doğruman-Al F, Tuncer C, Çamurdan-Duyan A, Dalgıç B, Alagözlü H, et al. Investigation of some protozoans with different diagnostic methods in patients with gastrointestinal discomfort. Turkiye Klinikleri J Med Sci 2009;29(5):1260-6.
9. Yalçın G, Bayram-Delibaş S. [Comparison of different staining methods for detecting Blastocystis hominis cysts]. Turkiye Klinikleri J Med Sci 2009;29(6):1370-5.
10. Nimri LF, Meqdam M. Enteropathogens associated with cases of gastroenteritis in a rural population in Jordan. Clin Microbiol Infect 2004;10(7):634-9.
11. Yakoob J, Jafri W, Jafri N, Khan R, Islam M, Beg MA, et al. Irritable bowel syndrome: in search of an etiology: role of Blastocystis hominis. Am J Trop Med Hyg 2004;70(4):383-5.
12. Leder K, Hellard ME, Sinclair MI, Fairley CK, Wolfe R. No correlation between clinical symptoms and Blastocystis hominis in immunocompetent individuals. J Gastroenterol Hepatol 2005;20(9):1390-4.
13. Chen TL, Chan CC, Chen HP, Fung CP, Lin CP, Chan WL, et al. Clinical characteristics and endoscopic findings associated with Blastocystis hominis in healthy adults. Am J Trop Med Hyg 2003;69(2):213-6.
14. Clark CG. Cryptic genetic variation in parasitic protozoa. J Med Microbiol 2000;49(6):489-91.
15. Clark CG. Extensive genetic diversity in Blastocystis hominis. Mol Biochem Parasitol 1997;87(1):79-83.
16. Noël C, Dufernez F, Gerbod D, Edgcomb VP, Delgado-Viscogliosi P, Ho LC, et al. Molecular phylogenies of Blastocystis isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. J Clin Microbiol 2005;43(1):348-55.
17. Stensvold CR, Suresh GK, Tan KS, Thompson RC, Traub RJ, Viscogliosi E, et al. Terminology for Blastocystis subtypes--a consensus. Trends Parasitol 2007;23(3):93-6.
18. Parkar U, Traub RJ, Vitali S, Elliot A, Levecke B, Robertson I, et al. Molecular characterization of Blastocystis isolates from zoo animals and their animal-keepers. Vet Parasitol 2010;169(1-2):8-17.
19. Yoshikawa H, Abe N, Iwasawa M, Kitano S, Nagano I, Wu Z, et al. Genomic analysis of Blastocystis hominis strains isolated from two long-term health care facilities. J Clin Microbiol 2000;38(4):1324-30.
20. Yoshikawa H, Abe N, Wu Z. PCR-based identification of zoonotic isolates of Blastocystis from mammals and birds. Microbiology 2004;150(Pt 5):1147-51.
21. Yoshikawa H, Wu Z, Nagano I, Takahashi Y. Molecular comparative studies among Blastocystis isolates obtained from humans and animals. J Parasitol 2003;89(3):585-94.
22. Yoshikawa H, Nagano I, Wu Z, Yap EH, Singh M, Takahashi Y. Genomic polymorphism among Blastocystis hominis strains and development of subtype-specific diagnostic primers. Mol Cell Probes 1998;12(3):153-9.
23. Kaneda Y, Horiki N, Cheng XJ, Fujita Y, Maruyama M, Tachibana H. Ribodemes of Blastocystis hominis isolated in Japan. Am J Trop Med Hyg 2001;65(4):393-6.
24. Ozyurt M, Kurt O, Mølbak K, Nielsen HV, Haznedaroglu T, Stensvold CR. Molecular epidemiology of Blastocystis infections in Turkey. Parasitol Int 2008;57(3):300-6.
25. Yoshikawa H, Wu Z, Kimata I, Iseki M, Ali IK, Hossain MB, et al. Polymerase chain reaction-based genotype classification among human Blastocystis hominis populations isolated from different countries. Parasitol Res 2004;92(1):22-9.
26. Eroglu F, Koltas IS. Evaluation of the transmission mode of B. hominis by using PCR method. Parasitol Res 2010;107(4):841-5.
27. Dogruman-Al F, Dagci H, Yoshikawa H, Kurt O, Demirel M. A possible link between subtype 2 and asymptomatic infections of Blastocystis hominis. Parasitol Res 2008;103(3):685-9.
28. Hussein EM, Hussein AM, Eida MM, Atwa MM. Pathophysiological variability of different genotypes of human Blastocystis hominis Egyptian isolates in experimentally infected rats. Parasitol Res 2008;102(5):853-60.
29. Eroglu F, Genc A, Elgun G, Koltas IS. Identification of Blastocystis hominis isolates from asymptomatic and symptomatic patients by PCR. Parasitol Res 2009;105(6):1589-92.
30. Vardar R, Ersöz G, Dağcı H, Yurdagül C, Ak M, Mandıracıoğlu A, et al. The prevalence of intestinal parasites is lower than expected in patients with decompensated cirrhosis. Turkiye Klinikleri J Gastroenterohepatol 2011;18(1):9-13.