

## Original Article

Subtype distribution and molecular characterization of *Blastocystis* from hemodialysis patients in Turkey

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**Abstract**

**Introduction:** The aim of this study was to determine the *Blastocystis* prevalence and subtypes in hemodialysis patients in Turkey.

**Methodology:** Eighty-four patients diagnosed with end-stage renal failure who were undergoing hemodialysis and 20 healthy volunteers were enrolled. *Blastocystis* presence was investigated by native-Lugol, trichrome staining, PCR using STS primers, and DNA sequencing analysis.

**Results:** Among the stool samples from the hemodialysis patients, 9.52% (8/84) were found to be *Blastocystis*-positive with the native-Lugol and trichrome staining. Seven of the eight *Blastocystis* isolates were subtyped using STS primers. *Blastocystis* subtype distribution was as follows: one had ST1, two had ST2, two had ST3, two had ST3+ST6, and one was not subtyped. *Blastocystis* positivity was detected in two healthy control (2/20, %10), one subject had ST1, and the other was not subtyped. All subtypes identified by PCR were confirmed by the sequencing analysis. In the two samples that had mixed subtypes (ST3+ST6) when using the STS primers, only ST3 was detected in the sequencing analysis. Although some patients have multiple symptoms, the most common symptoms in *Blastocystis* positive patients were bloating (5/8), diarrhea (4/8), nausea and vomiting (2/8), and gas and weight loss (1/8). Also, only one patient had *Giardia intestinalis*.

**Conclusions:** This was the first study to determine the *Blastocystis* subtypes in hemodialysis patients. A rare subtype, ST6, was identified in two of the patients. Thus, the ST6 infections were attributable to transmission from poultry infections. The presence of this unusual subtype suggests the need for further extensive studies of hemodialysis patients.

**Key words:** *Blastocystis*; hemodialysis patients; molecular characterization; ST6; subtype.

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**Introduction**

Infections caused by intestinal parasites are important causes of morbidity and mortality, and these infections are more severe in immunosuppressed patients [1]. *Blastocystis* is anaerobic intestinal microorganisms that are transmitted through the fecal-oral route, and they frequently cause infections in human beings and other animals [2]. A *Blastocystis* infection can be symptomatic, manifesting itself with abdominal pain, diarrhea, weight loss, and bloating, or it can be asymptotically present in humans [2,3]. Although the *Blastocystis* infection prevalence varies between countries, depending on the level of development, factors such as poor hygienic conditions, close contact with colonized animals, and the

consumption of contaminated water and food also contribute to the spread of this infection [4,5].

Based on the comparison of small subunit (SSU) rRNA gene sequences of microorganisms from the genus *Blastocystis*, seventeen subtypes (ST) have been defined, nine of which (ST1–ST9) cause infections in humans [3,6,7]. ST1 has been isolated from humans and other mammals, ST2 has been isolated from primates and pigs [3,4]. ST3 has been isolated from humans (this is also the subtype that has been isolated most often in epidemiological studies), ST4 has been isolated from rodents, ST5 has been isolated from cattle and pigs, ST6 and ST7 have been isolated from birds, certain primates, and their caregivers, and ST9 has been isolated from humans [6-10]. Despite the availability of

studies demonstrating the associations between various symptoms and the different *Blastocystis* subtypes in recent years, the relationships between the subtypes and pathogenicity have not yet been elucidated [5,11,12].

Hemodialysis patients are immunosuppressed for a variety of reasons, such as the disruption of granulocyte and lymphocyte functions by uremic toxins and malnutrition. However, the risk of developing severe infections as a result of immunosuppressive treatment is relevantly higher in dialysis patients. These patients are prone to many infectious diseases due to their weakened cellular and humoral immunity. As with other infectious agents in hemodialysis patients, parasitic infections can negatively affect their quality of life [13-15]. Studies on the parasite infection prevalences of hemodialysis patients have shown that the most commonly identified microorganism is *Blastocystis spp.* [16-18]. The other parasites found in this population included *Cryptosporidium*, *Endolimax nana*, *Entamoeba coli*, *Entamoeba histolytica*, *Entamoeba dispar*, and *Giardia intestinalis* [14,16-20]. Although research has been conducted on the prevalence of *Blastocystis*, there have been no studies published on *Blastocystis* subtyping in hemodialysis patients [14-18]. Therefore, in this study, we aimed to investigate the *Blastocystis* prevalence and subtypes in hemodialysis patients.

## Methodology

### *Patients and study design*

The study was carried out from May to July 2014 in Erzincan, in northeast Turkey. This cross-sectional study involved 84 end-stage renal disease patients (33 females, 51 males;  $59.80 \pm 16.92$ ) who had been undergoing hemodialysis for  $\geq 6$  months in the dialysis unit of Erzincan University. Twenty healthy volunteers who were not suffering from kidney problems were also enrolled as a control group.

### *Ethical approval and questionnaire*

This study was approved by the Ethics Committee of Erzincan University, and written informed consent was obtained from all of the participants included in this study. Each participant was asked to complete a short questionnaire using a face-to-face interview method to collect the following information: gender, age, renal replacement treatment duration (hemodialysis), drinking water source, the presence of animals in or around the house, and the presence of abdominal complaints (gas, nausea, vomiting, abdominal pain, diarrhea, constipation, bloating, and weight loss).

### *Stool samples and microscopy*

Stool samples were obtained from the study participants, and they were examined using native-Lugol's iodine staining for the detection of protozoan cysts and trophozoites and helminth ova. Then, all of the samples underwent trichrome staining in order to exclude enteric protozoa, such as *Entamoeba histolytica* and *Giardia lamblia*, and Kinyoun acid-fast staining to exclude *Cryptosporidium*, *Isospora*, and *Cyclospora* oocysts.

### *DNA isolation and subtyping*

The genomic DNA was extracted from the stool samples using a QIAamp DNA Stool Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. The DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA), and then, the samples were stored at  $-20\text{ }^{\circ}\text{C}$  for further use.

The PCR analyses were performed using subtype-specific sequence-tagged site (STS) primers (SB83, SB155, SB227, SB332, SB340, SB336, and SB337), as described by Yoshikawa *et al.* [21]. The PCR reaction mixtures (total volume of  $25\text{ }\mu\text{L}$ ) were comprised of  $12.5\text{ }\mu\text{L}$  of the DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc., Waltham, Massachusetts),  $0.5\text{ pmol}$  of each primer,  $4.5\text{ }\mu\text{L}$  of nuclease-free water, and  $5\text{ }\mu\text{L}$  of the DNA template. The PCR was carried out using the following reaction conditions: one cycle of initial denaturation at  $94\text{ }^{\circ}\text{C}$  for 5 min, followed by 40 cycles of denaturation at  $94\text{ }^{\circ}\text{C}$  for 30 seconds, annealing at  $57\text{ }^{\circ}\text{C}$  for 30 seconds, and extension at  $72\text{ }^{\circ}\text{C}$  for 60 seconds, with a final extension step at  $72\text{ }^{\circ}\text{C}$  for 5 minutes.

### *PCR amplification and DNA sequencing*

In order to amplify the barcoding region (600 bp) of the SSU-rRNA gene sequences of the *Blastocystis* isolates, the RD5 (5'-ATC TGG TTG ATC CTG CCAG T-3') and BhrDr (5'-GAG CTT TTT AAC TGC AAC G-3') primers were used [22]. The PCR was performed using a total volume of  $25\text{ }\mu\text{L}$ , which was comprised of  $12.5\text{ }\mu\text{L}$  of the DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA),  $1.5\text{ }\mu\text{L}$  of each primer ( $10\text{ pmol}$ ), and  $2\text{--}5\text{ }\mu\text{L}$  of the DNA. The amplification was performed under the following conditions: initial denaturation at  $94\text{ }^{\circ}\text{C}$  for 1 minute, followed by 40 cycles of denaturation at  $94\text{ }^{\circ}\text{C}$  for 1 minute, annealing at  $59\text{ }^{\circ}\text{C}$  for 1 minute, elongation at  $72\text{ }^{\circ}\text{C}$  for 1 minute, and a final extension at  $72\text{ }^{\circ}\text{C}$  for 10 minutes.

The PCR products and a size marker (GeneRuler 100 bp DNA Ladder; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) were electrophoresed in 2% agarose gels with Tris-Borate-EDTA buffer. The gels were stained with ethidium bromide, and then, they were photographed under an ultraviolet transilluminator.

All of the PCR products were purified using an Agencourt AMPure XP Beads PCR purification kit (Beckman Coulter Inc., Brea, California), and they were forward and reverse-sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California, USA). The sequencing products were run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, California, USA).

*Phylogenetic analysis*

The SSU-rRNA sequences were compared with the sequences in the National Center for Biotechnology Information (NCBI) nucleotide database using the Basic Local Alignment Search Tool algorithm. All of the sequences were aligned with the previously published data on the SSU-rRNA gene sequences of the *Blastocystis* isolates using the ClustalW program (UCD Conway Institute, University College Dublin, Ireland), and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Pennsylvania State University, University Park, Pennsylvania, USA) [23]. The maximum likelihood algorithm and Tamura-Nei model were used, all of the gaps were excluded from the analysis, and *Proteromonas lacerate* (AY224080) was used as the outgroup in the phylogenetic analysis.

*Statistical Analysis*

IBM SPSS Statistics for Windows (version 20.0; IBM Corp., Armonk, NY, USA) was used to evaluate the data. The relationships between the categorical variables were analyzed using Fisher’s exact test and the chi-squared test. In those cases in which the expected frequencies were less than 20%, the Monte Carlo simulation method was used to include those frequencies in the analysis. p values of < 0.05 and < 0.001 were considered to be statistically significant.

**Results**

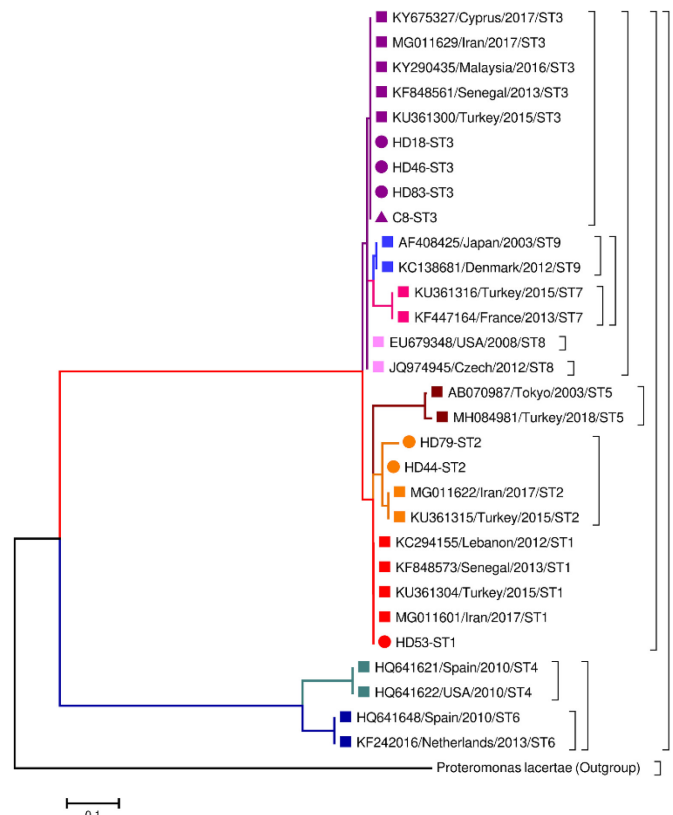
In the patient group, there were 51 males (60.71%) and 33 females (39.29%), with an age range of 13 to 90 years (mean: 59.80 ± 16.92 years). The control group was comprised of 15 males (75%) and five females

(25%), with an age range of 23 to 48 years (mean: 33.35 ± 7.33 years).

*Blastocystis* positivity was found in eight of the 84 patient stool samples (9.52%) that were evaluated using the native-Lugol’s iodine and trichrome staining. Based on the STS primers, seven of the eight *Blastocystis* isolates were subtyped. Of the seven patients with subtyped *Blastocystis* isolates, one had ST1, two had ST2, two had ST3, and two had ST3+ST6. The two patients that were ST3+ST6 positive were engaged in chicken farming.

Among the stool samples from the 20 healthy control subjects, two (10%) were *Blastocystis*-positive: one had ST1, but the other could not be subtyped. There was no statistically significant difference between the hemodialysis patients and the control group in terms of the *Blastocystis* prevalence (p = 0.95).

**Figure 1.** Phylogenetic tree constructed using the maximum likelihood method for the small subunit rRNA region gene sequences of the *Blastocystis* isolates. The hemodialysis patient and control subject isolates are indicated by “HD” and “C,” respectively. The sequences of the strains isolated from the hemodialysis patients and the control subjects are represented by circles and triangles, respectively. The squares represent the reference sequences of each subtype. *Proteromonas lacerate* (AY224080) was used as an outgroup during phylogenetic analysis.



All of the subtypes identified using PCR were confirmed by the sequencing analysis. Based on the STS primers, two of the patient samples exhibited mixed subtypes (ST3+ST6), but when the sequencing analysis was performed, only ST3 was detected (Figure 1).

All of the patients with *Blastocystis*-positive stool samples (8/8) were males, with a mean age of 60.13 ± 19.15 years. The two individuals with *Blastocystis*-positive stool samples in the control group were also males, with a mean age of 40.50 ± 4.95 years. In the patient group, a statistically significant difference was found between *Blastocystis*-positive and *Blastocystis*-negative individuals with regard to the male gender (p < 0.05) (Table 1).

Seventy-one (84.52%) of the 84 patients, including the eight *Blastocystis*-positive patients, reported drinking tap water, whereas the remaining 13 (15.48%) drank bottled spring water. Seventeen (85%) of the 20 healthy volunteers drank tap water, and three (15%) drank spring water. Similar to the patient group, both of the individuals in the control group that were *Blastocystis*-positive drank tap water. However, within the patient and control groups, there were no statistically significant differences between the individuals with regard to the drinking water source (p = 0.347 and p = 0.999, respectively) (Table 1).

In the patient group, the most common symptoms among the *Blastocystis*-positive individuals were bloating (5/8), diarrhea (4/8), nausea and vomiting (2/8), and gas and weight loss (1/8). None of the patients had abdominal pain or constipation (Table 1). *Giardia intestinalis* was detected in one patient. All of

the *Blastocystis*-positive individuals in the patient and control groups, including the Giardiasis-positive patient, were started on 750 mg of metronidazole orally 3 times a day for 10 days. After 2 weeks of treatment, no parasites were detected in any of the stool samples of these 11 individuals.

**Discussion**

The *Blastocystis* prevalences have been reported to range from 4.38% to 51% in Turkey [3,24-26], 1.5% to 20% in developed countries, and 30% to 60% in developing countries [3,27]. However, the highest prevalence (100%) was reported by El Safadi et al. [6] in a Senegalese population. In the review of the studies of investigating *Blastocystis* with conventional methods in hemodialysis patients, the prevalence of *Blastocystis* spp. has been reported to range from 4.4% to 24.5%, as given Table 2 [14-20]. However, to date, no studies have been conducted to investigate the distribution of *Blastocystis* subtypes with molecular methods in hemodialysis patients. In the current study, the prevalence of *Blastocystis* spp. was found to be 9.52%. We believe that the methodological differences affect the findings of the studies resulting in a wide range of prevalence.

In the patient group, when the *Blastocystis*-positive and *Blastocystis*-negative individuals were compared, statistically significant differences were found with regard to the male gender, diarrhea, bloating, and animal contact (p < 0.05); however, no statistically significant differences were found with regard to the other symptoms (gas, nausea, vomiting, abdominal pain, constipation, and weight loss) or the drinking

**Table 1.** Demographic, epidemiological, and clinical characteristics of the hemodialysis patients.

Characteristics	Hemodialysis patients		p value
	<i>Blastocystis</i> -positive n = 8	<i>Blastocystis</i> -negative n = 76	
Gender			
Female	0	33	0.020*
Male	8	43	
Gas	1	23	0.29
Nausea	2	20	0.936
Vomiting	2	14	0.652
Abdominal pain	0	12	0.358
Diarrhea	4	10	0.008**
Constipation	0	15	0.34
Bloating	5	20	0.047*
Weight loss	1	13	0.74
Source of water			
Tap water	8	63	0.347
Bottled spring water	0	13	
Contact with animals	2	0	0.001**

\*p < 0.05 was considered statistically significant. \*\*p < 0.001 was considered statistically highly significant.



water source ( $p > 0.05$ ). In the control group, there were no statistically significant differences between the *Blastocystis*-positive and *Blastocystis*-negative individuals in any of the parameters that were evaluated ( $p > 0.05$ ). When compared to other studies, although the symptom frequencies were similar, we did not observe abdominal pain and constipation in the *Blastocystis*-infected individuals [5,28,29].

Although it has been reported that maleness creates a statistically significant difference in the *Blastocystis* prevalence [29], there have also been studies demonstrating that there was a higher prevalence in females [30], or that there was no difference between the genders [25,26]. Similar to the study by Ertug *et al.* [29], we identified a statistically significant relationship between the male gender and the *Blastocystis*-positivity ( $p = 0.020$ ).

The majority of human *Blastocystis* infections are attributable to ST3, but ST1, ST2, and ST4 infections are also common. In their study of 51 asymptomatic and symptomatic patients, Vassalos *et al.* [28] detected *Blastocystis* in 31 patients, with ST3 being the most common genotype (61%). Yoshikawa *et al.* [31] analyzed 102 isolates from humans, and they reported ST3, ST1, ST4, ST2, ST7, and ST5 at frequencies of 60.8%, 12.7%, 12.7%, 4.9%, 3.9%, and 2%, respectively. El Safadi *et al.* [6] analyzed 93 samples in which they detected ST3, ST1, ST2, and ST4 at percentages of 49.5%, 28.2%, 20.4%, and 1.9%, respectively. In contrast, Sardarian *et al.* [32] conducted a study in western Iran in 2013, and they reported that the most frequently seen genotype among 41 *Blastocystis* isolates was ST1 (56.1%), followed by ST3 (22%), ST1+ST3 (14.63%), and ST2 (7.3%).

In Turkey, Ertug *et al.* [29] subtyped 44 of 61 *Blastocystis* isolates (72.1%), with 17 (38.6%) being identified as ST3, 13 (29.5%) as ST2, and 9 (20.5%) as ST1. In four of the samples (9.1%), ST1 and ST3 were found together, while ST1 and ST2 were present in 1 sample (2.3%). In 29 patients, Sakalar *et al.* [33]

identified ST1, ST2, ST3, and ST4 at frequencies of 37.9%, 13.8%, 55.2%, and 6.8%, respectively.

Ramirez *et al.* [34] suggested that ST6 had a cosmopolitan profile and broad geographic distribution. Although seven subtypes have been identified in birds (ST1, ST2, ST4, ST5, ST6, ST7, and ST10), ST6 and ST7 remain to be the most common subtypes in birds and are generally considered bird subtypes. Besides birds, these two subtypes are found in certain mammals; for example, ST6 in pigs, cattle, goats and dogs and ST7 in pigs, goats, cynomolgus monkeys, lemurs, and dogs. Wang *et al.* [35] reported that ST6 and ST7 were usually isolated from the reservoir hosts of birds. The authors isolated ST6 and ST7 from chickens and red-crowned cranes, while ST6 was detected in one pigeon.

Ramirez *et al.* [36] reported ST6 using the amplification and sequencing of the ‘barcode region’ in the rural and urban population. Based on previous studies, in which ST6 was detected from bird hosts, the authors proposed a zoonotic transmission between birds and humans [4]. Greige *et al.* [37] found high prevalence of *Blastocystis* in the samples taken from chickens and reported that ST6 and ST7 represented avian-adapted STs. Furthermore, the detection of ST6 in slaughterhouse personnel confirmed that this subtype occurred with zoonotic transmission between chickens and their processors whether directly or through repeated contact.

In many studies, ST6 was not detected (or rarely detected) in humans [38-40]. In humans, ST6 and ST7 constitute only a small proportion (approximately 9%) of *Blastocystis* cases [35]. In 2015, for the first time in Turkey, Adiyaman Korkmaz *et al.* [9] reported the presence of *Blastocystis* ST6 (1/43, 2.3%), which is known to come from birds (such as chickens). Mattiucci *et al.* [39] reported, for the first time in humans, *Blastocystis* ST6 prevalence of 3.2% in Italy.

As in previous studies, ST1, ST2, and ST3 were detected in our study. In addition, ST6, a rare subtype in Turkey and other countries, was isolated from two

**Table 2.** Summary of studies with case–control investigating the prevalence of *Blastocystis* in hemodialysis patients and control subjects.

First author	Publication Year	Country	Methods	Hemodialysis patients			Control		
				Samples (n)	Positive (n)	Percentage (%)	Samples (n)	Positive (n)	Percentage (%)
Kulik <i>et al.</i> [16]	2008	Brazil	NL, FECT	86	18	20.9	146	0	0
Gil <i>et al.</i> [14]	2013	Brazil	NL, FECT	110	27	24.5	86	36	41.8
Karadag <i>et al.</i> [17]	2013	Turkey	NL, FECT, TS	142	34	23.9	150	16	10.7
Hawash <i>et al.</i> [19]	2015	Saudi Arabia	NL, FECT, TS	50	8	16	50	4	8
Barazesh <i>et al.</i> [15]	2015	Iran	NL, FECT	88	8	9.1	ND	ND	ND
Fallah Omrani <i>et al.</i> [18]	2015	Iran	NL	78	11	14.1	140	4	2.9
Rasti <i>et al.</i> [20]	2017	Iran	NL, FECT	135	6	4.4	50	0	0

NL: Native-Lugol Method; FECT: Formalin Ether Concentration Technique; TS: Trichrome Staining, ND: Not-Determined.

patients. Two of the patient samples exhibited mixed subtypes (ST3+ST6) with STS primers, but when the sequencing analysis was performed, only ST3 was detected. When the risk factors of ST3 + ST6 identified two patients were retrospectively examined, it was seen that these hemodialysis patients raised poultry in their gardens. However, we were surprised to see rare ST6 in the PCR of STS primers. Since this study was not designed as a zoonotic transmission study, and the detection of a rare subtype, such as ST6 could not have been foreseen, we did not obtain samples from the poultry animals kept by our patients at that time. Therefore, the lack of sampling from poultry and failure to revealed ST6 with sequencing analysis as a limitation of our study. Based on the literature, it is possible that ST6 can be transmitted to hemodialysis patients from poultry.

## Conclusion

In conclusion, we recommended providing suitable personal hygiene and periodic stool examinations to screen parasites as part of the regular medical care of hemodialysis patients. To the best of our knowledge, the present study is the first conducted in Turkey and the world to determine the subtype distribution of *Blastocystis* in hemodialysis patients using molecular tools. Of the subtypes identified, ST3 was the most common, followed by ST1, ST2, and ST6 (a rare subtype in Turkey and in other countries). However, these results cannot be generalized to hemodialysis patients. Nevertheless, our study is a contribution to a better understanding of molecular epidemiology, genetic diversity, and host-parasite interaction of *Blastocystis* at a regional and global scale. Also, the current results will constitute a basis for future research. There is a need for further studies designed with a broader sample using high-resolution molecular markers in human populations in the world.

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## Authors' Contribution

The conception and design of the study: BG, KT, MA. Collected samples: BG, MA, AC, KT. Acquisition of data: BG, MA, MD, ISK, AC. Analysis and interpretation of data: BG, MA, MD, TD. Critical revision of the manuscript for important intellectual content and final approval of the manuscript: BG, MA, ISK, TD. All authors read and approved the final manuscript.

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