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ORIGINAL PAPER

Investigation of Isolated *Blastocystis* Subtypes from Cancer Patients in Turkey

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Abstract

Purpose It is not clear that *Blastocystis* remains without damage to the digestive tract or has a pathogenic effect in relation to subtypes in immunocompromised people, such as cancer patients. The present study aimed to investigate the frequency and subtype distribution of *Blastocystis* in cancer patients who were followed-up and treated in the Oncology clinic of Firat University Hospital and to determine the clinical signs of infected sufferers.

Methods 201 patients aged ≥ 18 with a diagnosis of cancer were enrolled in this cross-sectional study. Patients' stool samples were examined between September 2017 and August 2019 by native-Lugol, trichrome staining. Microscopy-positive stool samples were subjected to DNA isolation and subtyped by Sequence Tagged Site (STS)-PCR analysis. The symptoms and demographic characteristics of the patients were also evaluated.

Results Totally, 29 (14.4%) samples were positive for *Blastocystis* after all methods. 15 (51.7%) out of 29 samples were successfully subtyped by the sequenced-tagged site(STS)-PCR, while 14 (48.3%) could not be typed. Three subtypes of *Blastocystis* were detected: ST3 (40%), ST2 (33%), ST1 (20%), and one mixed infections with ST1/ST2 (6%). There was no statistically significant difference in terms of clinical findings and demographic characteristics.

Conclusion The outcomes of our study promote the idea that *Blastocystis* could be an asymptomatic and harmless commensal organism. However, more comprehensive molecular and clinical studies are needed to fully determine the pathogenicity and epidemiology of *Blastocystis* in cancer patients.

Keywords *Blastocystis* · Cancer · Immunocompromised · Subtype · Turkey

Introduction

Blastocystis is an anaerobic unicellular eukaryotic parasite located in the large intestine that can be commonly found in humans and animals that is associated with various gastrointestinal and extraintestinal disorders [1, 2]. *Blastocystis* has been classified in the phylum Stramenopiles [3–5]. At the morphological level, four main forms of *Blastocystis* have been identified, including the vacuole, granular, multi-vacuolar, and ameboid form, by culture and direct microscopy (DM) [6]. The prevalence of *Blastocystis* has been reported to be between 1.5 and 20% in developed countries and 30–60% in developing countries worldwide [7, 8]. In a recent study on children living in rural areas of Senegal, the prevalence of the parasite was found to be 100% [9]. This has been related to unsuitable substructure conditions, low sanitation, contact to animals, and uptake of contaminated food or water [3, 10]. Recently, *Blastocystis* prevalence has been determined at rates ranging from 1.4–23.5% in studies in Turkey [1].

Blastocystis has a highly polymorphic genome and there are many genotypes called subtype (ST) by molecular phylogenetic analyzes [11]. One of the two commonly preferred approaches to identify *Blastocystis* subtypes is the partial sequencing of the small-subunit ribosomal RNA (SSU-rRNA) encoding gene, and the other is the sequence-tagged site polymerase chain reaction (STS-PCR), using primers specific to the subtypes [12]. Recently, there have been 17 established subtypes, along with possibly five new subtypes

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(ST21, ST23–26), based on the SSU rRNA gene analysis of *Blastocystis* that were mostly identified in domesticated and animal wildlife species [7, 12–15]. Although the host specificity of each subtype remains unclear, reporting of nine of the 22 subtypes, ST1-ST8 and ST12, in both humans and animals indicates the zoonotic transmission of this parasite [14, 16]. It has been revealed that ST9 is only isolated from humans [17]. ST1-ST4 includes more than 90% of all reported subtypes. Besides, these four subtypes have been reported more widely in humans than in other hosts. Other subtypes (ST5–ST9) are uncommon in humans [18]. ST3 was reported to be the most common in human *Blastocystis* isolates [19].

Although the presence of *Blastocystis* in both symptomatic and asymptomatic patients leads to a dilemma as to whether it is a pathogen, the parasite is now accepted as a pathogen as well as a parasite included in "Water Sanitation and Health Program" of the World Health Organization [20, 21]. *Blastocystis* pathogenicity and clinical findings have been tried to be explained with many factors. The most important of these are subtypes, presence of ameboid form, and parasitic load [5, 6]. Despite conflicting results between subtypes and clinical findings, ST1, ST4, and ST7 are generally reported to be associated with symptoms, while ST2 and ST3 are reported as nonpathogenic subtypes [22].

Blastocystis has also been suggested to be an opportunistic pathogen in transplant recipients, acquired immune deficiency syndrome (AIDS) and cancer patients [23]. *Blastocystis* studies on ST distribution and pathogenic roles in immunocompromised individuals have been limited to AIDS patients, and there are quite insufficient studies related to these parameters in other immunocompromised individuals such as cancer patients [7, 23]. A study with cancer patients revealed that the infection indicated itself with abdominal pain, diarrhea, and bloating [24]. Subtypes 3 and 4 isolates were reported to be quite common in cancer patients [22, 23, 25]. In another study conducted with cancer patients with *Blastocystis* subtype distribution in Turkey, ST3, ST1, and ST2 were found to be the most common subgroups, respectively [7]. Chandramathi et al. reported that *Blastocystis* had also been in breast and colorectal cancer patients receiving chemotherapy. In the light of they demonstrated that the parasite can resist high cytotoxic drugs and increases colonization by weakening the immunosuppressive state induced by chemotherapy [26]. In a study, severe *Blastocystis* infection was detected in four people with bowel obstruction due to neoplasm. In addition it was recommended in the same study that the intestinal obstruction and concomitant stool retention, in addition to hemorrhage from the neoplasm, may have allowed hypergenesis of *Blastocystis* [7]. All these findings suggest that the parasite develops in cancer patients [22].

Until now, no studies have been conducted on the distribution of *Blastocystis* subtypes in the province of Elazığ located in southern part of Turkey. This is the first study investigating the subtype distribution of *Blastocystis* in cancer patients in our region. In the light of the information presented above, the main aim of the present study was to determine the frequency and subtype distribution of *Blastocystis* in cancer patients. The present study also aims at investigating their relation to demographic factors and the clinical symptoms of *Blastocystis* infection.

Materials and Methods

Design of Experiment

The design of the experiment is shown in Fig. 1.

Sample Collection and Microscopical Examination

This cross-sectional study was carried out between September 2017 and August 2019. Stool specimens of 201 cancer patients (one each) were collected from the Medical Oncology Department of Fırat University Faculty of Medicine, Elazığ, Turkey. Written informed consent was obtained from each of the cancer patients receiving inpatient and outpatient

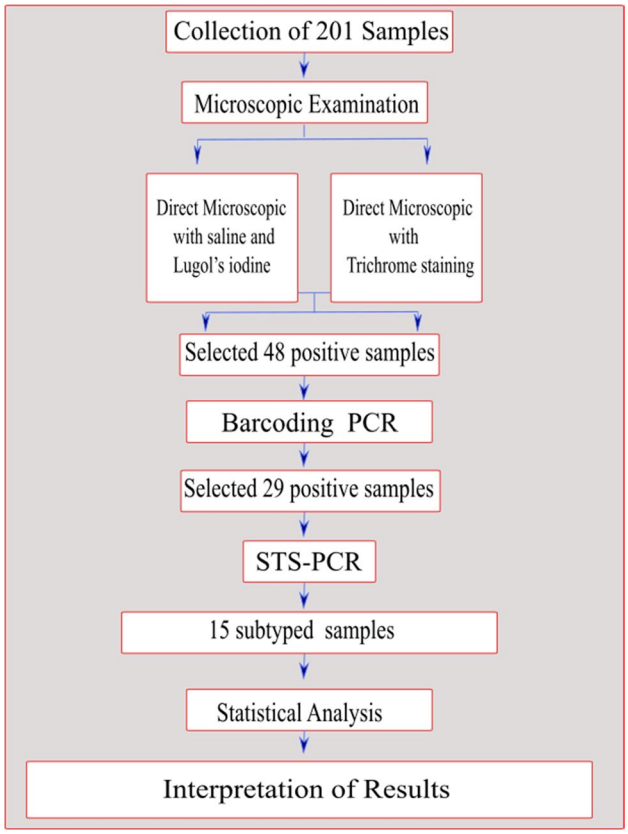

Fig. 1 The design of the experiment

treatment. Each patient was filled a form including demographic information, patient oncological characteristics, and gastrointestinal symptoms. These cancer patients were diagnosed with breast cancer, lung cancer, gastrointestinal cancer, ovarian cancer, and other cancers. These samples were examined by DM with saline and Lugol's iodine and using trichrome staining. Besides, the remaining fresh stool samples were stored at − 20 °C until DNA isolation.

DNA Extraction

Total DNA from stool samples was conducted using the Presto™ Stool DNA Extraction Kit (Genaid, Taiwan) in line with the manufacturer's instructions. Briefly, stool samples homogenization and lysis were performed with Bead Beating Tube and ST1 Buffer. ST2 Buffer was added to the removal step of PCR inhibitors. Residual inhibitors were further removed by passing through a specialized PCR inhibitor removal column. The flow-through was saved in the 2 ml centrifuge tube for DNA Binding. ST3 Buffer was added to flow-through and then was placed a GD Column in a 2 ml Collection Tube. The sample mixture was added to GD Column for DNA binding. It was added to Wash Buffer for removal of contaminants while DNA remains bound to membrane. The dry GD Column was transferred to a new 1.5 ml microcentrifuge tube and pre-warmed elution buffer was added. The resultant DNA was stored at − 20 °C until PCR analysis.

Detection of Subtypes Using PCR Method

In this study, SSU rDNA (barcoding) PCR was performed with to demonstrate the presence of the barcode region of *Blastocystis* [3, 4]. Furthermore, it was demonstrated with sequence-tagged site-PCR using seven pairs of the sequence-tagged site (STS) to investigate the presence of seven subtypes of *Blastocystis* [27]. The primer pairs names, sequences, and predicted product sizes used in this study are shown in Table 1.

PCR targeting 600 bp product of *Blastocystis* SSU-rDNA barcoding region was performed as described using the primers BhRDr and RD5 [12]. The 20 µl reaction mixture included 2.5 µl template DNA, the primers (0.5 µM each), 1U Taq DNA polymerase (Thermo Scientific) 200 mM dNTPs, 3.5 mM $MgCl_2$ and 1 × Taq reaction buffer. PCR was carried out in Arktik™ Thermal Cycler (Thermo Scientific, Massachusetts, ABD) using the following condition: 2 min initial denaturation step at 95 °C followed by 35 cycles of 30 s at 94 °C 30 s 60 °C and 30 s 72 °C and final extension of 5 min at 72 °C. All PCR amplifications with ST-specific primers were carried out Arktik™ Thermal Cycler (Thermo Scientific, Massachusetts, ABD) using the following condition: 2 min initial denaturation step at 95 °C followed by 35 cycles of 30 s at 94 °C 30 s 60 °C and 1 min 72 °C and final extension of 7 min at 72 °C. All PCR products were separated by %2 agarose gel electrophoresis stained by SafeView Classic (Applied Biological Materials Inc., Richmond, Canada) and visualized under Alpha Imager HP (Alpha Innotech).

Statistical Analysis

Statistical analyses of the collected data were performed using Statistical Package for Social Science (SPSS) 22. The level of significance of the relationship between the incidence of *Blastocystis* and the demographic characteristics, clinical findings, location of cancer was determined by the chi-square test or Fischer's exact test. For discrete and continuous variables, descriptive statistics (mean, standard deviation, median, minimum value, maximum value, and

Table 1 Sequencing of specific seven subtypes and *Blastocystis* sp. primers

Primer pair	Subtype	Sequence (5′–3′)	Size (bp)
SB83	ST1	F: AAGGACTCCTGACGATGA R: GTCCAAATGAAAGGCAGC	351
SB340	ST2	F: TGTTCTTGTGTCTTCTCAGCTC R: TTCTTTCACACTCCCGTCAT	704
SB227	ST3	F: TAGGATTTGGTGTTTGGAGA R: TTAGAAGTGAAGGAGATGGAAG	526
SB337	ST4	F: GTCTTTCCCTGTCTATTCTGCA R: AATTCGGTCTGCTTCTTCTG	487
SB336	ST5	F: GTGGGTAGAGGAAGGAAAACA R: AGAACAAGTCGATGAAGTGAGAT	317
SB332	ST6	F: GCATCCAGACTACTATCAACATT R: CCATTTTCAGACAACCACTTA	338
SB155	ST7	F: ATCAGCCTACAATCTCCTC R: ATCGCCACTTCTCCAAT	650
Barcode	*Blastoscystis* sp.	RD5: ATCTGGTTGATCCTGCCAGT BhRDr:AGCTTTTTAACTGCAACAACG	600

percentile) were performed. A value of $p < 0.05$ was considered as statistically significant.

Results

A total of 201 stool samples were enrolled from the cancer patients who were 123 males (61.19%) and 78 females (38.8%) with a mean age (± standard deviation) of 55.44 ± 12.07 years (range 27–84 years). In our study, 48 (23.8%) of the stool samples were found positive by native-Lugol and trichrome staining (Fig. 2). All the microscopy-positive stool samples were examined using barcoding PCR and amplification of expected size (~ 600 bp) was observed in 29 (60.4%) samples. Some of the *Blastocystis* isolates in the present study are shown in Fig. 3. These samples that were found to be positive by barcoding PCR method were screened with the PCR using specific seven types of STS primers to identify subtypes and amplification with subtype-specific primers was observed in 15 (51.7%) of 29 isolates. The remaining 14 isolates found to be positive for *barcoding PCR* could not be subtyped. The most common subtype was ST3 ($n = 6$, 40%) followed by ST2 ($n = 5$, 33%) and ST1 ($n = 3$, 20%), respectively. In addition, one patient revealed combined infections with ST1/ST2 ($n = 1$, 6%) and ST4-7 could not be detected in our study. (Fig. 4).

Demographic characteristics of cancer patients with *Blastocystis* infection are presented in Table 2 and no significant difference was found ($p > 0.05$). When *Blastocystis* infected cancer patients were analyzed in terms of symptomatology, similarly, there was no statistically significant difference (Table 3) ($p > 0.05$). In our study, due to an insufficient number of subtypes, statistical analysis could not be performed between subtypes and symptoms. The incidence of *Blastocystis* infection according to cancer location is presented in Table 4. The gastrointestinal tract, breast, respiratory tract cancers, and urogenital tract cancers accounted for the majority (90%) of the studied patients. In the present study, the rate of *Blastocystis* infection was higher in urogenital tract cancers(32%) as compared to other types.

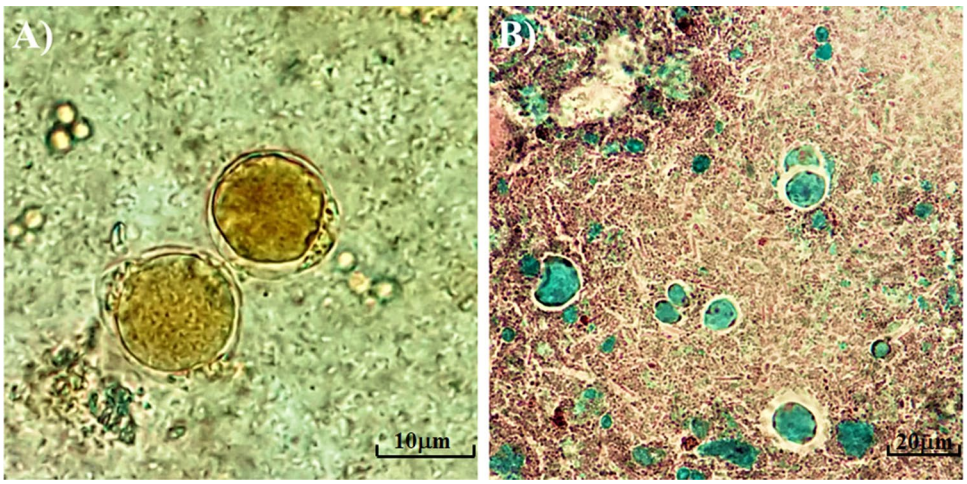

Fig. 2 **a** *Blastocystis* (vacuolar forms) detected by microscopy using Lugol staining (400 × magnification). **b** *Blastocystis* (vacuolar forms) detected by microscopy using Wheatley's Trichrome staining (1000 × magnification)

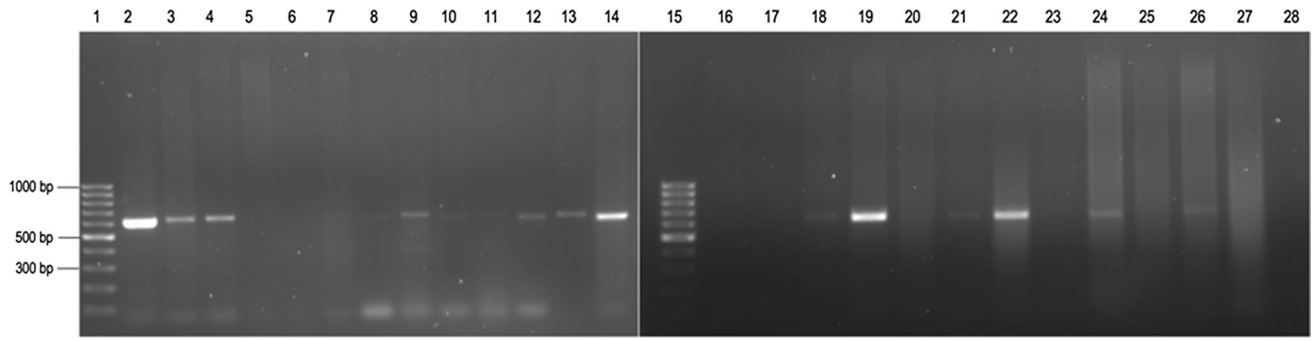

Fig. 3 Partial SSU rRNA gene of *Blastocystis* were electrophoresed on 2% agarose gel. All samples amplified 600 bp amplicon. (Lane 1) shows 100 bp DNA ladder; (Lane 2) represents positive control; (Lanes 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 21, 22, 24, 26) represent positive results for *Blastocystis*; (Lanes 5, 6, 15, 16, 17, 20, 23, 25, 27) represent negative results for *Blastocystis*; (Lane 28) represents negative control

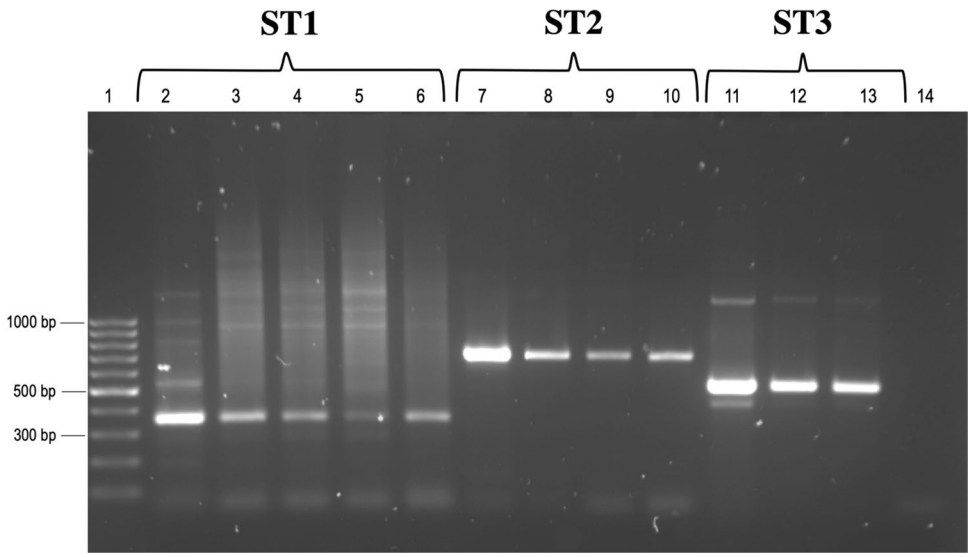

Fig. 4 Isolated of *Blastocystis* were electrophoresed on 2% agarose gel. All samples amplified by the sequence-tagged site (STS) primers. Lanes 1, 100 bp DNA ladder; Lane 2, 7, 11 positive control; Lanes 3–6, subtype 1 (351 bp); Lanes 8–10, subtype 2 (704 bp); Lanes 12–13 subtype 3 (526 bp); Lane 14 negative control

Table 2 Demographic characteristics of cancer patients examined for *Blastocystis* infection

Risk factor	No. examined	No. (%) infected	*p* value
Gender			$p=0.092$
Male	123	15 (12.2)	
Female	78	14 (17.9)	
Age groups			$p=0.3$
27–44 years	32	5 (15.6)	
45–64 years	127	15 (11.8)	
65 +	42	9 (21.4)	
Educational status[a]			$p=0.161$
Low	150	19 (12.7)	
High	51	10 (19.6)	
Residence			$p=0.177$
Urban	123	15 (12.2)	
Rural	78	14 (17.9)	
Animal raising			$p=0.227$
Yes	37	8 (21.6)	
No	164	21 (12.8)	
Drinking water			$p=0.216$
Mains	67	12 (17.9)	
Spring	134	17 (12.7)	
Frequency[b] of eating outside			$p=0.089$
High	46	10 (21.7)	
Low	155	19 (12.3)	

[a]*Low* illiterate or primary school, *High* high school or university

[b]Eating ≥ 3 times a week outside the home was considered "high"

Table 3 Symptomatology and *Blastocystis* infection

Symptoms	All patients	No. (%) infected	*p* value
Abdominal pain			
Yes	163	25 (%15.3)	$p=0.318$
No	38	4 (%10.5)	
Constipation			
Yes	119	15 (%12.6)	$p=0.246$
No	82	14 (%17.07)	
Diarrhea			
Yes	35	4 (%11.4)	$p=0.401$
No	166	25 (%15.1)	
Nausea			
Yes	172	26 (15.1)	$p=0.365$
No	29	3 (10.3)	
Vomiting			
Yes	150	22 (14.7)	$p=0.537$
No	51	7 (13.7)	
Itching			
Yes	88	13 (14.8)	$p=0.529$
No	113	16 (14.2)	
Bloating			
Yes	168	22 (13.1)	$p=0.171$
No	33	7 (21.2)	

Discussion

Cancer is the second leading cause of death globally and there were 18 million cancer cases and 9.6 million cancer deaths worldwide in 2018. It is estimated that by 2040 there will be 254,179 new cancer cases and 22,609 cancer deaths in Turkey [28]. Protozoa, such as *Cryptosporidium parvum, Microsporidia* (*Encephalitozoon spp.*, *Enterocytozoon bieneusi*)., *Cyclospora cayetanensis*, *Isospora belli,* and *Giardia intestinalis* are opportunistic microorganisms that cause serious manifestation in immunosuppressed patients such as cancer, HIV/AIDS patients, and transplant recipients [23].

Table 4 Cancer location and *Blastocystis* infection

Cancer location	*Blastocystis*		Subtype				
	No examined	No. (%) infected	ST1	ST2	ST3	ST1 + ST2	unknown ST
Gastrointestinal tract	69.5 (7.2%)		–	1	–	–	4
Breast	60.11 (18.3%)		3	3	2	–	3
Respiratory tract	27.3 (11.1%)		–	–	–	–	3
Urogenital tract	25.8 (32%)		–	1	4	1	2
Hematological tract	15.1 (6.7%)		–	–	–	–	1
Others	5.1 (20%)		–	–	–	–	1
Total	201.29 (14.4%)		3	5	6	1	14

Recently, the number of studies reporting the prevalence of *Blastocystis* infection in immunocompromised individuals has increased [29]. In addition, *Blastocystis*' impact on immunosuppressed patients has been rebated in the recent years, primarily due to its unknown pathogenicity and incidence frequency [23]. In the present study, *Blastocystis* was investigated among cancer patients using two different methods: DM and PCR based diagnosis. Although *Blastocystis* was seen in 48 of the samples by direct microscopy in our study, 29 (60.4%) of these samples by barcoding PCR gave amplicon of the expected size. A negative result was obtained in 15 isolates (31.3%). This can be attributed to the fact that some living or non-living factors such as fungi, macrophages, neutrophils, and *Cyclospora sp*. or fat globules in the stool cause false positivity in the DM method [30, 31]. Furthermore, the lack of consensus on which *Blastocystis* to be reported above which threshold value (> 5 cells, in a 40X objective area) also reduces the reliability of the results obtained from direct microscopy [11]. Some studies have also reported false-negative results with PCR due to inhibitors in the feces [32, 33]. It is also considered that the sensitivity of the PCR method is greatly influenced by the DNA extraction procedure [34].

In 2011, Yoshikawa et al. used different commercial DNA extraction kits in stool samples and reported that their detection sensitivity was different [35, 36]. The DNA isolation kit we used in our study contains an inhibitor removal column that absorbs PCR inhibitors. For this reason, the DNA isolation kit we use is thought to reduce or eliminate negative results from PCR inhibition. Based on this, in our study, it is thought that some patients who were found positive with the DM method were found to be negative with PCR due to false positives in the DM method. However, the BhRDr-RD5 primer pair used in the barcoding PCR method is not completely specific to *Blastocystis* since they amplify *Blastocystis* SSU-rDNA and SSU-rDNA from other eukaryotes, especially fungi, in the absence of *Blastocystis*. If these primers are used to screen fecal DNAs for *Blastocystis*, a certain false-positive rate may be predicted, and sequencing should often confirm positivity [37]. In sum, the whole prevalence of *Blastocystis* with barcoding PCR in our study was 14.4%, regardless of subtypes. This result is in accord with the prior studies declared a prevalence of 13–16% in cancer patients [24, 25]. Other studies in cancer patients in different regions of Turkey and the world, the prevalence ranged from 7.1–21.1% [7, 23, 38–40]. According to the results of the aforementioned studies, the rates are not similar in different regions. This may be due to weather conditions, public health, food, and different cultural habits, demographic factors as well as differences in diagnostic tests [41].

The *Blastocystis* subtype distribution in 29 isolates were listed as ST3 (6 samples 40%), ST2 (5 samples 33%), ST1 (3 samples 20%). Besides, more than one subtype (ST1 + ST2 6.6%) was detected in one sample. A thorough evaluation of the studies in the literature demonstrated that ST1, ST2, ST3, and ST4 constitute 90% of the *Blastocystis* infections in humans and, the most common subtype was ST3 [33, 42, 43]. Similar to the studies in the literature, the subtype distribution of our study consisted of ST1, ST2, and ST3. Some studies on *Blastocystis* subtype distribution in cancer patients have shown that ST3 is more common [7, 23, 40]. Consistent with these studies reported in cancer patients, the most common subtype ($n = 6$, 40%) was ST3 in our study. In line with the studies in the literature, Poirier et al. (2011) reported in their study that ST4 was the most common subtype followed by ST3 and ST7 [25]. Furthermore, Mohamed RT et al. (2017) reported that that ST2 was the most common subtype, followed by ST1 and ST5 [44]. However, ST4, ST5, ST6, and ST7 subtypes were not detected in our research though they were reported in the previous studies [7, 19, 32, 45].

One of the significant results is that no subtype could be detected in 14 samples (48.3%) which were found to be positive with barcoding PCR. In Turkey, this rate was found to be 27.9% by Ertug et al. [1] 35% by Korkmaz et al. [32] and 12% by Yersal et al. [7]. However, it was reported to be 3% by Yoshikawa et al. [27] 4% by Li et al. [46] and 42% by Zhan et al. [47] in different parts of the world. The researchers pointed out the PCR inhibitors in the stool as

the most important factor in creating this condition [32]. Furthermore, it was thought that in the presence of new subtypes, STS primers may be due to disability [32, 37]. DNA sequence analysis is a more useful method in determining whether there is a different subtype in samples that cannot be subtyped [4]. However, DNA sequence analysis could not be performed in our study due to financial insufficiencies.

Similar to the results obtained in cancer patients by Zhang et al. [39], we found no significant correlation between the rate of infection and the demographic factors (age, gender, residence et al.) and also the rate of infection was higher in women and patients from rural in both studies. However, according to another study in cancer patients, it has been shown that infection rates are remarkably higher in men and patients in urban areas [7]. According to the survey data in our study, it was found that the patients with *Blastocystis* had symptoms of abdominal pain, nausea, itching, vomiting, bloating, constipation, and diarrhea, respectively. In another study conducted in patients in the oncology service, similar to our study, it was reported that abdominal pain, bloating and constipation observed more frequently in cases with *Blastocystis* [7]. However, in contrast to our study, Zhang et al. showed that *Blastocystis* was found more frequently in cancer patients with diarrhea [39]. It is accepted that the reason for this difference in clinical findings in people with *Blastocystis* depends on many factors such as genotype of infection, microbiota, host immune response, *Blastocystis* density, and other concomitant infections [11, 48, 49].

Similar to the previous reports, statistical analysis could not be performed between subtypes and symptoms due to the insufficient number of cases whose subtypes were determined in our study [7, 50]. Also, there was no significant difference between cancer type and the rate of *Blastocystis* infection, which was supported by previous studies [39, 44, 51]. However, Yersal et al. [7]. reported that the rate of infection in patients with lung cancer was significantly higher than in other types of cancer [41]. Besides, in this study, the highest and lowest *Blastocystis* infection rate was reported in urogenital system (32%) and hematological system (6.7%) cancer patients, respectively.

Conclusion

In summary, we showed that cancer patients in the present study had a *Blastocystis* prevalence of 14.4% and that ST3 was the most predominant subtype. In addition, the present study provides the first subtype distribution of *Blastocystis* detected in cancer patients residing in our region. The absence of a relationship between *Blastocystis* infection and any gastrointestinal symptoms supports the idea that *Blastocystis* infections are asymptomatic and that this protozoan parasite can exist as normal flora. However, molecular-based studies in cancer patients are very limited. Therefore, to determine *Blastocystis* subtype profile in cancer patients and to fully elucidate the relationship between subtypes and pathogenicity, more studies should be done in different regions of Turkey.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval The present study was approved by Fırat University Clinical Research Ethics Committee (Protocol No: 2017/14-03).

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