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ORIGINAL PAPER

Determination the Subtypes of *Blastocystis* sp. and Evaluate the Effect of These Subtypes on Pathogenicity

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Abstract

Purpose The present study aimed to determine the frequency of subtypes of *Blastocystis* sp. in the immunosuppressed individuals, in patients with chronic urticaria, and in patients with GIS complaints to investigate the difference of *Blastocystis* sp. subtype distribution between patient and control groups.

Methods A total of 345 stool samples were collected from the patients and samples were studied by native-Lugol, trichrome staining, and Jones medium culture method. Positively detected samples were subjected to PCR to determine the subtypes.

Results This is the first study of nine subtypes of *Blastocystis* sp. investigated in our country and the most frequently found subtype was ST3, and then, the other subtypes were ST1, ST2, ST5, and ST6, respectively. Mix subtype was detected in the 11.6% and no subtype was detected in the 17.4% of the samples. The ST5 was detected first time in the control group and ST6, which is reported limitedly in our country, was found in patients with GIS complaints. ST1 and ST2 were found higher in the patient group.

Conclusion This study confirmed that the subtype (ST) differences are an important factor affecting the pathogenesis of *Blastocystis* sp.

Keywords *Blastocystis* sp. · Subtypes of *Blastocystis* · Pathogenicity · Polymerase chain reaction

Introduction

Blastocystis is an agent widely found in the world, living in the large intestine of humans and animals. Taxonomic classification is still controversial even today. As a result of recent studies and the application of modern phylogenetic techniques such as the study of 18S rRNA, *Blastocystis* has been placed into Stramenopile species [5]. It has been determined that humans and many animal species carry this parasite as a natural host and that animals infect certain subtypes to humans. Nine (subtype 1–9) out of 17 subtypes that were also stated by other articles and subtype 12 that was specified by a group have been detected in this study. Those subtypes have found to cause infection in humans [22, 25]. There are six different morphological forms of *Blastocystis* sp. These are vacuolar, avacuolar, multivacuolar, granular, ameboid and cyst forms [3].

Today, the discussions continue as to whether *Blastocystis* sp. is a pathogenic agent or not. Studies conducted in recent years support the view that *Blastocystis* sp. is a pathogenic agent [3, 31]. *Blastocystis* sp. has been reported to be associated with certain patient groups, especially gastrointestinal system (GIS) complaints, urticaria and irritable bowel syndrome [3, 6, 21, 31, 37].

One of the most discussed issues in this area is the identification of the factors affecting the pathogenicity of parasites [3, 27]. Currently, the factors affecting the pathogenesis of *Blastocystis* sp. are shown by various enzymes, various structures with immunomodulatory effect and cell surface structures [2, 31]. The question of why these structures, which destroy the connective tissues of human intestinal cells and activate the mechanisms of the immune system, do not have the same effect on all infected people with *Blastocystis* sp. is one of the most controversial topics for the researchers today. Here are two basic

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approaches. The first is that different morphological forms may be associated with pathogenicity. Although studies have reported that certain morphological forms such as the ameboid form are more infectious, it is reported that all forms may cause diseases [31]. The second is that certain subtypes are more related to infection. At present, the most accepted approach to the pathogenicity of *Blastocystis* sp. is that subtypes are the most important determinant of this pathogenicity [32]. The number of the studies that were carried out in this area has been increasing day by day. However, this issue has not been finalised yet; therefore, it is necessary to support this claim with new studies.

Our aim in this study is to investigate the relationship between subtype differences and pathogenicity, one of the controversial areas related to the pathogenicity of *Blastocystis* sp. The aim of this study was to investigate the frequency of *Blastocystis* sp. in three groups of patients with GIS complaints, chronic urticaria patients and immunodeficient individuals; and to determine whether there is a difference in the distribution of *Blastocystis* sp. subtypes between the symptomatic patient group and the control group.

Materials and Methods

This study was approved by the local Ethical Committee of Diyarbakır Dicle University Medical Faculty (No. 2015/188) and written informed consent was obtained from all patients. Between January 01, 2015 and September 30, 2016, the stool samples of 264 patients who applied to the Dicle University Medical School Gastroenterology, Dermatology and Oncology policlinic, and as the control group, 81 stool samples of patients who did not have any gastroenteritis and urticaria complaints and who had no immunosuppressive disease were included.

The collected stool specimens were first screened by the native-Lugol method. As a second method, positive samples were examined by trichrome staining method. In addition, each sample was added to Modified Jones Medium, and a second validation method was applied. The samples, which were positive for direct observation and culture, were stored at − 20 °C until DNA was obtained.

DNA Isolation Process

DNA was obtained from the faeces using a DNA isolation kit (Gene MATRIX Stool DNA Purification Kit, Poland). The insulation procedure was carried out in accordance with the operating procedures specified in the insulation kit.

Detection of Subtypes Using PCR Method

Samples that were positive for direct observation and culture were studied with the general primer *Blastocystis* sp., and the presence of Blastocyst was determined at the genus level [36]. *Blastocystis* sp. subtypes were detected from samples with general positive primer. For the detection of *Blastocystis* subtypes, STS primers were used, which were developed by Yoshikawa et al., and used in nine types of subspecies which were found to infect human beings [40].

All PCR amplifications with ST-specific primers were accomplished in a 10-µl volume including 2 µl of the template DNA (5 µg/ml), 1 × Ex Taq buffer, 0.2 U of TaKaRa Ex Taq® (Takara Bio Inc., Japan), 0.5 pM primers, and 0.2 mM dNTP mixture. The PCR conditions used were 94 °C for 5 min, followed by 38 cycles at 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, then 72 °C for 10 min for the final extension. The amplicons were examined in 1.5% agarose gels stained with ethidium bromide with a 50-bp ladder marker (50-bp to 1.5-kbp; GeneDirex Taiwan). DNAs processed according to agarose gel weights were transferred to a UV gel imaging system (Quantum ST4). The bands formed according to the weights of the DNAs were examined under the UV light. The detected bands are shown in Fig. 1.

Statistical evaluation was not performed because the numerical values of the subtypes obtained at the end of the study were insufficient for statistical evaluation. The results are considered fold values when compared to each other.

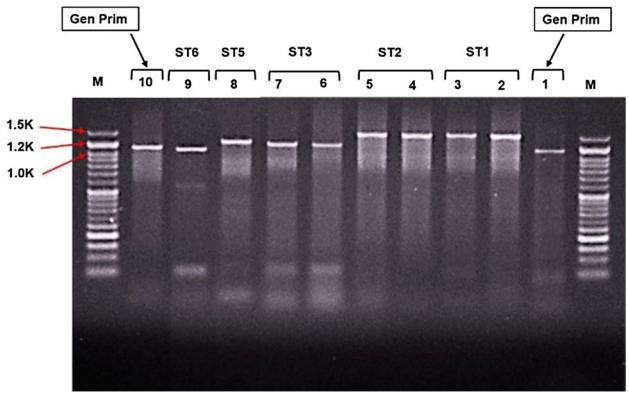

Fig. 1 Agarose gel images of DNA fragments. Lane M, DNA ladder (50 bp); DNA fragments in lanes 1 and 10 generated by general primers (1100 bp), PCR products monitorized in other lanes generated by subtype-specific primer pairs: lanes 2 and 3, subtype 1 (1500 bp). Lanes 4 and 5, subtype 2 (1500 bp). Lanes 6 and 7, subtype 3 (1200 bp). Lane 8, subtype 5 (1200 bp). Lane 9 subtype 6 (1050 bp)

Results

In the study, 69 (20%) of the 345 samples collected from Gastroenterology policlinics, Dermatology policlinics, Oncology clinics and healthy subjects were found positive.

21 (16.15%) of the 130 stool samples collected from the gastroenterology polyclinic of patients with GI complaints and 18 (22.5%) of the 80 stool specimens collected from patients with urticaria—diagnosed from the dermatology policlinic—and 12 of the 54 stool samples collected from patients receiving radiotherapy in the oncology clinic (22.2%) were positive in terms of *Blastocystis* sp. Of the 81 stool samples collected from the control group of healthy subjects without any complaint, 18 (14.6%) were found to be positive.

In our study, 69 samples, which were found to be positive in native-Lugol, trichrome and culture examinations, were studied using PCR method with general primers of *Blastocystis* and all were found positive.

The distribution of 69 *Blastocystis* sp. primers as positive for the subtypes was as follows: ST3 (60.9%) was detected as the most common; after ST3, ST1 (17.4%), ST2 (17.4%), ST5 (1.4%) and ST6 (1.4%) were detected, respectively.

More than one subtypes were detected in seven (10.1%) of the samples. Four samples had ST1 and ST3, one had ST1 and ST2, one had ST2 and ST3, and one had ST1, ST2, and ST3 among these seven samples that were observed to have more than one subtype. No subtypes were found in 12 (17.4%) of the 69 samples that were found to be positive for *Blastocystis* sp. general primers.

Comparisons of the *Blastocystis* sp. subtypes of the symptomatic patient group with the control group of the healthy subjects revealed the following results.

ST 1: There were three (25%) in the group with GIS complaints, four (33.3%) in the chemotherapy group, four (33.3%) in the urticaria group and one (8.3%) in the control group (Table 1).

ST 2: There were four (33.3%) in the GIS complaints group, five (41.7%) in the chemotherapy group, two (16.7%) in the urticaria group and one (8.3%) in the control group (Table 1).

ST 3: There were 13 (31.0%) in the group with GIS complaints, 5 (11.9%) in the chemotherapy group, 10 (23.8%) in the Urticaria group and 14 (33.3%) in the control group (Table 1).

One ST5 was seen in the control group, and one ST6 was detected in the group of patients with GIS complaints. Because these subtypes were inadequate in numbers, no comparison could be made with the control group. ST4, ST7, ST8 and ST9 could not be detected in our study.

In this study, comparing the symptomatic patient group with the control group, ST1 and ST2 were found higher in the symptomatic group of patients and ST3 was found higher in the control group.

Discussion

The rate of *Blastocystis* sp. was 20% in our study. *Blastocystis* incidence rates ranged from 2.1 to 34.16% in studies in our country [9, 15, 16, 30, 38]. When the carried-out studies are evaluated, it is seen that the rate of *Blastocystis* sp. changes according to the level of development levels of the provinces and whether the study is performed in the province centre or in the rural areas. In terms of the prevalence of the *Blastocystis* sp. in the society, we have a similar result between the ratios determined by the other studies that are carried out in the city centres of our country. When studies on the world are examined, *Blastocystis* sp. is more common in developing countries than in developed countries [29].

Table 1 Distribution of *Blastocystis* sp. subtypes in patients and control groups

	Gastro		Chemotherapy		Dermatology		Control		Total	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%
ST1	3	25.0	4	33.3	4	33.3	1	8.3	12	100
ST2	4	33.3	5	41.7	2	16.7	1	8.3	12	100
ST3	13	31.0	5	11.9	10	23.8	14	33.3	42	100
ST5	0	0	0	0	0	0	1	100	1	100
ST6	1	100	0	0	0	0	0	0	1	100
ST1 ST2	0	0	0	0	1	100	0	0	1	100
ST1 ST3	1	20	0	0	3	60	1	20	5	100
ST2 ST3	1	100	0	0	0	0	0	0	1	100
ST1 ST2 ST3	0	0	1	100	0	0	0	0	1	100
Non ST	4	33.3	0	0	6	50	2	16.7	12	100
Total	27	30.7	15	17	26	29.5	20	22.7	88	100

The results we achieved in our study are consistent with the rates seen in developing countries [7, 31, 41].

The most important point in the evaluation of epidemiological data of *Blastocystis* sp is to determine the distribution at the subtype level. Because there are certain subtypes in *Blastocystis* sp., studied subjects were evaluated as apathogenic and pathogenic [18, 31]. Investigation of the prevalence of these subtypes is more meaningful in determining the risk levels of that region.

In our study, the frequencies of *Blastocystis* subtypes were determined as ST3, ST1, ST2, ST5, and ST6, respectively. In other studies, ST3, ST1, ST2 and ST4, respectively, have been identified as the most common subtypes. These findings that we have achieved in our study are similar to the other studies carried out in our country and in the world [8, 10–12, 23, 24, 34].

In our study, similar results were obtained for ST3, ST1 and ST2, while ST4 could not be determined. This may be due to regional differences in the distribution of subtypes of *Blastocystis* sp. In our country, there are studies where ST1 and ST2 are detected more than ST3. In their study, Eroglu et al. [13] found ST2 and Koltas et al. [19] found ST1 as the most common subtype. Although ST3 is the most common subtype in our country, it is understood that ST1 and ST2 may be the most common subtypes according to the region in which the study group resides and according to the symptomatic or asymptomatic states of the individuals in this group. One ST6 was found in the patient group with GIS complaints. ST5 and ST6 have been reported in very limited numbers in our country and they have been detected for the first time in our region. These data are important to show the distribution of *Blastocystis* sp. subtypes in our country.

In our study, although we found positivity with *Blastocystis* sp. general primers, it is one of the interesting results that no subtype can be detected in 12 specimens. No subtypes were detected in 6 out of 18 patients with chronic urticaria complaints, 4 out of 21 patients with GIS complaints, and 2 out of 18 healthy control group, who were identified to be positive in terms of *Blastocystis* sp. general primers. All patients in the chemotherapy-treated group were infected with at least one subtype. In studies conducted in our country and in the world, different non-subtype ratios are seen. This rate was found to be 27.9% by Ertug et al. [14] and 12.7% by Lee et al. [20]. Tan et al. found two non-subtype species in a study of patients with immunosuppression, and that the cause of this condition might be a wrong isolation process or unknown subtypes [33].

In this study, nine of the ten known subtypes that mostly infect humans were examined. In 17.4% of the studied groups, which is remarkably high, no subtype was detected. Studies in this area show the problems that can occur in DNA isolation from the stool [31, 33]. We consider that there is no problem in the isolation process in our study since all samples were detected to be positive in terms of *Blastocystis* sp. general primers. This gives us an idea that there could be other subtypes becoming infectious in humans, and there might be a higher number of the subtypes of *Blastocystis* sp. that could be infectious in humans in the future studies. Furthermore, in our study, the non-subtype ratio in the urticaria group was seen at a very high rate of 50%. However, no comparison was made in the investigations, as there was no study investigating the non-subtype ratio in certain patient groups. However, in a disease where immune modulators such as urticaria are very effective, there is a high probability of subtypes carrying different mechanisms that can alter the human immune mechanism.

In our study, ST1 and ST2 were higher in the symptomatic group when the symptomatic group and the control group were compared (Table 1). Stensvold et al. and Tan et al. reported that ST1 is associated with symptomatic cases, in their study that they investigated the relationship of subtypes with symptomatic cases [28, 32]. Our findings also support this result.

In ST2-related studies, different results were found. In a study in which subtypes were investigated for pathogenicity, Dogruman et al. [10] reported that ST2 was an asymptomatic subtype. Hussein et al. [17] concluded that ST2 was not a potential pathogen in a study investigating the relationship of certain subtypes to pathogenicity. In contrast, Vogelberg et al. concluded that ST2 was involved in the development of urticaria and GIS complaints. Again, they indicated that the DNA sequence analyses of ST2 that they isolated from this patient group were similar to the DNA sequence analyses of ST2 from the same patient group in the Danish gene bank. In the discussion of this study, Vogelberk stated that the reason for the association of ST2 with symptomatic and asymptomatic cases in different studies may be due to differences in genetic and/or morphological variation in ST2 [35].

At present, one of the most discussed topics related to *Blastocystis* sp. is pathogenicity and subtype relation. These data we have obtained support ST1 as it is done by Tan et al. and Stensvold et al. and that ST2 may also be associated with pathogenicity, as reported by Vogelberg.

In our study, there is no indifference found in the distribution of ST3 between symptomatic and asymptomatic groups (Table 1). Studies have indicated that ST3 is the most frequently isolated, more anthropogenic subtype of humans, and may be present in symptomatic and asymptomatic individuals [7, 23, 24, 31]. The findings we have in our study are similar to those of our country and the world.

In this study, one ST5 was detected in the control group. ST5 has been reported in a very limited number of countries. It was first identified in a study conducted by Koçtaş et al. in 2016 and no ST5 notification was made [19]. ST5 was generally associated with symptomatic patients [4]. However, in our study, ST5 was detected in the control group.

The ST5 we identified was a person who lived in rural areas and was in close contact with animals for this reason. This strengthens the possibility that ST5, which we have identified, is contaminated in an animal-derived manner. Studies in the world show that ST5 is an animal-derived subtype and that bad hygiene plays a very important role in contagion [32, 39].

ST6 was identified in a group of patients with a GIS complaint. In our country, ST6 was detected in symptomatic patients by Dağcı et al., in patients with GIS complaints by Adıyaman et al. and in symptomatic patients by Koltas et al. [1, 8, 19]. Rene et al. [26] found an ST6 in a patient complaining of constipation in a study of patients with cysts in faeces. These studies are consistent with our work. When all these studies are evaluated together, it is understood that ST6 is seen in a group of patients with a GIS complaint, and has a potential for pathogenesis.

In the study, when the control group was compared with the patient group, ST1 and ST2 were determined high in the patient group. In ST3 distribution among the groups, close results were obtained. To determine the association of these subtypes with diseases and the virulence factors, further study in this area and subtypes should be investigated by animal experiments.

In this study, no subtypes were detected in 17.3% of the positive samples of *Blastocystis* sp. general primers, despite studying nine subtypes infecting in-house. In the studied samples, it was evaluated that the reason for not being able to detect the subtype at a high rate of 17.3% was that it could be the subtypes that are currently infecting human but not detected. Non-subtype samples should be evaluated by sequence analysis methods as to whether they are a different subtype that cannot be detected today.

In conclusion, in many in vivo and in vitro studies performed in this area, serious findings have been obtained that *Blastocystis* sp. carries various virulence factors that may cause infection and is a pathogenic agent. However, even nowadays, it is seen that there are studies that contradict this situation and contradict one another. Therefore, to avoid conflicting reports on the pathogenesis of *Blastocystis* sp., it is necessary to work with better standardised animal models and techniques by making axenic cultures of *Blastocystis* sp.

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