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Original Article

Molecular Identification, Subtypes Distribution, and Alleles Discrimination of *Blastocystis* sp., Isolated from Immunocompromised Subjects in Iran

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Received 15 Oct 2021

Accepted 06 Jan 2022

Keywords:

Blastocystis sp.;
Immunodeficiency;
Opportunistic infection;
Subtypes;
Allele discrimination;
Iran

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Abstract

Background: *Blastocystis* sp., is a prevalent protist isolated from humans and animals, which its opportunistic role in immunocompromised patients is still controversial. The current study aimed to evaluate the subtype and alleles distribution of *Blastocystis* sp., among immunocompromised patients.

Methods: Totally, 33 microscopically *Blastocystis*-positive stool samples, isolated from Guilan province during April 2018 to May 2019 were investigated. Total DNA extraction was performed and the barcoding region of the small subunit ribosomal RNA (SSU rRNA) gene was amplified. Targeted fragments were sequenced to characterize subtypes and relevant alleles. Phylogenetic tree was constructed using Maximum-likelihood and Tamura 3-parameter to illustrate the correlation between subtypes and certain immunodeficiency.

Results: Subtype analysis revealed the presence of ST1, ST2, ST3, and ST7 among 13/33 (39.4%), 5 (15.2%), 14/33 (42.4%), and 1/33 (3%), of samples, respectively. ST1 was the major subtype among cancer patients 5/7 (71.42%), while ST3 was the predominant subtype among rheumatoid arthritis (RA) patients 3/6 (50%), internal ward patients 5/10 (50%), and asthma and allergy patients 2/3 (66.66%). ST7 was isolated from a patient hospitalized in internal ward. No significant correlation was seen between the type of immunodeficiency and subtypes (P -value = 0.771). The phylogenetic tree showed no separation regarding the type of immunodeficiency.

Conclusion: Among studied immunocompromised patients, ST3 was the most prevalent subtype followed by ST1. There was no specific correlation between subtypes and alleles with type of immunodeficiency. Putative zoonotic alleles were highlighted the probability of zoonotic transmission for *Blastocystis* sp.



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Introduction

Blastocystis sp., is one of the most common parasite, which colonizes the intestine of its hosts. It is a cosmopolitan protist with a potential for pandemic distribution (1, 2). This parasite is reported from wide range of animals including amphibians, reptiles, invertebrates, birds, non-human primates, and artiodactyls, as well as humans (3, 4). Four major forms of this parasite including vacuolar, granular, amoeboid, and cyst have been reported from stool samples and cultured mediums (2). *Blastocystis* sp., was classified initially as a cyst of a flagellate, vegetable, fungus, and harmless yeast for many years, but it is now considered as an agent, which may lead to intestinal or extra-intestinal manifestations (5, 6). The fecal-oral route is considered as the main transmission mode of infection (7).

A couple of clinical features have been linked to *Blastocystis* sp., ranging from mild and chronic diarrhea to acute gastroenteritis, anemia, and urticarial (6, 8, 9). During recent years, the reports of *Blastocystis* sp., in symptomatic patients without any known causative agents signified the pathogenic role of *Blastocystis* sp., (10, 11). However, the pathogenicity of this parasite is still matter of debate and it mostly leads to a generally self-limiting gastrointestinal disorders (12, 13).

By study on the small subunit ribosomal RNA (SSU-rRNA) gene, 17 specific subtypes (ST) of *Blastocystis* sp., have been identified in humans and a wide range of animals (14, 15). *Blastocystis* sp., subtypes (ST1-ST9 and ST12) are isolated from human and animals, while the reports of ST9 is limited to humans (1, 16, 17). Subtypes ST10 to ST17 have been identified exclusively in non-human hosts including non-human primates (NHPs), mammals, birds and insects (15). The recent updates suggested the presence of at least 22 subtypes including ST21 and ST23-ST26 in mammals (18).

The pathogenicity of *Blastocystis* sp. seems to be affected by both host and parasite factors; however, evaluation of the pathogenic potential of certain subtypes is difficult (19). A probable correlation between specific subtypes and clinical manifestations has been proposed (20), and few studies suggested the presence/majority of certain subtypes in patients with background diseases or specific symptoms (21, 22).

In the current study, we aimed to evaluate the subtype distribution and frequency of relevant alleles among *Blastocystis* sp., isolated from different hospitalized immunocompromised patients receiving corticosteroids.

Materials and Methods

Ethics approval

This study received ethical approval from the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran (no. IR.SBMU.RIGLD.REC.1398.048).

Sample collection and DNA extraction

Totally, 33 microscopically *Blastocystis*-positive stool samples, isolated from Guilan province during April 2018 to May 2019, were investigated (23). Briefly, 250 μ L of stool suspensions was centrifuged at $2500 \times g$ for 5 min, supernatant was discarded and total DNA was extracted using stool DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran). Extracted DNA was stored at -20°C until further molecular analysis.

PCR amplification

The ~ 620 -bp fragment of the barcoding region of the SSU rRNA gene of *Blastocystis* sp., was amplified using primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') and

BhRDr (5'-GAGCTTTTAACTGCAACAACG-3') (24). PCR amplifications were done at standard conditions: an initial denaturing step of 95 °C for 5 min and 35 cycles consisting of 94 °C for 30 s, 58 °C for 30 s, and 30 s at 72 °C. A final extension at 72 °C was performed for 5 min. Finally, 5 µL of amplification products was fractionated on 1.5% agarose electrophoresis gel stained with ethidium bromide and then visualized by the UV transilluminator (Cleaver scientific Ltd., Warwickshire, United Kingdom).

Subtype analysis and allele discrimination

PCR products were purified and then sequenced with ABI 3130 sequencer. The obtained sequences were edited and trimmed by Chromas and BioEdit software. Homology analysis of the sequences was done using the basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/>) for the most similar reference sequences to determine the relevant subtypes. Finally, the new DNA sequences have been submitted into the genetic sequence database at the national center for biotechnical information (NCBI) by the Sequin program (version 10.3). All sequences are available in the GenBank database with accession numbers MT645783 to MT645814. To identify the relevant alleles, the edited sequences were submitted to (<https://pubmlst.org/organisms/blastocystis-spp/>).

Phylogenetic analysis

After alignment and trimming of the sequences, a ~ 530-bp fragment was utilized for analyses. The phylogenetic tree was constructed using Maximum-likelihood algorithm and tamura-3- parameter model in MEGA10 software (<http://www.megasoftware.net/>) (25). To evaluate the reliability of the tree, bootstrap with 1000 replications was considered.

Statistical analysis

The frequency of subtypes and gender, and the mean of age \pm standard deviation (SD) were calculated. In addition, to analyze the possible correlation between immunodeficiency and subtypes, the Fisher's exact test incorporated in SPSS Statistics software for Windows, v22 (IBM Corp., Armonk, NY, USA) was used. A probability (*P*) value < 0.05 was considered statistically significant.

Results

The studied patients were 16 (48.5%) males and 17 (51.5%) females. The average age \pm SD was 51.82 ± 13.17 . *Blastocystis* sp., was isolated from hospitalized patients, who received either corticosteroids or chemotherapy agents, including one (3%) transplantation recipient, six (18.2%) rheumatoid arthritis (RA), three (9.1%) gastrointestinal disorders, three (9.1%) systemic lupus erythematosus (SLE), seven (21.2%) cancer patients, three (9.1%) asthma and allergy patients, and 10 (30.3%) patients hospitalized in internal ward with undiagnosed immunodeficiency disorder.

Molecular detection, subtyping, and allele discriminations

Target fragment was amplified among all 33 microscopically *Blastocystis*-positive samples. Subtypes analysis revealed the presence of ST1, ST2, ST3, and ST7 among 13/33 (39.4%), 5/33 (15.2%), 14/33 (42.4%), and 1/33 (3%), of samples, respectively. The subtypes distribution among patients showed the majority of ST1 among cancer patients 5/7 (71.42%), while ST3 was the predominant subtype among RA patients 3/6 (50%), internal patients 5/10 (50%), and asthma and allergy patients 2/3 (66.66%). ST2 was seen among RA, internal, gastrointestinal, SLE, and transplantation patients. ST7 was isolated from a patient hospitalized in internal ward (Table 1).

The results of allele discrimination revealed the presence of alleles 4 (10/13) and 88 (3/13) in ST1. ST2 exhibited alleles 9 and 10 in four and one isolates, respectively (Table 2). ST3

represented alleles 34 and 36 with majority of allele 34 (10/13). The only ST7 was allele 99 (Fig. 1).

Table 1: *Blastocystis* sp., subtype distribution among immunocompromised patients

<i>Immunodeficiency disorders</i>	<i>Subtypes/No.33</i>			
	ST1	ST2	ST3	ST7
RA	2	1	3	-
Internal	3	1	5	1
Gastrointestinal disorders	1	1	1	-
SLE	1	1	1	-
Cancer patients	5	-	2	-
Asthma and allergy	1	-	2	-
Transplantation	-	1	-	-

Table 2: *Blastocystis* sp., and its subtypes among immunocompromised patients

<i>No.</i>	<i>Gender</i>	<i>Age(yr)</i>	<i>Blastocystis sp., (subtypes)</i>	<i>Alleles</i>	<i>Acc. No.</i>
1	Female	48	ST2	9	MT645783
2	Female	59	ST3	36	MT645784
3	Male	59	ST7	99	MT645785
4	Male	71	ST2	9	MT645786
5	Female	58	ST3	34	MT645787
6	Male	38	ST2	10	MT645788
7	Female	48	ST3	34	MT645789
8	Female	53	ST1	4	MT645790
9	Female	43	ST3	36	MT645791
10	Female	36	ST3	34	MT645792
11	Male	38	ST2	9	MT645793
12	Female	58	ST1	88	MT645794
13	Male	29	ST3	36	MT645795
14	Male	70	ST1	4	MT645796
15	Female	47	ST2	9	MT645797
16	Male	59	ST1	4	MT645798
17	Female	52	ST3	34	MT645799
18	Female	35	ST3	34	MT645800
19	Female	45	ST1	4	MT645801
20	Male	59	ST3	34	MT645802
21	Male	61	ST3	34	MT645803
22	Male	71	ST1	4	MT645804
23	Female	58	ST1	88	MT645805
24	Female	50	ST1	4	MT645806
25	Male	52	ST3		Not provided
26	Male	57	ST3	34	MT645807
27	Female	26	ST1	4	MT645808
28	Male	34	ST1	4	MT645809
29	Male	58	ST1	4	MT645810
30	Female	52	ST1	4	MT645811
31	Female	61	ST3	34	MT645812
32	Male	86	ST3	34	MT645813
33	Male	39	ST1	88	MT645814

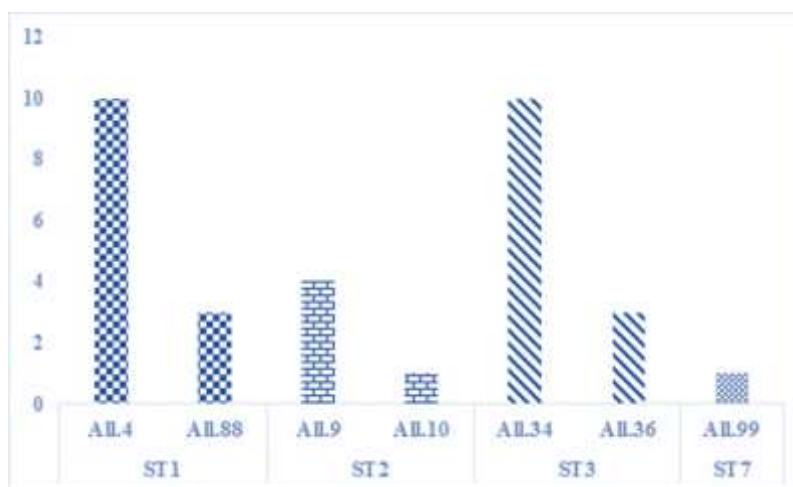


Fig. 1: Allele distribution among each studied subtype

Phylogenetic analysis

Phylogenetic analysis of the SSU rRNA gene sequences revealed that all subtypes were clearly separated into four clades regarding the currently characterized subtypes, with boot-

straps ranging from 77 to 99% (Fig. 2). The phylogenetic tree also showed that there was no separation regarding the type of immune diseases or hospitalization's wards.

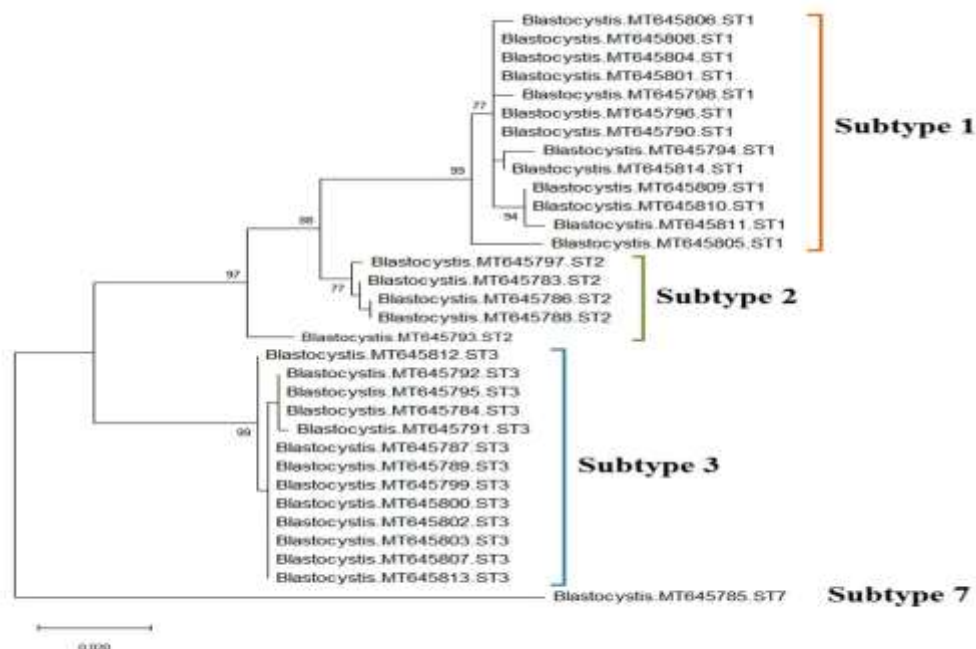


Fig. 2: The phylogenetic position of *Blastocystis* sp., ST1-3 and 7 isolated from immunocompromised patients. The phylogenetic tree was assembled based on the Maximum-likelihood and Tamura 3-parameter algorithms. Bootstrap lower than 75% were deleted

Discussion

Blastocystis sp., is a mysterious protist with a lot of unknown features in its life cycle, infectivity, and pathogenicity. The distribution of this protist is thought to be linked with the socioeconomic conditions (7), but a couple of studies indicated a high prevalence of *Blastocystis* sp., in developed countries with high levels of standard of living (26-30).

The pathogenicity of *Blastocystis* sp., is also unclear. Although some studies showed a correlation between the presence of this eukaryote and gastrointestinal, as well as extra-intestinal symptoms (6, 8, 9), most of researches have been failed to provide a strong correlation between symptoms and the presence of *Blastocystis* sp. In this line, Jalallu et al., (31) investigated the frequency of *Blastocystis* sp., and its subtypes in symptomatic and asymptomatic human subjects and claimed no significant coexistence between the protist and clinical manifestations. Dogan et al., (32) studied the prevalence of *Blastocystis* sp. among symptomatic and asymptomatic children and reported no statistical correlation between the presence of symptoms and *Blastocystis* sp. In a large-scale study conducted in France, a statistical significant correlation was not seen between the colonization of *Blastocystis* sp., and clinical manifestations (33). However, there are studies that suggested a linkage between the presence of *Blastocystis* sp., or a specific subtype with clinical symptoms. Abdulsalam et al. (34) screened outpatients who were referred to a laboratory and claimed that the prevalence of *Blastocystis* sp., in symptomatic group was higher than asymptomatic group. In addition, in a study in Lebanon the correlation between *Blastocystis* sp., subtype 1 and clinical manifestations was pointed out (20).

Although the pathogenic role of *Blastocystis* sp., in immunocompetent subjects has been remained controversial, a couple of studies suggested *Blastocystis* sp., to be an opportunistic infection in immunocompromised patients.

One of the first studies highlighting the opportunistic role of *Blastocystis* sp. was performed by Ghosh et al, (35) who presented a case of myeloid leukemia underwent bone marrow transplantation, which was colonized by *Blastocystis* sp. In the current study we did not access to stool samples of immunocompromised patient hospitalized in different wards to evaluate the prevalence of the protist; nevertheless, *Blastocystis* sp., ST2 was the only subtype detected in the transplant recipient.

The correlation between colonization of *Blastocystis* sp., and its subtypes with cancer has been evaluated. Kumarasamy et al (36), showed a significant correlation between *Blastocystis* sp., and colorectal cancer (CRC) and presented a significant association between the presence of ST3 and CRC. A high frequency of *Blastocystis* sp., ST3 was determined in cancer patients in Turkey (37), as well. Indeed, Mohamed et al., (38) analyzed the correlation between colonization of *Blastocystis* sp., and its subtypes with cancer and showed that not only colonization of *Blastocystis* sp., in cancer patients was higher than controls, but also there was a significant correlation between the presence of subtype 1 and CRC. Zhang et al. (39), characterized the prevalence rate of *Blastocystis* sp., subtypes among cancer patients and although there was no statistically significant correlation between subtypes and cancer, ST3 was the most prevalent subtype in these patients followed by ST1. In the current study, a significant correlation was not found between *Blastocystis* sp., and its subtypes with type of immunodeficiency; however, in line of study performed by Mohamed et al. (38), *Blastocystis* sp., ST1 was the most prevalent subtype in cancer patients followed by ST3.

Although some studies linked the presence of *Blastocystis* sp., particularly ST3, with urticarial and cutaneous rashes (22, 40, 41), there is no study evaluating subtype distribution of *Blastocystis* sp., among patients who suffered from asthma and allergy, and SLE. In the current study, only ST1 and ST3 were detected in asthma and allergy patients, while all three

subtypes ST1-3 were characterized in SLE patients. However, due to the low number of evaluated subjects, establishing a subtype pattern in these patients, particularly among asthma and allergy patients, needs to be validated.

To the best of our knowledge, there is no study evaluating the subtype distribution of *Blastocystis* sp., among RA patients. Nonetheless, Lee et al. (42) presented a case of RA with acute diarrhea and increased inflammation harboring *Blastocystis* sp., who after successful treatment of the protist with metronidazole, the symptoms such as diarrhea, abdominal pain, and inflammation of patient's knee were ameliorated. In the current study, three subtypes ST1-3 were characterized in RA patients; however, due to the low number of samples, this study was failed to propose a probable connection between certain subtype and RA.

As a result, ST1 represented alleles 4 and 88. Allele 4 seems to be the most prevalent allele related to ST1 (43). However, there is a little data presenting allele 88 from ST1 in the world (44). For ST2, alleles 9 and 10 were identified among samples. Allele 9 was characterized as one of the most prevalent alleles reported from humans and also dogs in Iran (43, 45). This allele was also reported from other countries and seems to be the common characterized allele from ST2. In contrast, allele 10 is not common in human subjects and there is no report of this allele in Iran, while it was reported from South America (4). For ST3, alleles 34 and 36 were identified among samples. These alleles are among the most frequently reported alleles in human and animal subjects. However, in the line of previous studies, allele 34 was the most prevalent allele in our subjects. ST7 represented allele 99. There are reports of this allele in humans and animals. Melo et al. (46) successfully cultivated microscopically *Blastocystis*-positive fecal samples and isolated *Blastocystis* ST7 allele 99 from asymptomatic Brazilian subjects. In Iran, Mohammadpour et al. (45) investigated the preva-

lence of *Blastocystis* sp., from cats, dogs, and brown rats, and isolated ST7 allele 99 from dog samples. Most recently, ST7 allele 99 was isolated from chicken sample in Iran (47). Although reports of this allele is limited to couple of studies, the presence of that in both animal and human isolates may provide a clue of zoonotic transmission of this allele.

Conclusion

Blastocystis sp., ST3 and ST1 were characterized as the most prevalent subtypes among studied immunocompromised patients of which ST3 was the most prevalent followed by ST1. Although ST1 was more prevalent in cancer patients and ST3 was the major subtype in RA patients, asthma and allergy patients, and those who were hospitalized in internal ward, a significant correlation between certain subtypes and type of immunodeficiency was not seen. The allele discrimination showed none specific alleles among immunocompromised patients, while putative zoonotic alleles were also detected.

Acknowledgements

The authors thank all members of the Foodborne and Waterborne Diseases Research Center for their supports. This study was financially supported by the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences with grant number: RIGLD-1072.

Conflict of interest

The authors declare that they have no conflict of interest

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