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Blastocystis subtype 1 (allele 4); Predominant subtype among tuberculosis patients in Iran

Ali Taghipour[a,b], Ehsan Javanmard[c], Hamed Mirjalali[c,*], Ali Haghighi[d,*], Payam Tabarsi[e], Mohamad Reza Sohrabi[f], Mohammad Reza Zali[g]

[a] Student Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[c] Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Department of Medical Parasitology and Mycology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Department of Infectious Diseases, Mycobacteriology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[f] Social determinants of Health Research Center and Department of Community Medicine, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[g] Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: *Blastocystis* and tuberculosis are two public health issues that are frequently reported in regions with low level of hygiene. Therefore, the current study aimed to investigate *Blastocystis* subtype and allele distribution in TB patients.

Methods: Totally, 161 stool samples were taken from TB patients who were undergoing anti-MTB treatment. Stool samples were concentrated using conventional formalin-ether technique and examined using Lugol's iodine staining under light microscopy. DNA extraction was carried out and discriminative fragment was amplified and sequenced. With comparison in GenBank database, relevant subtypes and alleles were characterized and phylogenetically analyzed using MEGA v.7 and Tamura 3-parameter model.

Results: In total, from 161 stool samples, 19 samples were suspected to be *Blastocystis*-positive. The expected fragment was amplified in 13 (8.07%) of samples. Accordingly, 11/13 (84.62%) of *Blastocystis* cases settled in urban and 2/13 (15.38%) were villagers. Close-contact with animals was also seen among 7/13 (53.84%) of samples. Subtype 1 (7/13; 53.84%) was the most prevalent followed by subtype 2 (5/13; 38.46%) and subtype 3 (1/13, 7.69%). All ST1 were allele 4, while alleles 9, 11 and 12 were seen in ST2 and allele 34 was the only allele observed in ST3. All three subtypes were clearly separated, while there was no separation between sequences from TB and non-TB patients.

Conclusion: *Blastocystis* ST1 was the most prevalent subtype in TB patients and there was no difference between *Blastocystis* isolates from TB and non-TB human subjects.

1. Introduction

Tuberculosis (TB) resulted from *Mycobacterium tuberculosis*, is one of the most important life-threating public health issues in the world, particularly in regions with low level of sanitation [1–3]. It is estimated that the incidence of this disease in Iran is about 16 cases per 100,000 in each year [4]. Epidemiological studies have shown that unsanitary living conditions can enhance the risk of both TB and intestinal parasites [5–7]. *Blastocystis* is a prevalent intestinal parasite in humans and animals [8–10] that transmits via fecal-contaminated food and water [10–12]. However, prevalence of this protozoan parasite reflects sanitary conditions of a community [10,11].

Blastocystis is a molecularly divergent microorganism that based on the signature region of small subunit ribosomal RNA (SSU rRNA) gene, 17 subtypes (STs) have been characterized in human and animal hosts [13,14]. Despite numerus studies on the distribution of *Blastocystis* and related risk factors, there is no strong evidence signifying the pathogenic role of this protozoan in human cases [15–18]. It seems that non-

* Corresponding authors.
E-mail addresses: hamed_mirjalali@hotmail.com, hamedmirjalali@sbmu.ac.ir (H. Mirjalali), ahaghighi110@yahoo.com (A. Haghighi).

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specific gastrointestinal symptoms such as diarrhea, constipation, abdominal pain, nausea and vomiting together with extra-intestinal manifestations, particularly urticarial, are the main complications reported from infected patients [17,19–22]. However, there is no convincing evidence establishing linkage between presence of certain subtype and clinical manifestations [15–17].

More recently, it was suggested that the levels of sanitation, income and education of a community affect the prevalence rate of *Blastocystis* [23]. On the other hand, the incidence rate of TB shows that this infection is more common in regions that suffer from poverty, malnutrition and low level of hygiene [2,24,25]. Although couple of studies highlighted the negative-correlation between some intestinal protozoan parasites and TB infection [6,7], there is no data about the co-incidence as well as subtypes and allele distribution of *Blastocystis* and TB. Therefore, the current study aimed to investigate the presence of *Blastocystis* in TB patients and characterize subtypes and alleles of *Blastocystis* in these patients.

2. Materials and methods

2.1. Study population

This study had received ethical approval from the Ethics Committee of the Shahid Beheshti University of Medical Science (SBMU), Tehran, Iran. (No. IR.SBMU.MSP.REC.1395.323). During Apr 2016 to Oct 2017, stool samples were taken from 161 patients admitted to Masih Daneshvari Hospital, referral tuberculosis center in Iran, with confirmed TB infection who were undergoing anti-MTB treatment [26,27]. In order to confirm TB infection, Ziel-Neelsen staining and sputum culture in Lowenstein-Jensen were performed. Those TB patients who had received anti-parasitic drugs during the month prior to the study were excluded from study. All patients filled and signed a consent form and standardized questionnaire regarding the socio-demographic features, risk factors and clinical symptoms related with *Blastocystis*.

2.2. Sample collection and parasitological analysis

To assess presence of *Blastocystis*, all stool samples were concentrated using conventional formalin-ether technique and examined using Lugol's iodine staining. Prepared slides were examined under light microscopy (Zeiss, Germany) with 10X, 40X and 100X objective magnification.

2.3. DNA extraction PCR and subtyping

DNA extraction was carried out for samples that were determined positive for *Blastocystis* with light microscope. For this propose, total DNA was extracted from 200 mg of stool specimens using the DNA isolation stool mini kit (Yekta Tajhiz Azma Co., Iran), according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until PCR analysis.

Blastocystis-specific DNA was amplified primers RD5 (5′-ATCTGGT TGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGCAACA ACG-3′) [28]. For amplification of targeted fragment, the following protocol was used: denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. A positive sequenced isolate as positive and sterile distillated water as negative controls were used in each PCR run together with all samples.

Subsequently, 10 µL of each PCR product was electrophoresed on a 1.5% agarose gel. Amplicons were stained with 0.5 µg/mL ethidium bromide and visualized using a UV Transilluminator (Cleaver scientific Ltd., Warwickshire, United Kingdom).

2.4. Phylogenetic analysis

All PCR products were sequenced using BHRD5 primer and ABI 3130 sequencer. The obtained sequences were edited and trimmed using Chromas and BioEdit software. To characterize the subtypes and relevant alleles, all sequences were compared in Basic Local Alignment Search Tool (BLAST) and Sequence Typing (MLST) database (http://pubmlst.org/blastocystis/), respectively. In addition, in order to identify potential correlation between *Blastocystis* isolates from TB patients with those from non-tuberculosis subjects, phylogenetic analysis was performed using Molecular and Evolutionary Genetic Analysis (MEGA v.7) software [29]. For this propose, Maximum-Likelihood algorithm and Tamura-3-parameter model were employed to draw phylogenetic tree. In order to assess reliability of the tree, bootstrap with 1000 replications was employed.

2.5. Statistical analysis

To evaluate the statistical correlation between subtypes and socio-economic factors, Fisher's exact test and Chi-square incorporated in SPSS v.22 (SPSS, Chicago, IL, USA) were employed. *P*-value > 0.05 was considered as statistically significant.

3. Results

The study population consisted of 161 confirmed TB patients (43.5% male, 56.5% female), with a mean age of 53.2 ± 19.1 years. From 19 (11.8%) of microscopically positive samples [27], 13 (68/42%) were molecularly positive. In addition, 11/13 (84.62%) of *Blastocystis*-positive cases settled in urban and 2/13 (15.38%) were villagers. Close-contact with animals was also evaluated and the results showed that 7/13 (53.84%) of *Blastocystis*-carriers with tuberculosis were in close-contact with animals. Risk factors and clinical symptoms related to *Blastocystis*-carriers suffered from tuberculosis are

Table 1
Distribution of *Blastocystis* subtypes and alleles in patients with tuberculosis based on demographic data, socio-economic factors, clinical symptoms.

No.	Sex/ Age	Residence	Occupation	Contact with soil	Contact with animals	Symptoms	Subtype	Alleles	Acc. No.
1	Male/ 59	Urban	Self-employed	No	No	Anemia	1	4	MH656710
2	Male/ 51	Urban	Self-employed	No	Yes	Nausea and vomiting	1	4	MH656713
3	Female/ 54	Urban	Housewife	No	No	Asymptomatic	1	4	MH656715
4	Male/ 62	Rural	Farmer	Yes	Yes	Diarrhea	1	4	MH656708
5	Female/ 35	Urban	Housewife	No	No	Asymptomatic	1	4	MH656717
6	Male/ 39	Urban	Self-employed	No	Yes	Anemia	1	4	MH656714
7	Female/ 55	Urban	Housewife	No	Yes	Abdominal pain	1	4	MH656707
8	Male/ 63	Rural	Shepherd	Yes	Yes	Asymptomatic	2	9	MH656709
9	Male/ 38	Urban	Self-employed	No	No	Constipation	2	9	MH656711
10	Female/28	Urban	Housewife	No	Yes	Diarrhea	2	9	MH656716
11	Male/ 42	Urban	Self-employed	No	Yes	Asymptomatic	2	11	MH656712
12	Female/ 30	Urban	Self-employed	No	No	Asymptomatic	2	12	MH656718
13	Female/ 52	Urban	Housewife	Yes	No	Diarrhea	3	34	MH656719

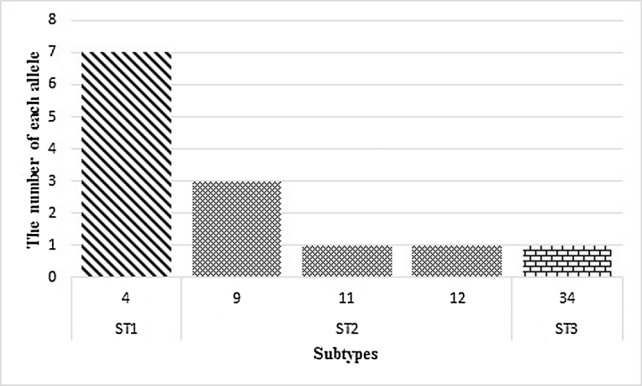

Fig. 1. Allele distribution among *Blastocystis* subtypes 1–3 isolated from TB patients.

summarized in Table 1.

3.1. Subtyping and allele analysis

All positive samples were successfully sequenced. The results of comparison of sequences in BLAST showed that subtype 1 (7/13; 53.84%) was the most prevalent followed by subtype 2 (5/13; 38.46%) and subtype 3 (1/13, 7.69%). Furthermore, allele analysis showed that all ST1 were allele 4, while alleles 9, 11 and 12 were seen in ST2 and allele 34 was the only allele observed in ST3 (Fig. 1). Analysis the correlation between socio-economic factors and subtypes showed that there was no statistically significant association between certain subtypes and the factors (Table 2).

3.2. Phylogenetic analysis

To calculate within subtype similarity, multiple alignment was performed that the results showed similarity 99.56% and 99.36% among ST1 and ST2, respectively. Phylogenetic analysis and comparison of the current sequences with those previously reported from non-TB patients revealed that all three subtypes were clearly separated (Fig. 2). Accordingly, it was no separation between sequences from TB and non-TB patients.

4. Discussion

During the past two decades, studies on molecular epidemiology, pathogenicity and subtype distribution of *Blastocystis* have dramatically increased that the various hosts, high prevalence even in developed countries and unknown facts in patho-physiology of this protozoan were the main reasons [30–34]. The frequency of *Blastocystis* infection varies from country to country, different regions of a country and studied populations. However, it seems that the prevalence of this protozoan is more than 50% of studied populations, particularly in undeveloped region with low-level of income and hygiene [35]. Nonetheless, concerning high prevalence of this protozoan in some European countries [36–40], apparently, blastocystosis is not a poverty-related infection. Therefore, apart from socio-economic status, some other factors such as geographic area, culture, and lifestyle of a community may play key role in distribution of this protozoan [23,41,42].

Table 2
Frequency of *Blastocystis* subtypes in relation with demographic and socio-economic factors among tuberculosis patients.

Variables		ST1	ST2	ST3	*P*-value
Gender	Male	4	3	–	1
	Female	3	2	1	
Residence	Urban	6	4	1	1
	Rural	1	1	–	
Occupation	Housewife	3	1	1	0.621
	Self-employed	3	3	–	
	Farmer	1	–	–	
	Shepherd	–	1	–	
Contact with soil	Yes	1	1	1	0.388
	No	6	4	–	
Contact with animals	Yes	4	3	–	1
	No	3	2	1	
Symptoms	Yes	5	2	1	0.728
	No	2	3	–	

P-value < 0.05 was statistically significant.

Co-infection of TB and intestinal parasites was evaluated during recent years [5–7]. Notably, most of studies suggested that infection with parasitic helminth may increase the risk of development of TB infection, and vice versa [43,44]. Contrastingly, almost all of these studies concluded that protozoan parasites such as *Giardia* and *Blastocystis* have negative-correlation with TB [7]. In a case-control study in Peru, Franke et al. [7] showed that presence of *Blastocystis* strongly reduced the risk of TB infection. Nonetheless, they claimed that either chronic asymptomatic infection with *Blastocystis* may support immune system against TB infection or immune response resulted from TB infection may protect the patients from *Blastocystis*. Controversially, in another study by Li et al, [6] *Blastocystis* was the most prevalent protozoan reported in TB patients and they showed that presence of *Blastocystis* and other intestinal parasites did not have negative- or positive-correlation with TB infection. In the current study, 13/161 samples were molecularly positive for *Blastocystis* that was significantly lower than the prevalence rate of this protist among healthy subjects in Iran [15–17].

In the current study, we assessed subtype and allele distribution of *Blastocystis* among TB patients. Interestingly, in this study ST1 was the most prevalent subtype, while ST3 was only reported from one TB patient. In Iran, several studies assessed subtype distribution of *Blastocystis* among healthy controls [15,16,32], patients with inflammatory bowel syndrome (IBS) [45] and patients who suffer from inflammatory bowel diseases (IBD) [46]. However, it seems that ST3 is the most prevalent subtype in Iran, followed by ST2 and ST1 [23]. Importantly, some studies have statistically concluded that ST1 in symptomatic and IBS patients is higher than other STs and thus, this subtype was described as the pathogenic subtype [21]. In this study, *Blastocystis* ST1 was isolated from both symptomatic and asymptomatic subjects. However, it does not seem that there was correlation between subtype and clinical manifestations in TB patients. Therefore, these hypothesizes may arise that 1) some *Blastocystis* subtypes probably are more resistant to drugs that are routinely prescribed in TB patients and 2) TB infection and prescribed drugs can alter gut microbiota community towards providing a niche favoring for certain subtypes.

In the current study, phylogenetic analysis was employed to assess the similarity between *Blastocystis* isolates from TB patients and those obtained from non-TB patients. Based on our findings, phylogenetic tree did not show classification between subtypes isolated from TB patients and non-TB subjects. These results are in line with previous studies that indicated no linkage between genetic diversity across discriminative fragment of SSU rRNA gene and pathogenic potential of the subtypes [16,32].

Furthermore, allele's characterization showed that alleles 4, 9 and 34 were the most prevalent alleles among ST1, ST2 and ST3, respectively. As explained by previous studies, alleles may provide more data about pathogenicity, geographical distribution as well as hosts specificity of isolated *Blastocystis* [8,47–49]. It was suggested that allele 4 is the most prevalent allele reported from ST1. Alfellani et al. [8] claimed that allele 4 was responsible for more than 95% of all human cases of ST1. After that, Rezaei Riabi et al. [32] reported alleles 4 among 92.4% of ST1s that were obtained from symptomatic and asymptomatic human subjects in Iran. In the current study, allele 4 was the only allele isolated from ST1 in TB patients. In ST2, allele 9 was the major allele in

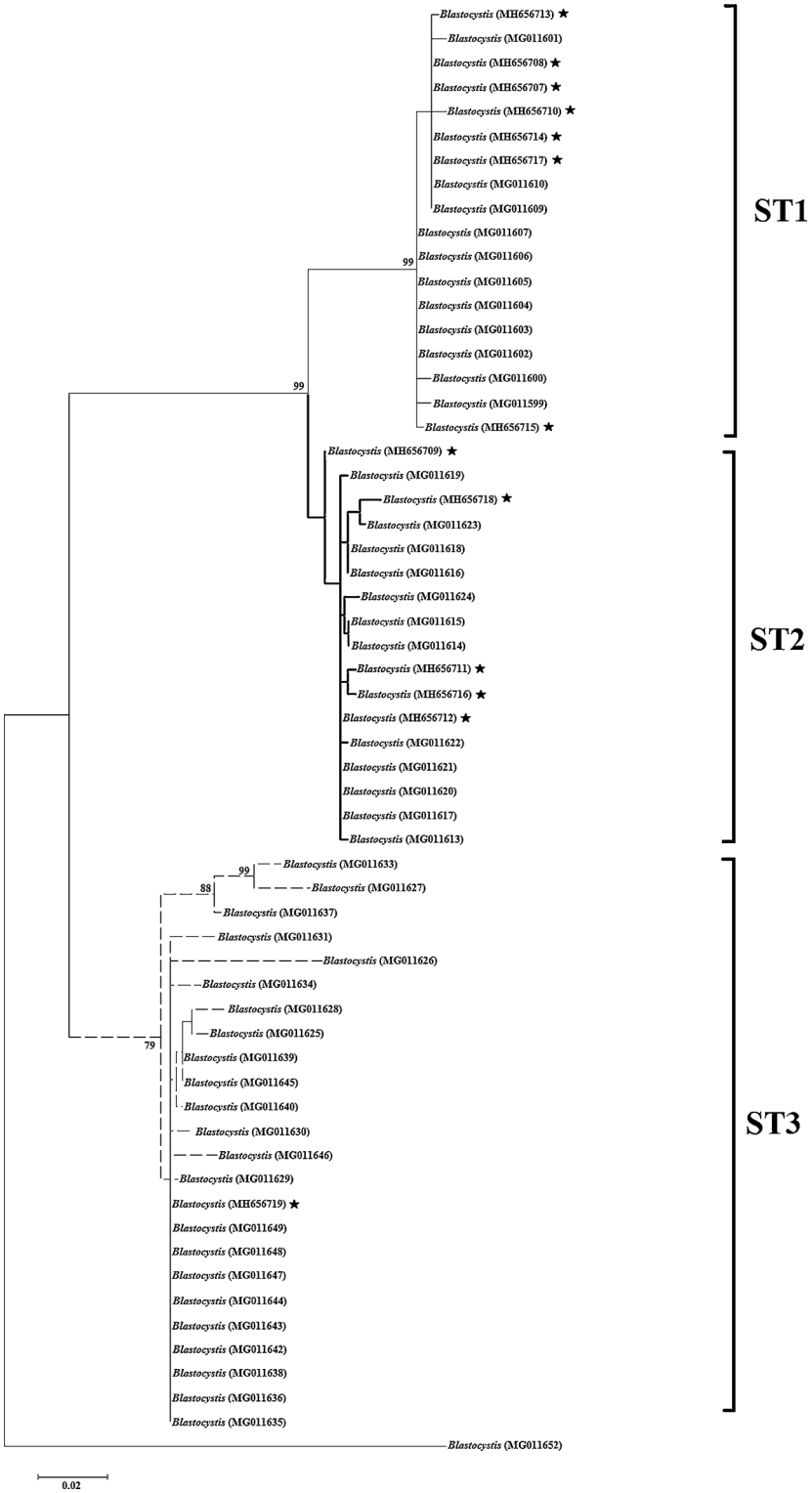

Fig. 2. Phylogenetic analysis of "barcoding region "from the SSU rRNA gene of *Blastocystis* isolated from TB patients and non-TB subjects. The tree was generated based on the Maximum-Likelihood test and the Tamura 3-parameter model using MEGA7 software. The number above the branch mentions the percentage of bootstrap. Branches without numbers have bootstrap values 75%. Tuberculosis isolates are indicated black-filled triangle.

TB patients. Although, more recently, Rezaei Riabi stated that allele 11 is probably the major allele in ST2s in Iran [32], in another studies allele 9 was the most prevalent allele retrieved from ST1s [47–49]. Interestingly, it was suggested that allele 9 is responsible for zoonotic transmission of *Blastocystis* ST2 [49]. Finally, allele 34 was the only allele isolated from ST3 in the current study. However, this allele seems to be the major allele reported from ST3 all over the world [32,47–50]. In the line with other studies, all reported alleles in this study probably did not have host-specificity that this finding may reflect the poor hygiene conditions and low levels of socio-economic factor in living areas of carriers.

In the current study, from nineteen microscopically positive

samples, thirteen samples were amplified using used primers. It was suggested that suggested primers for amplifying the barcoding region of SSU rRNA gene probably work better for extracted DNA from cultivated samples in comparison with stool samples [51]. This result was previously reported by Melo et al. [52] who could amplify targeted gene of *Blastocystis* from only 47/60 microscopically positive. However, it seems that destruction of DNA during DNA extraction, the low number of *Blastocystis* and also misdiagnosis of the protozoan were the main reasons of false-negative in our study [18,38].

However, hygiene conditions together with socio-economic factors of a living area are likely the most important co-factor for co-existence of *Blastocystis* and TB infection in a population.

5. Conclusion

According the results of the current study, *Blastocystis* subtype 1 was the most prevalent subtype obtained from TB patients. Based on phylogenetic analysis, there was no difference between *Blastocystis* isolates from TB and non-TB human subjects. Furthermore, molecular analysis showed that probably, hygiene conditions and socio-economic factors are the most important co-factor for co-existence of *Blastocystis* and TB infection in an area.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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