Original Article

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Subtypes Identification and Frequency of *Blastocystis* Isolated from Patients in Kashan, Central Iran

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Abstract

Aims: *Blastocystis* species are one of the most common enteric protist infections in humans and some animals worldwide. Molecular studies have shown that there is a high level of genetic variation among *Blastocystis* isolates. The aim of this study was to identify the subtypes and frequency of *Blastocystis* isolates in patients who referred to the medical diagnostic laboratories in Kashan, Central Iran. **Materials and Methods:** This cross-sectional study was conducted on 1118 patients, from December 2017 to June 2018. Fecal specimens were evaluated by the microscopic examination. Positive samples were cultivated in Robinson media. After massive growth and DNA extraction, a 550 bp from the small subunit ribosomal RNA gene was amplified by the polymerase chain reaction (PCR) for subtype identification. The PCR products have been sequenced, identified, and compared at the NCBI site. The results were analyzed using the SPSS software version 16. **Results:** The frequency of *Blastocystis* sp. was 8.58%, (confidence interval = 6.94%–10.22%) from which 76% were men and 24% were women. Of the 51 PCR positive samples, ST3 (41.2%), ST1 (39.2%), ST2 (11.8%), and 7.8% isolates were identified as mixed. ST3 and ST1 have been more common. The highest levels of infection were observed in the food-handlers, the age group of 31–40, and people with high school education. **Conclusion:** The results showed that the frequency of *Blastocystis* was lower than other studies and the most common *Blastocystis* subtype was subtype 3, followed by subtype 1, and subtype 2.

Keywords: *Blastocystis,* human, Iran, Kashan, polymerase chain reaction, subtype

Introduction

Blastocystis sp. is a prevalent parasite across the world and as the most frequent eukaryotic organism, it is identified in the stool samples of humans and other animals.[1] The disease caused by *Blastocystis* sp. (Blastocystosis) is largely dependent on its reproduction and population number in the intestine. It is also nonpathogenic in low traces.[2] In terms of life cycle, *Blastocystis* as a monoxenous parasite and infection in human and animals happens by eating its cyst form (fecal-oral route). The prevalence of the parasite is reported to be between 0.5% and 23% in developed countries,[3] while it is more than 60% in developing countries.[4,5] A recent study in Iran reported a prevalence of *Blastocystis* sp. 19.76% in patients with inflammatory bowel syndrome (IBS) and 17.2% in healthy controls.[6]

Molecular studies of *Blastocystis* sp. isolated from the human and animals and also the research on the small subunit ribosomal RNA (SSU-rRNA) gene of this organism announced the presence of different subtypes of this organism. Using amplification and sequencing of a discriminative fragment of SSU-rRNA gene, 17 subtypes of *Blastocystis* named ST1 to ST17 are isolated and identified.[7] The findings of these studies reported the isolation

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of ST1 to ST9 subtypes in human. Moreover, a recent study on human subtypes of *Blastocystis* in South America showed that ST12 may occur in humans.[8] Except ST9, all other subtypes have been found in animals. ST10 to ST17 subtypes are only for animals, and they have not been isolated from human. It seems that the prevalence of subtypes is under the control of climate, weather, animal diversity, and some other epidemiologic factors.[7,9] Some clinical and epidemiological studies have proven their pathogenicity, while some other studies disagree with this matter.[10] In recent years, by conducting exponential studies on this parasite, the potential pathogenicity of the parasite has been emphasized in healthy individuals,[11,12] as well as in patients who suffer from IBS, urticarial, anemia, and gastrointestinal disorders,[6,10,12] and also in patients with kidney transplant treated by immune-suppressing drugs.[13] Considering its genetic diversity, it seems that the pathogenicity of the parasite is related to a specific subtype of the organism, but there is no strong evidence of the correlation between certain subtypes and clinical manifestations.[6,7,9,12]

Kashan is one of the most touristic cities in Iran. The number of tourists visiting this city is high, thus considering the transmission of this parasite and the identification of its subtypes is important in this city. The present study was conducted to determine the frequency and type of *Blastocystis* subtypes in the Kashan, Central Iran.

Materials and Methods

Area study

With a total area of 4415.07 km^2, Kashan is located in the Central Iran and north of the Isfahan province, between 50° 55' and 52° 29' East longitude and 33° 30'–34° 27' North latitude next to the Kavir desert (dasht-e Kavir). Due to its altitude, this region has two relatively different climates: Moderate climate in the mountainous areas and hot and arid climate in the deserts. The city's annual precipitation is 45.61 mm, and its average annual temperature is 19.7°C.

Sampling

This cross-sectional study was conducted on 1118 patients who referred to 12 different medical diagnostic laboratories in Kashan from December 2017 to June 2018. The samples were transferred to the Department of Parasitology of Kashan University of Medical Sciences and examined.

Culture

The fresh stool samples were examined using microscopic observations with direct smear and formalin–ethyl acetate concentration method.[3] *Blastocystis* sp. that infected specimens without associating with other parasites was cultivated in the Robinson culture medium.[14]

After mass culture, the parasite was isolated from the culture medium, washed off with a sterile ringer solution and centrifuged for 1.5 min at 2000 rpm three times. Approximately 2 ml of each positive sample sediment was stored at − 20°C for the molecular analysis.

DNA extraction

Genomic DNA has been extracted from the samples using a DNA extraction kit DNP (Cinaclon, Iran). The spectrophotometer was used to determine the amount of DNA extracted.

Polymerase chain reaction amplification

One set of oligonucleotide primer[15,16] was used to perform the polymerase chain reaction (PCR) on the extracted DNA. Using the pair primer (Forward: 5′-GGA GGT AGT GAC AAT AAA TC-3′-Reverse: 5′-TAA GAC TAC GAG GGT ATC TA-3′), a 550 bp fragment of SSU rDNA length was obtained to confirm the *Blastocystis* genus.

PCR in the final volume of 25 µl contained 0.5 µl of parasite DNA, 10 picomol of forward and Reverse primers, 10 µl of master mixes (1.5 Mm Mgcl2), (Pishgam, Iran) and 12.5 µl double distilled water. The PCR program in 35 cycles (Flex Cycler2, Germany) was performed as follows: Initial denaturation for 5 min at 95°C (1 cycle), denaturation for 1 min at 94°C, annealing for 45s at 57°C, extension for 45s at 72°C (35 cycles), and final extension for 5 min at 72°C (1 cycle).

To ensure the amplification of the desired fragment, 1.5% gel agarose electrophoresis stained with ethidium bromide was applied. In addition, gel doc machine (Slite 200W, Bioimager Company) was used to assay the bands and photography.

Sequencing and subtyping

In order to identify the subtypes, PCR products were transferred to South Korea (Bioneer, Daejeon, South Korea) by GenFanAvaran Company, Iran. All PCR products were single stranded sequenced using the Sanger method on an ABI 3730 sequencer.

The sequences were compared with the sequences registered in the GenBank library, NCBI (https://www.ncbi.nlm.nih.gov/).

Statistical analysis

The results of subtypes with demographic data were analyzed using the SPSS software version 16 (SPSS Inc., Chicago, IL, USA) and descriptive statistics were obtained using the Chi-square and Fisher and probability $P < 0.05$.

Ethical statement

The manuscript does not contain clinical and laboratory animal studies or patient data. The participants' consent was obtained by the Deputy of Kashan University of Medical Sciences research committee. Furthermore, the authors attained the ethics code: IR.KAUMS.MEDNT. REC.1396.118.

Results

Out of 1118 studied cases, 883 individuals were female (79%) and 235 were male (21%). In microscopic observations, a total of 96 individuals were infected with *Blastocystis* sp. The prevalence of *Blastocystis* sp. was 8.58% (confidence interval [CI] = 6.94%–10.22%) in the studied society.

Moreover, considering the significance level ($P < 0.05$), there was no relationship between sex and infection to *Blastocystis* sp. ($P = 0.46$). The average age of patients infected with *Blastocystis* sp. was 40.32 ± 1.59 years old (age range: 3–80). Forty-eight (50%) individuals infected with *Blastocystis* sp. were high school graduates, 6 (6.2%) individuals were illiterate, and 6 (6.2%) individuals attended elementary school. Most individuals were settled in cities (88.5%), and the rest were villagers [Table 1]. Furthermore, 83 out of 96 stool samples were only infected with *Blastocystis* sp. and 13 samples had *Blastocystis* and other parasites too [Table 2]. DNA extraction was performed, and the PCR method was successfully applied for 51 cultured samples with *Blastocystis* sp. infection [Figure 1]. After electrophoresis and the emergence of bands in 51 positive samples, sequencing and the subtype determination were performed in the Blast program (NCBI). Totally, 47 samples were related to three subtypes (ST1, ST2, and ST3) and four samples did not provide appropriate sequence (mixed) to include in subtype analysis [Table 3]. The sequences obtained for the 47 isolates were deposited and recorded in Genbank (accession no: LC412909, LC413241, LC413621-22, LC413851-53, LC413889-94, LC 413922-28, LC414127-53).

Table 1: The demographic characteristics of patients infected to *Blastocystis* sp. who referred to Kashan medical diagnostic laboratories, 2017-2018

Demographic characteristics	*n* (%)
Sex	
Male	73 (76)
Female	23 (24)
Total	96 (100)
Age categories	
<10	3 (93.1)
11-20	3 (3.1)
21-30	18 (18.8)
31-40	25 (26)
41-50	20 (20.8)
51-60	18 (18.8)
>60	9 (9.4)
Total	96 (100)
Job	
Self-employment	24 (25)
Food-handlers	51 (53.1)
Homemakers	13 (13.5)
Retired	2 (2.1)
Unemployed	6 (6.2)
Total	96 (100)
Education	
Illiterate	6 (6.2)
Elementary school	6 (6.2)
Middle school	29 (29)
High school graduates	48 (50)
College education	7 (7.3)
Total	96 (100)
Location	
Cities	85 (88.5)
Villagers	11 (11.5)
Total	96 (100)

In this survey, ST3 (21 cases) and then ST1 (20 cases) were the dominant subtypes. The most frequent subtype was ST3 in the 21–30 age group and the least frequent subtype was ST2 in the 31–40 years' age group. There was no significant relationship between the types of *Blastocystis* subtypes and sex ($P = 0.159$). Food-handlers 12 (57.1%) were the most individuals who infected with *Blastocystis* (ST3).

The sequenced SSU rRNA gene fragment, multiple alignments, and analysis of intra-subtype variation in the BLAST program (NCBI) showed the maximum similarity of 98.37%, 98.11%, and 98.31%, respectively, for ST1, ST2, and ST3.

Discussion

Several studies have been conducted on the prevalence of *Blastocystis* subtypes in different regions of Iran; however, no studies have investigated this issue in Kashan, Isfahan province, Iran. In different regions of Iran, the prevalence of *Blastocystis* sp. infection was reported between 0.2% and 54.5%.[5,17-19] The results of this study showed that the frequency of *Blastocystis* sp. in Kashan was estimated to be 8.58% (CI = 6.94%–10.22%) which is similar to the results of a systematic review and meta-analysis study that showed the pool prevalence of *Blastocystis* sp. in Iran was 9.1%.[7] However, it was different from the results of some other regions of Iran such as Hamadan 18.5%[20] and Tehran 21%.[21]

Based on previous studies, the prevalence of *Blastocystis* sp. is also less common in Iran in comparison with other countries. Our study was consistent with the results of a survey conducted in Thailand 10%,[22] while it was different with Poland 15.3%,[23] Brazil 41.9%,[24] and Colombia 36.4%.[25]

Some factors such as variations in climate, geographic conditions, population density, lack of awareness, and health facilities affect the incidence of *Blastocystis* sp. and other human intestinal parasites.[7]

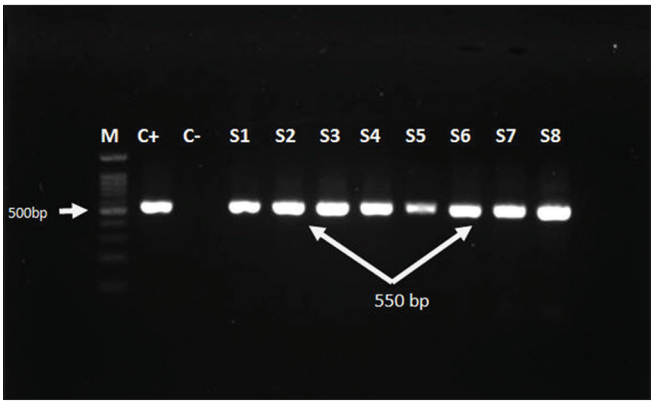

Figure 1: 1.5% Agarose gel electrophoresis of the polymerase chain reaction product of *Blastocystis* samples, M: 100-bpDNA ladder, C + positive control, C-negative control, S1–S8 positive samples

Table 2: The frequency of multiple simultaneous parasite in patients infected to *Blastocystis* Sp. who referred to Kashan Medical Diagnostic Laboratories, 2017-2018

Parasites number	Parasites associated	Frequency, *n* (%)
Single parasite	*Blastocystis* sp.	83 (86.6)
Double parasite	*Blastocystis* sp. + *E. coli*	4 (4.2)
	Blastocystis sp.+ *D. fragilis*	1 (1.0)
	Blastocystis sp. + *E. nana*	5 (5.2)
Triple parasite	*Blastocystis* sp. + *E. nana*+*E. histolytica*/*E. dispar*	1 (1.0)
	Blastocystis sp.+ *I. butschlii*+*E. histolytica*/*E. dispar*	1 (1.0)
Quadruple parasitic	*Blastocystis* sp. + *E. nana*+*E. histolytica*/*E. dispar*+*E. hartmanni*	1 (1.0)
Total		96 (100)

E. coli: Escherichia coli, D. fragilis: Dientamoeba fragilis, E. nana: Endolimax nana, E. histolytica: Entamoeba histolytica, E. dispar: Entamoeba dispar, I. buetschlii: Iodamoeba buetschlii, E. hartmanni: Entamoeba hartmanni

Table 3: The distribution of Blastocystis sp. subtypes according to sex of patients who referred to Kashan medical diagnostic laboratories, 2017-2018

Variable	Subtype				
	ST1	ST2	ST3	ST mixed	Total
Frequency	20 (39.2)	6 (11.8)	21 (41.2)	4 (7.8)	51 (100)
Sex					
Male	14 (35.9)	3 (7.7)	19 (48.7)	3 (7.7)	39 (100)
Female	6 (50)	3 (25)	2 (16.7)	1 (8.3)	12 (100)

The prevalence of this protozoan in healthy controls is about 10%–20%.[6,7,21] The reason for the low prevalence of *Blastocystis* sp. in present and similar studies is probably related to the high degree of phenotypic diversity, which led to missed a great number of positive cases. In addition, in most cases, the number of the organism is very low to be detected by light microscopy.

The results of the present study showed that majority of participants infected with *Blastocystis* sp. had with a high school education (50%) and were in the age group. In Northern Iran, the majority of infected people were illiterate (30.1%) and were in the age group (30–40 years).[32] In a study in Turkey, however, the highest infection rates was among people with secondary school education (33.3%) and in the age group (20–29).[33] In the present study, the highest frequency of *Blastocystis* sp. was found among food-handlers and restaurant workers. Sharif *et al*. reported the highest frequency (19.2%) among restaurant workers in Northern Iran.[32] Food-handlers can act as an important carrier and source of distribution and transmission of intestinal parasitic infections, including *Blastocystis* sp. to humans. Contaminated food and water as the most important sources of distribution of parasitic diseases can transmit more than 72 species of protozoan and helminthic parasites to humans through the consuming contaminated food and water.[34] Some of this parasite such as *Blastocystis* sp. shows a cosmopolitan distribution.

In human studies, ST1, ST2, ST3, and ST4 are often isolated. Moreover, ST3 is the predominant subtype in humans.[2,9] Different subtypes of *Blastocystis*, especially ST3, followed by ST1 and ST2 are found in asymptomatic hosts, and hence, it was proposed that this organism should not only be considered as a true pathogen but also as a possible commensal. This might be related to the specific human intestinal microbial communities.[35,36]

In the present study, three types of *Blastocystis* subtypes (ST1, ST2, and ST3) were identified in Kashan. The most common subtypes in Kashan were ST3, then ST1 and ST2. Four isolates have been reported as mixed subtypes. The present study is in line with Salehi *et al*. that identified three subtypes with ST3 as the most common in Ahvaz in southern Iran.[4] Furthermore, in a study in Tehran, the capital of Iran, four subtypes were identified, with subtype 3 being the most frequent.[26]

Study of Sardarian *et al*. (2013) showed three subtypes (1-3) in Hamadan and ST1 was dominant.[27] These above-mentioned findings show that subtype 3 is prevalent in human in these regions. Thus, presumably, the infection route has a human origin.

Moreover, in studies conducted in other countries, including, the center of Mexico, 3 subtypes were identified and ST3 was the dominant one.[28]

The majority of *Blastocystis* isolates reported in European countries, such as France, belonged to ST1, ST2, and ST3.[11] Furthermore, Mattiucci *et al*. used sequence analysis of the SSU rRNA gene method in Italy and indicated that six subtypes were detected and the most common subtype was ST3, followed by ST4, ST1, ST2, ST6, and ST8.[29] According to a study in Egypt, ST3 is the most common subtype, and then, ST1 and ST2 were the dominant ones among symptomatic patients.[30] However, in a study in Brazil, Oliveira *et al*. (2018) identified three subtypes among which ST1 was the dominant one,[24] while in another study conducted in Rio de Janeiro, Brazil, ST3 was reported as the dominant subtype in three rural valleys.[31] It seems that the distribution of subtypes depends on the factors such as weather, animals contact, sanitation levels, and economic and social circumstances in each region. In this study, no significant relation was observed between the frequency of *Blastocystis* subtypes and sex or age. This observation was consistent with the results of some other studies in Iran and worldwide.[22,26,29]

More studies should be conducted on human and animal populations in the different regions of Iran for finding the epidemiological pattern of *Blastocystis*.

Conclusion

The most common subtype found in this study, which was similar to most studies conducted in Iran and the world, was subtype 3, followed by subtype 1. Infected food-handlers may play an important role in the transmission of this parasite to others through the handling of food sources. Molecular epidemiological study of *Blastocystis* subtypes for the identification of human or animal origin of *Blastocystis* will be important for the local and global prevention and control of this parasite.

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Conflicts of interest

There are no conflicts of interest.

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