Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 160–164

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Higher prevalence of *Blastocystis hominis* in healthy individuals than patients with gastrointestinal symptoms from Ahvaz, southwestern Iran

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ARTICLE INFO

Keywords:
Blastocystis
Subtype
Healthy individuals
Gastrointestinal symptoms
Iran

ABSTRACT

Background: *Blastocystis*, a common intestinal protozoan of humans and animals, infected more than 1 billion people around the world. This enteric protozoan is frequently reported in both healthy individuals and patients with gastrointestinal (GI) symptoms.
Methods: Three hundred and forty-five fecal samples including 151 GI patients and 194 healthy individuals were examined by microscopy, culture and PCR-sequencing techniques to determine *Blastocystis* frequency and subtype (ST) variation.
Results: The occurrence of *Blastocystis* was detected 56 (16.2%) and 85 (24.6%) by microscopy, culture and PCR methods, respectively. Out of the 85 positive patients, 60 (70.6%) were asymptomatic and 25 (29.4%) were symptomatic. The results of 41 successfully sequenced isolates identified 8 (19.5%), 8 (19.5%), and 25 (61.0%) ST1, ST2, and ST3, respectively.
Conclusion: This study has found that *Blastocystis* was more common in healthy individuals than GI patients. Another finding was that no correlation was found between clinical symptoms and *Blastocystis* STs.

1. Introduction

Blastocystis is a common intestinal protozoan of humans and wide range of animals [1]. It is estimated that more than 1 billion people are infected with *Blastocystis* worldwide [2]. Molecular studies on the small-subunit (*SSU*) *rRNA* gene has classified *Blastocystis* in 17 subtypes (ST1-ST17), of which subtypes 1 to 9 have been reported from humans [3]. In a recent study from South America, subtype 12 has also been reported from humans [4]. Report of the same subtypes in animals and humans lead us to imagine that it has a zoonotic potential; however, molecular studies on *Blastocystis* subtypes in animals and their keepers have not indicated any share subtypes [5]. Among the identified subtypes in humans, subtypes 1, 2, 3, and 4 are more common but the geographic distribution of subtype 4 is more restricted to Europe, and rarely reported from other continents [6]. This enteric protozoan is frequently reported in both healthy individuals and patients with gastrointestinal (GI) symptoms [7]. Subtypes 1, 2, and 3 are equally reported from both groups, but recent studies in Europe indicate that subtype 4 apparently is more associated with diarrhea and irritable bowel syndrome (IBS) [4]. Although the recent *in vitro* studies showed the induced pathogenicity of this enteric protozoan, the pathogenicity of *Blastocystis* is still under debate [3]. *Blastocystis* is known as an associated factor with IBS, however, recent studies consider it as health marker of GI tract [8]. These contradictory findings have led to unanimity on pathogenicity of the protozoan in its hosts.

In Iran, there is a growing body of literature that indicates subtype 1, 2, and 3 as the identified subtypes among symptomatic and asymptomatic subjects [9–11], however, in a recent study from southwest Iran, the subtypes 4 and 5 have been reported from the examined patients [12]. Thus, the present study aimed to investigate the subtype variation of *Blastocystis* among patients admitted to the Ahvaz hospitals due to gastroenteritis and compare with healthy individuals, who referred to the Ahvaz health centers to collect health care card by microscopy, culture, and PCR-sequencing.

2. Materials and methods

2.1. Ethical statement

The study design and questionnaire of this research have been

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https://doi.org/10.1016/j.cimid.2019.05.018
Received 7 February 2019; Received in revised form 17 May 2019; Accepted 21 May 2019

approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (approval number IR.AJUMS.REC.1397.560). The aims of the study and requested information were provided to the participants; thereafter, they were requested to read and signed the informed consent form.

2.2. Study area

This cross-sectional study was carried out from October 2017 to February 2018 in Ahvaz County, southwest Iran. Ahvaz, the capital of Khuzestan Province, with a population of 1,136,989 persons and an area of 815 km^2 located in the southwest Iran [13]. In recent years, the county has faced dust storms, and was one of the hottest places of the world during the summer [14]. The average annual rainfall is around 250 mm [15]. Ahvaz County is located at 31°19′ N 40°09′ E and has an elevation of 20 m above sea level.

2.3. Sample collection

In the present study, the sample size was determined on the basis of a previous study carried out in Ahvaz [10]. Three hundred and forty-five fecal samples were collected from patients admitted to the Ahvaz hospitals, due to GI disorders (151 samples), and healthy subjects referred to the Ahvaz health centers, (194 samples) to collect health care card. The healthy group were examined by the health center physician and enrolled in the study if they met the following inclusion criteria: no history of bacterial, viral, and parasitic infections other than *Blastocystis*, and also gastrointestinal diseases. All participants completed a questionnaire containing items about socio-demographic and clinical data such as age, gender, clinical manifestations, occupation, educational level, history of disease, keeping animals at home, hands washing before eating, and source of drinking water. Fecal samples were transported same-day to the parasitology department, Ahvaz Jundishapur University of Medical Sciences, for further analyses.

2.4. Microscopic examination and culture

All fecal samples were examined using saline and lugol-iodine stain microscopically at 100X and 400X magnifications for the presence of *B. hominis*. Furthermore, approximately 100 mg of each fecal sample was inoculated into the Jones' medium as described previously [6,16]. The sediments of samples cultured were examined microscopically every 48 h for 10 days, and then 200 µl of each positive samples were transferred to 2 ml microtube, and stored at −20 °C for DNA extraction.

2.5. DNA extraction

First, samples were washed three times in sterile phosphate-buffered saline (PBS), then they were placed in water bath for 10 min at 100 °C. The genomic DNA was extracted by the phenol-chloroform method as already described by Barker et al, [17]. The diluted DNA was stored at −20 °C until further examinations.

2.6. PCR-sequencing

PCR was performed on extracted DNA to amplify the *Blastocystis SSU rDNA* gene. The 600 bp fragment of the *SSUr DNA* gene was amplified using the primers BhRDr (5′-GAG CTT TTT AAC TGC AAC AAC G-3′), RD5 (5′-ATC TGG TTGATC CTG CCAGTA-3′) [6]. The PCR reaction was performed in a final volume of 20 µl containing 10 µl of Taq DNA Polymerase 2× Master Mix RED (Ampliqon-Biomol, Hamburg, Germany), 3 µl of nuclease-free water, 2 µl of genomic DNA, and 2.5 µl of each primer (1.25 µm concentration). The PCR conditions included a primary denaturation step at 95 °C for 5 min, followed by 30 cycles including denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. The final extension was 10 min at 72 °C [6].

Table 1
Socio-demographic information of symptomatic and asymptomatic participants (N = 345) in the study.

Category	Group	Frequency	%
Gender	Male	236	68.4
	Female	109	31.6
Age groups (years)	< 10	84	24.3
	10–20	26	7.5
	21–30	65	18.5
	31–40	88	25.5
	41–50	40	11.6
	> 50	42	12.2
Educational status	Illiteracy	81	23.5
	Primary school	91	26.4
	Middle school	109	31.6
	High school	4	1.1
	Academic	60	17.4
Drinking water source	Tap water	6	1.7
	Refined home	265	76.8
	Mineral water	74	21.5
Residency area	Rural	11	3.2
	Urban	334	96.8
Occupation	Unemployed	86	24.9
	Governmental	72	20.9
	Part-time	187	54.2
Hands washing before eating	Yes	284	82.3
	No	61	17.7
Contact with animal	Yes	18	5.2
	No	327	94.8
Clinical symptoms	Asymptomatic	194	56.2
	Diarrhea	55	15.9
	Abdominal pain	70	20.3
	Constipation	19	5.5
	Nausea	4	1.2
	Itching	3	0.9

Table 2
Number and frequency (%) of *Blastocystis* in symptomatic (N = 151) and asymptomatic (N = 194) participants based on microscopy, culture and PCR analyses.

Test	Symptomatic		p value	Asymptomatic		Total (%)
	Frequency	%		Frequency	%	
Microscopy	19	33.9	0.86	37	66.1	56 (16.2)
Culture	25	29.4	0.002	60	70.6	85 (24.6)
PCR	25	29.4	0.002	60	70.6	85 (24.6)

Table 3
Frequency (%) of *Blastocystis* subtypes in symptomatic (N = 10) and asymptomatic (N = 31) participants.

Subtypes	Symptomatic		Asymptomatic		Total (%)
	Frequency	%	Frequency	%	
ST1	2	25.0	6	75.0	8 (19.5)
ST2	0	0.0	8	100.0	8 (19.5)
ST3	8	32.0	17	68.0	25 (61.0)
Total (%)	10	24.4	31	75.6	41 (100.0)

The amplified 600 bp fragments of *SSUr DNA* of *Blastocystis* were visualized on a UV transilluminator after electrophoresis in 2% agarose gels and staining with ethidium bromide. The PCR products were directly sequenced at Microsynth Co. (Switzerland) in one direction using the BhRDr primer. The obtained sequences were edited and aligned using ClustalW, and Phylogenetic tree was drawn by the neighbor-joining and maximum composite likelihood methods in MEGA 6.0 software.

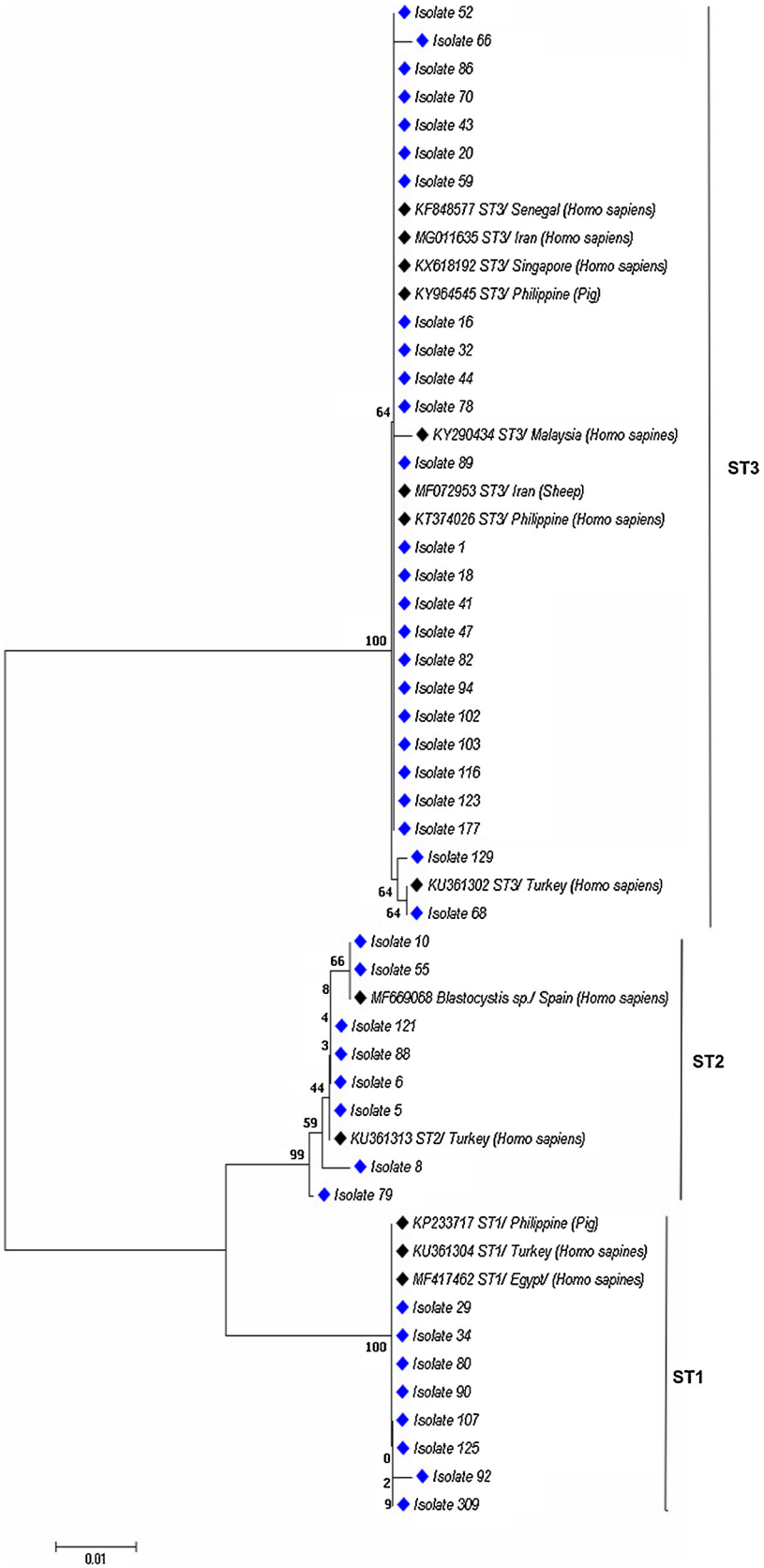

Fig. 1. The evolutionary relationships among *Blastocystis hominis* sequences at the *SSU rDNA* gene. Analysis was performed with the Neighbor-Joining method. The obtained sequences from this study are marked in blue.

2.7. Statistical analysis

The χ^2 and independent *t*-test were used to compare infection rates of *Blastocystis* in healthy individuals and patients with GI symptoms. Statistical analyses were conducted using the SPSS software.

3. Results

Three hundred and forty-five fecal samples were collected from 236 males and 109 females. The age ranged from 1 to 87 years, and the mean age was 28.3 years (standard deviation: 18.53). The majority (25.5%) of the participants in the study belonged to the age group of 31–40. Regarding educational status, 17.4% of the participants were college graduates and 23.5% of them were illiterate. The current study found that 98.3% of the participants declared that they use treated water. Among the participants, 11 (3.2%) were from rural areas and 334 (96.8%) were urban inhabitants. Collected data showed that only 18 (5.2%) had contact with animals. GI symptoms including diarrhea, abdominal pain, constipation, nausea, and itching were reported in 151 (43.8%) of the participants and 194 (56.2%) were asymptomatic (Table 1). Microscopy examinations identified 56 (16.2%) *Blastocystis* infections. The frequency of *Blastocystis* reached 85 (24.6%) by culture and PCR. The highest frequency of *Blastocystis* infection was found in the age group of 31–40 (34.1%; 29/85), and the age group of 10–20 showed the lowest frequency (2.4%; 2/85). Of the 29 positive isolates in the age group of 31–40, clinical manifestations were observed in 6 (20.7%) and 23 (70.3%) were asymptomatic. Among the positive isolates, 60 (70.6%) were asymptomatic and 25 (29.4%) showed clinical symptoms, including diarrhea (14; 56.0%), abdominal pain (7; 28.0%), constipation (1;4.0%), itching (1; 4.0%), and bloating (3; 12.0%). A significant difference was observed between infection and clinical symptoms (Table 2). The obtained results indicated that of the 25 symptomatic positive cases, 7 (28.0%) were female and 18 (72.0%) were male. The results of 41 successfully sequenced isolates identified 8 (19.5%), 8 (19.5%), and 25 (61.0%) ST1, ST2, and ST3, respectively (Table 3). Fig. 1 shows the evolutionary relationships among *B. hominis* sequences at the SSUr DNA gene locus from the present study with representative reference sequences retrieved from Gen-Bank.

4. Discussion

Blastocystis was described over 100 years ago by Alexeieff; however, there are still many unknowns about it, whether it is a pathogen or a beneficial agent of gut microbiota [8,18]. The aim of the current study was to assess the frequency and subtype variation of *Blastocystis* in healthy individuals and patients with GI symptoms. It was hypothesized that patients with GI symptoms may be more infected with *Blastocystis* than healthy subjects.

The current study found that, 85 (24.6%) of the participants were infected with *Blastocystis* by PCR. In a meta-analysis study by Badparva et al., *B. hominis* prevalence ranged from 0.5% to 54.5% in Iran [19]. The global prevalence of *B. hominis* has been recorded between 20% in Europe to 100% in a rural population of Senegal [20]. In developing countries, the high prevalence of *Blastocystis* is probably associated with the poor sanitary conditions and consumption of contaminated food or water [20]. It is somewhat surprising that in 98.3% of the participants, treated water was used as a drinking water source, and only 1.7% of them used tap water. Thus, this study has been unable to demonstrate that drinking water is a source of infection.

In a study by Rafiei et al. in 2014, the presence of intestinal parasites was assessed in surface waters from Ahvaz County and *Blastocystis* was observed in 13.6% of the examined water samples [21]. The obtained result may be explained by the fact that the majority of Ahvaz inhabitants use house water filtration system to purify water. It is possible; therefore, that *Blastocystis* infections occur through contaminated food. Contrary to expectations, this study showed that 70.6% of the infected cases belonged to the healthy group and only 29.4% belonged to the patients with GI symptoms. The results of this study are in line with previous studies, which reported higher infection rate among healthy subjects [10,22–27], but differ from those of published studies, which showed more infection in symptomatic patients [25,28,29]. This observation may support the hypothesis that the higher presence of *Blastocystis* in subjects without clinical signs could be a marker for intestinal or general health [3]. In recent years, some scientific evidence has linked various gut diseases to gut microbiota composition. *Blastocystis* is one of the intestinal protozoan, which is more reported in healthy individuals than patients with GI symptoms [30].

In this study, among the 41 sequenced isolates, ST3 (61.0%) was more prevalent than ST1 (19.5%) and ST2 (19.5%). The small size of sequenced samples did not allow us to confirm ST3 as a predominance ST in Ahvaz inhabitants, but these findings are consistent with those of Beiromvand [10], Moosavi [31], Badparva [32], and Khademvatan [12], who found that ST3 was the predominant ST, but the findings do not support the two previous research studies by Jalallou [23], and Alinaghizade [9], in which ST2 was reported as the most common ST. Although it is difficult to explain this difference, it might be related to geographical distribution of *Blastocystis*. Subtype 3 is considered as the most common ST in humans, globally [33]; however, *Blastocystis* STs distribution varies from country to country. Moreover, the distribution of *Blastocystis* in different regions of a country is not similar [34]. The most obvious finding to emerge from the study is that 70.6% of infected cases were healthy individuals, therefore, it is difficult to correlate clinical symptoms with frequency and ST variation of *Blastocystis*. However, some studies has linked the clinical symptoms with ST variation [35–39]. Thus, the question on the association between ST variation and pathogenicity of *Blastocystis* remains unanswered at present and further studies should focus on this issue.

5. Conclusion

The main goal of the current study was to determine frequency and ST variation in healthy individuals and patients with clinical symptoms. The most obvious finding to emerge from the current study is that *Blastocystis* was more common in healthy individuals than GI patients. The second major finding was that no correlation was found between clinical symptoms and *Blastocystis* ST. Therefore, the present study provides additional evidence with respect to the idea that *Blastocystis* might be a marker for intestinal or general health. Due to limited budget, the current study was unable to sequence all PCR-positive samples; therefore, further studies need to be carried out in order to validate predominance of ST3.

Conflict of interest

The authors have no conflict of interest.

Funding

This project was supported by the Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (96S97).

Acknowledgments

The authors are very grateful to all the participants who have voluntarily participated in this study. This study was the PhD project of M Mardani Kataki.

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