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Original paper

Prevalence and characterization of *Blastocystis* spp. in central southwest of Iran

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ABSTRACT. *Blastocystis* is one of the most common intestinal protozoan parasites of human all over the world. Due to high prevalence and widespread species diversity of this parasite and the possibility of potential transmission of them to human, this study was carried out to investigate the prevalence of *Blastocystis* and characterize its subtypes (genotypes) in Shahrekord, central southwest of Iran. Microscopic examination showed that 6.4% (55/864) of the subjects were infected with *Blastocystis* spp. All of positive samples were sequenced successfully. The molecular methods showed that the infection was caused by four subtypes, including ST1, ST2, ST3 and ST7. The most common identified genotype of *Blastocystis* was ST3 (36.4%). The statistical analysis of data showed that there was no significant correlation between the prevalence of *Blastocystis* or its subtypes with age, gender, job, level of education, contact with animals, and clinical manifestations of infection in the patients. While the frequency of blastocystosis in this population seems to be less than many parts of Iran, but it can be argued that *Blastocystis* is still the most common intestinal parasite of human in this region compared with other intestinal parasites and it seems that the transmission of infection has an anthroponotic pattern.

Keywords: *Blastocystis*, prevalence, polymerase chain reaction, sequencing

Introduction

Blastocystis is considered to be one of the most common single-celled, anaerobic protozoan parasite isolated from vertebrates and invertebrates in different parts of the world [1]. It is estimated that one billion people infected by this enigmatic parasite globally [2]. The prevalence of *Blastocystis* spp. in human varies from region to region of the world and it is more prevalent in developing countries than developed countries due to some factors such as poor hygiene practices and consumption of contaminated food or water [3,4]. It is unknown whether *B. hominis* is a truly pathogenic organism or a commensal or probably is capable of being a pathogen in specific situation. It is generally supposed that *B. hominis* is transmitted by the faecal-oral route, in the same way to other gastrointestinal protozoa. Clinical features such as nausea, anorexia, abdominal pain, bloating, flatulence, and acute or chronic diarrhea that have been assigned to *Blastocystis* spp. are nonspecific [5]. Available reports also suggest a relation between *Blastocystis* and irritable bowel syndrome (IBS) so that a serological study showed that there is significantly higher IgG2 level against *Blastocystis* in patients with IBS [6]. Based on the analysis of the SSU rRNA gene, different genotypes of the parasite have been identified and isolates from humans and animals can be potentially divided into 17 or more genotypes. The ST3 subtype of this parasite is considered as the most common isolated genotype in human and is probably the only genotype of human origin. It has been claimed that

different genotypes have different hosts, geographic distribution and different transmission modes [7]. Studies showed that amplification of a 600 bp region of the small subunit ribosomal RNA gene with BhRDr and RD5 primers and sequencing of the PCR product will provide sufficient information for accurate subtyping of *Blastocystis* [8]. Determining the prevalence and distribution of *Blastocystis* spp. can help us for better understanding of epidemiology and clinical indices of infection, including the potential transmission routes and clinical outcomes of human blastocystosis.

The aim of this study was to determine the prevalence and distribution of *Blastocystis* spp. and characterization of the parasites by molecular methods.

Materials and Methods

Sample collection and stool examination

The cross-sectional study was carried out on 864 stool samples collected from individuals who referred to Shahrekord's clinics for their recent illness or periodic check-ups. The subjects were selected based on a non-randomly simple sampling method. The stool samples were prepared as wet mount preparations (with normal saline and Lugol's solution) and formalin-ether sediments. The preparations were

Table 1. Prevalence of positive *Blastocystis* isolates based on demographic variable and at least on clinical symptom of individuals referred to Shahrekords' Imam Ali Polyclinic

Variable	No. examined	Positive No. (%)	P-value
Gender			0.890
Male	369	23(6.2%)	
Female	495	32(6.5%)	
Age			0.216
≤10	224	8(3.6%)	
11-20	62	4(6.5%)	
21-30	172	11(6.4%)	
31-40	150	11(7.3%)	
41-50	107	10(9.3%)	
51-60	88	9(10.2%)	
≥60	61	2(3.3%)	
Job			0.113
Unemployed	229	10(4.4%)	
Housewife	295	21(7.1%)	
Farmer	5	2(40.0%)	
Rancher	5	0(0.0%)	
Self-employment	160	11(6.9%)	
Clerk	38	4(10.5%)	
Student	132	7(5.3%)	
Location			0.157
Rural	118	11(9.3%)	
Urban	746	44(5.9%)	
Contact with animals	0.534		
Yes	176	13(7.4%)	
No	688	42(6.1%)	
Clinical symptom			0.702
Yes	477	29(6.1%)	
No	287	26(6.7%)	

examined microscopically for protozoan trophozoites /cysts or ova/larvae of intestinal helminths. The socio-demographic characteristics of the subjects including, age, gender, job, location, contact with animals, and common clinical symptoms (abdominal pain, diarrhea, vomiting, flatulence, nausea, fever and constipation) of the subjects were also collected through questionnaires.

Culture

After microscopic observation of the parasite in faecal samples, the positive samples were cultivated in culture tubes containing HSr+S medium (horse serum, ringer and starch rice; Razi Serum Institute, Iran) and the cultures incubated at 37°C for 48–72 hours [9]. After the incubation period, cultures were studied by microscopic examination.

DNA extraction

Genomic DNA of *Blastocystis* isolates was extracted from positive cultures using the QIAamp DNA Stool Mini Kit (QIAamp Fast DNA stool mini kit, Germany) according to the manufacturer's recommendations.

Genotyping

The method explained by Scicluna et al. [8] was used for genotype analysis. Briefly, the method includes a usual PCR with the primers RD5 (ATCTGGTTGATCCTGCCAGT) and BhRDr (GAGCTTTTTAACTGCAACAACG) amplifying and 600 base pair (bp) fragment of the small subunit ribosomal RNA gene (SSU rDNA). This section of the SSU rDNA has been shown to provide enough information for differentiating genotypes of *Blastocystis*. PCR was done using the Master Mix Red (Amplicon, Denmark). The reaction compound included 5µl of distilled water, 10 µl master mix, 0.5 µl forward primer and 0.5 µl reverse primers and 4 µl of extracted DNA in a final content of 20 µl. The reaction circumstance was set as following: 95°C for 5 min, and 35 cycles of denaturation at 95°C for one min then 58°C for 1min in order to annealing and extension at 72°C for 6 min. Distilled water used as negative control. Finally, the PCR products were electrophoresed on 1% agarose gel and were stained with Gel red (Biutum, USA), and finally, bands of the expected size (620bp) were obtained.

The Ab1 files of sequences were manually edited using the software Chromas 2.4.4 and compared with *Blastocystis* sequences in GenBank by BLAST searches at the National Center for Biotechnology Information (NCBI). Genotypes were identified by determining the exact match or closest similarity. The SSU rDNA sequences of the *Blastocystis* isolates, obtained in this study, have been deposited in GenBank under accession Nos MG987013 to MG987067.

Statistical analysis

The analysis of data was carried out by using the SPSS ver. 20 software and Chi-squared and Fisher's exact tests. The values less than <0.05 were considered significant.

Results

In this study the age of individuals ranged from one month to 80 years (mean, 29.6±20). Out of 864 samples, 495 samples (57.3%) were belonged to females and 369(42.7%) to males. The parasi - tological methods (wet mount preparations with normal saline and Lugol's solution and formalin-ether sediments) showed that 55 individuals (6.4%) were infected with *Blastocystis* spp. Table 1 shows the frequency of positive *Blastocystis* isolates based on demographic variable including age, gender, job, contact with animals, location and at least one clinical symptom including, abdominal pain, diarrhea, dysentery, vomiting, flatulence, nausea, or constipation. All of microscopic positive samples were grown on the culture medium. the expected 620 bp fragments of the SSU rDNA in all 55 samples were amplified and successfully sequenced (Figure 1). In *Blastocystis*-infected subjects, the sequencing showed that the infection was caused by four subtypes (genotypes) of the parasite, including ST1, ST2, ST3 and ST7 subtypes (Table 2). Among these, ST3 was the most common genotype of the parasite (36.4%). The statistical analysis of data indicated that

Table 2. Prevalence of *Blastocystis* genotypes in stool samples of individuals referred to Imam Ali polyclinic in Shahrekord

Genotype	No.	Prevalence %
ST1	16	29.1
ST2	15	27.3
ST3	20	36.4
ST7	4	7.3

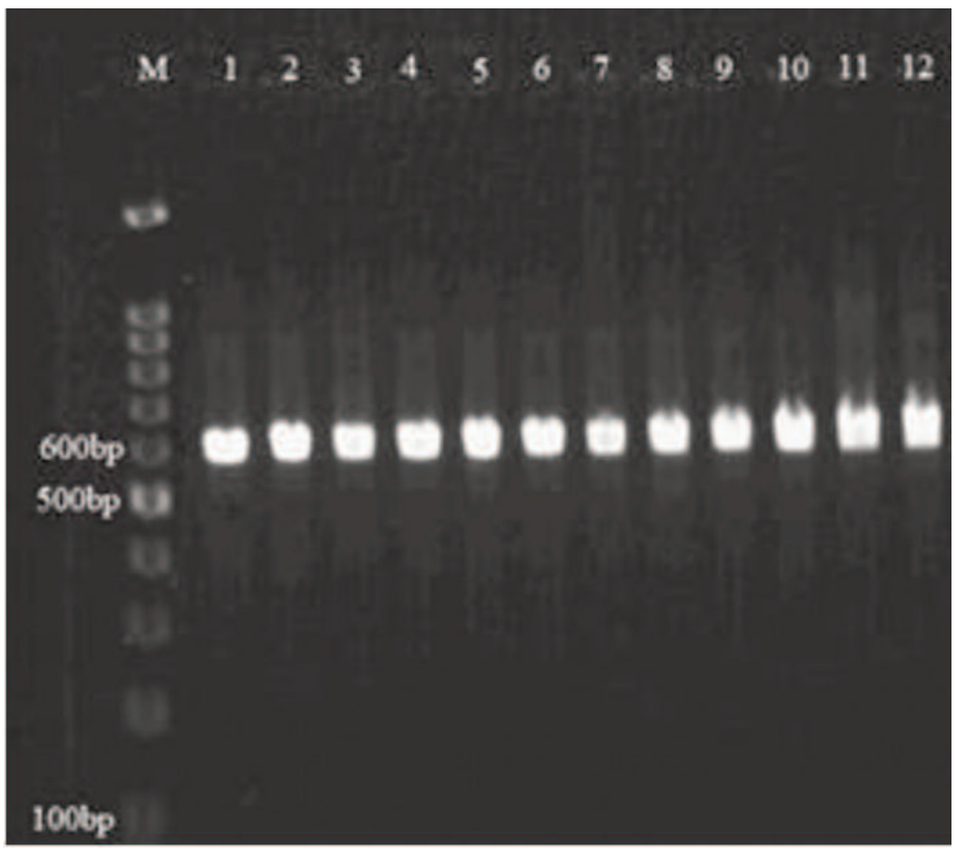

Figure 1. The PCR amplification of *Blastocystis hominis* genotypes (lanes 1–12). Lanes: M, molecular marker of a 100-bp ladder; Subtype 3 in lanes 3,4,6,9 and 10. Subtype 2 in lane 1 and 2. Subtype 1 in lanes 5, 7,8 and 11. Subtype 7 in lane 8 and 12.

there was no significant correlation between the frequency of *Blastocystis* and the variables including, age, gender, job, location, contact with animals, and clinical manifestations of infection (P>0.05). Moreover, in microscopic examination of all stool samples only protozoan parasites, whether pathogenic or non-pathogenic were found and no form of intestinal helminths were detected (Table 3).

Table 3. Prevalence of intestinal parasites found in faecal samples of individuals referred to Imam Ali Shahrekord Polyclinic

Parasite	No.	Prevalence %
Blastocystis spp.	55	6.4
Giardia lamblia	16	1.9
Chilomastix mesnelii	6	0.7
Entamoeba coli	2	0.2
Endolimax nana	2	0.2
Iodamoeba butschlii	1	0.1
E. histolytica/E. dispar	1	0.1

Discussion

Blastocystis hominis is one of the most common intestinal protozoan parasites of human, both from symptomatic patients and from healthy individuals all over the world [7]. Currently, there is little information about the prevalence of *Blastocystis* spp. and its genetic diversity in Iranian patients. The prevalence of *Blastocystis* spp. in this study is somewhat consistent with other studies carried out in some parts of Iran, the United States of America, and Denmark [10–16]. However, our information showed that the prevalence rate of *B. hominis* in Iran, is much lower than some other countries such as Europian countries and Brazil [17–20] However, the prevalence of blastocystosis is higher in developing countries (about 30 to 50%) than developed countries (about 1.5 to 10%) and this variation has been related to poor hygiene, exposure to animals, consumption of contaminated food or water and weather conditions of the area. Also, different diagnostic methods employed may

influence the results of different studies [21]. In this study, like many other studies, the statistical analysis by Chi-squared and Fisher's exact test showed that there was no significant relationship between socio-demographic characteristics of the patients such as age, gender, job, location, contact with animals and *Blastocystis* infection. So, it may be concluded that other factors such as the level of hygienic behavior effect on *Blastocystis* infection [22,23]. Although in this study no significant correlation was observed between *Blastocystis* infection and clinical signs of the subjects, but some studies indicated that there is an association between the parasite and signs [7,24,25], while there also have been a number of studies to the opposite [26,27]. Studies have shown that the role of *Blastocystis* in human disease is controversial, also clinical outcome of *Blastocystis* infection is multifactorial and is influenced by host and parasite factors [28]. The distribution of *Blastocystis* genotypes in human faecal samples differs from country to country. In this study ST1, ST2, ST3 and ST7 genotypes of *Blastocystis* were identified. This result is consistent with findings of a number of other studies that carried out in other parts of the world such as Sweden [29]. In our study the ST3 (36.4%) genotype of the parasite was the most common subtype found. In comparison with other subtypes, this high prevalence of ST3 subtype of *Blastocystis* has been found in many parts of the world, including some parts of Iran (Tehran, Khoramabad) [26], Bangladesh, Germany, Thailand [30], Turkey [31], Singapore [32], Malaysia [33] and France [34]. This subtype of the parasite is perhaps the only subtype of human origin. Also, the detected subtypes are influenced by populations studied and the applied method. However, according to studies conducted in Denmark and Spain, subtype 4 has been introduced as the most prevalent subtype of *Blastocystis* [35,36]. On the other hand, in China, genotype 1 of this parasite has been introduced as the common genotype [37]. Also, in some parts of Iran (Khuzestan and Shiraz) the genotypes 4 and 1 of *Blastocystis* were introduced as the most common genotype of the parasite, respectively [38,39]. It is believed that genotype 3 is specific to humans and other genotypes of the parasite are zoonotic and has been reported in patients with chronic gastrointestinal symptoms. A recent study revealed that acute urticaria and cutaneous rashes was associated with amoeboid forms belonging to genotype 3 [7,40]. In the present study ST1 subtype of *Blastocystis* was the second most prevalent genotype, in agree with Tehran and China studies [26,37], being found in 29.1% and 37.1% of the *Blastocystis* positive samples, respectively. The ST1 is said to be pathogenic and transmitted from domestic animals to humans [7,34]. In addition, other genotypes have been reported as a pathogenic genotype of the parasite, such as genotype 4. It is thought that this genotype is transmitted from domesticated rodents to humans [30,34,36]. In many studies, ST2 is known as the second most common genotype, but in the present study, the prevalence of this genotype was the third most prevalent genotype and It is suggested that ST2 is a non-pathogenic genotype [31]. In addition, other genotypes such as genotype 5 is related to cattle and pigs, and humans acquire infection from these animals, but in general, the infection of this genotype is less observed in humans [41]. In the Middle East ST6 and ST7 genotypes of *Blastocystis* are reported both in individuals with irritable bowel syndrome (IBS) and in healthy individuals. It is thought that these two genotypes are transmitted from birds to humans [42,43]. ST8 has been isolated in one Italian case. This subtype is normally very infrequent in humans but is common in primates. Interestingly, ST8 is also common in primate handlers, suggesting a zoonotic transmission from primates to humans [44]. Genotype 9 has also been reported only from Japan [30] and subtypes 11–13 are separated from zoo animals [45]. It seems that this diversity in the reported various genotypes can be due to the variety of cultural and nutritional habits and the climate and geographic conditions of the regions in different part of the world [7]. According to the findings of this study, the most common intestinal parasite among the subjects was *Blastocystis*. This finding is consistent with a number of other studies such as Spain [46].

It may be concluded that in comparison with other parts of the world, the prevalence of this parasite in this area, is low to moderate and due to potential pathogenesis of this parasite preventive measures should be developed. *Blastocystis hominis* is the most common intestinal parasite in this region and the transmission of the parasite occurs via human to human and the infection can be accounted as an anthroponotic infection.

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