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Original article

Prevalence and subtype identification of *Blastocystis* isolated from human in Shiraz city, southern Iran

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ABSTRACT

Background: *Blastocystis* is the most common parasite in humans and animals. Molecular studies based on the small subunit ribosomal RNA gene (SSU-rRNA) have shown that *Blastocystis* isolates have 17 subtypes and nine of these subtypes have been reported in human. The objective of the present study was to identify the subtype of *Blastocystis*. isolated from human in Shiraz city, southern Iran.

Materials and methods: A cross-sectional study was conducted from March to August 2019. A total of 802 fecal samples from persons who were referred to health centers of Shiraz University of medical sciences were collected and tested by wet mount method to find *Blastocystis*. Genomic DNA was extracted from the fecal samples to be positive for *Blastocystis*. SSU-rRNA gene was amplified by the polymerase chain reaction. The amplicons were sequenced and compared with published sequences in GenBank using BLAST system. Phylogenetic analysis was performed using the maximum likelihood method in the MEGA 5.0 software.

Results: The microscopy method showed that 39 out of the 802 fecal samples were positive to *Blastocystis*. A 500 bp fragment of SSU-rRNA was amplified from isolates after the PCR. Sequence analysis identified four subtypes (STs) of *Blastocystis* consisted of ST1 (32.43%), ST2 (24.32%), ST3 (35.14%) and ST7 (8.11%).

Conclusions: Four subtypes (STs) of *Blastocystis* in human was identified in our study and subtype 3 was the most prevalent subtype. The common subtype (ST3) in this study was identical to the reports from other regions of Iran. For identification of the more subtypes of *Blastocystis*, comprehensive molecular studies with a large number of *Blastocystis* isolates are suggested.

1. Introduction

Blastocystis is one of the most common protozoan parasite with worldwide distribution which inhabits in the intestine of humans and numerous animals such as farm animals, fishes, amphibians, birds, reptiles, rodents and cockroaches.[1,2] The main mode of transmission of *Blastocystis* is fecal-oral direct route and human can be infected through the consumption of water and food contaminated with *Blastocystis* cysts.[3] The pathogenicity of *Blastocystis* in human is controversial because of this protozoa reported in both asymptomatic and symptomatic individuals.[4] Symptoms such as abdominal pain, diarrhea, bloating, constipation, nausea, vomiting, fatigue, headaches, skin rash, joint pain, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) attributed to *Blastocystis*.[5] Different morphological forms of *Blastocystis* have been identified and four commonly forms are granular, vacuolar, amoeboid and cystic forms. The vacuolar form is the most common form and often observed under microscopic examination.[6] The amoeboid form is pathogenic form and detected only in symptomatic patients.[7] The prevalence rate of *Blastocystis* is 1.5%–20% in industrialized countries, while it is 30%–50% in developing countries.[8] Epidemiological studies have indicated that the prevalence of *Blastocystis* in human has been estimated from 0.5 to 54.4% in different parts of Iran.[8–14]

Based on the sequence analysis of the small subunit ribosomal ribonucleic acid (SSU-rRNA) gene, at least 17 subtypes (STs) of *Blastocystis* have been identified in humans and animals.[15,16] ST1-ST8 and ST12 are found both in humans and animals; ST9 was particularly identified in humans, while ST10, ST11 and ST13-ST17 are identified only in animals and have not been found in humans.[17,18] The majority of infections with *Blastocystis* in human is attributed to ST3, followed by ST1, ST2 and ST4, whereas ST5-ST9 and ST12 are rarely reported.[16] The molecular studies conducted for the genotype identification of *Blastocystis* isolated from humans in Iran have reported the ST1, ST2, ST3, ST4, ST5, ST6 and ST7 genotypes.[19–27] The result of many studies

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that were performed in Iran have showed that subtype 3 is the most common subtype of *Blastocystis*.[19,21,23–25,27]

Considering the fact that different subtypes of *Blastocystis* may be associated with geographical distribution, hosts and pathogenicity of parasite,[19,28] therefore, identification of the subtypes of *Blastocystis* is necessary for epidemiological studies. The current study aimed to estimate the prevalence rate of *Blastocystis* isolated from human in Shiraz city, southern Iran and identify its subtypes by PCR-sequencing method.

2. Material and methods

2.1. Study area

Shiraz city, capital of Fars province which is located in the south of Iran (29°37′N 52°32′E), is the fifth-most-populous city of the country. It has a moderate climate, and around 300 mm of rain falls each year in the city, almost entirely in the winter months.

2.2. Samples collection

A cross-sectional study was conducted from March to August 2019. A total of 802 fecal samples were collected from persons who were referred to health centers of Shiraz University of medical sciences, Shiraz city, Southern Iran. The stool specimens were placed in a clean plastic container and transferred to the parasitology laboratory of Shiraz University of medical sciences for microscopic examination. A structured questionnaire was filled out for each sample. The design of the study, including ethical aspects, was reviewed and approved by the ethics committee at the Shiraz University of Medical Sciences (code: IR.SUMS.MED.REC.1398.012).

2.3. Microscopy method

All the stool specimens were tested by wet mount (normal saline or Lugol's iodine) method to find *Blastocystis*. The samples contained *Blastocystis* were stored at −20 °C until DNA extraction.

2.4. Molecular method

2.4.1. DNA extraction and PCR

The genomic DNA from positive stool samples of *Blastocystis* was extracted using Stool DNA Isolation mini kit, with Proteinase (Yekta Tajhiz Azma kit, Cat. No. YT9032) according to the manufacturer's instructions. The small subunit ribosomal RNA (SSU-rRNA) gene was amplified using the PCR reaction. The forward (Blast 505–532: 5′-GGAGGTAGTGACAATAAATC-3′) and reverse (Blast 998–1017: 5′-TGCTTTCGCACTTGTTCATC -3′) primers were used for amplification of the SSU-rRNA gene.[22] The PCR reaction was performed in a final volume of 25 μl. Each reaction contained 12.5 μl of the PCR master mix (2x Master Mix RED (Ampliqon, Denmark), (1.25 U Taq DNA polymerase, 200 μM of dNTPs and 1.5 mM of MgCl2), 0.5 μl of each primer (12.5 pmol), 5 μl of template DNA and 6.5 μl of double-distilled water. The temperature profile was one cycle of 95 °C for 4 min to denature the double stranded DNA, followed by 35 cycles of 95 °C for 30 s (denaturation), 54 °C for 30 s (annealing), 72 °C for 30 s (extension), and a final extension of 72 °C for 5 min. Double-distilled water (DDW) instead of template DNA was included in each set of the PCR reaction as negative control and DNA extracted from *Blastocystis* as positive control. Amplicons were visualized by electrophoresis on a 1.5% agarose gel and stained with Gel Red (GelRed™ Nucleic Acid Gel Stain, 10,000X in Water, cat. No. 41003).

2.4.2. Sequencing and subtype identification

The PCR products were sequenced in two directions using the same forward and reverse primers used in the PCR by Sanger sequencing method. The SSU-rRNA sequence results were edited by the Geneious software (www.geneious.com) and the consensus sequences were compared with the reference sequences available in GenBank using BLAST (Basic Local Alignment Search Tool) system (http://www.ncbi.nlm.nih.gov/) for subtype identification. A phylogenetic tree was constructed with sequences obtained in the present study along with the reference sequences deposited in GenBank using the maximum

Table 1
Demographic characterizations, subtype and accession number of the positive samples.

	Isolate	Sex	Age	Subtype	Accession no.
1	H1	Female	38	3	MN396293
2	H2	Male	35	3	MN396294
3	H3	Female	72	3	MN396295
4	H4	Female	12	3	MN396296
5	H5	Female	67	1	MN396272
6	H6	Female	51	7	MN396306
7	H7	Male	75	3	MN396297
8	H8	Male	44	2	MN396284
9	H9	Male	44	3	MN396298
10	H10	Male	52	3	MN396299
11	H11	Female	30	3	MN396300
12	H12	Male	35	1	MN396273
13	H13	Male	20	1	MN396274
14	H14	Male	73	2	MN396285
15	H22	Female	49	2	MN396286
16	H23	Male	45	2	MN396287
17	H24	Female	67	–	–
18	H27	Female	57	2	MN396288
19	L1	Male	40	1	MN396275
20	L2	Female	63	7	MN396307
21	L3	Female	20	1	MN396276
22	L4	Female	9	3	MN396301
23	L5	Female	75	1	MN396277
24	L6	Female	44	1	MN396278
25	L7	Female	31	1	MN396279
26	L8	Female	47	2	MN396289
27	L9	Female	41	3	MN396302
28	L10	Male	64	3	MN396303
29	L11	Female	35	–	–
30	L12	Male	29	2	MN396290
31	L13	Female	46	1	MN396280
32	L14	Male	49	2	MN396291
33	L15	Female	44	1	MN396281
34	L16	Female	18	3	MN396304
35	L17	Female	41	7	MN396308
36	L18	Male	67	1	MN396282
37	L19	Male	57	3	MN396305
38	L20	Male	50	2	MN396292
39	L31	Male	40	1	MN396283

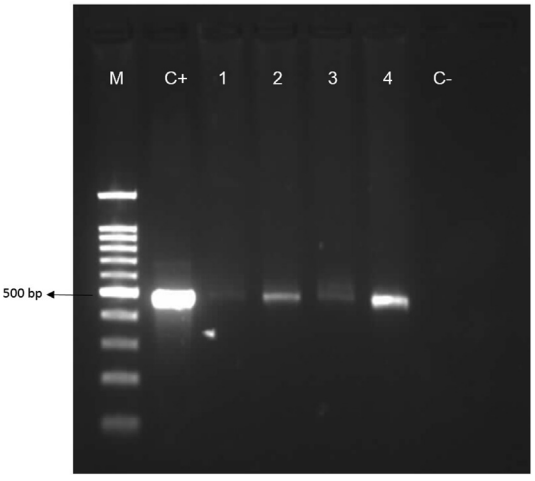

Fig. 1. Agarose gel electrophoresis of PCR products. M: 100 bp DNA marker; C+: positive control; C-: negative control; lanes 1–4: samples.

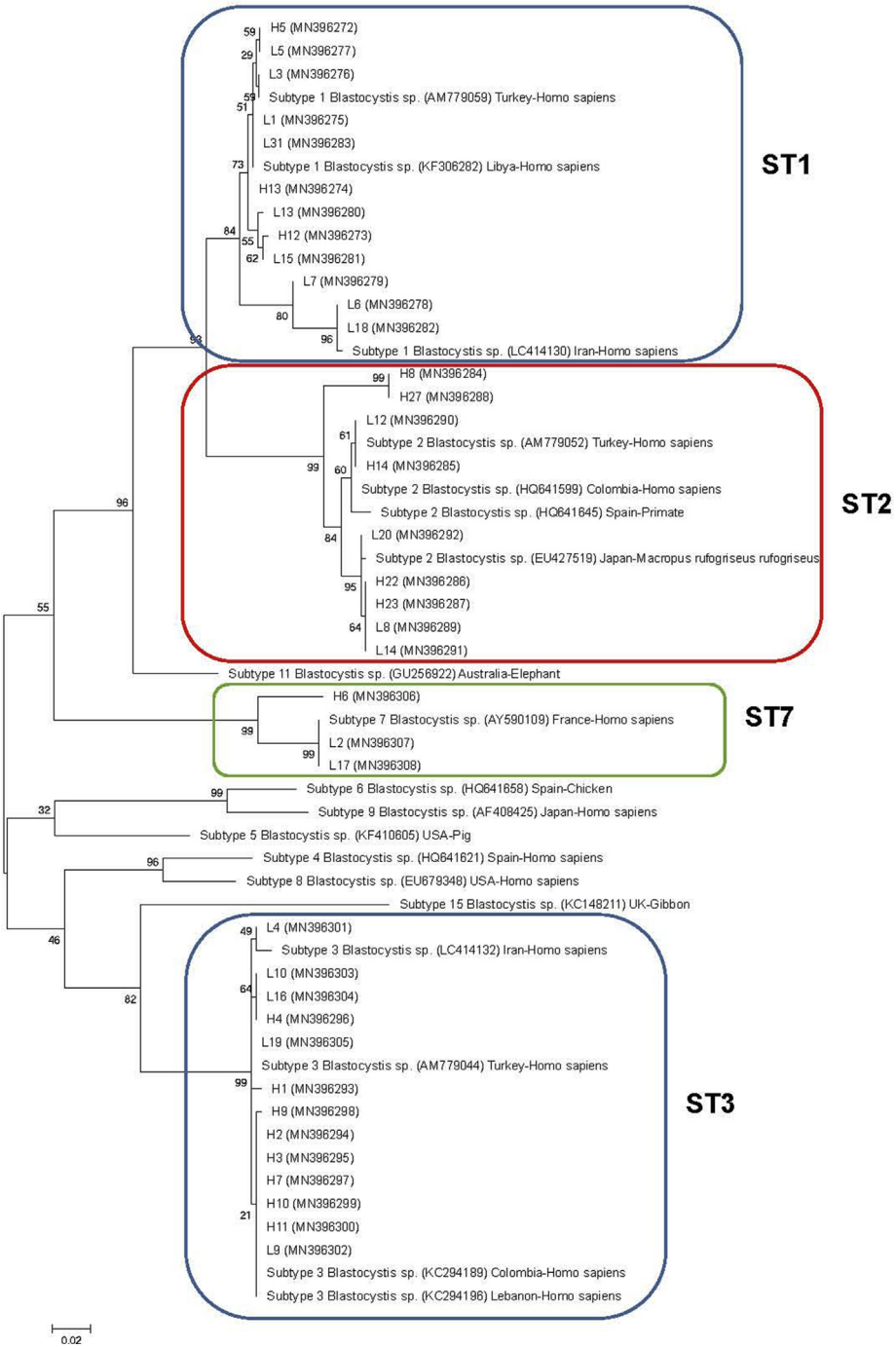

Fig. 2. Phylogenetic relationship of SSU-rRNA sequences of *Blastocystis* isolates obtained in this study and reference sequences retrieved from GenBank.

likelihood method in the Molecular evolutionary genetic analysis version 5 (MEGA 5.0) software.[29] Bootstrap analyses (using 1,000 replicates) were carried out to determine the robustness of the finding.

3. Results

A total of 39 (4.86%) out of 802 stool samples were positive to *Blastocystis* by microscopy method. The individuals infected with *Blastocystis* consisted of 17 males (43.6%) and 22 females (56.4%) with the mean age of 45.54 ± 17.29 years old (Table 1).

The positive stool samples were examined by the PCR method and a 500 bp fragment of SSU-rRNA was amplified from 39 isolates and the positive control, and no amplification was observed in the negative control (Fig. 1). Sequence analysis was performed for 37 PCR products to identify the subtypes of *Blastocystis*. The consensus sequences determined in this study were deposited in GenBank with accession numbers MN396272 to MN396308. BLAST analysis confirmed the presence of four subtypes (STs) of *Blastocystis*: ST1 (n = 12), ST2 (n = 9), ST3 (n = 13) and ST7 (n = 3). ST3 was the most prevalent subtype in this study with a prevalence of 35.14%, followed by ST1 (32.43%), ST2 (24.32%) and ST7 (8.11%). The consensus phylogenetic tree indicated that 13 ST3 of *Blastocystis* obtained in the current study based on the SSU-rRNA sequences were divided into 6 haplotypes, 12 ST1 of *Blastocystis* into 9 haplotypes, 9 ST2 of *Blastocystis* into 4 haplotypes and 3 ST7 of *Blastocystis* into 2 haplotypes (Fig. 2).

4. Discussion

The results of the current study reported a prevalence of 4.86% for *Blastocystis* isolated from humans in Shiraz city, southern Iran. Neghab et al. (2006) reported that the prevalence rate of *Blastocystis* was 25.4% among 39 catering staff of a university canteen in the city of Shiraz, which was higher than the rate in our study.[9] This may be due to the type of the diagnostic method. We used the wet mount (normal saline or Lugol's iodine) method to find *Blastocystis* while they used formalin-ether concentration technique for three stool samples collected from each person on three consecutive days. Study conducted on randomly 4200 stool samples obtained from patients with gastroenteritis reported a lower prevalence rate (0.5%) than that in our study.[11] Similar to our study, the infection rate of *Blastocystis* among 4788 individuals referred to health centers in Mazandaran Province was 5.2%.[14] Prevalence analysis of *Blastocystis* in Iran by Badparva et al. demonstrated that the total prevalence of *Blastocystis sp*. in Iran was 3% between 2003 and 2015, and the results showed that prevalence of *Blastocystosis* had a decreasing trend in Iran.[8] The survived population, sample size, diagnostic method and personal and community hygiene could affect the prevalence of *Blastocystis*.

In this study, the subtypes of *Blastocystis* were identified using the PCR-sequencing, while Motazedian et al. reported the genomic diversity of *Blastocystis* from patients in Shiraz by PCR-RFLP, which is not a suitable method for the genotype identification of *Blastocystis*.[30] Four subtypes of *Blastocystis* (ST1, ST2, ST3 and ST7) were identified in our study and ST3 was the most prevalent subtype. Molecular studies on *Blastocystis* previously conducted in Iran showed ST1, ST2, ST3, ST4, ST5, ST6 and ST7 in humans, and ST3 was reported in all studies performed in Iran.[19–27] The subtype 3 was the most prevalent subtype of *Blastocystis* in our study, which is similar to the several studies performed in Iran about the subtype distribution of *Blastocystis sp.* isolated from humans,[19,21,23–25] whereas two studies reported a higher incidence of subtype 1 in humans.[20,26] In contrast to our study, Khoshnood et al. reported the ST4 was the most prevalent subtype in human samples.[22] Generally, subtypes 3 has been identified as the most common subtype in human.[16]

In the present study, 13 ST3 of *Blastocystis* obtained based on the SSU-rRNA sequences were divided into 6 haplotypes, and 6 isolates (H2, H3, H7, H10, H11 and L9) were identical and exhibiting 100% homology with an isolate from human in Colombia (accession no. KC294189) and Lebanon (accession no. KC294196), also one ST3 sequence (L19) showed 100% identity with *Blastocystis* isolated from human in Turkey (accession no. AM779044). Twelve ST1 of *Blastocystis* were divided into 9 haplotypes, isolate L3 showed 100% homology to an isolate from Turkey (accession no. AM779059), another two ST1 sequences (L1 and L31) were identical to those from Libya (accession nos. KF306282). Nine ST2 of *Blastocystis* were divided into 4 haplotypes and two isolates (H14 and L12) did not differ from an isolate collected from human in Turkey (accession no. AM779052). Two sequences identified as ST7 (L2 and L17) had the similarity with those from human in France (accession no. AY591109).

5. Conclusion

Blastocystis sp. is the most common intestinal parasite in stool sample of both symptomatic and asymptomatic individuals worldwide. The ST1, ST2, ST3 and ST7 of *Blastocystis* can be found both in humans and animals. In this study, ST3 was the most common subtype, followed by ST1, ST2 and ST7. However, these results cannot be generalized to the all Shiraz population. Further studies are required to determine the distribution of subtypes of *Blastocystis* in the more population. Because of the zoonosis of *Blastocystis*, the prevention and control programs in human populations are necessary to help identify the sources of *Blastocystis* transmission to humans. Further studies will be needed to identify the subtypes of *Blastocystis* in different hosts and areas.

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Declaration of competing interest

The authors declare that there is no conflict of interests.

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