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Original Article

Molecular Detection and Subtype Characterization of *Blastocystis* spp. in Symptomatic Children Using PCR and Sequencing

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Abstract

Background: *Blastocystis* spp. is a globally distributed intestinal protozoan that infects humans and various animals. This protozoan is a common pathogen among humans and animals and can be transmitted from animal species to humans. Therefore, the present study aimed to identify and characterize *Blastocystis* subtypes in children with gastrointestinal symptoms using PCR and sequencing.

Methods: Overall, 150 stool samples were collected from children referred to academic hospitals in Mashhad, Iran over a period of seven months in 2020. Participants were divided into two groups: symptomatic cases (n = 50) and asymptomatic controls (n = 100). DNA was extracted from *Blastocystis*-positive samples and analyzed by PCR followed by sequencing for subtype identification.

Results: *Blastocystis* spp. was found in 6 (12%) of the symptomatic children and 7 (7%) of the asymptomatic group. All isolates from symptomatic cases belonged to subtype ST1, whereas isolates from the control group included ST1, ST2, ST3, and ST7. ST1 was the most frequently detected subtype overall. Among symptomatic children, abdominal pain was the most frequent symptom.

Conclusion: ST1 was the predominant subtype in both symptomatic and asymptomatic children, though it was the only subtype observed in symptomatic cases. These findings suggest a potential link between ST1 and gastrointestinal symptoms. Further studies with larger populations and in diverse geographic regions are warranted to investigate the clinical significance and distribution patterns of *Blastocystis* subtypes.

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Introduction

Blastocystis spp. is an anaerobic, unicellular intestinal protozoan that colonizes the gastrointestinal tract of humans and numerous animal species. It is considered one of the most frequently detected non-fungal eukaryotic organisms in human stool specimens (1). For the first time, this parasite was introduced by Alexieff as a protozoan cyst (2).

Based on the phylogenetic investigations of its small ribosomal subunit SSUrRNA, *Blastocystis* has been classified within the order Stramenopile, which includes a group of unicellular and multicellular eukaryotes (3, 4). Its prevalence exceeds 60% in developing countries and ranges between 5%-20% in developed nations (5, 6). This parasite has a fecal-oral transmission route and can cause gastrointestinal symptoms, including heartburn, diarrhea, anorexia, bloating, nausea, and vomiting (7). In addition to contaminated water and food, factors like season, age, low hygiene, contact with animals, and inadequate sanitation systems also contribute to its transmission (8).

As of 2024, at least 34 subtypes (ST1-ST34) of *Blastocystis* have been identified using molecular methods, though only ST1-ST9 are commonly reported in humans. Subtypes ST10 and above are more frequently associated with non-human hosts (9, 10). Of the 17 subtypes found in humans, subtypes 1 to 4 have been reported more frequently than subtypes 5 to 9, which are usually asymptomatic (11, 12). In addition to humans, Subtype 3 has been found in pigs and cattle (13, 14). Moreover, Subtype 5 is more prevalent in pigs and cattle and less prevalent in humans (15). Subtype 4 has been reported more frequently in rodents (10). Recent studies have expanded the number of identified *Blastocystis* subtypes to over 30, with ST1 to ST9 commonly found in humans, and others primarily in animals such as pigs, cattle, and rodents. This growing subtype diversity highlights the importance of molecular surveillance and zoonotic considerations (9, 10). Due to the limitations of direct microscopic examination, molecular techniques such as PCR are preferred for detecting *Blastocystis* and identifying its subtypes (16). The pathogenic role of *Blastocystis* remains controversial. Moreover, specific genotypes of this parasite may be associated with gastrointestinal symptoms, and identifying these subtypes may help develop strategies for prevention and treatment.

We aimed to investigate the subtype distribution of *Blastocystis* spp. in symptomatic children referred to academic hospitals in Mashhad, Iran, using PCR and sequencing techniques.

Materials and Methods

Study Population and Design

This case-control study included 150 pediatric patients (aged 2-10 yr) referred to university-affiliated hospitals of Mashhad University of Medical Sciences (Imam Reza, Ghaem, Dr. Sheikh, and Akbar), Mashhad, Iran over a seven-month period in 2020 during routine stool examinations. Participants were divided into two groups: cases (n = 50), children with gastrointestinal symptoms (mainly diarrhea), and controls (n = 100), children without such symptoms.

Inclusion criteria were age 2-10 yr, presence or absence of gastrointestinal symptoms (for case and control groups, respectively), and parental consent. Exclusion criteria included recent antibiotic use and incomplete questionnaires.

Parents completed a demographic questionnaire including age, gender, residence, animal contact, and clinical symptoms.

Microscopic Examination

Stool samples were examined within 30 min of collection using direct smear with normal saline and Lugol's iodine staining. The parasite load in positive samples was semi-quantitatively assessed under 10X magnification.

Sample Concentration and Storage

Positive samples were suspended in normal saline for 30 min, filtered through sterile gauze, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the pellet was stored at –20 °C in 1.5 ml microtubes until molecular analysis.

DNA extraction and PCR amplification

Genomic DNA was extracted from the concentrated stool samples (pellets) obtained after filtration and centrifugation, rather than from raw stool, using the DNA Isolation Kit (Norgen Biotek Corp., Canada), according to the manufacturer's instructions. PCR amplification was performed with the specific primers BL18SPPF1 (5´-AGTAGTCATACGCTCGTCTCTCAAA-3´) and BL18SR2PP (5´-TCTTCGTTACCCGTTACTGC-3´) (17).

The thermal cycling conditions consisted of an initial denaturation at 95 °C for 5 min. Then, 35 cycles were performed in 3 stages: 94 °C for 1 min, 57° C for 45 sec, and 72 °C for 45 sec. Eventually, one cycle at 72 °C was performed for 7 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with a DNA dye. The band lengths of 320-342 bp compared with the ladder were considered positive cases in the PCR diagnostic method. PCR-positive samples were subjected to Sanger sequencing using a commercial sequencing service. The obtained sequences were manually edited and aligned using BioEdit software. Subtype identification was performed by comparing the sequences with reference sequences available in the NCBI GenBank database using the BLAST algorithm. Sequences showing ≥98% similarity with reference sequences were assigned to the corresponding *Blastocystis* subtype.

Ethical clearance

The study protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1399.107). Written informed consent was obtained from parents or guardians before sample collection.

Statistical Analysis

All statistical analyses were performed using SPSS software ver. 26 (IBM Corp., Armonk, NY, USA). A P-value < 0.05 was considered as statistically significant. However, as no statistically significant results were found between the parameters, we omitted the P-values.

Results

Demographic Observations

Microscopic and molecular analysis identified *Blastocystis* spp. in 6/50 (12%) of symptomatic and 7/100 (7%) of asymptomatic children. Among the infected children in the case group, 2 (33%) were female and 4 (67%) were male. In the control group, 2 (30%) were female and 5 (70%) were male. No statistically significant associations were observed between *Blastocystis* infection and demographic factors such as age, sex, place of residence, or animal contact (Table 1).

Subtype Distribution

All 13 *Blastocystis* isolates successfully sequenced were identified by comparing their Small Subunit ribosomal RNA (SSU rRNA) gene sequences against reference sequences in the NCBI database. This comparison revealed a high similarity (≥ 98%) between the clinical isolates and the respective reference strains (Table 2).

The distribution of the identified subtypes is as follows:

Table 1: Frequency of *Blastocystis* spp. in Mashhad City by demographic data and risk factors

Some Risk factor	Category	Case (n=6) n (%)	Control (n=7) n (%)	Total (n=13) n (%)
Sex	Male	4 (66.7)	5 (71.4)	9 (69.2)
	Female	2 (33.3)	2 (28.6)	4 (30.8)
Residence	Urban	5 (83.3)	6 (85.7)	11 (84.6)
	Rural	1 (16.7)	1 (14.3)	2 (15.4)
Animal Contact	No	5 (83.3)	6 (85.7)	11 (84.6)
	Yes	1 (16.7)	1 (14.3)	2 (15.4)
Age Group	2–4	1 (16.7)	1 (14.3)	2 (15.4)
	5–6	0 (0)	2 (28.6)	2 (15.4)
	7–8	2 (33.3)	1 (14.3)	3 (23.1)
	>8	3 (50.0)	3 (42.9)	6 (46.2)

- Symptomatic Children (n = 6): All six positive samples belonged exclusively to Subtype 1 (ST1).
- Asymptomatic Children (n = 7): Four distinct subtypes were identified: ST1 (n = 3), ST2 (n = 2), ST3 (n = 1), and ST7 (n = 1).

ST1 was the predominant subtype overall. The complete SSU rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers MZ734401-MZ734396.

Table 2: Genotyping of *Blastocystis* Isolates in Mashhad City by Sequence Identity with Reference Strains

Subtype (ST)	Case (n = 6)	Control (n = 7)	Total Isolates (n = 13)	Sequence Identity to NCBI Reference
ST1	6 (100)	3 (42.85)	9	≥ 98\%
ST2	0	2 (28.57)	2	≥ 98\%
ST3	0	1 (14.28)	1	≥ 98\%
ST7	0	1 (14.28)	1	≥ 98\%
Total	6	7	13	-

Clinical Presentation

Among ST1-positive symptomatic children, the most frequently reported symptom was abdominal pain (83.3%), followed by anorexia and bloating (16.7% each). None of the symptomatic children reported nausea or vomiting.

Discussion

Blastocystis spp. represents a widely distributed parasitic protozoan exhibiting significant genetic heterogeneity across the globe. While the clinical significance of this organism is still debated, accumulating data indicate a potential link between certain subtypes and gastrointestinal disorders (18).

In the present study, *Blastocystis* occurred in both symptomatic and asymptomatic children, in a higher proportion in symptomatic patients. While this difference was not statistically tested, ST1 was exclusively identified in symptomatic individuals, suggesting a possible association with gastrointestinal complaints including abdominal pain, anorexia, and bloating. Abdominal pain was the most frequently reported symptom, consistent with previous findings (19, 20). Recent studies have also supported the symptomatic role of ST1. An Egyptian study

demonstrated a high association between ST1 and irritable bowel syndrome (IBS), abdominal pain, and bloating in children (21). Kumarasamy et al. have also speculated a possible link with colorectal cancer and, thereby, raised concerns regarding its pathogenicity (22).

In contrast, the control group exhibited greater subtype diversity, including ST1, ST2, ST3, and ST7. Notably, ST7 was found only in asymptomatic individuals, consistent with previous studies that reported its presence in asymptomatic hosts, suggesting it may be a non-pathogenic or host-adapted subtype (23). The most common isolated subtypes in the present study included ST1, followed by ST2. Moreover, ST3 and ST7 had equal prevalence. These results are consistent with the findings of Rahimi et al. in northwestern Iran (Qazvin), reporting the ST1 as the predominant subtype in this region, with a rate of 56% (24). Similarly, in Nigeria, the most common subtype in this region was ST1 (55.5%) (25). However, in Saudi Arabia and Egypt, reported the ST3 as the most common subtype of *Blastocystis* spp. in the mentioned regions (26, 27). The prevalence of different subtypes of this protozoan underscore the influence of geographic, climatic, and cultural factors in subtype distribution.

The findings of the present study demonstrated a gender-related disparity in *Blastocystis* prevalence, with a higher infection rate among male participants (67%) compared to females (33%). Additionally, the highest prevalence was noted among children over eight years old, which may be attributed to increased outdoor activity and reduced attention to personal hygiene in this age group. These behavioral factors likely contribute to greater exposure to sources of infection. The observed patterns align with the findings reported by Khalili et al., who similarly identified a higher prevalence of *Blastocystis* among older children and male subjects (28).

Despite these correlations, *Blastocystis* pathogenesis is multifactorial. Host immunity, gut microbiota, coinfections, and parasite burden likely impact symptom genesis. In this account, sparse clinical evidence and small numbers preclude conclusions. Nevertheless, the limited subtype profile of symptomatic cases confirms the hypothesis that ST1 may be more pathogenic than other subtypes.

Limitation

There were several limitations in this study. The relatively small sample size may have limited the statistical power to detect significant differences between groups. Subtype identification was limited to PCR-positive samples, and the parasite load was not quantified. Moreover, gut microbiota composition and co-infections with other enteric pathogens were not assessed, which may confound the interpretation of clinical symptoms. These points reduce the ability to infer causality between *Blastocystis* subtypes and clinical symptoms. Although the phylogenetic tree was constructed with 1000 bootstrap replicates, the bootstrap values were not shown in the figure, which limits the visual interpretation of branch support.

Conclusion

ST1 was the most frequently detected *Blastocystis* subtype among both symptomatic and asymptomatic children in Mashhad. However, the frequency of ST1 alone in symptomatic cases leaves the possibility for a subtype-specific pathogenic role. Subtypes ST2, ST3, and ST7 were exclusively identified in asymptomatic individuals, raising the need for further investigation of their clinical significance. Future research would require larger sample sizes, statistical analysis, microbial typing, and longitudinal follow-up to study the pathogenicity of individual *Blastocystis* subtypes more exhaustively.

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Conflict of interest

The authors declare that there is no conflict of interests.

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