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Original Article

Clinical Significance and Subtype Distribution of *Blastocystis* Infection with Colonoscopy Findings

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Abstract

Background: As the role of *Blastocystis* in the pathogenesis of intestinal disorders remains controversial, we aimed to clarify its clinical significance and subtype (ST) distribution in relation to colonoscopic findings.

Methods: Fecal samples were collected from adult patients scheduled for colonoscopy in Aydin Adnan Menderes University, Gastroenterology Department in 2023, 125 consecutive patients with colonic abnormalities and 125 with normal mucosa. *Blastocystis* positivity was determined with direct microscopy and amplification of the small subunit ribosomal RNA (*SSU rRNA*) gene. Subtypes were determined based on exact database matches. Demographic characteristics and clinical findings of participants were statistically analyzed.

Results: *Blastocystis* was detected in 26 fecal samples (10.4%) by direct microscopy and 32 samples (12.8%) by molecular test. Positivity was 16.0% (n=20) in controls and 9.6% (n=12) in patients with colonic abnormalities, with no significant difference ($P>0.05$). In males, *Blastocystis* was more prevalent in those with normal findings than with abnormalities (n=11, 19.6% vs. n=6, 7.7%, $P<0.05$), whereas no difference was observed in females ($P>0.05$). Subtype distribution was ST3 (59.4%, n=19), ST2 (21.8%, n=7), and ST1 (18.7%, n=6). No significant association was found between the overall *Blastocystis* positivity and age, gender, or gastrointestinal symptoms.

Conclusion: Although limited to routine colonoscopic findings, these results raise questions about the potential association of *Blastocystis* with a healthy gut or colonic abnormalities. ST3 was the predominant subtype, consistent with national and global reports.

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Introduction

Blastocystis is a common single-celled eukaryote in fecal samples of humans and a variety of other hosts. Higher prevalences have been reported in low-income countries because of inadequate sanitation and water treatment facilities. The diagnostic method and characteristics of the study populations may affect the reported *Blastocystis* prevalence (1, 2). High polymorphism in small subunit RNA (*SSU rRNA*) gene of *Blastocystis* has led to the identification of numerous subtypes (STs) (3). Following the identification of two novel *Blastocystis* subtypes (ST47 and ST48) in non-human primates (NHPs), the number of defined subtypes has reached 48 (4). The shared genotypes between humans and various animal hosts have suggested possible zoonotic transmission (5).

Although most individuals infected with *Blastocystis* remain asymptomatic, a subset may experience nonspecific gastrointestinal symptoms, such as diarrhea and abdominal pain (6). The findings from experimental murine models are largely restricted to murine-adapted subtypes, particularly ST4. Therefore, their relevance to human infections may be limited for mammalian or avian adapted subtypes. The role of *Blastocystis* in gastrointestinal diseases such as ulcerative colitis (UC) and irritable bowel syndrome (IBS) has recently become a topic of great interest (7, 8). Colonoscopy is the gold standard for screening the colon for abnormalities and the diagnosis of UC, CRC, and IBS. It provides great insights for the association of microbes with colon pathologies such as mucosal redness, ulcers, and erosion (9). In addition, colonic aspirates could be used to test *Blastocystis* positivity (8).

The primary aim of this study was to compare *Blastocystis* positivity between patients with colon abnormalities and those with normal colon mucosa. We also aimed to analyze the subtype distribution of isolates, as well as the demographic characteristics and clinical findings of the patients.

Materials and Methods

Study design

This research was designed as a cross-sectional and observational study. Adult patients scheduled for colonoscopic examination at the Division of Gastroenterology and Hepatology, University Hospital, in 2023 were included in the study. Since pediatric patients were not examined in this clinic, all participants were over 18 yr of age. Voluntariness was the only inclusion criterion. In addition, patients who tested positive for other protozoa or helminths were excluded from the study.

Ethical approval

The Non-Interventional Research Ethics Board in the same university approved the study (Approval No. 2022/154). Written informed consent was obtained from all patients.

Patients and fecal samples

Fecal samples from all patients were collected before colonoscopy cleaning process (i.e., administration of laxatives or bowel-cleansing agents) or medications. The routine colon preparation diet is initiated three days before the colonoscopy, and bowel cleansing is completed the night before the procedure using oral (Sennoside A + B Calcium) and rectal laxatives (sodium dihydrogen phosphate + disodium hydrogen phosphate).

The study (n=125) and control (n=125) groups were defined based on the colonoscopy findings of the participants. The study group was composed of consecutive patients with any of the colonic abnormalities, including polyps, ulcerative colitis, CRC, diverticulosis coli, nonspecific colitis, Crohn's disease, and non-specific ileitis. In addition, polyps were categorized

into adenomatous, hyperplastic, or serrated types.

The control group consisted of patients with a grossly normal colon with no evidence of mucosal abnormalities, masses, or inflammatory changes. Normal colonic mucosa appeared smooth with a uniform pink coloration and a visible vascular pattern. The clinical features and demographic data of the patients were assessed by a gastroenterologist or obtained from the hospital information system. The patients were screened for abdominal/pelvic pain, fecal consistency, diarrhea, iron deficiency anemia (IDA), bleeding per rectum/hemorrhoids, constipation, and weight loss/lack of appetite.

Determination of Blastocystis positivity

Fecal samples were screened with direct microscopy of wet mount preparations (0.9% NaCl and Lugol's iodine). We used a commercial kit (Magen Biotechnology, China) to extract fecal genomic DNA and stored at -20 °C. The final elution volume was 150 µL. The amplification of partial *Blastocystis SSU rRNA* gene was in a 30 µl volume: 1.5 mM $MgCl_2$, 0.2 µM primers (BhRDr and RD5), 0.2 mM dNTP mix, 0.2 U Taq DNA polymerase (Thermo Fisher Scientific, USA), and 1-2 µl template DNA. The reaction was set as follows: pre-denaturation at 94 ºC for 3 min, 30 cycles (30 sec at 94 ºC, 30 sec at 59 ºC, and 60 sec at 72 ºC), and a final extension phase at 72 ºC for 5 min. A 5-µl of amplified DNA fragments was run on a 1.5% agarose (Biomax, Québec, Canada) gel electrophoresis in 1× TBE buffer, with a 100 bp Opti-DNA marker (ABM, Richmond, Canada). Agarose gels were stained with SafeView (ABM, Richmond, Canada) for electrophoresis and visualized under UV (Vilber, Collégien, France).

Determination of Blastocystis subtypes

The samples with the expected amplification size (~600 bp) were unidirectionally sequenced by a commercial company (Medsantek, Istanbul, Turkey). The sequences were aligned with the multiple sequence alignment tool (ClustalW) and edited using BioEdit software (Ibis Biosciences, Carlsbad, USA).

The edited sequences were queried against the *Blastocystis* PubMLST database (pubmlst.org/*Blastocystis*) to determine subtypes and aligned with reference sequences for ST1–7 using MEGA X (Molecular Evolutionary Genetics Analysis). Evolutionary relationships or the taxa were inferred using the Neighbor-Joining method in MEGA. Bootstrap analysis with 1000 replicates was performed and evolutionary distances were calculated using the Maximum Composite Likelihood method. The partial SSU rRNA gene sequence of a lizard flagellate, *Proteromonas lacerate* (Acc. No. AY224080) was included as the outgroup. To identify potential mixed subtypes, positive samples were re-analyzed using subtype-specific (STS) primers. Seven pairs of STS primers (SB83, SB155, SB227, SB332, SB340, SB336, and SB337) were used as previously reported (10). Haplotype and nucleotide diversity analyses were performed separately for each subtype and diversity indices were calculated using the DnaSP v6.0 software.

Analysis of colonoscopic and clinical findings

The data was analyzed with SPSS 23.0 software package (IBM Corp., Armonk, NY, USA). The categorical variables, including colonoscopy results and the presence of common clinical findings were analyzed with the Chi-square test. The mean ages of *Blastocystis*-infected and non-infected patients were compared with the Student's T-test. In addition, logistic regression analysis with was performed to identify factors independently associated with *Blastocystis* positivity. The *P*-value of equal to or less than 0.05 was statistically significant.

Results

Blastocystis positivity and subtypes

Of the 250 fecal specimens from colonoscopy patients, 26 (10.4%) were positive for the presence of *Blastocystis* forms with DM. The positivity of *Blastocystis* increased to 32 (12.8%)

with PCR method (Fig. 1). DM-negative six samples were identified as positive by PCR. Cohen's kappa (ϰ) was approximately 0.82, indicating a strong agreement between microscopy and PCR results. The most frequent subtype was ST3 (n=19, 59.4%), followed by ST2 (n=7, 21.8%), and ST1 (n=6, 18.7). The distinct partial SSU rDNA sequences from the present study were submitted to GenBank® with the following accession numbers: PQ578236-45. The evolutionary relationships and genetic diversity of *Blastocystis* subtypes with reference sequences were also presented in a constructed phylogenetic tree (Fig. 2).

Genetic diversity analysis of *Blastocystis* subtypes revealed varying levels of polymorphism and haplotype diversity. ST1 (n=6) showed five polymorphic sites and three haplotypes (Hd = 0.600; π = 0.00310). ST2 (n=7) exhibited three polymorphic sites and four haplotypes (Hd = 0.857; π = 0.00317), indicating the highest diversity. ST3 (n=19) had only one polymorphic site and two haplotypes, with the lowest diversity (Hd = 0.351; π = 0.00066).

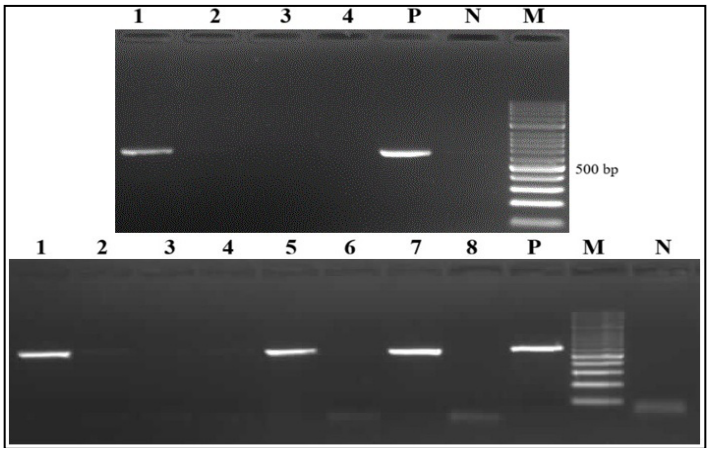

Fig. 1: Agarose gel electrophoresis of PCR-positive samples. P, positive control; N, negative control; M, 100 bp DNA marker. Lanes in the upper gel: 1; lanes in the lower gel: 1, 5, and 7, PCR products of the expected size (~600 bp)

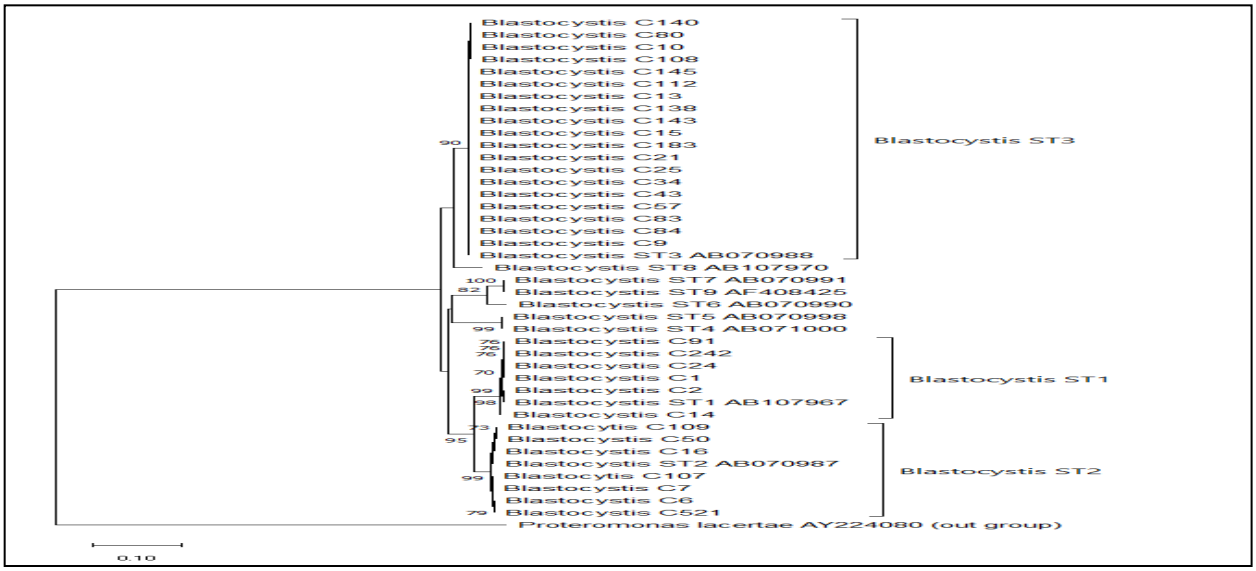

Fig. 2: The phylogenetic tree based on the genetic distance of *Blastocystis* isolates

Analysis of colonoscopic findings

Blastocystis positivity and colonoscopic findings are presented in Table 1. The most common finding in study group patients was colon polyps (n=58, 46.4%), followed by ulcerative colitis (n=29, 23.2%). The polyp types were 38 adenomatous (65.5%), 16 hyperplastic (27.6%), and 4 serrated (6.9%). *Blastocystis* positivity among the individuals with normal colon (n=20, 16.0%) and patients with any findings (n=12, 9.6%) was not statistically different (*P*>0.05). However, a significant difference was observed among males: *Blastocystis* was more frequent in the

control group (n=11, 19.6%) than in the study group (n=6, 7.7%) (P<0.05). No such difference was noted among female patients.

Table 1: *Blastocystis* positivity and colonoscopic findings

	Blastocystis			Subtypes		
	Positive (%)	Negative (%)	Total	ST1 (%)	ST2 (%)	ST3 (%)
Normal colon	20 (16.0)	105 (84.0)	125	4 (20.0)	4 (20.0)	12 (60.0)
Any of the findings	12 (9.6)	113 (90.4)	125	2 (16.7)	3 (25.0)	7 (58.3)
Colon polyps, types	8 (13.8)	50 (86.2)	58	1 (12.5)	2 (25.0)	5 (62.5)
Adenomatous	6 (75.0)	32 (64.0)	38	1 (20.0)	2 (40.0)	2 (40.0)
Hyperplastic	2 (25.0)	14 (28.0)	16	-	-	3 (100)
Serrated	-	4 (8.0)	4	-	-	-
UC	3 (10.3)	26 (91.4)	29	1 (33.3)	-	2 (66.6)
Diverticulosis coli	2 (12.5)	14 (87.5)	16	-	1 (50.0)	1 (50.0)
Non-specific colitis	2 (11.8)	15 (88.2)	17	-	1 (50.0)	1 (50.0)
Other*	-	16 (100.0)	16	-	-	-

***** Crohn's disease (n=4), colorectal cancer (n=9), and non-specific ileitis (n=2); UC: ulcerative colitis

Demographic characteristics and clinical Findings

Blastocystis positivity was not different between the genders and age groups (Table 2). Most patients were between the ages of 61 and 70. The average of *Blastocystis*-positive patients was 58.3±11.7, and it was 57.9±13.1 for negative ones (t=0.152, P=0.879). The most common symptom among participants was abdominal or pelvic pain (n=90, 36%), followed by iron deficiency anemia (n=49, 19.6%). Other symptoms, including constipation (n=24, 9.6%), diarrhea (n=21, 8.4%), bleeding per rectum or hemorrhoids (n=19, 7.6%), and weight loss or lack of appetite (n=18, 7.2%), were less frequent. Nearly one-fifth of participants (n=48, 19.2%) were asymptomatic at the time of colon screening. None of these clinical findings was statistically different between the *Blastocystis*-infected and non-infected groups (Table 3).

Table 2: *Blastocystis* positivity by gender and age groups

	Blastocystis			Statistics		
Gender	Positive (%)	Negative (%)	Total	P*	OR (95% CI)	P**
Female	17 (12.7)	117 (87.3)	134 (53.6)	0.954	0.465-2.058	0.954
Male	15 (12.9)	101 (87.1)	116 (46.4)			
Age Groups (yr)						
20-30	-	11 (100.0)	11 (4.4)	0.475	0.971-1.029	0.970
31-40	4 (25.0)	12 (75.0)	16 (6.4)			
41-50	6 (15.0)	34 (85.0)	40 (16)			
51-60	6 (11.5)	46 (88.5)	52 (20.8)			
61-70	10 (10.9)	82 (89.1)	92 (36.8)			
70+	6 (15.4)	33 (84.6)	39 (15.6)			
Total	32 (12.8)	218 (87.2)	250 (100.0)			

P*, chi-square test; P** logistic regression analysis

Table 3: *Blastocystis* positivity and analysis of clinical findings

		Blastocystis			Statistics		
Variable		Positive (%)	Negative (%)	Total	*P**	OR (95% CI)	P**
Abdominal/ pelvic pain	Yes	11 (12.2)	79 (87.8)	90	0.838	0.422-2.011	0.838
	No	21 (13.1)	139 (86.9)	160			
Iron Def. Anemia	Yes	7 (14.3)	42 (85.7)	49	0.728	0.476-2.895	0.729
	No	25 (12.4)	176 (87.6)	201			
Bleeding per rectum/ haemorrhoids	Yes	1(5.3)	18 (94.7)	19	0.306	0.046-2.781	0.326
	No	31(13.4)	200 (86.6)	231			
Constipation	Yes	2 (8.3)	22 (91.7)	24	0.491	0.133-2.656	0.495
	No	30 (13.3)	196 (86.7)	226			
Weight loss/ lack of appetite	Yes	1 (5.6)	17 (94.4)	18	0.340	0.049-2.968	0.357
	No	31 (13.4)	201 (86.6)	232			
Diarrhoea	Yes	0	21	21	0.670	N.A.	N.A.
	No	32 (14)	197 (86)	229			
None of the symptoms/ Screening	Yes	6 (12.5)	42 (87.5)	48	0.945	0.404-2.432	0.985
	No	26 (12.9)	176 (87.1)	202			

*P**, chi-square test; *P*** logistic regression analysis; N.A, not applicable for logistic regression, maximum number of iterations has been reached

Discussion

The present study was primarily designed to evaluate the clinical significance of *Blastocystis* in relation to colonoscopic and clinical findings. Perhaps the most important finding was that *Blastocystis* positivity was 16.0% (n=20) in cases with no apparent change in the mucosa, compared to 9.6% (n=12) in patients with colonic abnormalities, although the difference was not statistically significant. However, when patients were grouped by gender, a significant difference was observed among males: *Blastocystis* was more frequently detected in the control group (n=11, 19.6%) than in the study group (n=6, 7.7%). This difference was not observed in females. These colonoscopic findings align with recent evidence suggesting that *Blastocystis* may not be characteristically pathogenic but instead may act as a commensal or even beneficial component of the gut microbiome. A large-scale metagenomic study found that the presence of *Blastocystis* was associated with healthier diets, more favorable cardiometabolic profiles, and increased microbial diversity (11). *Blastocystis*-infected patients had normal mucosa, and ST2 was the most common subtype in this group. In addition, the most frequent finding was ulcerative colitis in *Blastocystis*-positive patients (12). Another study involving IBS patients and healthy individuals reported 17.0% and 5.5% positivity of *Blastocystis*, respectively (8).

Emerging evidence suggests a potential association between *Blastocystis* infection and CRC, with several studies reporting higher prevalence rates of the parasite in CRC patients compared to healthy individuals. In the present study, *Blastocystis* was not detected in patients with Crohn's disease, colorectal cancer, or non-

specific ileitis; however, the limited number of these findings or patients (n=15) should be noted. Colonic polyps accounted for the majority of colonoscopic abnormalities observed; although generally benign, these lesions carry a potential risk for colorectal cancer over time. A study from Poland found a significantly higher frequency of *Blastocystis* in CRC patients compared to the control group (13). The majority (75%) of CRC patient isolates were ST3 (n=9, 75.0%). *Blastocystis* was detected in 8.0% of UC patients; the subtypes were ST3 (66.7%), ST1, ST2, and ST7 (7). In our study, *Blastocystis* was positive in 10.3% of UC patients. A systematic review reported that *Blastocystis* positivity in CRC patients ranged from 2.0% to 28.0%, with ST1 and ST3 being the predominant subtypes (14). A case-control study from the UAE reported that *Blastocystis* frequency was significantly higher among CRC patients compared to cancer cancer-free group. In addition, ST2 was the most common among the cancer group and ST3 in the other group (15). The potential role of *Blastocystis* infection in CRC pathogenesis and the importance of its monitoring throughout disease management were highlighted (16). It was the most prevalent and was consistently detected at all stages of CRC diagnosis and treatment. Overall, the existing data in the literature remain controversial because of the variation in study area, study population and methods. *Blastocystis* does not invade colon mucosa, but it can interact with colonic mucosa, causing cytokine changes in certain *Blastocystis* subtypes in patients with IBS (17). *Blastocystis* can disrupt intestinal barrier function by increasing oxidative stress and causing inflammation, like bacterial pathogens; therefore, it may modulate the progression of CRC (18). *Blastocystis* can affect certain microbiota-related diseases, such as active stage-UC and IBS (19, 20).

No significant relation was found between *Blastocystis* infection and any clinical symptoms, including abdominal/pelvic pain, IDA, bleeding per rectum/hemorrhoids, constipation, weight loss/lack of appetite, and diarrhea. These were the primary characteristic symptoms of the patients scheduled for colonoscopic screening. Although most individuals infected with *Blastocystis* exhibit no clinical symptoms, some studies have reported non-specific findings, including dyspepsia, abdominal pain, vomiting, diarrhea, and urticarial symptoms (3,6). Another interesting finding that stands out from the present study is that none of the patients with diarrhea were *Blastocystis* positive. *Blastocystis* frequency was lower in these patients than in the control group (21). However, there were only 21 patients with diarrhea in our study; therefore, making a general statement was not reliable. Several case reports of *Blastocystis* and gastrointestinal bleeding were also found in the literature (22). The elimination of other potential pathogens is crucial for obtaining reliable results. For this purpose, individuals infected with other pathogens (e.g., *Entamoeba histolytica/dispar, Giardia intestinalis*) were excluded from the study. In the present study, *Blastocystis* was detected in 10.4% of samples by direct microscopy and 12.8% by PCR, with 6 additional PCR-positive cases among microscopy-negative samples. The conventional parasitological methods particularly direct microscopy shows lower sensitivity than molecular methods, causing underestimation of the actual parasite burden (3). Intermittent or irregular shedding, as reported for other protozoan parasites such as *Cryptosporidium* spp., can also lead to reduced *Blastocystis* detection with microscopy, particularly during low-shedding periods (23).

Blastocystis isolates in the present study were distributed into three subtypes: ST3 (n=19, 59.4%), ST2 (n=7, 21.8%), and ST1 (n=6, 18.7%). The subtype distribution appears to be consistent with many studies conducted both in Turkey and worldwide, as *Blastocystis* subtypes ST1 to ST7 have been identified in human fecal samples in Turkey (24-26). In a global scale, it was reported that the great majority of human *Blastocystis* isolates worldwide belong to ST1–4 (25). Subtype 3 was reported as the most frequent subtype in many studied populations and regions, including the

Netherlands, Italy, Turkey, China, Iran and North and South America (26-29). However, some reports indicate the predominance of other subtypes in humans, such as among Amazonian indigenous people (30). Geographical variation may influence subtype distribution. ST3 predominance in human samples was reported in Europe, with ST4, ST2, and ST1 as secondary subtypes, whereas in African countries, ST3 predominance was followed by ST1 and ST2 (31). However, the methods and target populations have varied significantly in epidemiological studies; therefore, it is challenging to make general statements. Standardization of subtyping methods and creating biobanks are essential for understanding the transmission dynamics, molecular epidemiology, and potential pathogenicity of different *Blastocystis* subtypes.

The potential influence of demographic factors on *Blastocystis* prevalence has been widely investigated, yet findings remain inconsistent. In the present study, no significant association was observed between *Blastocystis* positivity and either gender or age. Similarly, several studies have reported no correlation between gender and *Blastocystis* positivity (32). In contrast, other studies have reported gender-related differences, with one study showing a prevalence of 53.6% in men and 28.9% in women (33). Age-related variation was observed in a Peruvian cohort, with a rising frequency of *Blastocystis* among older individuals (34). Conversely, no association was observed between *Blastocystis* positivity and age or gender among CRC patients (13). These conflicting findings may be attributed to differences in population size, geographic variation, and study design. In our study, the higher prevalence observed among men in the control group may be attributed to lifestyle-related and occupational factors. In this region, male predominance in outdoor and agricultural activities may increase exposure to environmental sources of infection.

A limitation of the study was the lack of screening for viral and bacterial pathogens. Although these pathogens typically do not have a direct impact on colonoscopy findings, they may act as important confounders in the analysis of clinical findings. We could exclude individuals with other protozoan or helminth infections from the study. Another limitation related to the microscopic detection of *Blastocystis* was the use of a single fecal sample per individual, have limited detection sensitivity due to the intermittent shedding of *Blastocystis*. Finally, the control and study groups were not matched for gender or age, as participants were recruited consecutively during the study period. Comparison of cases and controls revealed no statistically significant difference in mean age, whereas a significant difference was observed in gender. Consequently, colonoscopy findings were analyzed separately by gender. Future studies should employ gender-matched groups to minimize potential confounding effects.

Conclusion

The present study provided an evaluation of the clinical role of *Blastocystis* in humans regarding colonoscopic findings and symptoms. *Blastocystis* positivity was 16.0% (n=20) among individuals with normal colonic appearance and 9.6% (n=12) among patients with colonic abnormalities, with no statistically significant difference. None of the symptoms were linked to *Blastocystis* infection. The predominance of ST3 aligned with national studies and global epidemiological reports in human samples. The use of a secondary diagnostic method, such as PCR, can be beneficial to reduce the number of misdiagnosed cases. Although limited to routine colonoscopic findings, these findings raised questions about whether *Blastocystis* colonization was associated with a healthy gut or was commonly observed in patients with colonic abnormalities. Thus, further research with larger study populations is needed for a comprehensive analysis of clinical relevance and potential impact of *Blastocystis* on intestinal health.

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Conflict of interest

The authors declare that there is no conflict of interests.

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