Original Article

Investigation of fecal occult blood as a marker of intestinal morbidity in blastocystosis

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ABSTRACT

Background: Because the testing for fecal occult blood (FOB) served as a non-invasive screening tool for intestinal pathological conditions, we hypothesized that FOB might correlate with pathogenic *Blastocystis* subtypes.
Objective: To evaluate the usefulness of FOB in blastocystosis as a marker of intestinal morbidity and its correlation with the detected subtypes.
Patients and Methods: This cross-sectional study recruited 51 patients infected with *Blastocystis* spp. Demographic, clinical data, and stool samples were collected. Rapid diagnostic tests for FOB and *H. pylori* antigens were performed, and *Blastocystis* strains were molecularly subtyped using the sequenced-tagged site-based PCR assay.
Results: Among the study population, gastrointestinal (GI) symptoms were reported in 64.71%, including abdominal pain (31.4%), diarrhea (13.7%), and irregular bowel habits (29.4%). Positive FOB was detected in 9 patients (17.6%). Two *Blastocystis* subtypes were identified: ST1 in 6 cases (11.8%), and ST3 in 45 (88.2%). Statistical analysis indicated a significant association between *Blastocystis* ST1 and alternating bowel habits. No significant association was detected between FOB and any *Blastocystis* subtypes, or the demographic and clinical parameters.
Conclusion: The study demonstrated FOB insignificant association with blastocystosis. Accordingly, we eliminated its possible role as an intestinal morbidity marker in blastocystosis.

Keywords: blastocystosis; fecal occult blood; gastrointestinal manifestations; molecular subtyping; morbidity marker.

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INTRODUCTION

Blastocystosis that affects a broad variety of animal hosts is caused by *Blastocystis* spp., an intestinal protozoan belonging to the Stramenopile line of Chromista kingdom inhabiting the lower digestive tract of humans[1-3]. While conventionally referred to as *Blastocystis hominis*; the term *Blastocystis* spp. is described as more appropriate due to the parasite high genetic heterogeneity and low host specificity[4]. Currently, *Blastocystis* spp. comprises over 20 identified heterogenous subtypes (ST) based on small-subunit ribosomal RNA (SSU-rRNA) gene analysis. In humans, nine *Blastocystis* subtypes (ST1-ST9) were frequently identified[1,5].

Globally, *Blastocystis* spp. is widely distributed, and considered the most prevalent intestinal protozoa with an estimate of 1 billion human infections, and a higher predominance in low-middle income countries[6]. In Egypt, infection rates as low as 16.5% and as high as 67.4% were reported in humans[7,8]. Clinically, the majority of cases are asymptomatic, or present with non-specific GI symptoms including diarrhea, vomiting, anorexia, flatulence and abdominal pain. In addition, blastocystosis was proposed to be strongly linked to irritable bowel syndrome, inflammatory bowel disease, as well as cutaneous disorders such as urticaria[9,10]. Though several studies[11-13] attempted to identify the pathogenesis of *Blastocystis*, its pathogenic potential in human hosts has remained uncertain. In fact, the parasite ability to cause disease is not fully elucidated and possible virulence factors are still explored[11,-13].

Since the introduction of rapid diagnostic tests, FOB was recognized as a tool for detection of minimal amounts of fecal blood. Although FOB test served primarily as a non-invasive screening method for colorectal cancer, various other conditions involving intestinal inflammation and tissue damage are associated with positive FOB[14]. From the same perspective, FOB test was used to indicate blood loss in enteric parasitic infections as a biomarker of intestinal pathological changes, including soil transmitted helminths[15,16], *E. histolytica*[17], and *S. mansoni*[18].

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Given its role as a marker of intestinal morbidity, the present study aims to investigate the presence of FOB in blastocystosis, and its association with the detected *Blastocystis* subtypes.

PATIENTS AND METHODS

This cross-sectional analytical study was carried at the Medical Parasitology Department, Faculty of Medicine, Cairo University during the period from May 2023 to January 2024.

Study design: The study microscopically examined stool samples to select patients with blastocystosis. Other intestinal parasites, and *H. pylori* were excluded. From each patient, relevant demographic, and medical data were obtained, and qualitative FOB detection was performed. Besides, molecular *Blastocystis* subtyping was identified. Results were interpretated to assess FOB as a marker for intestinal morbidity, and its relation to *Blastocystis* subtypes.

Study population: This consisted of 51 patients including both children and adults, attending the Diagnostic and Research Unit of Parasitic Diseases (DRUP), at the Medical Parasitology department, Faculty of Medicine, Cairo University. Participants were recruited based on predefined set of inclusion criteria: presence of *Blastocystis* spp. on microscopic examination of stool samples, and absence of co-infections including other intestinal parasites and *H. pylori*. Patients diagnosed with intestinal conditions associated with FOB including colorectal cancer, polyps, diverticulosis, Crohn's disease and ulcerative colitis, were excluded from the study.

Sample collection and processing: From each patient, relevant demographic and medical data were obtained, and a stool sample was collected in a clean plastic container with a tightly fitted lid. Each stool sample was separated into two aliquots. A part was subjected to macroscopic and microscopic examination, detection of FOB, and exclusion of *H. pylori*. The second part was kept in -20°C for the molecular subtyping of *Blastocystis* spp.

Microscopic examination: The diagnosis of blastocystosis was determined using wet mounts stained with iodine for detection of *Blastocystis* vacuolar forms. Microscopic examination was also performed to exclude presence of cysts, trophozoites, and ova. Modified Ziehl-Neelsen stained slides were prepared for exclusion of coccidian oocysts[19].

Coproimmunoassays: For each sample, detection of FOB and exclusion of *H. pylori* was performed using commercially available copro-immunoassays. The rapid diagnostic tests, ABON™ FOB One Step Fecal Occult Blood Test (Faeces) and ABON™ One Step *H. pylori* Antigen Test Device (Feces) (Abon Biopharm, Hangzhou Co., Ltd., China) were used respectively, according to the manufacturer's instructions[20,21].

Molecular identification of *Blastocystis* subtypes: The genomic DNA was isolated from ~200 mg of each frozen aliquot. The samples were subjected to thermal shock by freezing for 5 min in liquid nitrogen, and thawing in a 95°C water bath for 5 min, that was repeated for 10 cycles. The DNA extraction procedure was then completed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). According to the method described by Scicluna *et al*.[22], an initial PCR was conducted using the *Blastocystis* barcode primers RD5 and BhRDr to amplify a *Blastocystis* spp. DNA sequence of ~600 bp (Table 1). The PCR reaction mixture volume was 25 µl; formed of 12.5 µl master mix, 1 µl of each primer, 0.1 µl Taq polymerase, 7.4 µl distilled water and 3 µl of the extracted DNA. The cycling conditions included an initial denaturation at 94°C for 4 min,

Table 1. Primers' sequence used for molecular identification of *Blastocystis* subtyping, and the respective products size.

Primer	Sequence (5'-3')	Product size (bp)	Reference
RD5 **BhRDr**	**F:** ATCTGGTTGATCCTGCCAGT **R:** GAGCTTTTTAACTGCAACAACG	~600	[22]
SB83 ST 1	**F:** GAAGGACTCTCTGACGATGA **R:** GTCCAAATGAAAGGCAGC	351	
SB155 ST 2	**F:** ATCAGCCTACAATCTCCTC **R:** ATCGCCACTTCTCCAAT	650	
SB227 ST 3	**F:** TAGGATTTGGTGTTTGGAGA **R:** TTAGAAGTGAAGGAGATGGAAG	526	
SB332 ST 4	**F:** GCATCCAGACTACTATCAACATT **R:** CCATTTTCAGACAACCACTTA	338	[23]
SB340 ST 5	**F:** TGTTCTTGTGTCTTCTCAGCTC **R:** TTCTTTCACACTCCCGTCAT	704	
SB336 ST 6	**F:** GTGGGTAGAGGAAGGAAAACA **R:** AGAACAAGTCGATGAAGTGAGAT	317	
SB337 ST 7	**F:** GTCTTTCCCTGTCTATTCTGCA **R:** AATTCGGTCTGCTTCTTCTG	487	

F: Forward; **R:** Reverse.

then 35 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, extension at 72°C for 80 sec, and final extension for 5 min. The amplified products were stained with ethidium bromide, loaded in 1.5% agarose gel electrophoresis and visualized under UV illumination. *Blastocystis* subtype detection was achieved according to Yoshikawa *et al.*[23], by performing PCR on *Blastocystis* spp. genomic DNA using 7 sets of standardized primers targeting subtype-specific sequence-tagged-site gene, each set in a separate reaction (Table 1). The amplified products were visualized as aforementioned.

Statistical analysis: The collected data were analyzed using statistical package for the social science (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Quantitative data were presented as mean and standard deviation, and for categorical data frequency and percentages were used. Non-parametric Mann-Whitney test was used for quantitative variables analysis. Chi-square test was performed to compare categorical data, and substituted by Fisher's exact test when a frequency is less than 5. Statistical significance was considered when *P* value was less than 0.05.

Ethical considerations: The current study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University (N-114-2023/A-32-2024). Informed consent and assent were obtained accordingly from all the study participants. All participants were notified with their results and directed for the appropriate management.

RESULTS

Demographic and clinical data of the study population: Among 51 patients infected with blastocystosis, females constituted 70.6% while the males were 29.4%. Their age ranged from 10 to 82 years with a mean of 38.2±17.7 y. When they were categorized into three groups: <18 y, 18-65 y, and > 65 y, the second group formed the majority with a percentage of 80.4%. Regarding their residence, 35 cases (68.6%) were living in rural areas whereas 16 (31.4%) were residing in urban areas. Clinical manifestations in the form of GI symptoms were recorded in 33 (64.71%) including mainly abdominal pain (16, 31.4%), irregular bowel habits with alternating episodes of constipation and diarrhea (15, 29.4%), and diarrhea (7, 13.7%). Some patients experienced more than one symptom (Fig. 1).

Detection of FOB and its distribution: Occult blood was detected in 9 patients (17.6%), whereas 82.4% had negative samples. The mean age of patients with positive FOB test was higher than FOB negative patients. No statistically significant association was detected between FOB results and the different study parameters (Table 2).

***Blastocystis* subtypes and their distribution:** All the samples were successfully amplified. Two subtypes were determined: ST1 in 6 cases (11.8%) and ST3 in 45 cases (88.2%), i.e., no cases of mixed subtypes were detected (Fig. 2). The mean age of patients with ST1 was higher than those with ST3. No statistically significant association was detected between *Blastocystis* subtypes and the different study parameters except for the alternating bowel habits manifestation which was significantly associated with ST1 ($P<0.05$) (Table 3).

Distribution of *Blastocystis* subtypes according to FOB test result: Statistical analysis of FOB results revealed no statistically significant association with the detected *Blastocystis* subtypes; ST1 and ST3 (Table 4).

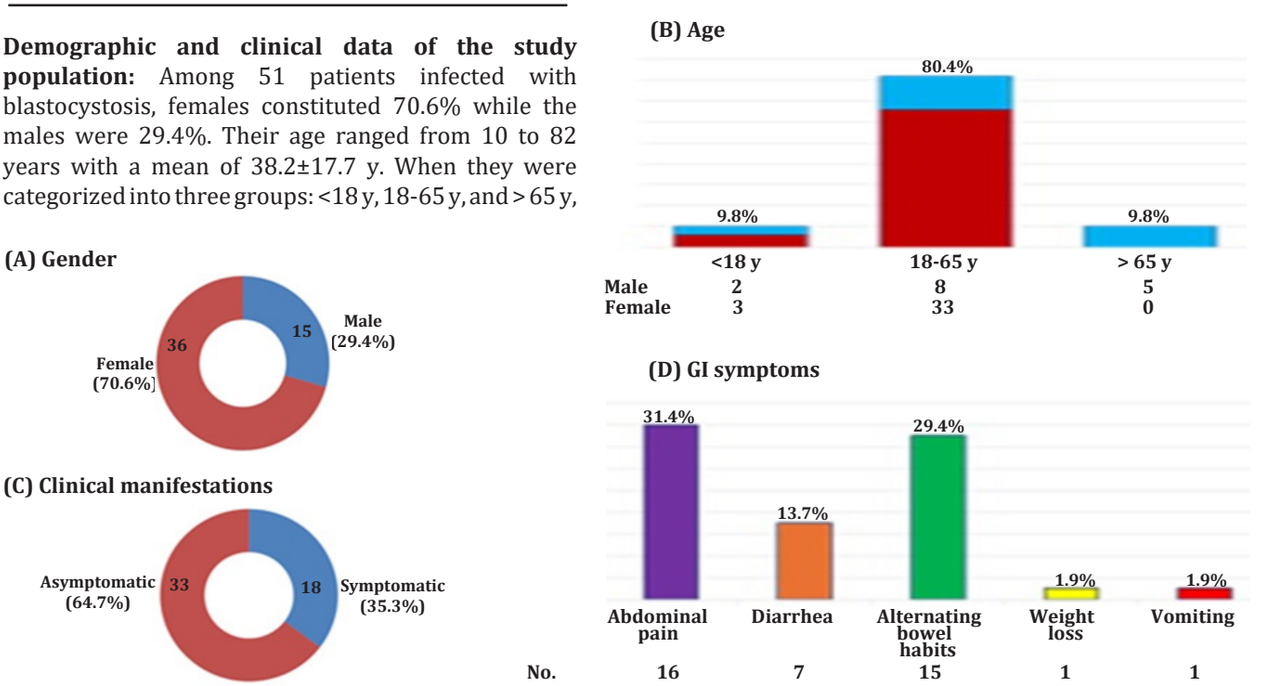

Fig. 1. Diagrams of the demographic and clinical data of the study population. **(A)** gender distribution, **(B)** age distribution including gender prevalence in each age group, **(C)** clinical manifestations, **(D)** distribution of the various GI symptoms reported in the study.

Table 2. Distribution of demographic and clinical data of the study population according to FOB test result.

Parameter			Positive FOB	Negative FOB	Statistical analysis
			No. = 9 (17.6%)	No. = 42 (82.4%)	*P* value
Age (Mean ± SD)			49.11 ±20.42	35.95 ±16.51	0.065
Age groups	**<18 y** **18-<65 y** **> 65 y**		0 (0.0%) 7 (17.1%) 2 (40.0%)	5 (100%) 34 (82.9%) 3 (60.0%)	0.386
Gender	**Male** **Female**		4 (26.7%) 5 (13.9%)	11 (73.3%) 31 (86.1%)	0.421
Residence	**Urban** **Rural**		1 (6.3%) 8 (22.9%)	15 (93.8%) 27 (77.1%)	0.242
Clinical manifestations	**Asymptomatic**	**Yes** **No**	3 (16.7%) 6 (18.2%)	15 (83.3%) 27 (81.8%)	1
	Abdominal pain	**Yes** **No**	2 (12.5%) 7 (20.0%)	14 (87.5%) 28 (80.0%)	0.701
	Diarrhea	**Yes** **No**	2 (28.6%) 7 (15.9%)	5 (71.4%) 37 (84.1%)	0.592
	Alternating bowel habits	**Yes** **No**	2 (13.3%) 7 (19.4%)	13 (86.7%) 29 (80.6%)	0.709
	Loss of weight	**Yes** **No**	0 (0.0%) 9 (18.0%)	1 (100%) 41 (82.0%)	1
	Vomiting	**Yes** **No**	0 (0.0%) 9 (18.0%)	1 (100%) 41 (82.0%)	1

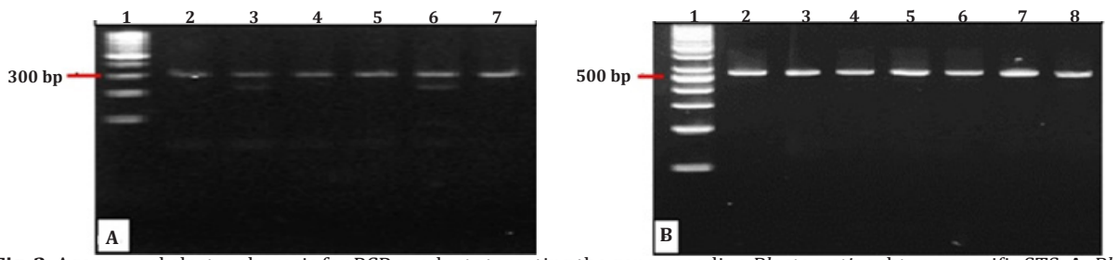

Fig. 2. Agarose gel electrophoresis for PCR products targeting the gene encoding *Blastocystis* subtype-specific STS. **A:** *Blastocystis* ST1 at 351 bp, and **B:** *Blastocystis* ST3 at 526 bp, DNA molecular weight marker of 100 bp in lane 1 in both A and B.

Table 3. Distribution of demographic and clinical data of the study population according to the detected *Blastocystis* subtypes.

Parameter			Subtype (ST) 1	Subtype (ST) 3	Statistical analysis
			No. = 6 (11.8%)	No. = 45 (88.2%)	*P* value
Age (Mean ± SD)			53.0 ± 20.07	36.31 ± 16.73	0.054
Age groups	**<18 y** **18-<65 y** **> 65 y**		0 (0.0%) 4 (9.8%) 2 (40.0%)	5 (100.0%) 37 (90.2%) 3 (60.0%)	0.194
Gender	**Male** **Female**		4 (26.7%) 2 (5.6%)	11 (73.3%) 34 (94.4%)	0.054
Residence	**Urban** **Rural**		1 (6.3%) 5 (14.3%)	15 (93.8%) 30 (85.7%)	0.651
Clinical manifestations	**Asymptomatic**	**Yes** **No**	1 (5.6%) 5 (15.2%)	17 (94.4%) 28 (84.8%)	0.405
	Abdominal pain	**Yes** **No**	0 (0.0%) 6 (17.1%)	16 (100.0%) 29 (82.9%)	0.159
	Diarrhea	**Yes** **No**	0 (0.0%) 6 (13.6%)	7 (100.0%) 38 (86.4%)	0.578
	Alternating bowel habits	**Yes** **No**	5 (33.3%) 1 (2.8%)	10 (66.7%) 35 (97.2%)	0.006*
	Loss of weight	**Yes** **No**	0 (0.0%) 6 (12.0%)	1 (100.0%) 44 (88.0%)	1
	Vomiting	**Yes** **No**	0 (0.0%) 6 (12.0%)	1 (100.0%) 44 (88.0%)	1

***:** Significant (*P*<0.05).

Table 4. Distribution of *Blastocystis* subtypes according to FOB results.

Blastocystis subtype	Positive FOB	Negative FOB	Statistical analysis
	No. = 9 (17.6%)	No. = 42 (82.4%)	*P* value
ST 1	2 (33.3%)	4 (66.7%)	0.284
ST 3	7 (15.5%)	38 (84.4%)	

DISCUSSION

In the present study, FOB test was applied as a measure of intestinal morbidity in blastocystosis, after excluding other parasitic and *H. pylori* infections. While FOB was detected in 17.6% of blastocystosis cases, no significant association was found between FOB and the study parameters including *Blastocystis* subtype, age, gender, residence and clinical manifestations. Aligned with our results, the study by Matiut and Hritcu[24] included 49 patients infected with *Blastocystis*, either alone or with other intestinal pathogens, and claimed the absence of significant correlation between parasitism and FOB. These findings were also supported by another study including 29 cases where no significant association was recorded between blastocystosis and FOB. Interestingly, among the various clinical and laboratory parameters examined in their study, the investigators reported a significant association between blastocystosis and only 2 parameters: the absence of diarrhea and high creatinine level[25]. On the contrary, a retrospective study on *Blastocystis* infected children conveyed different findings. The study analyzed a set of hematological indices in 6851 *Blastocystis* spp. infected cases versus 3615 controls visiting health care facilities for routine checkup. They concluded that erythrocyte sedimentation rate, C reactive protein and FOB were significantly higher in *Blastocystis* cases compared to the controls. Furthermore, the study pointed to the possible complicity in iron deficiency anemia, likely through colitis with intestinal mucosal inflammation and potential invasion[26].

Similar to *E. histolytica*, *Blastocystis* presumably secrete a hyaluronidase enzyme that can destroy the extracellular matrix, providing a route of entry for mucosal invasion[27]. Besides, it possesses proteases that can cleave the immune effector molecules, such as cysteine protease, anti-lactoferrin and anti-lysozyme. In addition, *Blastocystis* spp. is capable of becoming phagocytic with altered environmental conditions, and insufficient nutrients. Based on these activities, *Blastocystis* was described as predatory allowing its survival and persistence[28]. Nonetheless, the true identity of *Blastocystis*, whether it is a pathogen or commensal is still an unresolved debate. It is worth noting that the controversial pathogenicity of *Blastocystis* is largely attributed to the consistent existence of the parasite in both symptomatic and asymptomatic individuals. Our findings revealed that 35.3% of patients were asymptomatic while 64.71% had GI symptoms, mainly abdominal pain and irregular bowel. On the one hand, numerous reports indicated a significant association between blastocystosis and symptoms, where the greatest proportion of infected patients suffer from GI symptoms, notably abdominal pain[29-31]. Factors considered to be associated with occurrence of symptoms are high parasite load (≥5 parasites/field)[32] and liquid stools[33]. On the other hand, several studies reported blastocystosis to be more prevalent in asymptomatic individuals than in those with GI manifestations[34,35]. Likewise, Cinek *et al*.[36] described most *Blastocystis* infected cases as asymptomatic carriers.

Only 2 *Blastocystis* subtypes were detected in the present study; predominantly ST3 (88.2%) and ST1 (11.8%). Both subtypes were observed in either of asymptomatic and symptomatic patients, while no statistically significant association was detected between *Blastocystis* subtypes and the different study parameters except for the alternating bowel habit. In agreement with our findings, Cinek *et al*.[36] studied samples collected from 6 countries, and reported ST3 as the most frequent subtype followed by ST1 then ST2. Congruently, multiple studies[37-42] have established the ST3 predominance. Nevertheless, other subtypes were identified as the prevalent in two studies including ST1[43] and ST5[44]. While in their review, Popruk *et al*.[40] indicated that ST4 was more common in Europe and UK, and that collectively ST1-ST4 accounted for 91.65% of all the identified *Blastocystis* subtypes worldwide. In fact, the existence of various *Blastocystis* subtypes and strains was regarded as the most plausible explanation of its variable pathogenicity[45]. Phenotypically, the pathogenic subtypes were described as being larger in size, having coarse surface, growing rapidly and acquiring the amoeboid form in culture[27]. For instance, in Zhao *et al*.[46] report, ST1 and ST3 were identified in both symptomatic and asymptomatic blastocystosis, whereas ST2, ST4, ST7 were exclusively detected in diarrhea cases. Likewise, Muñoz-Sánchez *et al*.[47] concluded that ST2 was particularly associated with intestinal manifestations. Correspondingly, Kesuma *et al*.[48] reported significant association between ST1 and irritable bowel syndrome, while Mohamed *et al*.[49] reported ST1 dominance in colorectal cancer patients. Clearly, intra-subtype genetic variation analyses can further elucidate *Blastocystis* association with disease and its interaction with the intestinal ecosystem[50]. Currently, exploring the interplay between *Blastocystis* subtypes and the gut microbiota, and their correlation with pathogenesis and clinical manifestations are active areas of research[46].

Over the past years, fecal biomarkers were expanded into a large panel of proteins, DNA and RNA as well as microbiome shifts that are detected in stool samples. They offer a non-invasive tool to assess the gut health and predict the extent of intestinal pathology in GI diseases such as neoplasia, inflammation, allergy and malabsorption[51,52]. While many of these biomarkers are still under research, and not established for clinical use, FOB has been a mainstay in routine laboratory investigations being rapid, simple, sensitive and cost effective[53]. Presence of FOB in intestinal parasitic infections reflects mucosal pathological changes and blood loss; an association that may be applied in clinical and epidemiological settings. For instance, the use of FOB test correlated with *S. mansoni* infection patency and severity, since its positivity was significantly reduced after treatment. In addition, FOB tests are point-of-care, field-feasible, low cost compared to other fecal biomarkers as calprotectin, and can identify early mucosal changes before eggs become detectable in stool. Subsequently, FOB was proposed for incorporation in control programs as a reliable marker to monitor intestinal morbidity before and after praziquantel treatment[18,54].

It is worth mentioning that despite the lack of significant association, our findings revealed that 3 out of the 9 FOB positive cases were asymptomatic, denoting that absence of symptoms does not exclude intestinal pathology. This fact raises the question of the need for treatment, as therapy in blastocystosis is generally not recommended and limited to symptomatic patients passing a considerable number of the parasite in stool[11,55]. Accordingly, efficient intestinal morbidity markers can essentially influence the treatment decision of blastocystosis.

In conclusion, we found no association between FOB and blastocystosis, thus eliminating the potential role of FOB test as a marker of intestinal pathology in this infection. The search for reliable cost-effective morbidity markers is necessary to elucidate the extent of the pathologic impact attributed to this enigmatic parasite.

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