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Molecular assay and *in vitro* culture for *Blastocystis* prevalence in Dakahlia governorate, Egypt

Mohammed Y. Shakra[1], Gamal A. Abu-Sheishaa[2], Adel O. Hafez[2], and Fatma M. El-Lessy[3]

[1]Department of Medical Parasitology, Faculty of Medicine, Al-Azhar University, Damietta, Egypt.
[2]Department of Medical Parasitology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.
[3]Department of Medical Parasitology, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt.

Corresponding author: Mohammed Y. Shakra, Department of Medical Parasitology, Faculty of Medicine, Al-Azhar University, Damietta, Egypt.
Email: dr.mohammed_youssef@yahoo.com

Abstract

Blastocystis is a polymorphic enteric parasite with a worldwide distribution. It is one of the most common human intestinal protozoans in developing countries. The primary objective of this study was to determine the diagnostic value of microscopy, stool culture, and a polymerase chain reaction (PCR) technique for assessment of *Blastocystis* prevalence and risk factors. Human stool samples were collected from 110 individuals from Dakahlia governorate, Egypt as a part of a routine check-up or having gastrointestinal tract (GIT) symptoms. These samples were subjected to direct fecal smear microscopy, culture, and PCR for the detection of *Blastocystis sp*. Positive results for *Blastocystis* screening among the study population were 36 (32.7%), 41 (37.3%), and 43 (39.1%) by microscopy, PCR, and culture, respectively. Statistical analyses demonstrated that the agreement between the culture and PCR was perfect (K=0.925). Compared to culture, the sensitivity of PCR was 95% and the specificity was 97% while the sensitivity of microscopy was 84% and the specificity was 90.5%. We concluded that the *in vitro* culture and molecular assay have significant diagnostic value for the accurate detection and identification of *Blastocystis* in stool samples. The pathogenic potential of *Blastocystis* cannot be ruled out because our results found a link between *Blastocystis* carriage and gastrointestinal symptoms.

Keywords: *Blastocystis*, PCR, Culture, Dakahlia.

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Introduction

Blastocystosis is a disease caused by a protist parasite, namely *Blastocystis*. *Blastocystis sp.* is an anaerobic, enteric unicellular stramenopile. It colonizes the gastrointestinal tract of humans and animals (such as birds, rodents, fishes, and domestic animals). *Blastocystis hominis* is one of the most frequent species of *Blastocystis*.[1] *Blastocystis* is one of the most common enteric parasites found in human fecal samples, due to poor hygienic practices. The clinical presentation of *Blastocystis* infection may be an asymptomatic carrier or nonspecific symptoms such as abdominal pain, flatulence, nausea, vomiting, anorexia, weight loss, and acute or

chronic diarrhea.[2] Irritable bowel syndrome and inflammatory bowel disease have been reported to be associated with infection.[3]

The detection of *Blastocystis* in human fecal samples is usually based on microscopic examination of either wet mount preparations or trichrome-stained smears. *Blastocystis* is highly polymorphic e.g., vacuolar, granular, multi-vacuolar, amoeboid, and cyst. Also, the parasite may be difficult to distinguish from leucocytes or cysts and trophozoites of the protozoa, this makes the morphology-based diagnosis problematic.[4] Hence, various alternative diagnostic techniques such as culture and molecular methods have been developed for the identification of *Blastocystis*.[5] DNA-based detection methods have high sensitivity and specificity in low parasite prevalence populations, compared to microscopy approaches. DNA-based detection methods also allow multiplexing, which in turn facilitates the identification of co-infection and determination of species genetic variations.[6]

in-vitro stool culture is one of the most sensitive techniques for the detection of *Blastocystis* in comparison with microscopic methods. It has a significant value in large-scale screening purposes and obtaining a higher concentration of the requested genetic material for molecular testing.[7]

In the current study we evaluated the diagnostic value of microscopy, stool culture, and a polymerase chain reaction (PCR) technique for assessment of *Blastocystis* prevalence and risk factors among populations in Dakahlia Governorate, Egypt.

Subjects and Methods

Study area

The study was conducted in Dakahlia Governorate. The governorate is considered one of the major agricultural areas in Egypt lying northeast of Cairo. Its area is approximately 3,500 km^2 between 31 02'60.00" N and 31 22'59.99" E.

Study Subjects

This was a cross-sectional study, included 110 individuals from Dakahlia governorate, Egypt. Their ages ranged from 2 to 69 years (33.8 ± 13.65), and both sexes were included. They attended central hospitals and health care units, during July 2021 to April 2022, for screening for parasitic infection as part of a routine check-up or they suffered gastrointestinal tract (GIT) symptoms. A structured questionnaire concerning required demographic and clinical data was filled out for each studied case.

Collection of stool samples

Participants were asked to submit fresh stool specimens free from water and urine. Stool samples were collected in clean, dry, leak-proof, plastic containers and sent immediately to the laboratory. Each stool sample was divided into four portions to perform microscopy, culture, and a molecular assay.

Microscopy

The first part (50 mg) was microscopically examined by direct wet mount saline smear and Lugol's iodine smear.[8] Both smears were examined under low (×10) and high power (x40) magnification for visualization of ova, larvae, segments, trophozoites, cysts, and associated elements; pus cells, RBC, and yeast cells. Three smears from each stool sample were prepared and examined.

The second part of the specimens was preserved in tight containers using formalin saline fixative for the modified Kinyoun's Acid-Fast Stain (Cold Method), for detection of coccidian oocysts, and for Wheatley's modified Trichrome stain for detection of intestinal protozoa.[8]

Culture

The third part of all fresh stool samples was cultured on a modified Jones' medium for detection of *Blastocystis* spp. according to El-Badry et al., 2018.[9] Briefly, about 50 mg fresh stool was directly inoculated into tubes containing 5 ml Jones' medium enriched with 10% horse serum and incubated at 37 ºC for 48 – 72 hrs. The sediment was examined after 48 - 72 hrs. using light microscopy for the detection of *Blastocystis*. If no organisms were found, the samples checked every 48 hr. for 10 days after cultures, before reporting negative results. The

rest of the specimen was stored at -20°C in Eppendorf tubes for molecular studies.

Molecular assays

Extraction of genomic DNA from stool specimens was performed by using commercial kits (Cat. No. 51504, QIAamp® Fast DNA Stool Mini Kit, QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The spin column was transferred to a clean microcentrifuge tube then 50µl DNA elution buffer was added to the spin column, incubated for 2-5 minutes at room temperature, and then centrifuged in a microfuge at top speed for 30 seconds to elute the DNA. The eluted DNA was stored at -20°C for future use.

The extracted DNA was amplified using Reverse primer BhRDr (GAGCTTTTTAACTG CAACAACG) and Forward primer RD5 (ATCTGGTTGATCCTGCCAGT).[9] PCR amplification was performed in a final volume of 50 µL per reaction containing 25 µL TopTaq Master Mix provided in the kit (QI-AGEN, Hilden, Germany), 17 µL nuclease-free water, 4 µL template DNA, and 2 µL of each primer. Amplification was carried out in a thermal cycler (Nyx Technik, USA) and the following amplification profile was used: 4 min of initial denaturation at 95 °C, followed by 35 cycles, each of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.20 min. The final extension was conducted at 72 °C for 4 min. The amplified PCR products were separated on a 1.5% (w/v) agarose gel (Promega V 312A), at 70 V for 110 min (Bio-Rad Laboratories, USA), stained with ethidium bromide (Promega, G188A), and visualized under UV light.

Statistical analysis

Data were statistically analyzed by the SPSS software version 26 (Chicago, IL, USA). The mean, standard deviation, frequency, and percentage were used to establish descriptive statistics for both quantitative and qualitative factors. The Chi-square test was used to determine statistical significance, and data were considered statistically significant if the p value was less than 0.05. The receiver operating characteristic (ROC) curve analysis was used to determine the diagnostic tests' accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The 95% confidence intervals (CI) were calculated. The kappa coefficients were used to test the agreement between culture and microscopy / PCR results. No agreement was considered at Kappa <0; 0.01-0.20 indicated slight agreement; 0.21-0.40 fair agreement; 0.41-0.60 moderate agreement; 0.61-0.80 substantial agreement; and 0.81-1.00 almost perfect agreement.

Results

The correlation between the *Blastocystis*-positive samples and the residence of patients was statistically significant (*p*=0.047). It is more common in rural areas than in urban areas. The animal contact and low socioeconomic status were linked to *Blastocystis* infection (53.5% and 55.8%, respectively). *Blastocystis* was more detected in symptomatic (67.4%) than asymptomatic (32.6%) participants *p*=0.041, (Table 1).

The overall prevalence of parasitic infections in the 110 samples of human feces was 50.9%. The most prevalent parasite was *Blastocystis* sp. (32.7%), followed by *Giardia intestinalis* (9.1%), *Entamoeba histolytica/dispar* (6.3%), *Enterobius vermicularis* (3.6%), *Ascaris lumbricoides* (2.7%), *E. coli* (2.7%), *Hymenolepis. nana* (1.8%), and the least detected ones were *Cryptosporidium species* (0.9%) (Table 2).

Microscopical examination for detection of parasites in the stool samples of the 110 study subjects revealed that 36 cases (32.7%) were positive for *Blastocystis*, mono-infection 26 cases (23.9%) and multi-infection 10 cases (8.8%) (Table 2).

Table 1. Demographic, environmental, and clinical variables of the 110 study individuals.

Variables		*Blastocystis* sp. Culture						*p*-value
		Positive		Negative		Total		
		No.	%	No.	%	No.	%	
Age group (year)	2-6	2	4.7	4	5.9	6	5.6	NS
	7-18	19	44.2	17	25.4	36	32.7	
	19-40	11	25.6	30	44.8	41	37.3	
	41-60	7	16.3	10	14.9	17	15.6	
	>60	4	9.3	6	8.9	10	9.1	
Sex	Males	25	58.1	36	53.7	61	55.5	NS
	Females	18	41.9	31	46.3	49	44.5	
Residence	Rural	26	60.5	29	43.3	55	50.0	0.047
	Urban	17	39.5	38	56.7	55	50.0	
Animal contact	Present	23	53.5	41	61.2	64	58.2	NS
	Absent	20	46.5	26	38.8	46	41.8	
Socioeconomic standard	High	2	4.7	5	7.5	7	6.4	NS
	Medium	17	39.5	23	34.3	40	36.4	
	Low	24	55.8	39	58.2	63	57.3	
GIT-Symptoms	Symptomatic	29	67.4	33	49.3	64	58.2	0.041
	Asymptomatic	14	32.6	34	50.8	46	41.8	
Total		43	100	67	100	110	100	

Table 2. Detection of prevailing parasites by microscopy.

		Parasites	Frequency	Percent (%)
Microscopic examination	Mon infection	*Blastocystis* species	26	23.6
		G. intestinalis	8	7.3
		E. histolytica /dispar	5	4.5
		Cryptosporidium species	1	0.9
		Entrobious vermeculareis	3	2.7
		Ascaris lumbricoides	2	1.8
		Hymenolepis nana	1	0.9
		Parasites	Frequency	Percent (%)
	Multi infections	*Blastocystis species and G. intestinalis*	2	1.8
		Blastocystis species and E. histolytica/dispar	2	1.8
		Blastocystis species and E. coli	3	2.7
		Blastocystis species and Enterobius vermiculates	1	0.9
		Blastocystis species and Ascaris lumbricoides	1	0.9
		Blastocystis species and Hymenolepis nana	1	0.9
		Total	56	50.9
		No ova and parasites detected	54	49.1
		Total	110	100.0

The most common morphological form of the parasite was the vacuolar form in direct smears and culture. Our *in-vitro* cultured samples showed growth of *Blastocystis* in 43 samples (39.1%). Of these, 7 culture-positive samples were recorded as negative by microscopy (Table 3 and Figure 1). The PCR assay of DNA extracted directly from stools showed 41 positive samples

(37.3%) for *Blastocystis*. The molecular assay revealed 5 new positive samples, which were initially negative with microscopy (Table 3 and Figure 2).

Table 3. Detection of *Blastocystis* species using direct wet mount smears and the PCR assay among study populations.

				Blastocystis species (Microscopy)		PCR		
				Positive (%)	Negative (%)	Positive (%)	Negative (%)	Total (%)
Blastocystis species Culture	Positive	Single infection		26 (23.9)	7 (6.4)	31 (28.2)	2 (1.8)	33 (30.0)
		Multiple infections	*Blastocystis species and E. histolytica/dispar*	2 (1.8)	0 (0.0)	2 (1.8)	0 (0.0)	2 (1.8)
			Blastocystis species and Entrobious vermiculates	1 (0.9)	0 (0.0)	1 (0.9)	0 (0.0)	1 (0.9)
			Blastocystis species and E. coli	3 (2.7)	0 (0.0)	3 (2.7)	0 (0.0)	3 (2.7)
			Blastocystis species and Ascaris lumbricoides	1 (0.9)	0 (0.0)	1 (0.9)	0 (0.0)	1 (0.9)
			Blastocystis species and G. intestinalis	2 (1.8)	0 (0.0)	2 (1.8)	0 (0.0)	2 (1.8)
			Blastocystis species and Hymenolepis nana	1 (0.9)	0 (0.0)	1 (0.9)	0 (0.0)	1 (0.9)
			Total	36 (32.7)	7 (5.5)	41 (37.3)	2 (1.8)	43 (39.1)
			Negative	0 (0.0)	67 (60.9)	0 (0.0)	67 (60.9)	67 (60.9)
			Total	36 (32.7)	74 (67.3)	41 (37.3)	69 (62.7)	110 (100%)

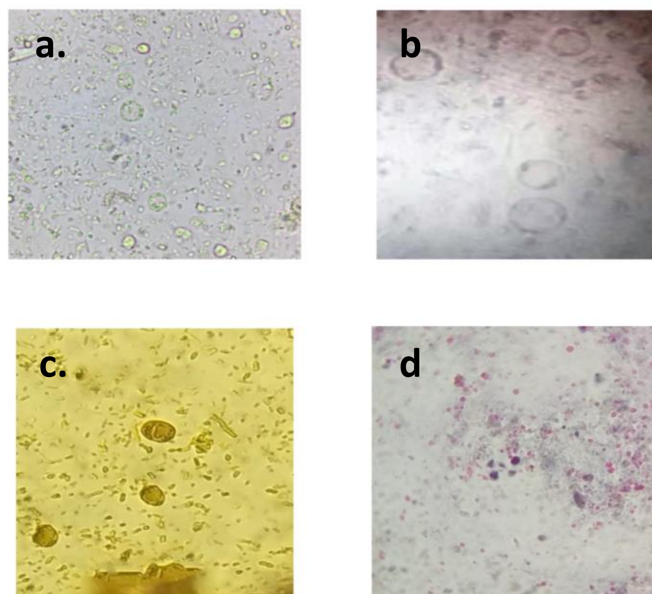

Figure 1. a-Direct wet mount of unstained *B.* sp. from a stool sample; central vacuole with a thin peripheral rim of cytoplasm & multiple nuclei b- Unstained vacuolar forms of *B.* sp. from culture c- vacuolar forms of B. sp. Iodine-stained d- Vacuolar forms of B. sp. Trichrome stained.

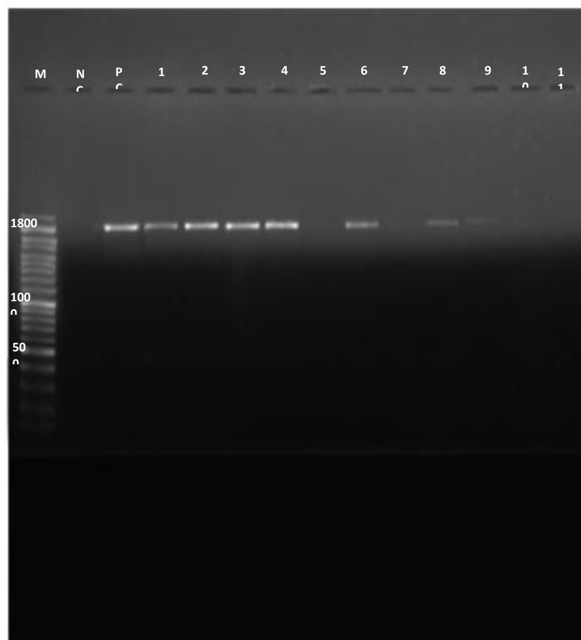

Figure 2. Analysis of PCR products on a 1.5% (w/v) agarose gel. (M): molecular weight marker (100-bp DNA ladder), (Nc): Negative control, (Pc): Positive control, lanes (1/2/3/4/6/8/9): PCR positive samples showing distinct band at1800bp; (5,7,10,11): PCR negative samples.

Patients reported many symptoms all of which belonged to the gastrointestinal system. The leading symptoms in *Blastocystis*-positive patients were abdominal pain (p<0.001), nausea (= 0.007), vomiting (p=0.002), and anorexia (p= 0.043) (Table 4).

Table 4. Frequency of symptoms among individuals infected with *Blastocystis* spp. as detected by culture.

		Blastocystis spp. (symptomatic cases) (n = 23)		p value*
		No.	%	
Abdominal pain	Yes	21	(91.3)	<0.001
	No	2	(8.7)	
Diarrhea	Yes	15	(65.2)	NS
	No	8	(34.8)	
Nausea	Yes	5	(21.7)	0.007
	No	18	(78.3)	
Constipation	Yes	13	(56.5)	NS
	No	10	(43.5)	
Vomiting	Yes	4	(17.4)	0.002
	No	19	(82.6)	
Distension	Yes	7	(30.4)	NS
	No	16	(69.6)	
Anorexia	Yes	6	(26.1)	0.043
	No	17	(73.9)	
Fever	Yes	9	(39.1)	NS
	No	14	(60.9)	
Loss of weight	Yes	7	(30.4)	NS
	No	16	(69.6)	

* Z-score test and P > 0.05 is not significant (NS).

Statistical analyses demonstrated that the agreement between the culture and PCR was perfect (K=0.925) while the agreement between the culture and microscopy was substantial agreement (K=0.715). Compared to culture, the sensitivity of the PCR was 95% and the specificity was 97% while the sensitivity of microscopy was 84% and the specificity was 90.5% (Table 5)

Table 5. Diagnostic performance (sensitivity, specificity, PPV, NPV, and accuracy) and Kappa agreement of Microscopy and PCR using in-vitro culture as the gold standard method.

		PCR	Microscopy
Culture	Positive (43)	41	**36**
	Negative (67)	69	**74**
Total		110	**110**
Goodness criteria		PCR	**Microscopy**
AUC (95% CI) (p-value)		0.977 (0.940-1.000) (<0.001)	**0.919 (0.852-0.985) (<0.001)**
Accuracy		96.5%	**88%**
Sensitivity		95%	**84%**
Specificity		97%	**90.5%**
PPV		97%	**91%**
NPV		95%	**83.5%**
Weighted Kappa Agreement		0.925 (p<0.001)	**0.715 (p=0.009)**
95% CI		0.852 – 0.997	**0.640 – 0.851**

AUC: area under the curve. Key for Kappa: <0 No agreement. 0.01-0.20 Slight agreement. 0.21-0.40 Fair agreement. 0.41-0.60 Moderate agreement. 0.61-0.80 Substantial agreement. 0.81-1.00 Almost perfect agreement. *$P \leq 0.05$ is significant.

Discussion

The present study intended to determine the diagnostic value of microscopy, stool culture, and a PCR technique for assessing the prevalence of *Blastocystis* infection in a selected sample from Dakahlia governorate. Utilizing direct microscopy, the current study's overall prevalence of parasitic infections in the 110 feces samples 50.9%. The prevalence of intestinal parasitic infections varied significantly between studies conducted in Egypt, with infection rates ranged from 22.4 to 63.8%.[10,11] This can be attributed to the unsanitary environmental conditions and a low level of public awareness.

In the current study, the most prevalent parasite was *Blastocystis*. sp. (32.7%), followed by *Giardia intestinalis* (9.1%) *E. histolytica*/dispar (6.3%), *E. vermicularis* (3.6%), *A. lumbricoides* and *E. coli* (2.7%), *H. nana* (1.8%), and the least detected ones were Cryptosporidium species (0.9%). In another study in Egypt, the authors found that *Blastocystis hominis* was the most common parasite, followed by *Dientamoeba fragilis* (19%), and *Hymenolepis nana, Entamoeba histolytica and Schistosoma mansoni* were the least common.[12]

With a rate of 2.7% of all co-infected patients in the current study, *Blastocystis* was shown to be associated mainly with E. coli, followed by *Blastocystis* with *G. intestinalis* (1.8%) and *E. histolytica*/dispar (1.8%). Similarly, authors of another study in Morocco reported that *Blastocystis* spp. was the most frequent gut protist associated with polyparasitism, mainly with gut parasites.[13] This co-infection may result from having similar environmental, social, transmission, and age prevalence curves circumstances.

Based on the current study, 39.1% stool samples were positive for *Blastocystis* sp. by culture, 37.3% by PCR, and 32.7% by light microscopy. According to previous studies conducted in 2018, 2020, and 2021, the authors

concluded that the occurrence of *Blastocystis* sp. infection was 35.7%, 39%, and 38.9% respectively.[14,16,15] *Blastocystis* sp. was detected in 52% of cases by light microscopy and in 65% of cases by cultures, and in 67% of cases by PCR.12 Low parasitic activity, sporadic shedding, and morphological variations in the specimens may make microscopic detection challenging even after staining, which increases the likelihood of false negative results from wet mount smears.

In our study, males had a higher prevalence of *Blastocystis* sp. (58.1%) than females (41.9%), however, the difference did not reach statistical significance. *Blastocystis* sp. infections were significantly more common in rural areas (60.5%) than in urban areas (39.5%; p=0.047). This agreed with another study in which a significant difference between the prevalence of *Blastocystis* sp. infection in rural areas (68%) and urban areas (32%) was reported.[16] More interaction with the earth and animals, poor sanitation, and contaminated water sources can explain such difference.

In the current study, there was a significant difference between *Blastocystis* detection in symptomatic (67.4%) and asymptomatic (32.6%) participants. This agreed with the results of previous research in which the authors reported that 70.2% of individuals with *Blastocystis* infection had symptoms.[17] While others found no connection between symptoms and *Blastocystis* infection.[18]

Concerning the association of infection with the various clinical manifestations of the studied individuals, the most common symptoms were abdominal pain (91.3%), diarrhea (65.2%), constipation (56.5 %), followed by fever, distension, and loss of weight. The general symptoms associated with *Blastocystis* infection were abdominal pain (76%), diarrhea (27%), and other gastrointestinal complaints in (16%) of the symptomatic patients.[17] In the current study, abdominal pain, nausea, vomiting, and anorexia were the manifestations significantly associated with *Blastocystis* infection (p<0.05). These symptoms were reported in symptomatic *Blastocystis* sp. infected individuals.[19, 20] The association of these manifestations with *Blastocystis* sp. suggests the pathogenic impact of this parasite.

The sensitivity of microscopy was 84%, specificity 90.5%, PPV 91%, NPV 83.5%, and accuracy 88% with substantial agreement as compared to culture on Jones' medium as a reference test in detecting *Blastocystis* sp. The sensitivity of microscopic examination for the detection of *Blastocystis* sp. was 62.2%, specificity 95.7%, positive predictive value 92.0%, negative predictive value 75.9%, and diagnostic accuracy of 83.1% for direct smear compared to PCR.[21] The low intensity of parasitic infection and intermittent shedding of gut parasites in stool specimens increased the number of false negative cases diagnosed by microscopic examination of wet mount fecal smears. However, in medical laboratories, direct microscopy is still considered easy, rapid, convenient, and economic for the diagnosis of *Blastocystis*.[22,9,12,15] The *in vitro* culture technique in this study was able to reveal more cases than the direct microscopy and PCR assay. The higher detection rates seen in cultures may be attributed to the organism needing more time to grow and replicate.

in vitro culture of *Blastocystis* sp. on modified Jones' medium was chosen in the present study for detecting *Blastocystis* sp. owing to its higher sensitivity and specificity as suggested by other authors.[23] The in-vitro culture of *Blastocystis* has many advantages e.g., increase the detection efficiency of parasites in human, can be used for epidemiological prevalence studies, determine the sensitivity of *Blastocystis* sp. to drugs, follow up of therapy effectiveness, isolation the parasitic antigens, understanding the pathogenesis and the potential virulence of different strains of different origin.[24] The sensitivity of the PCR assay was 95%, specificity 97%, PPV 97%, NPV 95%, and accuracy 96.5%. Other studies reported that the molecular assay was the most efficient method for the detection of *Blastocystis*.[25,26,9] The PCR assay has some disadvantages, e.g., un-successful DNA extraction and the choice of unsuitable primers could lead to no and/or over-amplification of PCR assay.[27]

In conclusion, the pathogenic potential of *Blastocystis* cannot be ruled out because our study found a link between *Blastocystis* carriage and gastrointestinal symptoms. *in vitro* culture, an inexpensive and simple to perform, and the PCR assay were both excellent confirmatory screening techniques.

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Author Contributions

MS and GA conceived and designed the research. GA, AO, FA, and MS conducted the experiments. MS and GA analyzed the data and wrote the manuscript. All authors made critical reviews and approved the final version.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Al-Azhar University (dated May 2021).

Informed consent

All study participants and their parents were told of the study purpose, its methods, and a written consent form was obtained. Providing a stool sample was considered a child assent.

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