Restriction fragment length polymorphism (RFLP) analysis of *Blastocystis* spp. in symptomatic and asymptomatic individuals from Alexandria, Egypt

Original Article

Hend A El-Taweel[1], Yasmine A Isaa[2], Ghada A Shehata[3], Ahmed Gaballah[4], Wael M Lotfy[5], Mona M Tolba[1]

Departments of Parasitology[1], Biochemistry[2,3], Microbiology[4], and Community Health[5], Medical Research Institute[1,4], Faculties of Medicine[2], Science[3] and Nursing[5], Alexandria University[1,2,3,4], and Matrouh University[5], Egypt

ABSTRACT

Background: *Blastocystis* spp. is a unique enteric parasite commonly found in the intestines of humans and animals. In humans, prevalence up to 60% has been reported in tropical, subtropical, and developing countries. Currently 26 subtypes (STs) of *Blastocystis* have been described based on sequence analysis, 9 were reported in humans.

Objectives: The aim of the work was to determine the different genotypes of *Blastocystis* spp. in symptomatic and asymptomatic individuals in Alexandria city, Egypt.

Subjects and Methods: Examination of 100 stool samples was performed to detect *Blastocystis* collected from patients complaining of gastrointestinal (GI) disturbances and asymptomatic individuals. PCR restriction fragment length polymorphism analysis (PCR-RFLP) and sequencing of amplified products was performed for *Blastocystis* subtyping.

Results: Out of 47 fecal samples positive only for *Blastocystis* spp. by microscopy, 39 patients presented with symptoms and 8 were asymptomatic participants. *Blastocystis* small subunit rRNA (SSU rRNA) was successfully amplified from 27 samples; 24/39 symptomatic and 3/8 asymptomatic. Among symptomatic patients, four STs were identified; ST3 was the most common (55%) followed by ST1 (20%), ST4 (15%) and ST2 (10%). While ST2 and ST4 were identified only in patients having GI symptoms, ST1 and ST3 were found in both symptomatic and asymptomatic participants. Amplicons of 7 samples from symptomatic patients were not digested after incubation with the restriction enzymes and could not be genotyped.

Conclusion: Genotyping of *Blastocystis* spp. from fecal samples revealed the presence of four different subtypes: ST1, ST2, ST3 and ST4 with predominance of ST3. No statistically significant association could be observed between *Blastocystis* STs and clinical presentation of the studied subjects.

Keywords: *Blastocystis* spp.; subtypes; gastrointestinal symptoms; RFLP; sequencing.

INTRODUCTION

Blastocystis spp. are anaerobic organisms colonizing the intestinal tract of humans and a wide range of animals[1,2]. An earlier study grouped this unicellular organism with yeasts, sporozoa or sarcodines[3]. However, sequence analysis of its complete SSU rRNA indicated that it should be included in Stramenopiles, a complex group of protozoa[4,5]. Globally, *Blastocystis* is one of the most common enteric organisms with higher prevalence rates in developing than in developed countries[6,7]. Faeco-oral route is believed to be the principle mode of transmission where low socioeconomic conditions, poor hygienic practice and consumption of contaminated water act as major risk factors[8,9].

Although *Blastocystis* has been identified in fecal samples since the early 1900s, it gained great attention only in recent decades when its pathogenic potential became a matter of debate[1]. Infection was demonstrated in both symptomatic and asymptomatic individuals[10]. While it was considered a commensal organism with no pathogenic role, its recording in parasitological stool reports was questioned[11]. Others linked *Blastocystis* to GI symptoms such as nausea, flatulence, and diarrhea[12,13]. Association with irritable bowel syndrome was also reported[14].

It was proposed that the duration and intensity of infection and host genetic factors may influence infection outcome[1]. Colonization by different STs is one of the possible explanations for the inconsistent pathogenesis of *Blastocystis*[12,13,15]. Molecular methods based on analysis of SSU rRNA revealed that genus *Blastocystis* comprises 26 different STs among which, only nine STs were reported in humans

(most prevalent are ST1-5 as well as other rarer STs, ST6-9)[16]. A previous study in Egypt detected ST1 in symptomatic patients and ST2 in asymptomatic persons while ST3 and ST4 were identified in both symptomatic and asymptomatic individuals[17]. Another study showed that ST3 is the most common one in fecal samples collected from Saudi patients with GI complaints[18]. Treatment of *Blastocystis* is generally recommended in symptomatic patients, regardless the infecting ST, when it is detected in the absence of other detectable enteric pathogens[10]. However, differences in sensitivity of *Blastocystis* STs to anti-microbial agents were described[19]. Identification of *Blastocystis* subtypes may, therefore, help in determining the need for treatment and prescription of the appropriate anti-parasitic drug.   Moreover, characterization of *Blastocystis* subtypes may provide a clue for predicting the likely source of infection and understanding the dynamics of transmission[3].

Recent reports on the distribution of *Blastocystis* subtypes are available from several countries and, in some cases, from different areas of the same country. In a large multi-center study in France, the distribution of *Blastocystis* subtypes in 141 positive stool samples concluded that most cases are caused by STs 1-4[20]. In a Brazilian rural community, subtyping of isolates from 85 *Blastocystis* isolates highlighted predominance of ST3 followed by ST1 and ST2 in equal percentages while ST4 and ST8 were less prevalent[21]. In an urban area of the same country, another study showed markedly lower prevalence of ST2 compared to ST3 and ST1 in 64 samples[22]. In Egypt, studies investigating *Blastocystis* subtypes are limited. Data is only available from Cairo[23,24], Ismailia[17] and El Mansoura[25]. Based on the standard terminology described by Stensvold *et al*.[26], a total of six STs (STs1-4 and STs 6,7) were identified in the mentioned Egyptian studies with dissimilar relative frequency.

The present work aimed to analyze STs of *Blastocystis* spp.in infected symptomatic and asymptomatic individuals from Alexandria, Egypt using PCR-RFLP analysis. Sequencing of the amplified products was performed on random samples for confirmation.

SUBJECTS AND METHODS

A cross sectional study was carried out to detect infection with *Blastocystis* spp. in 100 individuals referred to the Parasitology Laboratory of the Medical Research Institute, Alexandria University for routine parasitological examination during the period between April and August 2017. Subjects with *Blastocystis*-positive stool samples and with no other detectable parasite were enrolled in the study. Personal information as name, age, gender, and clinical history were recorded. Based on clinical presentation, participants were categorized as 'asymptomatic' if they had no GI symptoms or 'symptomatic' if they had any GI complaints such as diarrhea, abdominal pain, nausea, or flatulence.

Collection and processing of stool samples: Fresh stool specimens free of water or urine were collected in clean, dry sealed labelled plastic containers. At the Parasitology Department laboratory, each sample was mixed thoroughly and examined for *Blastocystis* spp. using saline and iodine wet mount smears and formalin ethyl-acetate sedimentation technique[27].

DNA extraction and PCR-RFLP: DNA was extracted from *Blastocystis* positive fecal samples using the extraction kit Bioline fecal isolate DNA (UK) according to the manufacturer's instructions[28]. A PCR protocol was used to amplify a region of *Blastocystis* SSU rRNA gene using the thermal cycler (Beco, Germany); amplification was performed using the forward and reverse primers; RD3 (GGGATCCTGATCCTTCCGCAGGTTCACCTAC) and RD5 (GGGATCCTGATCCTTCCGCAGGTTCACCTAC), respectively[28]. The PCR was carried out in a 50 µl reaction volume containing 10 µl DNA extract, 25 µl DreamTaq® GreenMaster Mix (Fermentas, USA), 1 µl forward primer, 1 µl reverse primer and 13 µl nuclease-free water. The amplification steps included a first cycle of denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 3 min, annealing at 58°C for 30 sec, and extension at 72°C for 2 min, and a final extension step at 72 °C for 5 min. Amplification products and molecular weight marker (100 base pair DNA ladder, Bioron, Germany) were electrophoresed in 0.8% agarose gel stained with ethidium bromide and were visualized under UV transilluminator. Each PCR run included a positive control of a known PCR positive sample approved by sequencing from previous study in the department and a negative control of nuclease-free water[29].

The amplified products for the SSU rRNA gene were digested to determine the strain subtype of *Blastocystis* spp. using 3 restriction enzymes; Hinfl, Rsal, and Sau3Al[28]. Restriction digestion was performed at 37°C for 15 minutes for the first two enzymes and for one hour for the third one. The total volume of the reaction mix was composed of 25 µl containing 10 µl PCR product, 2.5 µl 10 X buffer, 1 µl restriction enzyme and 11.5 µl sterile nuclease-free water. The restriction fragments were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized with a UV transilluminator. Interpretation of the resulting products of digestion and identification of B. *hominis* subtypes were performed by comparison to patterns described by Clark[28] and modified according to the consensus terminology described by Stensvold *et al*.[26].

Sequencing: For confirmation of RFLP typing results, the amplified products of randomly selected samples were sequenced. The amplicon bands were

excised, and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA sequencing of purified PCR products was performed using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany). Nucleotide sequences were determined on an ABI Prism 310 genetic Analyzer (Applied Biosystems, Germany). Sequences obtained in the present study were analyzed and aligned with previously recorded sequences of *Blastocystis* using standard software BioEdit Version 7.0.5 (Sequence Alignment Editor). Comparison of the identified samples with those accessible in the GenBank Database was held using the BLAST (Basic Local Alignment Search Tool) program located at the databases of the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) (www.blast.ncbi.nlm.nih.gov/Blast.cgi).
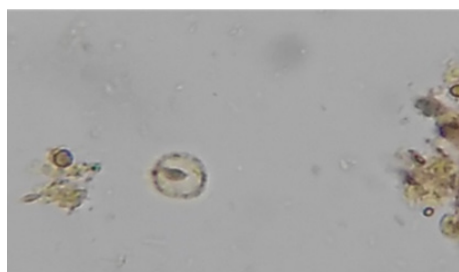
Statistical analysis: Data were statistically analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Chi-square, Fisher's exact test or Monte Carlo corrections were used to test the association between variables. A *P* value less than 0.05 was considered statistically significant.

Ethical Consideration: The protocol of the study was approved by the Research Ethics Committee of the Medical Research Institute (MRI), Alexandria University (IORG 0008812). An informed consent was obtained from each patient. Infected individuals were referred to a physician for treatment.

RESULTS

Out of 100 stool samples submitted to the Parasitology laboratory during the study period. *Blastocystis* spp. was detected microscopically in 63 samples (63%) using saline and iodine wet mounts (Figure 1). It was found alone in 47 (74.6%) of these samples and in conjunction with other protozoa (*E. coli, G. lamblia,* and *D. fragilis*) in 16 (25.4%) samples.

Among 47 *Blastocystis* positive individuals with no other detectable parasites, 39 subjects (83%) were symptomatic, and 8 subjects (17%) were asymptomatic. Twenty-one subjects (45%) were in the age group 20-<40 years. In the other two age groups (5-<20 and 40-55 years) lower percentages were recorded in 12 (25%) and in 14 (30%) cases,

respectively. In the youngest age group, 92% of the subjects were symptomatic compared to 86 % and 76% of the oldest and middle age participants respectively. However, there was no statistically significant association between subjects' age and presence of GI symptoms. Regarding gender, GI symptoms were present in 86% of males and 78% of females with no statistically significant difference. (Table 1).

Out of 47 samples, amplification of *Blastocystis* spp. SSU rRNA produced a PCR product with the expected length (1800 bp) in 27 samples (Figure 2); 24 were from symptomatic and 3 from asymptomatic patients. Distinct patterns were obtained after digestion by restriction enzymes in 20 samples. Based on patterns described by Clark[28] and modification according to the standard terminology described by Stensvold *et al*.[26], four *Blastocystis* subtypes were identified (Figure 3). ST3 was the most common, being detected in 11/20 samples (55%). This was followed by ST1 (4/20 samples: 20%), ST4 (3/20 samples: 15%) and ST2 (2/20 samples: 10%).

Sequencing of the amplified products was performed in six randomly chosen samples that included two samples assigned by RFLP to ST1 and four samples assigned to ST3. Analysis of sequencing results of the six samples revealed *Blastocystis* subtypes concordant with the corresponding RFLP subtypes.

The demographic and clinical criteria of participants in whom *Blastocystis* spp. could be subtyped are shown in table (2). While STs 1 and 3 were detected in symptomatic and asymptomatic persons, STs 2 and 4 were identified only in symptomatic participants. In the youngest age group, samples that could be subtyped belonged to STs 1, 3, and 4 (one sample each). In the middle age group, ST 3 was the most common (6 samples) followed by ST 1 and 2 (each in 2 samples) and ST4 (in one sample). Three STs (1, 3 and 4) were identified in the six genotyped samples of the older group with ST 3 being the most common (in 4 samples). Samples that could be genotyped belonged to 11 males and 9 females. The most common ST in males was ST 3 (9 cases), followed by STs 1 and 4 (each in one sample). Among females, the most common ST was ST1(3 samples) followed by STs 2, 3 and 4 (each in two samples).
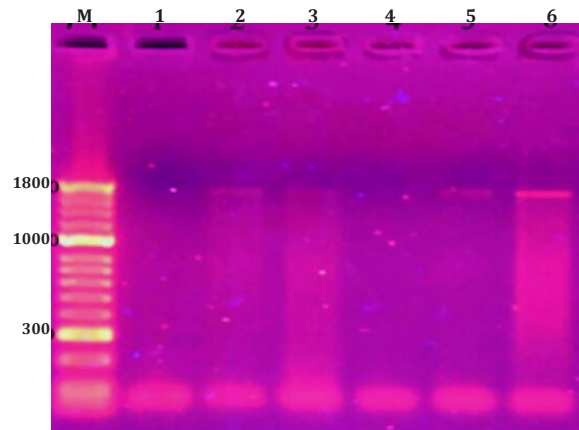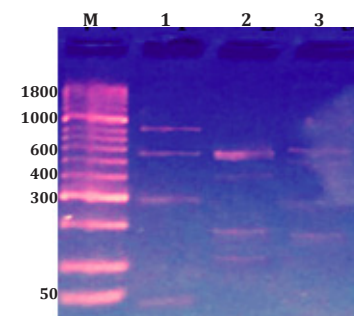

Fig. 1. *Blastocystis* spp. in iodine wet mount smear (400 x).

Table 1. Symptomatic and asymptomatic blastocystosis according to age and gender among 47 *Blastocystis*-positive individuals.

	Symptomatic No. (%)	Asymptomatic No. (%)	Total@ No. (%)	P value
Age in years				
5-	11 (91.7)	1 (8.3)	12 (25.5)	
20-	16 (76.2)	5 (23.8)	21 (44.7)	MCP = 0.621
40-55	12 (85.7)	2 (14.3)	14 (29.8)	
Gender				
Males	25 (86.2)	4 (13.8)	29 (61.7)	FEP = 0.621
Females	14 (77.8)	4 (22.2)	18 (38.3)	
Total*	39 (83.0)	8 (17.0)	47 (100)	

MC*P*: *P* value for Monte Carlo test, FE*P*: *P* value for Fisher's exact test.
*Percentages calculated from the total in each age or gender group.
@Percentages calculated from the total number of *Blastocystis* positive subjects with no other detectable parasites (n=47).

Fig. 2. Gel electrophoresis stained with ethidium bromide showing results of PCR amplification of *Blastocystis* SSU rRNA from microscopically positive fecal samples. **Lane 1:** Negative control; **Lanes 2, 3 and 5:** PCR positive samples showing one band at 1800 bp; **Lane 4:** PCR negative sample; **Lane 6:** positive control. M is the molecular weight marker (100-bp DNA ladder).

Fig. 3. RFLP pattern of a patient sample identified as ST1 after digestion with Hinfl enzyme (**lane 1**), Rsal enzyme (**lane 2**) and Sau3Al enzyme (**lane 3**). **M** is the molecular weight marker (100-bpDNA ladder).

Table 2. *Blastocystis* STs identified by SSU rRNA-RFLP and their associated demographic and clinical criteria among 20 participants with distinct patterns.

Criteria	*Blastocystis* subtypes			
	ST1	ST2	ST3	ST4
Age				
5-	1	0	1	1
20-	2	2	6	1
40-55	1	0	4	1
Gender				
Male	1	0	9	1
Female	3	2	2	2
Clinical presentation				
Symptomatic	3	2	9	3
Asymptomatic	1	0	2	0
Total	4	2	11	3

DISCUSSION

Blastocystis is a common intestinal eukaryote infecting several mammalian hosts. The wide genetic variation of *Blastocystis* isolates is thought to be linked to the clinical and epidemiological outcome of infection[30]. In the present study, parasitological examination of 100 fecal samples revealed *Blastocystis* detection rate of 63%. In most samples (47/63; 74.6%), *Blastocystis* was identified with no other intestinal parasitic infection. Previous studies in Egypt reported nearly similar high rates of *Blastocystis* infection. EL-Marhoumy *et al.*[31] stated that *Blastocystis* was found in 53% of studied pre-school children. Eassa *et al.*[32] detected *Blastocystis* in 54.5% of immunocompromised and 67.4% of immunocompetent individuals. Its prevalence is generally higher in developing countries compared to developed ones. It was suggested that travel to

tropical countries increases the risk of *Blastocystis* infection[33]. High prevalence was reported among immigrants (52.7%) in Naples city in Italy[34]. A prevalence of 18% was reported in a multi-center study in France[20]. Different factors influence transmission and prevalence of *Blastocystis* infection including socioeconomic state, personal hygiene, animal contact and climatic conditions[7-9].

In the present study, 45% of individuals infected solely with *Blastocystis* spp. were in the age group 20-<40 years and 61.7% were males. El-Safadi *et al.*[20] in France observed that the prevalence of *Blastocystis* sp. was significantly higher in the age group 15 to 49 years compared to older subjects. Paulos *et al.*[35] in Spain found that children were more commonly infected by *Blastocystis* spp. A study in China reported inconsistent age and gender variations in different areas. Such differences in *Blastocystis* infection may be related to the associated exposure risk and environmental conditions rather than host physiological properties[36].

Reports on the pathogenicity of *Blastocystis* spp. are contradictory[1,37]. The findings of our present study indicate that *Blastocystis* may be encountered in both symptomatic and asymptomatic individuals. In the absence of associated parasitic infection, the majority of *Blastocystis* infected subjects (39/47; 83%) were symptomatic. This agrees with Mohamed *et al.*[18] who found that 64% of *Blastocystis* positive subjects had GI symptoms. El Safadi *et al.*[20] did not find any correlation between symptoms and infection with *Blastocystis.* It is to be noted that the present study was hospital based and therefore, data may not reflect the population's real distribution of symptomatic and asymptomatic *Blastocystis* infection. Generally, it was recommended to consider *Blastocystis* the causative agent of patient symptoms so long as no other etiology is identified[10].

Our results also revealed that occurrence of GI symptoms in *Blastocystis* infection is not affected by the age or gender of infected persons. Jerez Puebla *et al.*[38] found that the rate of infection was significantly higher among school children having GI symptoms in comparison with asymptomatic ones while the group above 40 years old was more likely to have an asymptomatic infection. The authors attributed this to repeated infection with different STs as a result of bad hygienic practices in juvenile age. In contrast, Salvador *et al.*[39] studied the epidemiologic and clinical profile of *Blastocystis* infected patients and found that asymptomatic patients were significantly younger.

While Santos and Rivera[40] reported false negative results in only 2/21 (9.5%) of cases, in our study out of 47 microscopically positive samples, *Blastocystis* SSU rRNA gene was successfully amplified by PCR in only 27 (57.5%) samples. Our high record of false negative PCR result may be attributed to storage conditions of the samples prior to performing the PCR analysis which

might have resulted in degradation of the organisms and consequently the DNA[41]. Inhibitors in stool such as polysaccharide complex, bile salts, bilirubin and hemoglobin degradation products could be another cause, the amount of these interfering compounds varies according to host diet and gut microflora[42].

PCR-RELP analysis of *Blastocystis* subtypes was performed in the present study on DNA extracted directly from fecal samples. It was previously observed that this method is better than genotyping based on DNA extracted from stool culture which, in addition to being time consuming, may result in bias due to the possibility of preferential growth of some subtypes[30]. The RFLP pattern was confirmed in samples that could be sequenced denoting validity of the used protocol.

Our results showed that *Blastocystis* subtypes identified by RFLP included, in a descending order, ST3, ST1, ST4 and ST2. Confirmation of the results of subtyping was done by sequencing of some random samples. Nearly similar findings were previously reported using SSU rRNA RFLP or cloning and sequence analysis[23,24,43]. Another older Egyptian study using a PCR protocol based on sequence tagged site primers, reported similar predominance of ST3 (54.4%) followed by ST1 (18.2%) among 44 persons. However, this study reported the presence of two other subtypes namely ST6 and ST7 (18.2% and 9.1%, respectively)[5].

Worldwide, most *Blastocystis* infections in humans are attributed to STs 1-4. ST3 followed by ST1 were the most prevalent STs in in many countries as in Brazil[22], France[20], United Arab Emirates[44], Tunisia[45], Saudi Arabia[18], Indonesia[46] and Iran[15]. Few studies showed slightly higher prevalence of ST1 compared to ST3 as in Philippines[47] and Tanzania[48]. In Thailand, ST prevalence showed marked geographic variation with ST1 being the most common in the central region and ST3 in the north and northeast regions[49]. Variability in distribution of *Blastocystis* subtypes from one country to another and within the same country depend on the prevailing epidemiologic situation and climatic conditions[9,13,49]. Such variability may reflect difference in dynamics of transmission. Actually, a study in Malaysia illustrated that the prevalence of *Blastocystis* ST1 was higher during the wet season compared to the dry season while ST3 prevalence is nearly similar in both seasons suggesting subtype difference in susceptibility to adverse environmental conditions. Moreover, person to person transmission was found to play a role in transmission of ST1, ST2 and ST3, while the use of untreated water was identified as a risk factor for ST3 only[13].

A striking higher prevalence of ST2 (62%) compared to ST3 (17%) and ST1 (13%) was found in 63 samples in Spain[35]. Regional variation of ST2 distribution was described in a study conducted among migrant workers in Qatar where it was completely absent (0%)

from Western Asian *Blastocystis*-positive subjects but was detected in (5.3%) of Africans and (9.7%) of East Asian workers[50].

Regarding ST4, increased evidence indicates that it is either absent or accounts for less than 4% of human infections as in Iran[15], France[20], Brazil[22], United Arab Emirates[44], Tunisia[45] and Tanzania[48]. Although, ST4 is considered mainly a parasite of animals[51], a relatively high distribution was observed in some European studies[12,52,53]. On the other hand, STs: 5, 6 and 7 are relatively uncommon and have been reported mainly from rural areas in association with the other common STs. The potential for zoonotic transmission of these STs was proposed[51].

It is worth mentioning that *Blastocystis* ST1 and ST3 were detected in our study in both symptomatic and asymptomatic participants, ST2 and ST4 were found only in patients having GI symptoms. Studies investigating the association of STs with GI symptoms are contradictory. Our results are consistent with a large multicenter study conducted in France[20]. However, an association between ST1 infection and nausea and between ST3 infection and flatulence were reported in a Malaysian study[13]. A study in Italy found that ST4 was more common in patients with diarrhea[12]. It was proposed that intrinsic host factors in addition to parasite factors may play a role in determining the outcome of *Blastocystis* infection[13,48].

In conclusion, *Blastocystis* is commonly spacing in routine examination for parasitic ova and cysts. Infection by *Blastocystis* may be symptomatic or asymptomatic regardless the individual's age or gender. Infection is caused by STs 1-4 with predominance of ST3. Most infections are most probably mixed subtypes.

Authors contributions: El-Taweel HA designed and coordinated all field activities, experiments, analyses, and critically reviewed the manuscript. Issa YA organized bioassays, and drafted the manuscript. Shehata GA collected biological material organized bioassays, analyzed statistical data, and drafted the manuscript. Gaballah A performed genotyping and statistical analysis. Lofty WM assisted in the statistical analysis and critically reviewed the manuscript. Tolba MM coordinated collection of samples, biological analyses, and critically reviewed the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest.

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