DOI: 10.1515/ap-2015-0015

Acta Parasitologica, 2015, 60(1), 116–123; ISSN 1230-2821

VERSITA

The pathogenic role of *Blastocystis* isolated from patients with irritable bowel syndrome and colitis from Iasi, Romania

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Abstract

Blastocystis is a common parasite and regarded as one of the etiologic agents of irritable bowel syndrome, colitis and chronic diarrhea. Our study was undertaken in order to identify different subtypes of *Blastocystis* isolated in patients with irritable bowel syndrome and colitis, as well as with chronic diarrhea and to evaluate their pathogenic potential. Seventy-three subjects (10 asymptomatic infected subjects, 49 subjects harboring *Blastocystis* or associated with other etiologic agents like bacteria, yeasts, protozoa, helminthes and 14 subjects with unknown etiologic agents) were investigated by in vitro parasitological and bacteriological stool samples followed by PCR subtyping of *Blastocystis* using STS primers, immunological markers (total serum IgA and IgE antibody levels), *Helicobacter pylori* antigen rapid test and fecal occult blood test. Also, among 49 subjects, there were 12 subjects harboring *Blastocystis* as the single etiologic agent. Subtyping proved that only three subtypes of *Blastocystis* were identified as following: subtype II (66.66%) in single infected subjects, subtype I (16.66%) in mixed infection with subtype II and subtype IV (8.33%) in single infected subjects. Total serum IgA and IgE antibody levels were in normal range. Subtype II was the most frequent subtype followed by subtype I and subtype IV of *Blastocystis* isolates in patients with irritable bowel syndrome, colitis, and chronic diarrhea as well as in asymptomatic infected group. Our results suggest that the severity of clinical manifestations depend on factors involving the host and possible parasitic density and not necessarily by isolated subtype.

Keywords

Blastocystis subtype, host, irritable bowel syndrome, colitis, chronic diarrhea

Introduction

Blastocystis is now in attention of the parasitologists due to the increasing incidence of parasites and higher responsiveness of the human host. *Blastocystis* is an extremely common parasite with a wide distribution (Tan 2004). According with Stensvold *et al.* (2007), *Blastocystis hominis* is often shortened as *Blastocystis*, mainly to avoid a phylogenetic error among *Blastocystis* subtypes.

Blastocystis is currently the main dominant parasite frequently isolated, with the highest incidence among known parasites (Baldo *et al.* 2004; Cirioni *et al.* 1999; Florez *et al.* 2003; Herwaldt *et al.* 2001; Pegelow *et al.* 1997; Saksirisampant *et al.* 2003; Taamasri *et al.* 2000; Wang 2004; Windsor *et al.* 2002). The reasons through *Blastocystis* is currently the main dominant parasite found in human stool samples are multiple as following: the self-limited evolution and transient symptoms did not require treatment, increasing resistance to antibiotics; metabolism, lifestyle, and multiplication gives advantages over other protozoa; the health of the human host influenced by immune status, nutrition, allowing parasite infection and proliferation; its opportunistic nature leads to private exploitation of environmental conditions of life. Moreover, the zoonotic isolates genetically different identified from humans were combined under the name *Blastocystis* (Stensvold *et al.* 2007); this heterogeneity causing the controversial nature of its pathogenicity (Boorom *et al.* 2008). Although his study started long time ago, its pathogenic role remains controversial because it is found in significant numbers in both symptomatic and asymptomatic and do not consistently produce clinical manifestations (Stenzel and Boreham 1996).

Clinical manifestations associated with *Blastocystis* infection are characterized by diffuse abdominal pain and discom-

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fort, diarrhea, constipation or alternating diarrhea and constipation (Windsor *et al.* 2002).

Irritable bowel syndrome (IBS) is a common disorder producing abdominal pain, bloating and irregular defecation in the absence of organic causes (Brandt *et al.* 2002). Physicians diagnose IBS using symptom-based criteria known as Rome criteria developed in 1988 and were revised (Rome II) in 1999 and again in 2006 (Rome III) (Fouad *et al.* 2011). For diagnosis of IBS based on Rome III criteria the patient should suffer at least 3 month (Saito *et al.* 2000). Infectious origin of the disease is not yet final, it may be caused by a series of infectious agents: protozoa, bacteria and helminthes (Stark *et al.* 2007).

In recent studies, Fouad *et al.* (2011) has revealed a correlation between the presence of clinical manifestations (IBS and colitis), and certain subtype or combination of subtypes and the presence in asymptomatic patients of other subtypes than those found in patients with clinical manifestations (Bohm-Gloning *et al.* 1997; Clark 1997; Kaneda *et al.* 2001; Kukoschke and Muller 1991). Sometimes, the same subtype is observed in both symptomatic and the asymptomatic patients. Clinical manifestations appear due to the immune status of the host (Roberts *et al.* 2014).

Polymerase chain reaction (PCR) can be used to discriminate strains, species and pathogenic potential of *Blastocystis* isolates. Subtype-specific sequence-tagged-site (STS) primers have been developed and used in several studies for typing *Blastocystis* isolates from humans and animals (Li *et al.* 2007).

Materials and Methods

Patients

Praxis Laboratory Iasi received between August 2011 to September 2012, 73 consecutive patients recruited from the gastroenterology unit of St. Spiridon University Hospital and MedCenter unit, Iasi, Romania. Ten of them were asymptomatic infected subjects, 49 subjects harboring *Blastocystis* or associated with other etiologic agents like bacteria, yeasts, protozoa, helminthes and 14 subjects were with unknown etiologic agents. Among 49 subjects, there were 12 subjects harboring *Blastocystis* as the unique etiologic agent were evidenced. All patients exhibited intestinal discomfort characterized by bloating, accelerated transit and abdominal pain.

The study was conducted according to the provisions of the Helsinki Declaration and was approved by the St. Spiridon University Hospital ethics committee (No. 36598). All subjects gave their written informed consent to participate in the study. Also, the study protocol was conducted according to written consent of Praxis Laboratory Iasi and Laboratory of Diagnosis (SR EN-ISO 15189) and Investigation in Public Health (SR EN-ISO 17021), Romania which provide specific equipment and kits.

Coproparasitologic examination

Stool examination for *Blastocystis* as well as helminthes and protozoa was carried out in all samples according to Garcia (2001). One-two fecal samples were collected from each subject, on non-consecutive days. Stool samples were first examined microscopically for blood, mucus, consistency and worms following by microscopic examination. Approximately 1–2 mg of each fecal sample was thoroughly emulsified on the left part of glass slide in one drop of physiologic saline and covered with a cover slip. A similar preparation was made on the right part of the same slide using Lugol's iodine. These preparations were examined under both the low power (×10) and high dry (×40) objectives.

Bacteriological examination

For the pathogenic enterobacteria, in vitro cultivation was performed on solid culture media, Hektoen agar and McConkey (Merck) with the broth enriched with sodium selenite. For yeast cultivation in vitro cultivation was performed on Sabouraud agar medium (Merck) without any additives.

Determination of total serum IgA and IgE antibody levels

Serum levels of IgA and IgE antibody were measured by immuno-turbidimetric automated assay using the RX Imola system.

Helicobacter pylori antigen rapid test

The presence of *H. pylori* organisms in stool was determined by *H. pylori* antigen rapid test CE kit (BIOTECH). A visible two red lines (control and test lines) indicated the presence of *H. pylori*.

Test Fecal Occult Blood (FOB)

The presence of human occult blood in stool was determined by OneStep FOB RapiCard InstaTest (Cortez Diagnostics, Inc.). A pink colored band which appear in the test region indicated human occult blood in stool.

In vitro cultivation for *Blastocystis*

Only for 12 subjects harboring *Blastocystis* as the unique etiologic agent, cultures were done by inoculating approximately 50 mg of stool into Roswell Park Memorial Institute (RPMI 1640) medium (Gibco®RPMI 1640) (Zhang *et al.* 2012). The cultures were incubated at 37ºC and examined after 2–3 days. The sediment was examined using a low-power (100×) and high-power (400×) objectives. When vacuolar forms of *Blastocystis* were observed, they were subcultured in fresh medium for another 3–4 days.

Table I. Origin of *B. hominis* isolates (W-woman; M-man)

Isolates	Subjects (W/M)	Age (year)	Clinical diagnosis	Cultivation/growth
34	M	74	IBS	yes
35	W	68	Mild symptoms	yes
36	W	62	IBS	yes
37	M	22	IBS	yes
38	W	27	Colitis	yes
39	W	55	IBS	yes
40	W	43	IBS	yes
41	M	28	Colitis	yes
42	M	24	Flatulence	yes
43	M	3	Colitis	yes
44	W	4	Colitis	yes
45	W	58	IBS	yes

Genomic DNA extraction

Blastocystis in RPMI medium was isolated by centrifugation with 400 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4). This suspension was overlaid on to a Ficoll-Paque column and centrifuged at 2000 × g for 10 min. *Blastocystis* separated into a band approximately 1 cm from the surface. This layer was collected and resuspended in 8 ml of PBS and centrifuged at 500 × g for 5 min, which was repeated six times. The resultant pellet was resuspended in 1 ml of PBS and centrifuged at 500 × g for 5 min. and stored at –20ºC until required (Zaman 1997). DNA extraction was performed using GoTaq® Green Master Mix 2X (Promega).

Subtyping by PCR using STS primers

Standardized subtype-specific (STS) primers were used for subtyping of *Blastocystis* isolates (Tan *et al.* 2002). Subtype I was detected by SB83 using forward primer GAAGGACTCTCTGACGATGA and reverse primer GTCCAAATGAAAGGCAGC with product size 351 base pair (bp) (Gen Bank accession no. AF166086). Subtype II was detected by SB155 using forward primer ATCAGCCTACAATCT CCTC and reverse primer ATCGCCACTTCTCCAAT with product size 650 bp (Gen Bank accession no. AF166087). Subtype III was detected by SB227 using forward primer TAGGATTTGGTGTTTGGA GA and reverse primer TTAGAAGTGAAGGAGATGGAAG with product size 526 bp (Gen Bank accession no. AF166088). Subtype IV was detected by SB332 using forward primer GCATCCAGACTACTATCAACATT and reverse primer CCATTTTCAGAC AACCACTTA with product size 338 bp (Gen Bank accession no. AF166091). The amplifications round was performed in a total volume of 25 µl containing 12.5 µl of GoTaq® Green Master Mix, 2X; 2.5 µl of template DNA; 0.5 µl of upstream primer; 0.5 µl of downstream primer and 9 µl of Nuclease-Free Water. The PCR products were electrophorized in 2% agarose gel. The bands were visualized after being stained with ethidium bromide.

Results

Our results revealed 49 subjects harboring *Blastocystis* or associated with other etiologic agents. Among these subjects, there were evidenced 12 subjects harboring *Blastocystis* as the unique etiologic agent (Table I).

Table II. Etiological association

Blastocystis hominis and associated species	**Positive samples**	**Positive FOB**
Blastocystis hominis	12	6
Blastocystis hominis and *Endolimax nana*	5	0
Blastocystis hominis and *Entamoeba coli*	5	1
Blastocystis hominis and *Giardia duodenalis*	4	3
Blastocystis hominis and *Enterobius vermicularis*	2	2
Blastocystis hominis and *Ascaris lumbricoides*	1	1
Blastocystis hominis and levuri	16	2
Blastocystis hominis and *Hellicobacter pylori*	4	4
Salmonella/Shigella/Yersinia	0	0
Negatives samples	14	4

By microscopic examination (power 400x), in these 49 patients were evidenced more than 10 vacuolar and granular forms of *Blastocystis* as compared with control asymptomatic subjects with more than 5 vacuolar forms only evidenced.

Consequently, *Blastocystis* was associated with other etiologic agents such as yeasts (*Geotrichum candidum*) (Fig 1a), commensal (*Entamoeba coli*) (Fig 1b) and pathogen (*Giardia duodenalis*) (Fig 1c) protozoa, helminthes (*Enterobius vermicularis*, *Ascaris lumbricoides*) (Fig 1d and Fig 1e). Usually, in these aforementioned associations development and manifestation of one of the system components is promoted (Moglan and Popescu 2009).

In association with helminthes, clinical manifestations were attributed to helminthes, while *Blastocystis* is only associated

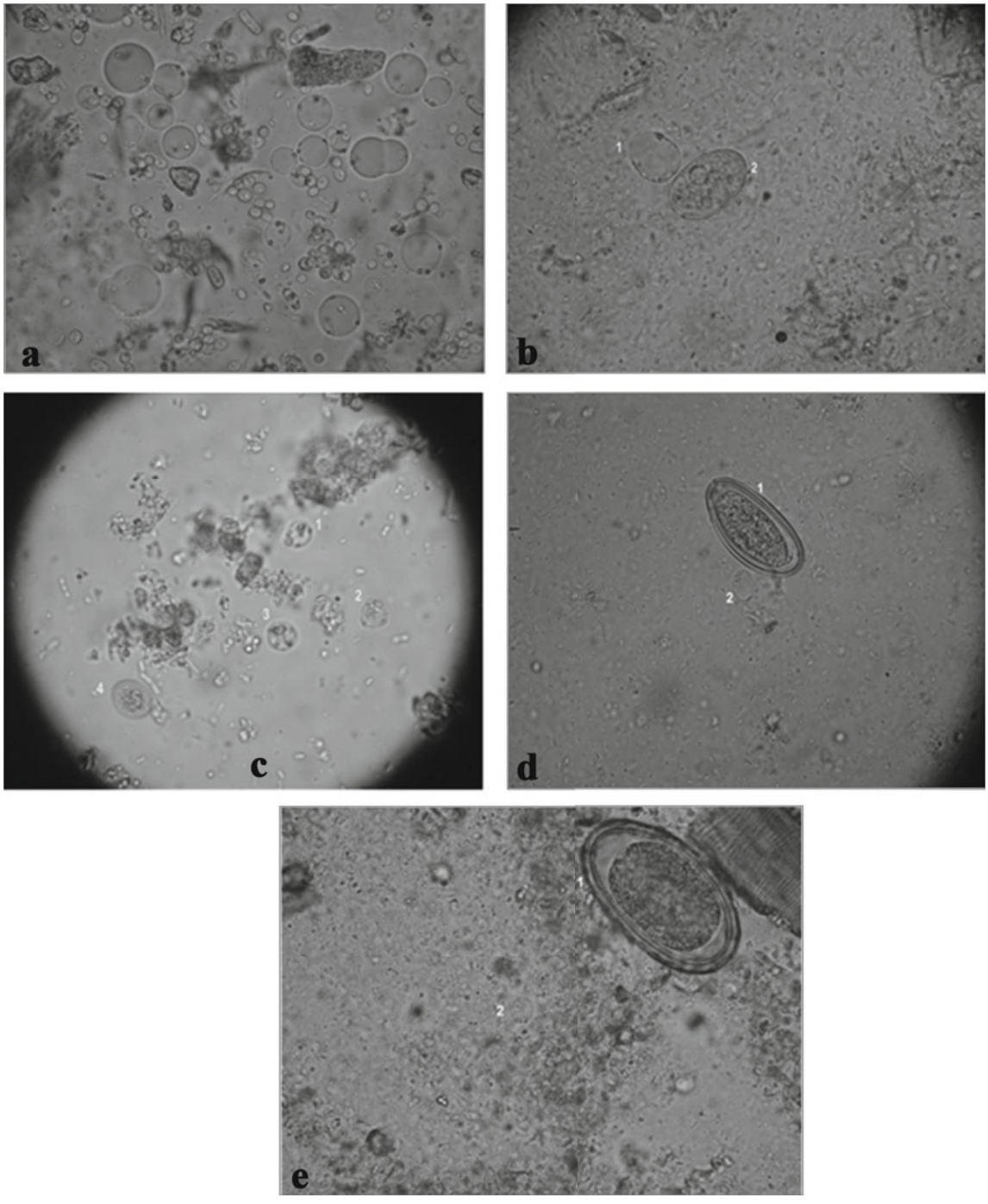

Fig 1. Microscopic examination (power 400x) of *Blastocystis* and associated etiological agents: **a –** *Blastocystis* and *Geotrichum candidum*; **b –** *Blastocystis* (1) and *Entamoeba coli* (2); **c –** *Blastocystis* (1,2,3) and *Giardia duodenalis* (4); **d –** *Blastocystis* (2) and *Enterobius vermicularis* (1*)* and **e –** *Blastocystis* (2) and *Ascaris lumbricoides* (1)

Table III. Total serum IgA and IgE antibody levels

No.	Subjects (W/M)	Age (year)	IgE	IgA
1(34)	M	74	87.32	168.60
2(35)	W	68	67.21	145.40
3(36)	W	62	82.27	210.20
4(37)	M	22	86.23	181.30
5(38)	W	27	68.21	129.60
6(39)	M	55	89.23	265.20
7(40)	M	43	81.00	259.30
8(41)	W	28	87.23	130.60
9(42)	W	24	84.20	160.20
10(43)	W	3	85.33	157.60
11(45)	W	58	82.29	184.20

Total serum IgE physiological range: 00.00 – 100.00 UI/ml
Total serum IgA physiological range: 90.00 – 450.00 mg/dl

Table IV. Different *B. hominis* genotypes in asymptomatic and symptomatic (IBS, colitis and flatulence) subjects

Subjects	Genotype I(GI)	Genotype II (GII)	Genotype IV (GIV)	Mixed infections
Asymptomatic	0	6	0	0
IBS	0	6	0	2 (GI+GII)
Colitis	0	2	1	0
Flatulance	0	1	0	0

agent but whose presence demonstrates its opportunist character. The most common association was with yeasts. No pathogenic bacteria was evidenced by bacteriological examination.

Since in 23 cases, test for faecal hemoglobin was positive both in the presence and absence of the parasite, FOB test cannot be significantly correlated with the presence of *Blastocystis* in stool samples. It was evidenced that *Blastocystis* is associated with infection induced by *Hellicobacter pylori* which may be a starting point for future investigations of IBS (Table II).

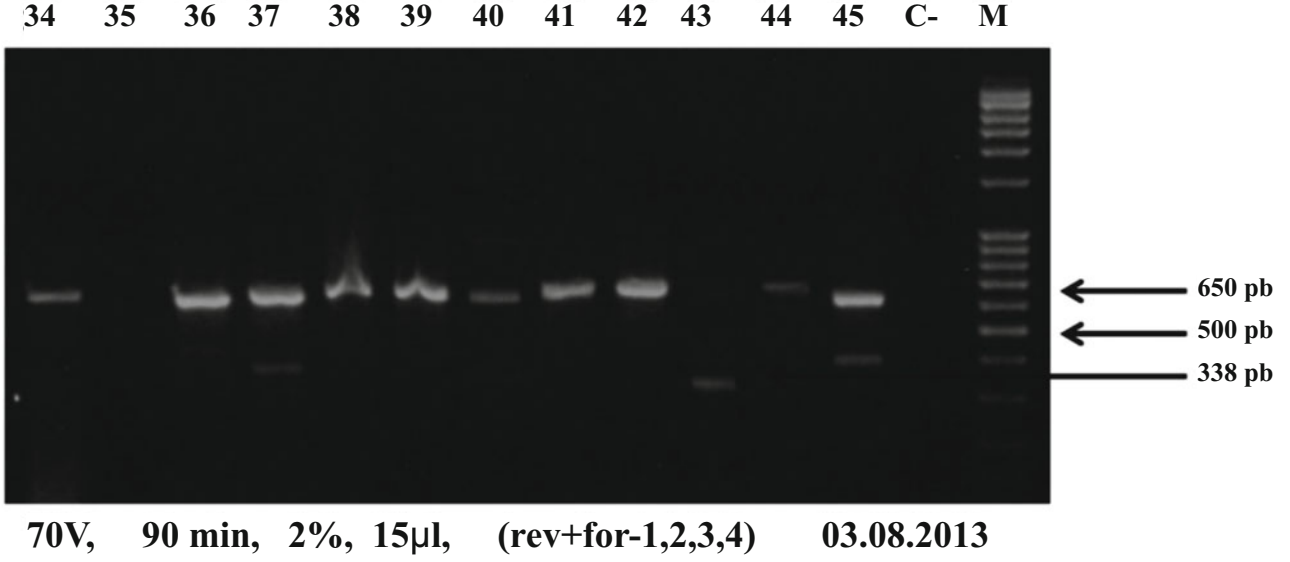

Fig 2. *Blastocystis* subtypes of symptomatic patients with IBS, colitis and chronic diarrhea using PCR. M is the ladder DNA at 100 bp. C- is negative control. Subtype II (650 bp) in lanes 34, 36, 38, 39, 40, 41, 42, 44 and 45. Subtype I (351 bp) in mixed infection with Subtype II (650 bp) in lanes 37 and 45. Subtype IV (338 bp) in single infected subjects in lane 43

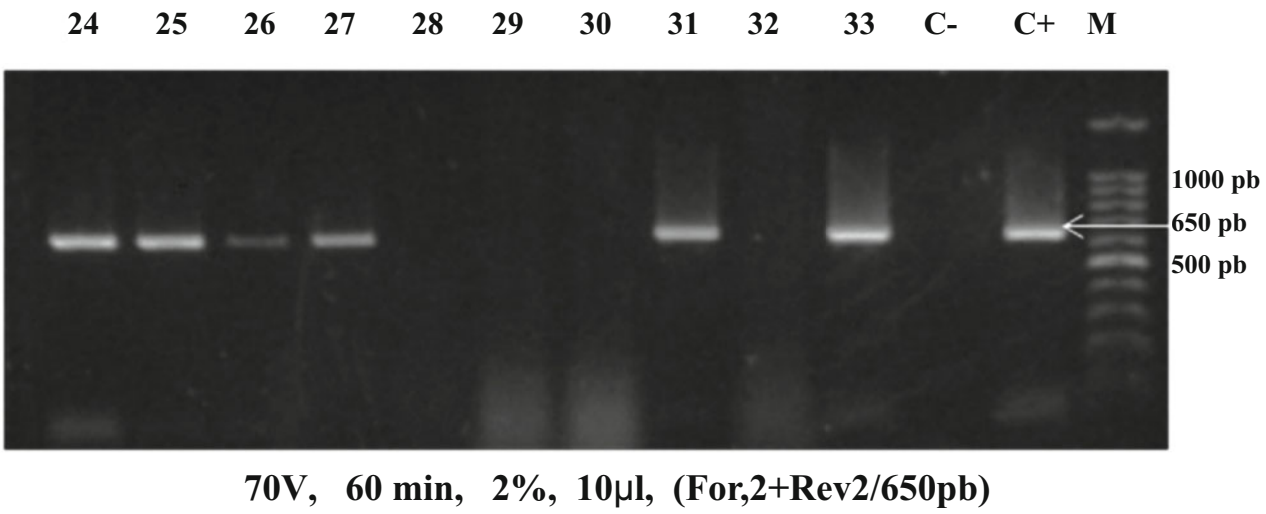

Fig 3. *Blastocystis* subtypes of asymptomatic patients using PCR. M is the ladder DNA at 100 bp. C- is negative control. C+ is positive control

Immunological markers (total serum IgA and IgE antibody levels) in 12 patients harboring only *Blastocystis* were in physiological ranges (Table III).

As shown in Table IV, 6 asymptomatic subjects, 2 subjects (IBS subjects) had mixed infections with subtype I and subtype II, 6 subjects with IBS, 2 subjects with colitis and 1 subject with flatulence had infections with subtype II and 1 subject with colitis had single infection with subtype IV.

Subtyping of *Blastocystis* isolates obtained from 12 patients harboring *Blastocystis* and 10 asymptomatic patients using PCR with STS primers proved that only three *Blastocystis* subtypes were identified (Fig. 2 and Fig. 3).

Subtype II was the commonest detected subtype evidenced in 66.66 % cases of single infection (in lanes 34, 36, 38, 39, 40, 41, 42, 44 and 45), subtype I (16.66%) in mixed infection with subtype II (in lanes 37 and 45) and subtype IV (8.33%) in single infected subjects (in lane 43) (Fig. 2). In asymptomatic patients only subtype II was exclusively identified (Fig. 3).

Discussion

It has been suggested that an intestinal tract that is abnormal for any reason may provide conditions suitable for proliferation of *Blastocystis* (Zaman 1997). Subtypic differences between isolates should assist in determining the pathogenicity of *Blastocystis* (Fouad *et al.* 2011).

Recently, Fouad *et al.* (2011) found that subtype I was the most pathogenic subtype of *Blastocystis* isolates in patients with IBS while subtype II was not detected among those patients. Also the authors reported the presence of pathogenic and non-pathogenic strains among subtypes III and IV.

In the present study, *Blastocystis* subtype II was detected both in asymptomatic and symptomatic patients with IBS, colitis and flatulence. This controversial aspect was also identified in patients infected with *Entamoeba histolytica* (Stanley Jr 2003). In another study registered in the population of San Francisco, USA, the authors found also the absence of clinical symptoms of infection with *Entamoaba histolytica* and *Giardia duodenalis* (Markell and Udkow 1986). Similar results were obtained for *Blastocystis* and a long time it is believed that it is non-pathogenic (Albrecht *et al.* 1995; Junod 1995; Shlim *et al.* 1995).

We firmly believe that it is possible that the high number of parasites/microscopic field over 5/400x and sometimes over 10/400x to determine the clinical symptoms for subtype II which means extensive studies are needed and repeated. The studies should be followed by mucosal IgA assessing and cytokine production to demonstrate the pathogenic potential of this subtype (prevailing subtype isolated from the patients).

Production of specific mucosal IgA seems to be the common factor in all symptomatic patients and missing in the asymptomatic patients (Mahmoud and Saleh 2003).

To understand the mechanisms of pathogenicity is necessary to study both components of biome host/parasite. It appears that the same subtype can cause different clinical manifestations (Roberts *et al.* 2014). This suggests that mainly the host is the factor involved in the occurrence of symptoms and the clinical manifestations represent the host response to this parasite and not its intrinsic pathogenicity. It is reported that *Blastocystis* secreted-proteins have the potential to modulate host defenses and to facilitate nutrient acquisition and parasite colonization. Moreover, *Blastocystis* would be able to alter integrity of gut epithelia and probably participate in dysbiosis (Poirier *et al.* 2012).

The response may be caused by the high number of parasitic elements, age of diagnosis (years), sex (female/male), the persistence of antigen (parasite) on the mucosa (Eida and Eida 2008), the metabolic activity of the parasite (Poirier *et al.* 2012). Moreover, the inflammatory changes of IBS patients harboring *Blastocystis* could suggest that pro-inflammatory

cytokines (IL-10, TNF-alpha, IL-4), at least in part, play a role in the pathogenesis of IBS (Fouad *et al.* 2011; Ramirez-Miranda *et al.* 2010).

In conclusion, in the present study, the presence of *Blastocystis* was detected in 77% of patients with IBS and colitis. In 12 cases, the parasite was found exclusively, without being associated with any other etiologic agent. The association of *Blastocystis* with other etiological agents reveals its opportunist character. The presence of *Blastocystis* cannot be significantly correlated with fecal occult. The levels of serum IgE and IgA antibody were in the range of normal values. Our results suggest the presence of subtypes II, I and IV, while the subtype II was the commonest detected subtype evidenced in single infection or mixed infection. Subtype II was isolated either from symptomatic or asymptomatic patients. The present study suggests that the host is the main factor involved in the occurrence of symptoms and clinical manifestations. However, further studies with larger number of patients are recommended in order to clarify this aspect.

Acknowledgments. The authors would also like to show their gratitude to Lucian Gorgan, PhD, Alexandru Ioan Cuza University of Iasi, Department of Biology, for his valuable support in subtyping of *Blastocystis* analyses. The authors declare that they have no conflict of interest.

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Received: March 6**,** 2014
Revised: September 10, 2014
Accepted for publication: September 19, 2014