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Genotyping of *Blastocystis* Species Revealed In Irritable Bowel Syndrome Patients

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ABSTRACT

Introduction: *Blastocystis* is a common gastrointestinal protozoan. Its association with irritable bowel syndrome (IBS) is debatable. This study was carried out to investigate the relation between IBS and *Blastocystis* sp. infection and to identify its subtypes. Methods: A cross-section study involved IBS group and control group. Microscopy stool examination and in vitro stool culture was done. Further subtyping for all positive *Blastocystis* samples was performed. Results: *Blastocystis* sp. infection were identified in 49 out of 160 (30.6%) and 11 out of 100 cases (11%) in IBS group and control group respectively with statistically significant difference between the two groups. Subtype 3 represented the only subtype in control group and the most common single isolated subtype (93.88%) in IBS group. Whereas, only 3 mixed infection (subtype 3 and subtype 1) were identified in IBS group. Conclusions: A significant association between *Blastocystis* sp. infection and IBS was identified. Further studies are recommended to investigate the pathological potential within the different *Blastocystis* sp. subtypes.

INTRODUCTION

Irritable bowel syndrome is a chronic, relapsing and remitting functional gastrointestinal disorder (Ragavan *et al*., 2015). Its prevalence has varied globally according to geographical location. Nevertheless, IBS is one of the most common diagnosed gastrointestinal (GIT) disorders worldwide with a negative affection on quality of patients' lives and being an economic burden for health authorities (Ford *et al*., 2009 and Canavan *et al*., 2014).

According to Rome III criteria, IBS is a symptom-based condition defined by abdominal pain or discomfort in association with an alteration in stool pattern (Longstreth *et al*., 2006). Its actual etiology is not yet well-identified; however, several underlying mechanisms have been suggested. Recent studies have proposed infectious diseases as etiological agent of IBS (Thompson, 2016).

The pathogenicity of *Blastocystis* sp. is yet debatable. Abdominal pain, nausea, flatulence, diarrhea and constipation are the common symptoms of *Blastocystis* sp. infection (Ragavan *et al*., 2015).These symptoms are comparable to those of IBS. Interestingly, imbalance of microbiota of the gut which is one of the proposed mechanisms of IBS is identified in *Blastocystis* sp. infection (Nourrisson *et al*., 2014).

Several studies have identified a significant relation between *Blastocystis* sp. infection and IBS. Conversely, other studies demonstrated that *Blastocystis* sp. were not involved in the pathogenesis of IBS. The aim of the present study is to assess the association between IBS and *Blastocystis* sp. infection and detection of involved subtypes (STs). Therefore, the aim of the current study was in one hand to investigate the association between IBS and *Blastocystis* sp. infection. On the other hand to identify the subtypes of the recovered *Blastocystis* sp.

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Methods:

A cross section study was designed that involved 260 participants attending outpatient clinic of Kasr Alainy School of medicine hospital, Cairo University, between May 2014 and October 2015. All participants were informed about the purpose of the study and a written consent was obtained. All participant were subjected to structured questionnaire and thorough clinical assessment.

The study population were categorized into 2 groups; IBS-group which included patients with symptoms suggestive of IBS according to the Rome (III) Criteria: repeated abdominal pain for at least 3 days monthly in the last 3 months, accompanied by a minimum two of the following: improvement with defecation, change in frequency of defecation (> 3 daily or < 3 weekly), or change in stool form, and control group which included apparently healthy individuals who were free of any gastrointestinal tract complaints. IBS-group were subcategorized according to their clinical symptoms into clinical-IBS subtypes; IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), IBS mixed type (IBS-M), and IBS unsubtyped (IBS-U) (Longstreth *et al*., 2006).

Sample collection:

Stool samples were collected from all study participants. As previously described by Garcia (2007), microscopy stool examination that included; direct stool examination using (saline wet mount and iodine wet mount) and formol ether concentration technique was done for detection of *Blastocystis* and other parasites. Moreover, Ziehl- Neelsen staining was also done. Any cases that were associated with any pathogens other than *Blastocystis* were excluded from the study.

Culture:

Fifty mg of each stool specimen was cultured in 3ml of Jones medium supplemented with 10% horse serum. Cultures were kept in incubator at 37°C and examined after 48, 72 and 96 hours. Positive samples were sub-cultured 2-3 times on another fresh media after which 200 mL was stored at -20°C until Genomic Deoxyribonucleic Acid (DNA) extraction was done (Jones 1946 and Zman and Khan, 1994).

Subtyping of Blastocystis:

DNA of all positive stool cultures was extracted using the QIAamp DNA stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. PCR amplification of small subunit ribosomal RNA (SSUrRNA) gene (about1800 bp) was done using two sets of oligonucleotide Primers RD5 (GGAAGCTTATCTGGTTGATCCTGCCAGTA) & RD3 (GGGATCCTGATCCTTCCGCAGGTCACCTAC) (Bioneer, USA) in a total volume of 50 µl containing 25µl GoTaq® Green Master Mix (Promega Corporation, Madison, U.S.A.), 2.5µl of each primer, 5 µl of template DNA and 15 µl DNase-RNase free deionized water as previously described by Clark (1997).

The amplified 1800 bp fragments were visualized after electrophoresis in 1.5% agarose gels stained with ethidium bromide using UV transilluminator. Further subtypes of *Blastocystis* were identified with the three restriction enzymes; *Hinf* I, *Rsa* I and *Sau*3A I (BioLabs New England) using restriction fragment length polymorphism (RFLP) technique. According to manufacture instructions, 3 eppendorf for each sample were prepared. Each of a total of 20 µl; each 6 µl DNA, 2 µl of the restriction enzyme, 2 µl of enzyme buffer and 10 µl distilled water. Incubation at 37˚C in heat block for 4 hours and inactivation at 80˚C for 15 min were done. Electrophoresis in 2% agarose gels of digested DNA was carried out and visualized by ethidium bromide staining along with 100 bp ladder. Identification of subtype was performed as described by Clark (1997).

Statistical analysis:

Data were coded and entered using the statistical package SPSS version 12. Data were summarized using mean and standard deviation for quantitative variables and percent for qualitative variables. Comparisons between groups were done using Chi square test and analysis of variants (ANOVA) for qualitative variables and T-test for quantitative variables. *p*- values < 0.05 were considered as statistically significant.

Results:

The study population included 260 participants; 160 cases of IBS who were diagnosed according to Rome III criteria (92 female and 68 males) and 100 healthy individuals as control group (54 females and 46 males).The mean age was 35.2 ± 14.7 for IBS group and was 37 ± 9.9 years for control group.

Table1: *Blastocystis* isolates in microscopically examined stool samples (direct stool smear and formol ether concentration technique) and in-vitro stool cultivation among IBS group and control group.

	Positive % of *Blastocystis* sp.		
	Microscopy stool examination		In-vitro stool cultivation
	Direct smear examination	Formol ether concentration technique	
IBS group (n=160)	28 (17.5%)	10 (6.25%)	49 (30.6%)
Control group (n=100)	5(5%)	2 (2%)	11(11%)

In-vitro stool cultivation for *Blastocystis* sp. showed statistically significant higher yield as it detected *Blastocystis sp.* in 49 cases (30.6%) of IBS patients and in 11 cases (11%) of control group compared to direct smear examination which was positive in 28 cases (17.5%) of IBS group and in 5 cases (5%) of the control group and to examination after formol ether concentration technique which was positive in 10 cases (6.25%) of IBS group and 2 cases (2%) of the controls (Figure 1& Table1).

All stool samples that were positive for *Blastocystis* sp. were further subtyped by (PCR-RFLP) technique and identified as described by Clark (1997). ST3 was the most frequent subtype identified. *Blastocysti*s ST3 was isolated in (46 out of 49) in IBS patients and all the 11 cases in the control group. Mixed ST1 and ST3 *Blastocystis* infection were identified in 3 cases in the IBS group (Figure 2& table2).

Table 2: Distribution of subtypes in positive *Blastocystis* cases in IBS and control group.

Subtype	IBS group (n=49)	Control group (n= 11)
ST3	46 (93.88%)	11 (100%)
Mixed ST1 and ST3	3 (6.12%)	0 (0%)

As regarding the correlation between *Blastocystis* sp. and clinical-IBS subtypes, out of total 49 IBS patients were positive for *Blastocystis* sp., 51 % (25/49) belonged to IBS-D. Twenty two of these cases were identified as *Blastocystis* ST3 and the other 3 cases were mixed ST1and ST3 *Blastocystis* infections. Whereas, 34.7% (17/49) was IBS-M and 14.3% (7/49) was IBS-C. All of which belonged to ST3. Statistical analysis showed a significant association between *Blastocystis* ST3 and IBS-D and no other association between with *Blastocystis* STs and other clinical-IBS subtypes was detected.

Discussion:

Blastocystis infection is common worldwide. Diagnosis of infection is usually made by direct microscopic stool examination (Cekin *et al*., 2012). However, in vitro culture increases the sensitivity of *Blastocystis* detection compared to microscopy (Sekar and Shanthi, 2015). In this study, regarding the techniques used for identification of *Blastocystis* sp., stool examination following formol ether concentration technique yielded the least positive cases in both groups; 10 cases (6.25%) and 2 cases (2%) in IBS group and control group respectively. In agreement, other studies considered that concentration technique is a low sensitivity method for *Blastocystis* detection (Suresh and Smith, 2004; Stensvold *et al*., 2006; Stensvold *et al*., 2007 and Sekar and Shanthi, 2015).Although concentration technique is the most frequently applied method in most laboratories for diagnosing intestinal parasites, yet it is not recommended for *Blastocystis* identification, because it can disrupt the characteristic appearance of the vacuolar form of *Blastocystis*, which is frequently used in its diagnosis (Saksirisampant *et al*., 2003). Moreover, cystic form of *Blastocystis*, which in some cases the main or the only form that can be observed, is not easily identified by formol ether concentration methods but require specific techniques to be applied as Ficoll-Paque column centrifugation (Suresh and Smith, 2004).

In-vitro stool cultivation proved superior sensitivity compared to direct wet stool examination in *Blastocystis* sp. identification. In-vitro stool cultivation detected 49 (30.6%) and 11 cases (11%) positive cases for *Blastocystis* in IBS group and control group respectively versus 28 cases (17.5%) positive cases in IBS group and in 5 cases (5%) in control group respectively that was identified by direct wet stool examination. Similar results were obtained in many studies where wet mount had lower sensitivity than in vitro-stool culture (Leelayoova *et al*., 2002; Thathaisong *et al*., 2003; Yakoob *et al*., 2004 and Dogruman-Al *et al*., 2009). In disagreement, Das *et al*. (2016) reported higher sensitivity of microscopy more than in-vitro stool culture, which was attributed to examination of three consecutive stool samples on three consecutive days in contrast to the present and other studies where microscopic examination of single stool sample was done.

In-vitro stool culture yielded the highest positive cases in this study and was considered as gold standard for identification of blastocystosis in various studies (Tungtrongchitr *et al*., 2004 and Yakoob *et al*., 2004). So, in the present study and as considering in-vitro stool culture as gold standard for identification of blastocystosis, 49 (30.6%) cases in IBS group and 11 cases (11%) in control group were identified as positive cases for *Blastocystis* sp. with statistically significant difference between the two groups.

Noticeably, not only *Blastocystis* sp. prevalence varied according to geographical location and even within different communities in the same country (Sekar and Shanthi, 2015) but also the relationship between *Blastocystis* sp. infection and IBS had been controversial (Ragavan *et al*., 2015). In agreement with the present study, several studies have also revealed a significant relationship between *Blastocystis* sp. infection and IBS. In Pakistan and using stool culture as diagnostic method, Yakoob *et al*. (2004) were able to detect *Blastocystis* sp. in 46% (44 of 95) of the IBS-group and 7% (4 of 55) of the control group with statistically significant difference. Later on, another study was done in Pakistan also, in vitro cultivation of *Blastocystis* was positive in 53% (90/171) in IBS group compared to 16% (25/159) in control group with statistically significant difference (Yakoob *et al*., 2010a).

Other studies used PCR as diagnostic method for detection for *Blastocystis* sp. In Mexico, Jimenez-Gonzalez *et al*. (2012) found 31.1% positive for *Blastocystis* in IBS group compared to 13.3% in control group

with statistically significant difference. In a study conducted in Malaysia, 17% (6/35) of IBS group were found positive for *Blastocystis* sp. versus 5.5% (4/74) in control group with statistically significant difference (Ragavan *et al*., 2015). Moreover, in India, Das *et al*. (2016) was able to identify *Blastocystis* sp. in 33.3% (50/150) in IBS group and 15% (15/100) in the control group with statistically significant difference.

In disagreement with the present study, some studies did not find significant association between *Blastocystis* sp. infection and IBS and suggested that *Blastocystis* sp. were not likely to have a direct role in the pathogenesis of IBS. In Thailand, Tungtrongchitr *et al*. (2004), using stool culture as a diagnostic method, *Blastocystis* detection rate was 13.6% (8 out of 59) in IBS group versus 12% (3 out 25) in the control group with no statistically significant difference. Later on, in Thailand also, in vitro stool cultivation for *Blastocystis* sp. was positive in 16.7% of IBS group and 10% in the control with statistically non- significant difference between groups (Surangsrirat *et al*., 2010).

Another study, in Turkey, using trichrome stain for detection of *Blastocystis sp.*, 5.74% (134 out of 2334) in IBS group versus 3.12% (6 out of 192) in the control group were positive for *Blastocystis sp.* with no statistically significant difference between groups (Cekin *et al*., 2012). Later on, other studies used PCR as a diagnostic method for *Blastocystis* sp. In french study done by Nourrisson *et al*. (2014), *Blastocystis* sp. was detected in 23.2% of IBS group versus 16.1% in the control group with no statistically significant difference. Unlikely and different from all previously mentioned studies, in Denmark, Krogsgaard *et al*. (2015) detected *Blastocystis* sp. in higher percentage in control group (22%) than in IBS-group (15%) with no statistically significant difference between groups.

In this study, DNA extraction was performed from positive in-vitro cultured stool samples and not directly from stool samples. Although PCR is rapid, highly sensitive and accurate technique (Stensvold *et al*., 2006 and Parkar *et al*., 2007), the presence of inhibitory substances in fecal samples may result in false negative results (Souppart *et al*., 2009). Moreover, in vitro cultivation result in enough DNA for subsequent PCR amplification and molecular typing (Suresh and Smith, 2004 and Termmathurapoj *et al*., 2004).

According to small subunit ribosomal RNA gene analysis, 17 *Blastocystis* subtypes were identified (Alfellani *et al*., 2013). ST1 to ST9 were recovered from human stool with ST3 being the most predominant human ST (Nourrisson *et al*., 2014). In the present study, ST3 represented the only subtype in control group and the most common single isolated ST (93.88%) in IBS group. Whereas, ST1was identified as mixed infection with ST3 in only three IBS patients.

In agreement with these results, several studies have also demonstrated that ST3 and ST1 were predominant in patients with IBS (Yakoob *et al*., 2010 b; Jimenez-Gonzalez *et al*., 2012 and Das *et al*., 2016). Nevertheless, ST4 was the most common ST in a study done by Nourrisson *et al*. (2014) who did not show any association between ST and IBS. In another study conducted by Ragavan *et al*. (2015), ST3 was found to be the most common ST (50%), but also ST4 (33.3%) and ST5 (16.6%) were identified.

The explanation for the variation in the reported differences in the STs distribution between countries and even within the same country was attributed to multiple parameters including various factors as environmental, parasite and host factors and the adopted parasitological diagnostic method that varied between researches. This in addition to the small number of study population that is unfortunately was met in this study (Poirier *et al*., 2012).

On studying relation between clinical symptoms and *Blastocystis* ST, statistical analysis showed a significant association between *Blastocystis* sp. and IBS-D only and not with the other IBS-clinical subtypes. Similar results were observed in studies done by Yakoob *et al*. (2010b) and Das *et al*. (2016). Whereas, Nourrisson *et al*. (2014) did not observe any association between *Blastocystis* sp. and any IBS-clinical subtypes. Possible explanation for the association between *Blastocystis* sp. and IBS-D was proposed by researchers who reported high levels of protease secretion by *Blastocystis* in symptomatic patients (Tan, 2008; Denoeud *et al*., 2011 and Poirier *et al*., 2012). The protease-activated receptors type 2 (PAR-2) are responsible of increasing of permeability of gut with a low grade inflammation that resulting in diarrhea (Poirier *et al*., 2012).

In the present study, higher percentage of *Blastocystis* infection was detected in IBS group than control group with statistical significant difference between groups. However, ST3 was identified in both IBS and control group. Thus, its pathogenic potential could not be confirmed and is yet questionable. However, recent genetic analyses studies showed that *Blastocystis* subtypes isolated from humans and other animals showed difference in pathological potential although sharing similar morphological features (Yan *et al*., 2006; Li *et al*., 2007 and Souppart *et al*., 2009). Intra-subtype variations in pathogenicity have also been observed and this probably explains the differences between pathogenic and non-pathogenic potential with the existence of wide intra ST variation in ST3 (Sekar and Shanthi, 2015). Also, proteases secreted from *Blastocystis* sp. could play a key role in IBS as it results in disturbance in intestinal homeostasis and low-grade inflammation of the mucosa that lead to IBS symptoms (Poirier *et al*., 2012). Moreover, pro-inflammatory cytokines secreted by *Blastocystis* sp. might be involved in pathophysiology of IBS also (Sekar and Shanthi, 2015). On contrast, another explanation that the occurrence of change in the intestinal environment in IBS favors the growth of *Blastocystis* sp. was postulated (Sekar and Shanthi, 2015).

Limitations faced this study involved the small number of the study population and uni-centered sample collection. Further multi-centered studies are recommended involving larger number of IBS cases. Sequencing the entire genome of *Blastocystis* STs is essential. Moreover, animals' studies to reveal pathological potential of different STs and the involved pathophysiology are demanded. Therapeutic trails for *Blastocystis* sp. infection with monitoring the alleviation or persistency of IBS symptoms will be helpful. Various researches are recommended to investigate these debatable issues.

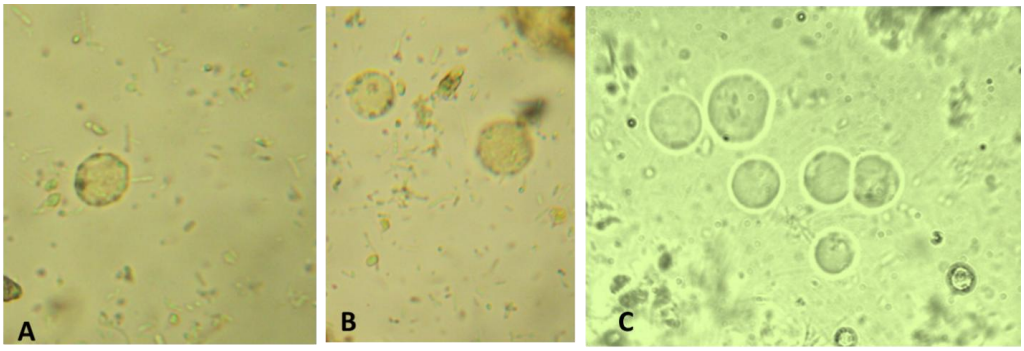

Fig. 1: A &B: *Blastocystis* (vacuolar form) as detected in direct stool wet preparation at a microscopic magnification of (x400). C: In vitro cultivation of *Blastocystis* after 48 hours of cultivation on Jones medium culture (x 400).

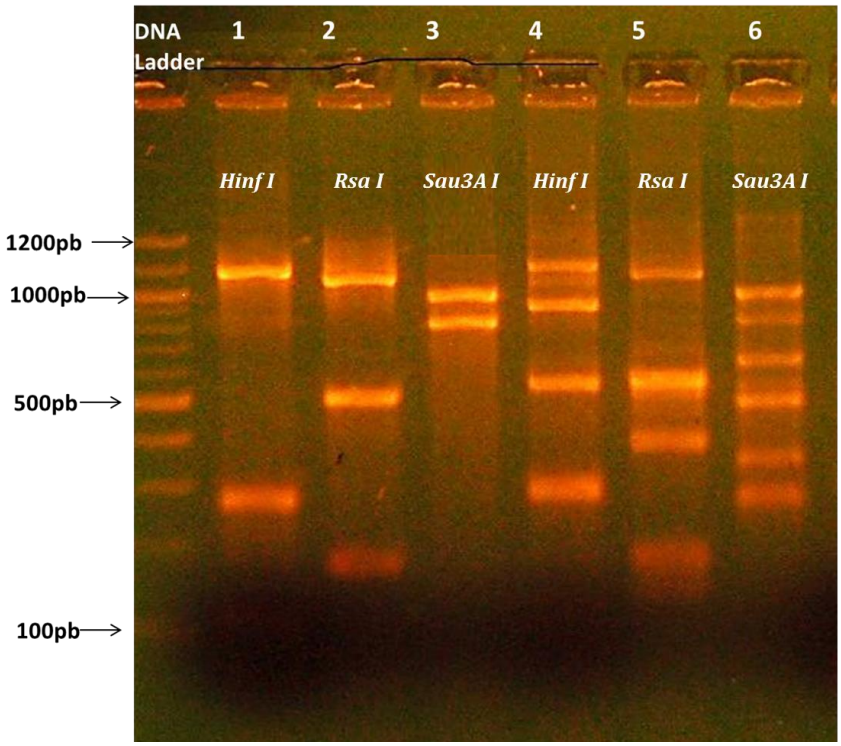

Fig. 2: Electrophoresis gel pattern of *Blastocystis* subtype 3 (from lane 1 to lane 3) and mixed *Blastocystis* subtype 1&3 (from lane 4 to lane 6) digested with *Hinf* I, *Rsa* I and *Sau*3A I. **Lane 1** showed digestion by *Hinf* I, at 1100 & 280 bp, **Lane 2** digestion by *Rsa* I at 1100, 500 and 180 bp, **Lane 3** digestion by *Sau*3A I at 900 and 800 bp which is characteristic pattern for subtype 3. **Lane 4** showed digestion by *Hinf* I at 900,580,280 bp, **lane 5** shows digestion by *Rsa* I at 600, 380,170 bp and **lane 6** expresses digestion by *Sau*3A I at 650, 490, 350 and 270 bp and this is the restriction pattern for subtype 1. While again **lane** 4 showed digestion by *Hinf* I at 1100 bp, **lane 5** digestion by *Rsa* I at 1100 bp and lane 6 digestion by *Sau*3A I at 900, 800 bp and this is restriction pattern for subtype 3 (according to Clark, 1997). So **lane 4, 5, 6** showed mixed subtype 1&3.

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