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Original paper

Prevalence and subtype diversity of *Blastocystis* sp. in an Iraqi population with and without irritable bowel syndrome (IBS)

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ABSTRACT. The parasites that belong to the genus *Blastocystis* are the most common intestinal parasite detected in a wide range of hosts including humans. Although the pathogenicity of these parasites remains controversial, many studies confirmed their pathogenicity and some researchers strongly believe that the pathogenicity may be linked with specific subtypes of these parasites. The current study investigated the *Blastocystis* sp. subtypes recovered from individuals suffering from the irritable bowel syndrome (IBS) in comparison with those recovered from non-IBS subjects. Fresh stool samples were collected from each participant and processed during the same day of collection. Iodine wet mounts and trichrome stained smears prepared from fresh stool and sediment concentrates were microscopically examined for *Blastocystis* parasites. In addition, Jones's medium has been used in order to confirm the identification and also to get the genomic DNA from positive samples for PCR and sequencing. The culture was significantly more sensitive (P=0.0035) than the other identification methods, especially in IBS patients. *Blastocystis* was detected in 60.0% of patients with IBS and in 22.0% of non-IBS individuals and the difference between two groups was statistically significant (P=0.0001). Regarding the impact of age and gender on the prevalence of infection with *Blastocystis*, no significant differences were observed between IBS patients and non-IBS subjects except for the age group (10–30 years) where the non-IBS subjects were significantly more prone (P=0.0223) to the infection with this parasite than IBS patients. The abdominal pain and bloating were the leading symptoms. DNA sequencing and phylogenetic analysis of Iraqi *Blastocystis* isolates identified three subtypes (ST1, ST2 and ST3). Among these three subtypes, ST3 was significantly more prevalent (OR=8.5; P=0.0058) among IBS patients (60%) than non-IBS subjects (25%). In contrast, the dominance of ST1 was significantly higher (OR=7.0; P=0.0062) in the non-IBS subjects (70%) than their IBS patients counterparts (15%). As far as we know, this study is the first to deal with the genetic characterization of *Blastocystis* subtypes in an Iraqi population with and without IBS.

Keywords: *Blastocystis* sp., irritable bowel syndrome (IBS), prevalence, PCR, subtypes, Iraq

Introduction

Some studies have introduced *Blastocystis* sp. as a potential pathogen, with digestive symptoms including diarrhea, abdominal pain, anorexia, bloat, fatigue, and extra gastrointestinal symptoms such as urticaria and itchy skin, as well as joint pain [1–3]. As infected people without symptoms are also found, its pathogenesis is unclear and controversial [4]. Recently, Badparva and Kheirandish [5] reported that the absence of symptoms in people infected with *Blastocystis* sp. does not mean, in any way, that the parasite is not pathogenic mainly because the results of some genetic studies revealed that some subtypes (STs) are non-pathogenic, such as ST2, while others are pathogenic, such as ST1 [6,7].

In addition to the genotype studies, phenotype studies also have provided important information about the diverse characters of *Blastocystis* parasites which are polymorphic in nature. For example, the subtypes that cause clinical symptoms in their hosts (but not the asymptomatic subtypes) can grow in culture media into ameboid forms which are the

only feeding forms and responsible for the pathogenicity of these parasites, as they are the only stages that can attack the mucosa of the large intestine of the infected hosts via the secretion of the hyaluronidase enzyme that destroys proteins in the extracellular matrix and paves the way for parasite attack [8,9]. It has been reported that the pathogenic subtypes are much bigger and have coarse surfaces and grow faster in culture media than the non-pathogenic subtypes/genotypes [10]. Moreover, differences in isoenzyme model, the shape of proteins, and serological properties between pathogenic and non-pathogenic subtypes have been found in some studies [11,12].

In addition, the hosts can also play a significant role in the pathogenicity of the different subtypes/genotypes of parasites that belong to the genus *Blastocystis*. For example, it has been shown that the prevalence of the infection with these parasites is higher in people with mental retardation in comparison with healthy subjects, which can be due to the lack of sanitary considerations in these people [13,14]. Another example, the immuno-deficient patients are more prone to the infection with *Blastocystis* parasites which can lead to severe symptoms and even death in immunocompromised patients [1]. It is well known that Irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) are digestive disorders which have been related to infection with *B. hominis* in some studies [7,15–17]. About 30–40% of the IBS patients were found infected with *B. hominis* in one study conducted in Europe and the Middle East [18], while in another study, it has been reported that 46% of the IBS patients were found infected with *B. hominis* in comparison with 7% in healthy non-IBS subjects [19].

It has been reported that serine protease which is secreted by the parasites that belong to the genus *Blastocystis* can cause IBS and IBM disorders, and the high levels of this enzyme can cause severe neural activity, abdominal pain, and muscle cramp, which are not found in bacterial and viral enteritis [20]. Moreover, the results of some studies have shown that some individuals who were infected with these parasites have symptoms of skin allergy such as erythema, itching, and urticaria, due to the secretion of IgE as a result of the immune system response of the infected host to the parasite's surface antigens [21–23].

As far as we know, only one previous *Blastocystis* genomic study has been done in Iraq [24], while so many genomic studies on humans and animals have been done in neigbouring countries and worldwide [25–27]. *Blastocystis* infection has been reported to be associated with irritable bowel syndrome (IBS) and other diseases and syndromes [3,5]. Consequently, the identification of the subtypes of *Blastocystis* found in IBS patients in comparison with non-IBS subjects would be of interest in clarifying the picture regarding the role of the infection with *Blastocystis* in development of IBS. Accordingly, the main objective of the present study was to investigate the prevalence and subtype diversity of *Blastocystis* parasites isolated from IBS patients in comparison with their counterparts isolated from apparently healthy individuals who do not suffer from IBS or any other bowel diseases (non-IBS subjects) living in the Middle of Iraq.

Materials and Methods

Isolation and identification of Blastocystis parasites

This study is another part of PhD thesis project of the first author. Stool samples were collected from 250 IBS patients living in Diyala Province, Iraq, and processed during the same day of collection using the research facilities provided by the Department of Biology and the Department of Biotechnology, College of Sciences, Diyala University, Diyala, Iraq. The patients who attended the gastrointestinal private clinics in Baquba City, the capital city of Diyala Province, middle of Iraq during 2021 and confirmed to have IBS by specialist doctor using Rome III criteria were enrolled as IBS patients. In addition, stool samples were collected from 100 non-IBS healthy subjects who attend the gastrointestinal clinics either for regular medical check-up or from those who accompany the patients. Exclusion criteria were: IBS symptoms and any type of cancer. In addition, any participant with a history of diabetes mellitus, infectious diseases and chronic inflammatory diseases was excluded.

The participants were asked to submit fresh stool specimens free from water and urine and processed as explained previously [2] with some modifications. Each participant was supplied with a standardized questionnaire in order to determine the risk factors and outcomes of *Blastocystis* infection, and containing inquiries regarding age, sex, underlying disease, gastrointestinal symptoms, and contact with animals. A verbal consent has been obtained from

each participant. Each stool sample was divided into two parts: one part (about 5 mg) was processed immediately by iodine wet mounts and trichrome staining techniques, while the second part (about 200 mg) was directly inoculated into tubes containing 5 ml of Jones' medium supplemented with 10% heat-inactivated horse serum and incubated at 37°C for 48–72 hours. The cultures were microscopically examined daily for the detection of *Blastocystis* parasites. Approximately 50 µl were removed from the sediment of positive cultures in order to prepare subcultures in fresh medium to remove fecal debris and minimize the contamination. Seven ml phosphate-buffered saline (PBS) were added to 3 ml of the subculture suspension and vortexed thoroughly then centrifuged at 12.000 g for one minute and the pellet was re-suspended in one ml of PBS solution and preserved at –20°C until used for extracting the DNA for PCR assay and sequencing process.

Extraction of genomic DNA and PCR assay

To determine if genetic diversity of *Blastocystis* spp. exists in Iraq, we monitored 40 *Blastocystis*-positive subjects: 20 IBS patients and 20 non-IBS healthy subjects. In the present study, we extracted DNA directly from culture rather than stool samples because it has been found that about 32% of the isolates could not be identified when extraction of DNA from stool samples followed by PCR due to the presence of PCR inhibitors so it has been recommended to use culture step before molecular genotyping [28]. Accordingly, the subcultures prepared from culture-positive samples (20 positive samples obtained from people with irritable bowel syndrome (IBS) and 20 positive samples obtained from non-IBS subjects) were centrifuged at 10.000 g for one minute and genomic DNA was extracted from the pellets using commercial DNA MiniPrestoTM kit (Geneaid, Taiwan) following the manufacturer's instructions.

In order to identify the STs of *Blastocystis* sp., a polymerase chain reaction (PCR) was carried out using nine pairs of primers targeting the SSU rDNA gene and sequenced. The PCR reaction was carried out utilizing the AccuPower PCR PreMix (Cat No: K-2012, Bioneer, Daejeon, South Korea) following the manufacturer's instructions.

Identification of Blastocystis subtypes

The sequences were compared to *Blastocystis* SSU-rDNA sequences in GenBank nucleotide database using BLAST tool at the National Centre for Biotechnology Information website [29]. In addition, the sequences were queried against the *Blastocystis s*equence typing website database (https://pubmlst.org/blastocystis/) [30]. The subtypes of the isolates were determined according to the exact or closest matches [31].

Statistical analysis

The statistical analyses were performed using Graph Pad Prison version 8 (Graph Pad Software Inc., La Jolla, CA). Student's t-test was used to determine whether group variance was significant or not. Chi-square was used to determine the differences between the three diagnostic methods used in the study. The differences between the age groups were estimated by the independent sample Student's t-test. Data were expressed as mean ± SE and statistical differences were considered significant when $P \leq 0.05$. The odds ratio (OR) is a measure of association between an exposure and an outcome and represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. The OR was calculated using: Med Calc Software Ltd. Odds ratio calculator. https://www.medcalc.org/calc/ odds_ratio.php (Version 20.013; accessed October 3, 2021).

Ethical statement

The study was approved by the Ethics Committee of the Biotechnology Department, College of Sciences, Diyala University, Diyala, Iraq (protocol number: 5/2020).

Results

Stool samples were examined with iodine and trichrome staining together with *in vitro* culture method for detection of *Blastocystis* and the results are shown in table 1. In stool samples collected from IBS patients, iodine and trichrome staining detected 112 (44.8%) and 100 (40.0%) positive samples for *Blastocystis*, respectively. In contrast, culture method by using Jones's medium detected 150 (60.0%) positive samples. In stool samples collected from non-IBS subjects, iodine and trichrome staining detected 17 (17.0%) and 15 (15.0%) positive samples, respectively, while the culture method detected 22 (22.0%) positive samples. In IBS patients, *in vitro* culture was significantly more sensitive than the wet iodine and trichrome staining

Table 1. Comparison between the laboratory techniques used for the detection of *Blastocystis* sp. in patients with irritable bowel syndrome (IBS) and non-IBS subjects

Participants	Number (%)			Chi-square	*P*-value
	Iodine wet mounts	Trichrome staining	*In vitro* culture		
IBS patients (n=250)	112 (44.8 %)	100 (40.0%)	150 (60.0%)	11.29	0.0035
Non-IBS controls (n=100)	17 (17.0%)	15 (15.0%)	22 (22.0%)	1.44	0.4857

Table 2. Distribution of the prevalence of the infection with the parasites that belong to the genus *Blastocystis* in patients with irritable bowel syndrome (IBS) in comparison with non-IBS subjects according to the gender and the age groups. The odds ratio (OR) was calculated to clarify the statistical differences between the two groups

Variables	IBS patients infected with *Blastocystis*/No(%)	Non-IBS subjects infected with *Blastocystis*/No(%)	*P*-value*
Males	68 (45.3%)	10 (45.5%)	0.9951
Females	82 (54.7%)	12 (54.6%)	0.9915
Total	150 (60.0%)	22 (22.0%)	0.0001
Average age (years ± SE)	36.8 ± 1.4	37.3 ± 0.98	0.4134
10-30	62 (41.3)	15 (68.2%)	0.0223
31-50	52 (34.7%)	6 (27.3)	0.4949
Over 50	36 (24.0%)	1 (4.6%)	0.0692

SE: Standard error; * level of significance as estimated by the independent sample Student's t-test

methods (X^2=11.29, *P*=0.0035). In contrast, in non-IBS subjects no significant differences were detected between the three identification methods (X^2=1.44, *P*= 0.4857) (Tab. 1).

It can be seen from table 2 that the highest prevalence of *Blastocystis* sp. in IBS patients was observed in the age group of 10–30 years (41.3%) followed by the age group of 31–50 years (34.7 %) and finally the age group of >50 years (24.0%). Similarly, in non-IBS subjects the highest prevalence of *Blastocystis* sp. was observed in the age group of 10–30 years (68.2%) followed by the age group 31–50 years (27.3 %) and finally the age group of >50 years (4.6%). The statistical analysis showed a significant difference between IBS patients and non-IBS subjects only in one age group which was 10–30 years (OR=0.33, *P*=0.0223).

In IBS patients who were infected with *Blastocystis* sp., bloating was the main symptom (90%), followed by abdominal pain (80%), fatigue (49.3%), constipation (45.3%), diarrhea (28%), joint pain (23.3%), vomiting (8%), and rash (5.3%). However, in non-IBS subject who were infected with *Blastocystis*, abdominal pain was the main symptom (54.5%), followed by bloating (40.9%), fatigue (27.3%), joint pain (18.2%), constipation (13.6%), diarrhea (9.1%), rash (9.1%), and vomiting (4.6) (Tab. 3). The statistical analysis showed significant differences in the frequency of the following symptoms: bloating (OR=3.3, *P*= 0.0111), abdominal pain (OR=7.8; *P*=0.0001), and constipation (OR=5.3; *P*=0.0098) between IBS patients and non-IBS subjects who were infected with *Blastocystis* parasites. In contrast, no significant differences have been observed between the two groups regarding the frequency of the remaining symptoms mentioned in table 3.

The detailed information (age, sex and subtypes) on the symptomatic status of IBS patients and non-IBS subjects from whom *Blastocystis* was isolated

Table 3. Comparison between patients with irritable bowel syndrome (IBS) and non-IBS individuals (control group) who were infected with *Blastocystis* sp. regarding the frequency of clinical symptoms

Symptoms	Numbers with symptoms (%)		Odds Ratio (OR)	*P*-value
	IBS patients (n=150)	Non-IBS subjects (n=22)		
Abdominal pain	120 (80%)	12 (54.5%)	3.3	0.0111
Bloating	135 (90%)	9 (40.9%)	7.8	0.0001
Fatigue	74 (49.3%)	6 (27.3%)	2.6	0.0592
Constipation	68 (45.3%)	3 (13.6%)	5.3	0.0098
Diarrhea	42 (28%)	2 (9.1%)	3.9	0.0753
Joint pain	35 (23.3%)	4 (18.2%)	1.3	0.6928
Vomiting	12 (8%)	1 (4.6%)	1.8	0.5725
Rash	8 (5.3%)	2 (9.1%)	0.86	0.4872

Table 4. Details of patients with irritable bowel syndrome (IBS) and non-IBS subjects from whom *Blastocystis* parasites were isolated

Participants	IBS patients			Non-IBS subjects		
	Age (years)	Gender	Subtype	Age (years)	Gender	Subtype
1	53	F	ST1+ ST3	9	M	ST1
2	47	M	ST1	18	F	ST1
3	27	F	ST3	24	M	ST1
4	31	F	ST3	37	M	ST1
5	38	M	ST1	25	F	ST1
6	24	M	ST1	20	F	ST3
7	62	F	ST3	29	F	ST3
8	8	F	ST1+ ST3	46	M	ST1
9	35	F	ST1	23	M	ST1
10	42	M	ST1	32	M	ST1
11	25	M	ST3	33	F	ST1
12	33	F	ST3	11	F	ST1
13	19	F	ST3	28	M	ST3
14	28	M	ST3	54	M	ST1+ ST3
15	45	F	ST3	19	M	ST2
16	37	F	ST3	25	F	ST1
17	53	M	ST1+ ST3	31	M	ST1
18	39	F	ST3	8	F	ST2
19	62	M	ST3	41	F	ST1
20	41	M	ST3	15	F	ST1

M: Male; F: Female

Table 5. Distribution of subtypes identified in irritable bowel syndrome (IBS) patients and non-IBS subjects

Subtype	IBS patients (n=20)	Non-IBS subjects (n=20)	OR	*P*-value
ST1	5 (25.0%)	14 (70.0%)	7.00	0.0062
ST2	0 (0.0%)	2 (10.0%)	0.181	0.2792
ST3	12 (60.0%)	3 (15.0%)	8.5	0.0058
Mixed (ST1 and ST3)	3 (15.0%)	1 (5.0%)	3.35	0.3142

are given in table 4. The *Blastocystis* subtypes (STs) isolated from IBS patients and non-IBS subjects are summarized in table 5. When 20 isolates from IBS patients were screened by PCR amplification with the STs primers, 12 were identified as ST3 (60%), 5 as ST1 (25%), and mixed infections (ST1 + ST3) were detected in 3 samples (15%). Of the 20 samples of non-IBS subjects tested by PCR/DNA sequencing, 14 were identified as ST1 (70%), two as ST2 (10%) and three as ST3 (15%), and only one sample (5%) showed mixed infections (ST1+ST3). ST3 was the dominant subtype in the samples of IBS patients, while ST1 was the dominant ST in non-IBS individuals (Tab. 5 and Fig. 1). It is interesting to mention that the non-IBS subjects were significantly more prone to the infection with ST1 than IBS patients (OR=7.0; *P*=0.0062). In contrast, the IBS patients were significantly more susceptible to the infection with ST3 than non-IBS individuals (OR=8.5; *P*=0.0058) (Tab. 5).

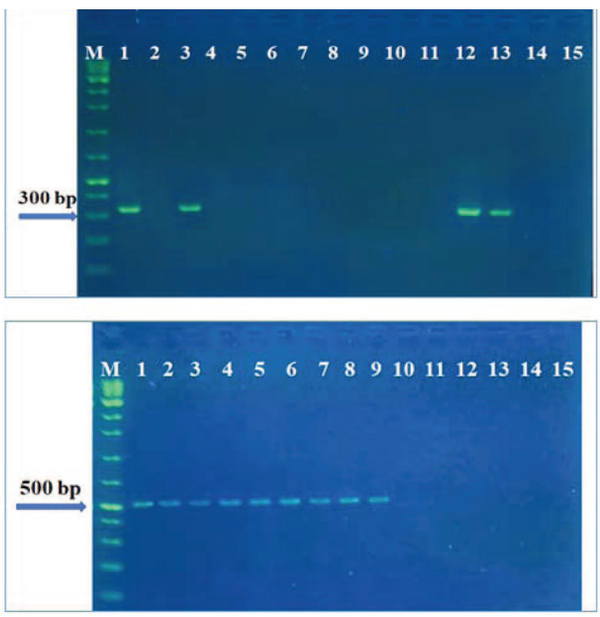

Figure 1. Agarose gel electrophoresis images show *Blastocystis* spp. subtypes (STs) from patients with irritable bowel syndrome (IBS). M is the ladder at 100–1000 bp. Top panel: isolates in lanes 1, 3, 12 and 13 show infections with ST1 (351bp). Bottom panel: isolates in lanes 1–9 show infections with ST3 (526 bp)

Discussion

Blastocystis was detected in 60.0% of patients with IBS and in 22.0% of non-IBS individuals and the difference between two groups was statistically significant (OR=5.32; *P*=0.0001). This means that the IBS patients are more prone to the infection with *Blastocystis* parasites than non-IBS subjects by a factor exceeds 5 folds. The overall prevalence of *Blastocystis* sp. in IBS samples is higher than the rates recorded in the previous studies conducted in Iraq, which ranged between 32.3% and 57% [3,24,32,33]. This prevalence rate is also higher than the rates recorded in Iran [34], Turkey [4], Saudi Arabia [35] and Egypt [2,36].

For the detection and differentiation of *Blasto - cystis* sp., three methodological approaches have been chosen in this study, direct wet iodine mounts, trichrome staining and *in vitro* culture and the positivity rate of *Blastocystis* parasites was 44.8% in direct wet mount, 40.0% in trichrome staining and 60% when the *in vitro* culture was used (Jones' medium). Accordingly, the present study revealed that the *in vitro* culture method was significantly more sensitive in detecting *Blastocystis* parasites than both the iodine and trichrome staining methods, especially in IBS patients. Similarly, many studies have revealed the superiority of culture method over the direct microscopy and trichrome staining in detection of *Blastocystis* parasites [21,36–39].

The principal finding of the present study was the identification of two subtypes (STs) of *Blastocystis* (ST1 and ST3) which are associated with the chronic infections in IBS patients and the ST3 was the dominant subtype. In Iraq, only one

Table 6. Subtypes (STs) of *Blastocystis* in people with irritable bowel syndrome (IBS) reported previously in Iraq (including the present study) and other countries

Country	Subtypes (STs)	Most common	References
Iran	ST1, ST2, ST3, ST4 and ST5	ST3	[40]
Iran	ST1, ST2, ST3 and ST7	ST3	[41]
Turkey	ST2 and ST3	ST3	[4]
Saudi Arabia	ST1, ST2 and ST3	ST3	[35]
Egypt	ST1 and ST3	ST3	[36]
India	ST1 and ST3	ST3	[38]
Indonesia	ST1, ST2 and ST3	ST1	[50]
Iraq	ST1, ST3 and ST7	ST1	[24]
Iraq	ST1 and ST3	ST3	Present study

previous genomic study has been conducted in which the authors identified three subtypes (ST1, ST3 and ST7) to be associated with chronic infection in IBS patients and the ST1 was the most prevalent one [24]. In Turkey, Dogruman-Al et al. [4] have identified *Blastocystis* sp. subtypes 2 and 3 in patients with IBS and that the ST3 was the most commonly found. Abaza et al. [37] identified 54 *Blastocystis* isolates with single infection and three isolates with mixed infections from three different groups of Egyptian patients (IBS and non-IBS with and without gastrointestinal tract symptoms) and reported that ST3 was the most common one in the studied Egyptian population followed by ST1 and ST2.

The dominance of ST3 in IBS patients also

Table 7. Subtypes (STs) of *Blastocystis* in people without irritable bowel syndrome (non-IBS) reported previously in Iraq (including the present study) and other countries

Country	Subtypes (STs)	Predominant ST	References
India	ST1 and ST3	ST3	[38]
Indonesia	ST1, ST2 and ST3	ST3	[50]
Iran	ST1, ST2, ST3 and ST7	ST3	[47]
Malaysia	ST1, ST2, ST3 and ST4	ST3	[54]
Nigeria	ST1, ST2, ST3 and ST7	ST1	[53]
Qatar	ST1, ST2 and ST3	ST3	[51]
Senegal	ST1, ST2, ST3 and ST4	ST3	[49]
Turkey	ST1, ST2, ST3 and ST7	ST3	[39]
Turkey	ST1, ST2 and ST3	ST3	[4]
Czech Republic	ST1–ST8	ST3	[45]
United Arab Emirates	ST1, ST2 and ST3	ST3	[52]
Iraq	ST1, ST2 and ST3	ST1	Present study

revealed in other studies conducted in other countries (Tab. 6). In a similar study conducted in India, Das et al. [38] studied the association between *Blastocystis* sp. and its subtypes with IBS and reported that only two subtypes have been detected (ST1 and ST3) and ST3 was the dominant subtype. In Egypt, El-Badry et al. [36] collected stool samples from 115 Egyptian IBS patients and microscopically examined and cultured on Jones' medium with further sequencing of positive *Blastocystis* isolates and found a predominance of *Blastocystis* ST3 followed by ST1. In a study conducted in Iran, Khademvatan et al. [40] identified 5 different subtypes and ST3 showed the highest prevalence. In addition, the authors reported that eleven PCR samples showed mixed subtypes. Recently, Salehi et al. [41] reported that the sequencing of PCR products derived from *Blastocystis* parasites isolated from Iranian IBS patients showed the dominance of ST3 over the other 3 STs (ST1, ST2, and ST7). Moreover, genomic studies revealed the presence of 22 subtypes within the genus *Blastocystis* and 10 of these subtypes (ST1-ST9, and ST12) were found in humans, and that the ST3 showed the highest genetic diversity and has a worldwide distribution in comparison with other genetic subtypes [4,25,27,31,42].

In the present study, most of the included samples showed single infections (17/20 in IBS patients and 19/20 in non-IBS subjects). In studies conducted in France and Egypt, it has been reported that the majority of the samples included in these studies represented single infections [28,37]. In the present study, mixed infections were detected in both IBS patients (3 samples) and non-IBS subjects (one sample). Similarly, mixed infections have been detected in both asymptomatic and symptomatic patients in other studies conducted in Turkey, Egypt and India [4,37,38].

The overall prevalence of *Blastocystis* in non-IBS samples was 22% and this rate corresponds well with prevalence rates recorded in Czech Republic and some other European countries in populations of healthy people ranging between 18% and 30% [43–45], but much higher than that recently reported in Iran and USA which were 6.4% and 7%, respectively [46,47]. In contrast, much higher prevalence rates were recorded in studies conducted in Ireland and Senegal which were 56% and 100%, respectively [48,49]. Various factors such as environmental, nutritional, and difference in study populations or inclusion criteria may be behind the differences between the findings of the studies conducted in different countries [3].

In non-IBS subjects, the results of the present study revealed the presence of three STs (ST1, ST2, and ST3) and the ST1 was the dominant ST followed by ST3 and ST2. Table 7 shows a comparison between the findings of the present study and other studies conducted in different countries regarding the types and prevalence of *Blastocystis* subtypes in non-IBS subjects. Similarly, these three subtypes have been found in non-IBS individuals living in Indonesia [50], Qatar [51] and in United Arab Emirates [52]. Subtype 1 was the predominant one in Nigeria [53]. In contrast, El Safadi et al. [49] reported that all apparently healthy children in a study conducted in the Senegal were found infected with *Blastocystis* and ST3 was the most abundant genotype followed by ST1, ST2, and ST4. Sankur et al. [39] identified four different subtypes of *Blastocystis* (ST1, ST2, ST3, and ST7) in Turkish children and ST3 was responsible for the great majority of infections. Kesuma et al. [50] investigated the risk factors of IBS and the association between IBS types with *Blastocystis* subtypes in Indonesian adolescent students and reported that ST1 was predominant in IBS-students, while ST3 was the predominant subtype in non- IBS students. Recently, Marali et al. [47] investigated the prevalence of *Blastocystis* and characterized its subtypes in central southwest of Iran and reported that 6.4% of the subjects were infected with *Blastocystis* sp. and the molecular methods showed that the infection was caused by four subtypes (ST1, ST2, ST3 and ST7) and the most common genotype was ST3. Moreover, subtype 3 was the predominant one in India [38] and Malaysia [54] (Tab. 7).

The differences between different countries regarding the prevalence rates, types and distribution of the *Blastocystis* subtypes may be due to differences in various epidemiological aspects such as life-style, contact with animals or diet [45]. For example, in most of the Islamic countries the contact with dogs, cats and other pets is limited (limited sharing household with pets) and consequently the distribution of the zoonotic subtypes is lower than that in Western countries [45]. In a recent study conducted in Czech Republic [45], it has been found that the prevalence of *Blastocystis* sp. in people living in villages was significantly higher than those living in towns and

big cities and that the most common STs in people living in villages were ST1, ST3 and ST2, while in urban areas, ST3, ST2, ST1 and ST4 were the common STs. These differences may be attributed to the close contact with animals in the rural areas and to the exposure to the sources of infection with these parasites such as contaminated food and water [45,55].

So far, only 10 different *Blastocystis* subtypes (ST1–ST9 and ST12) have been found in humans [56,57], of which we detected two (ST1 and ST3) in IBS patients and three (ST1, ST2 and ST3) in non-IBS subjects. This lower diversity of *Blastocystis* subtypes matches the findings from similar studies conducted in India, USA and Malaysia [38,46,54]. However, some studies conducted in France and Czech Republic showed very high diversity of subtypes (up to 8 subtypes) [43,45]. It has been reported that the comparison between different studies regarding the prevalence and distribution of *Blastocystis* subtypes is very difficult and may be impossible due to the huge inconsistency in structure of human populations whether symptomatic or asymptomatic people, in countries of origin whether developing or developed, in geographic localizations whether rural or urban, and/or in using different diagnostic methods [45,58,59].

In the present study, the highest prevalence of *Blastocystis* sp. in IBS patients and non-IBS subjects was found in the age group 10–30 years. The second highest prevalence of *Blastocystis* sp. in was detected in the 30–50 age group, while the lowest prevalence was detected in participants aged over 50. Similarly, El-Safadi et al. [43] reported that the lowest prevalence of *Blastocystis* sp. in French people was in people over 50. In contrast, in a study conducted in the Czech Republic it has been reported that the second highest prevalence was detected in the 50–60 age group [45].

Although the current study is the largest and the more comprehensive study being conducted so far in Iraq, some limitations related to the fact that the epidemiological scenario described here was restricted to only one province in Iraq, so further surveys should be planned to include more human samples from other geographical areas of Iraq, as well as from different animal species in order to evaluate the zoonotic importance of *Blastocystis* parasites.

In conclusion, in comparison with other parts of the world, the prevalence of *Blastocystis* parasites in the middle of Iraq is very high and preventive measures should be developed. DNA sequencing and phylogenetic analysis of Iraqi *Blastocystis* isolates identified two subtypes (ST1 and ST3) in IBS patients and three subtypes (ST1, ST2 and ST3) in non-IBS subjects. As far as we know, this study is the first to deal with the genetic characterization of *Blastocystis* subtypes in this part of Iraq. Further studies are necessary to determine the distribution of STs in humans and animals in all provinces of Iraq in order to evaluate the zoonotic importance of *Blastocystis* parasites.

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