

Original paper

Serological levels of cytokines in irritable bowel syndrome (IBS) patients and non-IBS subjects with and without *Blastocystis* spp. infection

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ABSTRACT. The objectives of the present study were to investigate the prevalence of *Blastocystis* spp. and to determine the levels of some pro-and anti-inflammatory cytokines in patients with irritable bowel syndrome (IBS) and without *Blastocystis* infection in comparison with non-IBS control individuals. Stool samples were collected from 250 IBS patients and 100 samples from controls, examined for the presence of *Blastocystis* and the percentage of infection was recorded. After that each group subdivided into two groups: non-IBS subjects not infected with *Blastocystis* (group 1), non-IBS subjects infected with *Blastocystis* (group 2), IBS patients not infected with *Blastocystis* (group 3), and IBS patients infected with *Blastocystis* (group 4). After that, blood samples were collected from 10 participant from each group and sera were separated and used for measuring the levels of the selected pro- and anti-inflammatory cytokines using ELISA method. The results revealed that about 60% of IBS patients were found infected with *Blastocystis* while 22% of the subjects in the control group were found infected with this parasite and the difference between the two groups was highly significant ($P=0.0001$). In addition, the results revealed that IBS patients with *Blastocystis* showed significantly higher In addition, the results revealed that IBS patients with *Blastocystis* showed significantly higher serum IL-6 ($P=0.0004$), IL-10 ($P=<0.0001$), IL-18 ($P=0.0001$), IFN- γ ($P=<0.0001$), and TNF- α ($P=<0.0001$) when compared with the subjects in the control group. Similarly, the non-IBS subjects infected with *Blastocystis* showed significantly higher serum IL-6 ($P=0.0032$), IL-10 ($P=0.0001$), IL-18 ($P=0.0002$), IFN- γ ($P=0.0001$), and TNF- α ($P=0.0001$) in comparison with the subjects in the control group. In conclusion, the infection with *Blastocystis* has a significant impact on the levels of all the selected cytokines.

Keywords: *Blastocystis*, prevalence, irritable bowel syndrome, levels of cytokines

Introduction

The parasites that belong to the genus *Blastocystis* are common intestinal zoonotic protozoan parasites infecting humans and a wide range of animal hosts and based on some epidemiological surveys, it is estimated that these parasites have colonized about 2 billion people worldwide [1]. The molecular studies regarding the small subunit (SSU) rRNA gene of *Blastocystis* identified 22 different subtypes/genotypes in humans and animals [2]. Among these, ST1–ST9 and ST12 have been reported in humans, but ST1–ST4 are the most common, accounting for more than 90% of human *Blastocystis* strains [3].

Clark et al. [4] reported in their review that the prevalence of the subtypes of *Blastocystis* vary significantly between regions and countries. Moreover, the subtypes show notable differences in biological activities such as drug resistance, immune response, pathogenicity, and their impact on microbiota [5–7].

Regarding the pathogenicity of the parasites that belong to the genus *Blastocystis*, the results of some studies suggested that these parasites are commonly associated with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [8–10]. However, most of these studies have not thoroughly analyzed the etiological role of *Blastocystis* in the development of IBS or IBD and, in fact, some IBS

and IBD patients are not *Blastocystis* carriers, which in itself should cast doubt on the etiological role of *Blastocystis* in these syndromes [11].

Irritable bowel syndrome (IBS) is a digestive disorder with a wide range of symptoms such as abdominal pain, diarrhoea, and constipation [12,13]. In a study conducted in Europe and the Middle East, 30–40% of the IBS patients were infected with *B. hominis* [14]. In another study, 46% of the patients with IBS were positive for *B. hominis*, while only 7% of the control group had this parasite [15]. It has been reported that the protease, which is secreted by *B. hominis*, can cause a wide range of diseases and syndromes, and its high level can cause intense neural activity, abdominal pain, muscle cramp, and generalized pains, which are not found in bacterial and viral infections [16]. Other studies have also demonstrated that some people infected with *B. hominis* have skin allergy symptoms such as erythema, itching, and urticaria, where the factor is believed to be IgE secreted due to the immune system response to the parasite's surface antigens [17–19].

The host's immune response against *Blastocystis* spp. infection has also not been defined yet [20]. Therefore, the objective of the current study was to assess the serological levels of both pro-inflammatory and anti-inflammatory cytokines in irritable bowel syndrome (IBS) patients and non-IBS subjects who were infected with *Blastocystis* spp. in comparison with their *Blastocystis*-negative counterparts.

Materials and Methods

Stool collection and examination

After obtaining verbal consent, 250 stool samples were obtained from patients who attended the gastrointestinal private clinics in Baquba City, the capital city of Diyala Province, middle of Iraq during 2021, and confirmed to be with irritable bowel syndrome (IBS). In addition, 100 stool samples from apparently healthy people (control group) were collected and transported to the Department of Biology, College of Sciences, University of Diyala, Iraq and examined for the presence of *Blastocystis* spp. The stool samples were examined in three different diagnostic methods including iodine-stained smears, trichrome stained smears and culture methods. Jones's medium was used for *in vitro* cultivation and after incubation for 48 to 72 h at 37°C, each sample was

examined by light microscopy for the presence of vacuolar, cyst, trophozoite or other forms of the parasite.

Each participant in the study 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.

Blood samples and cytokines measurement

After finishing the examination of the stool samples for *Blastocystis* and the percentage of infection has been recorded, it was decided to determine the serological levels of certain pro- and anti-inflammatory cytokines in the sera of both IBS patients and non-IBS control group with and without the infection of *Blastocystis*. Accordingly, each group has been subdivided into two groups and as follows: non-IBS subjects not infected with *Blastocystis* (controls/group 1), non-IBS subjects infected with *Blastocystis* (group 2), IBS patients not infected with *Blastocystis* (group 3), and IBS patients infected with *Blastocystis* (group 4).

After obtaining informed consent, ten participants from each group were selected randomly and asked to give blood. About 3 ml of venous blood were collected into sterile tubes without ethylenediaminetetraacetic acid (EDTA). Blood samples were used to separate sera by centrifugation at 1500 rpm for 10 minutes and then serum samples were stored at –20°C until used. The sera were used for the determination of the concentrations of five pro- and anti-inflammatory cytokines: interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α), interleukin IL-6, IL-10 and IL-18. The levels of the cytokines in the sera of the four different groups were measured by enzyme-linked immunosorbent assay (ELISA) using ELISA kits according to the manufacturer's instructions (ELISA kits, Bioassay Technology Laboratory (BT Lab), China).

The Human Ethics Committee at the College of Sciences, Diyala University has approved the study (Protocol 3/ 2021).

Statistical analysis

The data were analysed using Excel software (Microsoft, USA) and the student's t-test was used to test the significance differences between the different groups. A value of $P \leq 0.05$ was considered significant.

Table 1. Prevalence of *Blastocystis* spp. in patients with irritable bowel syndrome (IBS) in comparison with non-IBS control subjects

Parameters	Control group	IBS patients	Odds ratio (OR)	P-value
No. tested	100	250		
No. infected	22	150	5.32	<0.0001
% infection	22	60		
No. negative	78	100		

Results

It can be seen from table 1 that 150 IBS patients out of 250 (60%) were found infected with *Blastocystis* spp., while 22 control subjects out of 100 (22%) were found infected with this parasite and the difference between the two groups was highly significant ($P=<0.0001$). The results revealed that the odds ratio (OR) between the two groups was 5.32 which means that the IBS patients are more

than 5 times more prone to the infection with *Blastocystis* than non-IBS control subjects.

It can be seen from table 2 that in comparison with the control subjects (non-IBS and not infected with *Blastocystis*/group 1), the concentrations of IL-6 (mean 35.1 ± 4.56 pg/ml versus 56.62 ± 2.74 pg/ml, $P=0.0032$), IL-10 (mean 68.7 ± 3.6 pg/ml versus 143.7 ± 12.4 pg/ml, $P=0.0001$), IL-18 (mean 8.36 ± 0.31 pg/ml versus 13.84 ± 1.0 pg/ml, $P=0.0002$), TNF- α (mean 60.5 ± 1.54 pg/ml versus

Table 2. Concentrations of some selected cytokines in the sera of apparently healthy control subjects (non-IBS and not infected with *Blastocystis*/group 1) in comparison with non-IBS subjects but infected with *Blastocystis* (*Blastocystis*-positive/group 2)

Cytokines	Concentration (Pg/ml)/Mean \pm SE		P-value
	Controls (group 1)	Group 2 (Non-IBS with <i>Blastocystis</i>)	
IL-6	35.1 ± 4.56	56.62 ± 2.74	0.0032
IL-10	68.7 ± 3.6	143.7 ± 12.4	0.0001
IL-18	8.36 ± 0.31	13.84 ± 1.0	0.0002
TNF- α	60.5 ± 1.54	142.04 ± 12.4	0.0001
IFN- γ	22.2 ± 0.95	57.8 ± 3.0	0.0001

Pg: picogram

Table 3. Concentrations of some selected cytokines in the sera of apparently healthy control subjects (non-IBS and not infected with *Blastocystis*/group 1) in comparison with IBS patients who were not infected with *Blastocystis* (*Blastocystis*-negative/group 3)

Cytokines	Concentration (Pg/ml)/Mean \pm SE		P-value
	Controls (group 1)	Group 3 (IBS patients without <i>Blastocystis</i>)	
IL-6	35.1 ± 4.56	60.9 ± 4.18	0.0226
IL-10	68.7 ± 3.6	118.5 ± 16.7	<0.0001
IL-18	8.36 ± 0.31	14.4 ± 1.12	0.0003
TNF- α	60.5 ± 1.54	118.1 ± 15.1	0.0100
IFN- γ	22.2 ± 0.95	39.7 ± 3.88	0.0010

Table 4. Concentrations of some selected cytokines in the sera of apparently healthy control subjects (non-IBS and not infected with *Blastocystis*/group 1) in comparison with IBS patients who were infected with *Blastocystis* (*Blastocystis*-positive/group 4)

Cytokines	Concentration (Pg/ml)/Mean ± SE		P-value
	Controls (group 1)	Group 4 (IBS patients with <i>Blastocystis</i>)	
IL-6	35.1 ± 4.56	224.2 ± 32.6	0.0004
IL-10	68.7 ± 3.6	300.5 ± 27.7	<0.0001
IL-18	8.36 ± 0.31	46.7 ± 4.8	0.0001
TNF- α	60.5 ± 1.54	302.4 ± 23.5	<0.0001
IFN- γ	22.2 ± 0.95	79.5 ± 3.63	<0.0001

Table 5. Concentrations of some selected cytokines in the sera of non-IBS subjects infected with *Blastocystis* (*Blastocystis*-positive/group 2) in comparison with IBS patients who were not infected with *Blastocystis* (*Blastocystis*-negative/group 3)

Cytokines	Concentration (Pg/ml)/Mean ± SE		P-value
	Group 2 (Non-IBS with <i>Blastocystis</i>)	Group 3 (IBS without <i>Blastocystis</i>)	
IL-6	56.62 ± 2.74	60.9 ± 4.18	0.4471
IL-10	143.7 ± 12.4	118.5 ± 16.7	0.0881
IL-18	13.84 ± 1.0	14.4 ± 1.12	0.5346
TNF- α	141.94 ± 12.4	118.1 ± 15.9	0.1383
IFN- γ	57.8 ± 3.0	39.7 ± 4.1	0.0147

Table 6. Concentrations of some selected cytokines in the sera of non-IBS subjects infected with *Blastocystis* (*Blastocystis*-positive/group 2) in comparison with IBS patients infected with *Blastocystis* (*Blastocystis*-positive/group 4)

Cytokines	Concentration (Pg/ml)/Mean ± SE		P-value
	Group 2 (Non-IBS with <i>Blastocystis</i>)	Group 4 (IBS with <i>Blastocystis</i>)	
IL-6	56.62 ± 2.74	224.2 ± 32.6	0.0010
IL-10	143.7 ± 12.4	300.5 ± 27.7	0.0006
IL-18	13.84 ± 1.0	46.7 ± 4.8	0.0001
TNF- α	142.04 ± 12.4	302.4 ± 23.5	0.0001
IFN- γ	57.8 ± 3.0	79.5 ± 3.63	0.0024

142.04 ± 12.4 pg/ml, $P=0.0001$), and IFN- γ (mean 22.2 ± 0.95 pg/ml versus 57.8 ± 3.0 pg/ml, $P=0.0001$) in the sera of non-IBS individuals who were infected with *Blastocystis* (*Blastocystis*-positive/group 2) were significantly higher.

It can be seen from table 3 that the concentrations of all the selected cytokines (IL-6, IL-18, TNF- α and IFN- γ) in the sera of IBS patients who were not

infected with *Blastocystis* (*Blastocystis*-negative/group 3) were significantly higher ($P=<0.0001$ for each comparison) than that in the sera of control subjects (group 1).

Table 4 shows clearly that the concentrations of all the selected cytokines (IL-6, IL-18, TNF- α and IFN- γ) in the sera of IBS patients infected with *Blastocystis* (group 4) were significantly higher

Table 7. Concentrations of some selected cytokines in the sera of IBS patients not infected with *Blastocystis* (*Blastocystis*-negative/group 3) in comparison with their counterparts infected with this parasite (*Blastocystis*-positive/group 4)

Cytokines	Concentration (Pg/ml)/Mean \pm SE		
	Group 3 (IBS without <i>Blastocystis</i>)	Group 4 (IBS with <i>Blastocystis</i>)	P-value
IL-6	60.9 \pm 4.18	224.2 \pm 32.6	0.0014
IL-10	118.5 \pm 16.7	300.5 \pm 27.7	0.0001
IL-18	14.4 \pm 1.12	46.7 \pm 4.8	0.0001
TNF- α	118.1 \pm 15.1	302.4 \pm 23.5	0.0001
IFN- γ	39.7 \pm 3.88	79.5 \pm 3.63	0.0001

($P < 0.0001$ for each comparison) than that in the sera of control subjects (group 1).

It can be seen from table 5 that only the concentrations of IFN- γ in the sera of non-IBS individuals who were infected with *Blastocystis* (group 2) were significantly higher ($P = 0.0147$) than that in the sera of IBS patients who were not infected with the parasite (group 3). No significant differences in the levels of IL-6, IL-18 and TNF- α were found between the two groups.

It can be seen from table 6 that the concentrations of all the selected cytokines (IL-6, IL-18, TNF- α and IFN- γ) in the sera of IBS individuals who were infected with *Blastocystis* (group 4) were significantly higher ($P < 0.0001$ for each comparison) than that in the sera of non-IBS subjects infected with *Blastocystis* (group 2).

It can be seen from table 7 that the concentrations of all selected cytokines in the sera of IBS patients infected with *Blastocystis* (group 4) were significantly higher ($P < 0.0001$ for each comparison) than that in the sera of their counterparts who were not infected with this parasite (group 3).

Discussion

The results of the current study revealed that about 60% of the irritable bowel syndrome (IBS) patients were found infected with *Blastocystis* spp. This prevalence rate is almost similar to the prevalence rate reported by a recent study conducted in the same province as Wadi et al. [10] reported that about 57% of the examined IBS patients were found infected with this parasite. In contrast, the prevalence rate reported in the present study is much higher than the other previous studies conducted in other provinces of Iraq [21–24].

Similarly, the prevalence rate reported in this study is much higher than those recorded in some countries close to Iraq; 19.7% and 15% in Iran [25,26], 13.5% in Saudi Arabia [27], 14.7% in Jordan [28], and 1.8%, 5.8%, and 38% in Turkey [17,29,30]. Studies conducted in other parts of the world have also reported an association between the infection with *Blastocystis* spp. and IBS and urticaria [31–33], although some studies did not confirm this link [34,35]. Moreover, the immunosuppressed individuals, especially HIV and renal transplants, and in inflammatory bowel disease (IBD) were found to be more prone to the infection with *Blastocystis* [29,36–38]. A wide range of epidemiological factors are behind the variation in the reported prevalence rates and the link between the infection with *Blastocystis* and diseases, and these factors include geographic variations in parasite distribution and transmission patterns in the community, differences in the virulence of the different subtypes of the parasites, as well as differences in host genetic susceptibility [39–41]. In addition to the epidemiological factors, some technical factors could also play a significant role in reported prevalence rates such as sensitivity of the diagnostic method and expertise in microscopic detection of the different morphological forms of the parasites that belong to the genus *Blastocystis* [42,43]. A recent study conducted in Senegal reported a prevalence rate of 100% in Senegalese school children when PCR based screening was applied [44]. Some recent studies demonstrated that the prevalence of the infection with *Blastocystis* is probably under diagnosed by using conventional diagnostic tools because they are far less sensitive than the culture and PCR based screening diagnostic methods [26,44].

In addition, the results of the present study revealed that the infection with the parasites that belong to the genus *Blastocystis* has a significant impact on the levels of all the selected cytokines (IL-6, IL-10, IL-18, TNF- α and IFN- γ) as the concentrations of these cytokines increased significantly in the blood of IBS-subjects and non-IBS subjects who were infected with the parasites in comparison with their non-infected counterparts. Similarly, Mutlag et al. [45] investigated the impact of *Blastocystis hominis* infection on the levels of two interleukins (IL-10 and IL-18) in Iraqi healthy control subjects and individuals suffering from irritable bowel syndrome (IBS) and the authors reported a significant increase in the concentration of IL-18 and slight increase in the concentration of IL-10 in the sera of IBS patients infected with *B. hominis* in comparison with control healthy subjects.

In the present study, the comparison between IBS patients with *Blastocystis* spp. and IBS patients without *Blastocystis* showed that the blood levels of all the selected cytokines was significantly higher in the first group than in the blood of the second group. Similarly, Yakoob et al. [46] have determined some cytokines (interleukins-8, 10, 12 and TNF- α) expression by peripheral blood mononuclear cells (PBMCs) in diarrhoea-predominant irritable bowel syndrome (D-IBS) with *Blastocystis* spp. (two subtypes 1 and 3) in comparison with D-IBS without *Blastocystis* (control group) and reported that overall there were significant differences between the two groups regarding the levels of IL-10 and TNF- α but no significant differences were found between the two groups regarding the levels of the other cytokines (IL-8 and IL-12).

The differences observed in the blood levels of IL-10 observed in the present study may be attributable to an immune modifying effect of *Blastocystis* sp. infection in D-IBS [46]. The synthesized IL-10 might be produced by the host's immune system in response to infection and it has been reported that this anti-inflammatory cytokine acts as one of the regulatory cytokines that reduce inflammatory response [20].

Concerning the levels of blood TNF- α , the results of the present study revealed that the level of this cytokine was significantly higher in IBS patients with *Blastocystis* spp. than in IBS patients without *Blastocystis*. Similarly, Yakoob et al. [46] reported that there was a significant increase in the level of TNF- α in *Blastocystis* sp. ST1 but not ST3.

Abdel-Hafeez et al. [20] evaluated the potential pathogenicity of *Blastocystis* spp. and its capacity to modulate the immune response in experimentally infected immunocompetent and immunosuppressed mice and reported that the levels of blood TNF- α and IL-10 increased significantly in immunocompetent and immunosuppressed mice infected with *Blastocystis* spp. in comparison with naïve and immunosuppressed mice not infected with *Blastocystis* spp.

The results of the current study revealed a significant increase in the blood levels of IL-18 in IBS patients infected with *Blastocystis* in comparison with the subjects in the healthy control groups. This finding agrees with the findings of Mutlag et al. [45] who reported that the levels of IL-18 increased significantly in the blood of IBS patients who were infected with *Blastocystis* when compared with the subjects in the control group. It has been reported that the IL-18 is a pleiotropic cytokine which is involved in the regulation of innate and acquired immune response, and also is a potent inducer of IFN- γ in natural killer (NK) cells and CD4 T helper (Th) 1 lymphocytes [47].

In comparison with the control group, the results of the present study showed that the serum IFN- γ was significantly higher in IBS patients with *Blastocystis* infection, in non-IBS patients with *Blastocystis* infection, as well as in IBS patients infected with *Blastocystis*. It has been reported that *Blastocystis* spp. causes mucosal sloughing, increase in goblet cell mucin, increased intestinal permeability and to induce a pro-inflammatory cytokine response with upregulation of TNF- α , IFN- γ and IL-12 [6].

It can be concluded that the IBS patients are more prone to the infection with *Blastocystis* than non-IBS subjects. In addition, infection with the parasites that belong to the genus *Blastocystis* has a significant impact on the levels of all the selected pro-inflammatory and anti-inflammatory cytokines (IL-6, IL-10, IL-18, TNF- α and IFN- γ) as the concentrations of these cytokines increased significantly in the blood of IBS-subjects infected with the parasites in comparison with their non-infected counterparts.

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Received 15 September 2021

Accepted 30 November 2021