

## Case Control Study

**Protozoan parasites in irritable bowel syndrome: A case-control study**

Khaled A Jadallah, Laila F Nimri, Rola A Ghanem

Khaled A Jadallah, Department of Internal Medicine, King Abdullah University Hospital, Faculty of Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan

Laila F Nimri, Rola A Ghanem, Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan

**Author contributions:** Jadallah KA and Nimri LF designed the research; Jadallah KA recruited the cases; Ghanem RA recruited the healthy controls, gathered clinical data, and collected stool samples; Nimri LF and Ghanem RA performed stool processing and analysis; Jadallah KA and Nimri LF drafted the manuscript; all authors critically revised and approved the final version of the manuscript.

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**Correspondence to:** Khaled A Jadallah, MD, Associate Professor, Department of Internal Medicine, King Abdullah University Hospital, Faculty of Medicine, Jordan University of Science and Technology, Ramtha Str., Irbid 22110, Jordan. [khaled-j@just.edu.jo](mailto:khaled-j@just.edu.jo)  
Telephone: +962-79-6673884  
Fax: +962-2-7200624

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**Abstract****AIM**

To investigate the putative role of protozoan parasites in the development of irritable bowel syndrome (IBS).

**METHODS**

The study included 109 IBS consecutive adult patients fulfilling the Rome III criteria and 100 healthy control subjects. All study subjects filled a structured questionnaire, which covered demographic information and clinical data. Fresh stool samples were collected from patients and control subjects and processed within less than 2 h of collection. Iodine wet mounts and Trichrome stained smears prepared from fresh stool and sediment concentrate were microscopically examined for parasites. Blastocystis DNA was detected by polymerase chain reaction, and Cryptosporidium antigens were detected by ELISA.

**RESULTS**

A total of 109 IBS patients (31 males, 78 females) with a mean age  $\pm$  SD of 27.25  $\pm$  11.58 years (range: 16

-60 years) were enrolled in the study. The main IBS subtype based on the symptoms of these patients was constipation-predominant (88.7% of patients). A hundred healthy subjects (30 males, 70 females) with a mean  $\pm$  SD age of  $25.0 \pm 9.13$  years (range 18-66 years) were recruited as controls. In the IBS patients, Blastocystis DNA was detected in 25.7%, Cryptosporidium oocysts were observed in 9.2%, and Giardia cysts were observed in 11%. In the control subjects, Blastocystis, Cryptosporidium and Giardia were detected in 9%, 0%, and 1%, respectively. The difference in the presence of Blastocystis ( $P = 0.0034$ ), Cryptosporidium ( $P = 0.0003$ ), and Giardia ( $P = 0.0029$ ) between IBS patients and controls was statistically significant by all methods used in this study.

### CONCLUSION

Prevalence of Blastocystis, Cryptosporidium and Giardia is higher in IBS patients than in controls. These parasites are likely to have a role in the pathogenesis of IBS.

**Key words:** Blastocystis; Cryptosporidium; Protozoan parasites; Giardia; Irritable bowel syndrome

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**Core tip:** A mounting body of evidence suggests that infections with protozoan parasites may be implicated in the pathogenesis of irritable bowel syndrome (IBS). However, previous studies from different geographic regions have yielded conflicting results. The present study investigates the infection rate of protozoan parasites in Jordanian IBS patients. Our results indicate that infection with Blastocystis, Cryptosporidium, and Giardia spp. may play a role in the pathogenesis of IBS in a significant proportion of patients. Testing for these parasites in cases of presumed IBS may offer new insights into the pathogenesis of IBS and thus improve its management.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with a complex etiology and pathogenesis<sup>[1,2]</sup>. The cause of IBS is not well understood and a combination of genetic, physical, mental health problems and environmental factors have been proposed<sup>[3]</sup>. Triggers vary among patients, and they may include certain foods, hormones, medications, stress, or an acute episode of gastroenteritis<sup>[4,5]</sup>.

Previous infections and persistent low-grade inflammation may play an important role in the pathogenesis

of IBS<sup>[6]</sup>. Numerous IBS cases develop after acute enteric infection with different pathogens causing what is called post-infectious IBS (PI-IBS), where symptoms develop and persist in patients who had not met the Rome criteria before<sup>[7,8]</sup>. The PI-IBS symptoms have been reported in a significant number of patients following infections with bacteria and viruses<sup>[9]</sup>. The duration of diarrhea during enteritis has been identified as the strongest risk factor for subsequent IBS development<sup>[10]</sup>, which might reflect the severity of the initial inflammation.

*Blastocystis hominis* is one of the most common human intestinal protozoa in both developing and developed countries<sup>[11,12]</sup>. It is common in patients with diarrhea-predominant-IBS<sup>[1,13,14]</sup>, and its possible role in the pathogenesis of IBS has been suggested. Infections with this parasite might be responsible for bowel dysfunction due to the penetration of mucosal layer<sup>[14]</sup>.

*Cryptosporidium parvum* (*C. parvum*) accounts for about 20% of diarrheal episodes in children in developing countries and up to 9% of episodes in developed countries<sup>[15]</sup>. Infections are usually characterized by self-limiting diarrhea (1-2 wk) with severe abdominal pain. However, the duration of clinical symptoms is highly dependent on the person's immunological status<sup>[16]</sup>, and more severe and long lasting cases may develop in children, the elderly, and immunocompromised individuals<sup>[12,17]</sup>. *Cryptosporidium* has been reported in IBS patients, with the onset of gastrointestinal symptoms after an acute episode of cryptosporidiosis despite recovery and parasite clearance<sup>[18,19]</sup>. Symptoms following *C. parvum* infection are similar to those described by IBS patients, suggesting that this parasite is a potential cause of PI-IBS.

The aim of this study was to evaluate the possible association of protozoan parasites with IBS. The detection of these pathogens in IBS cases may offer new insights into the pathogenesis and management of this complex disorder.

## MATERIALS AND METHODS

### Study subjects

The study included 109 IBS consecutive adult patients fulfilling the Rome III criteria<sup>[5]</sup>. The patients were recruited at the gastroenterology outpatient clinics at King Abdullah University Hospital (KAUH) between September 2013 and April 2014.

The diagnosis of IBS was ascertained by an experienced gastroenterologist (KAJ). Patients who did not agree to sign an informed consent were excluded from the study. Other exclusion criteria were: Positive stool cultures for bacteria; steatorrhea; lactose intolerance; gluten sensitivity. Healthy subjects without current or previous gastrointestinal symptoms, attending the hospital during the same time period for routine examination or accompanying other patients were enrolled in the control group. All study participants gave written informed consent prior to study enrollment.

The study protocol was reviewed and approved by the Institutional Review Board at King Abdullah

University Hospital and by the Committee of Research on Human subjects at the Jordan University of Science and Technology.

### **Clinical samples**

Fresh stool samples were collected from patients and control subjects. All eligible subjects filled a structured questionnaire, which covered demographic information, and clinical data (*e.g.*, IBS history; IBS subtype; previous bacterial, viral, and/or parasitic infection, postinfection treatment and any antibiotic therapy received in the three months prior to enrollment).

### **Stool processing**

Stool samples were processed within less than 2 h of collection. Each sample was divided into two portions before processing. One portion was immediately processed for protozoan parasites and the second portion was kept refrigerated in Eppendorf tube preserved in absolute ethanol (ratio 3:1) for antigen and Polymerase Chain Reaction (PCR) testing.

### **Microscopy**

Collecting multiple stool samples per subject was not feasible. Therefore, as an alternative, a comprehensive examination of a single fresh stool sample from each subject was done using several laboratory techniques.

For each sample, normal saline wet mounts and Lugol's iodine wet mounts were microscopically examined to initially observe parasites. To maximize recovery of different parasites stages, all stool samples were concentrated using the formalin-ether acetate sedimentation technique (FEA)<sup>[20]</sup>, which is the Center for Disease Control (CDC) recommended stool concentration method for clinical laboratories. Iodine wet mounts; a trichrome stained smears and modified Kinyoun acid-fast stained smears were prepared from the concentrates. Slides were thoroughly screened (200 to 300 fields) for parasites by an experienced parasitologist using 40 × and 100 × oil immersion objectives, and results were recorded.

### **Detection of *Cryptosporidium* antigen by ELISA**

All stool samples were tested using an FDA-approved RIDASCREEN® *Cryptosporidium* ELISA kit (R-Biopharm AG, Darmstadt, Germany) following the manufacturer's instructions. This immunoassay is designed for the qualitative detection of *C. parvum* and *C. hominis* antigens in human stool samples.

### **Detection of *Blastocystis* spp. by polymerase chain reaction**

Genomic DNA was extracted from stool samples using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's instructions. DNA concentration and purity were measured using NanoDrop microvolume UV-Vis spectrophotometer (Thermo Scientific, United States), and DNA was stored at -20 °C for later

testing. The primers used were F1 (5'-GGAGGTAGT GACAATAATC-3') and BHCR seq3 (5'-TAAGACTACGAG GGT ATCTA-3') (Integrated DNA Technology, Coralville, United States) targeting the small subunit ribosomal RNA (SSU rRNA) gene of *Blastocystis* spp.<sup>[21]</sup>. Each PCR reaction contained 12.5 µL of GoTaq® Green Master Mix solution containing *Taq* DNA polymerase, dNTPs, MgCl<sub>2</sub> and reaction buffers (Promega, United States), 0.5 µL of each primer, 5 µL of sample genomic DNA extract, and 5.5 µL of nuclease free water in a total volume of 25 µL. All PCR runs included a positive control containing the same components with a *Blastocystis hominis* suspension as DNA template (Microbiologics, United States), and a negative control containing the same components, but without DNA.

PCR amplification was performed using a Perkin-Elmer GeneAmp 9600 thermal cycler. PCR amplification conditions were: Denaturation at 95 °C for 7 min, 35 cycles each consisting of denaturation 94 °C for 60 s, annealing at 56 °C for 45 s, followed by a final extension step at 72 °C for 7 min. PCR products and 100 bp DNA ladder (Promega, United States) were loaded into 2% agarose gel, in TBE buffer, and bands were visualized under UV transilluminator after staining with ethidium bromide. Positive samples for *Blastocystis* spp. had the DNA bands of 550-585 bp.

### **Statistical analysis**

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 19, Chicago, IL). Results are expressed as mean ± SD for continuous variables (*e.g.*, age), and proportions and percentages for categorical data (*e.g.*, sex). Fisher's exact test was used in the analysis of nominal variables to determine the significance of differences in parasite infection rates between the patients and the control subjects. A *P*-value ≤ 0.05 was considered statistically significant. All *P*-values were two sided.

## **RESULTS**

A total of 109 patients (31 males, 78 females) with a mean age ± SD of 27.25 ± 11.58 years (range: 16 -60 years) were enrolled in the study. A hundred healthy subjects (30 males, 70 females) with a mean ± SD age of 25.0 ± 9.13 years (range 18 - 66 years) were recruited as controls. The two groups were comparable in terms of age, sex and other demographic characteristics.

### **Microscopy of iodine wet mounts of fresh stool**

In the IBS cases, *Blastocystis* cyst-like form in moderate to many in numbers was observed in 16 (14.7%), *Cryptosporidium* oocysts were observed in 10 (9.2%) and *Giardia lamblia* cysts were observed in 9 (8.3%) (Table 1). Nonpathogenic *Entamoeba hartmanni* and *Endolimax nana* were each observed in 3 (2.8%) samples. Coinfections with 2 parasites were observed in 2 (1.8%) samples, one had *Entamoeba hartmanni* with *Endolimax nana*, and the second had *Blastocystis* spp. with *Endo-*

**Table 1 Parasites observed in iodine wet mounts of cases and controls before and after concentration *n* (%)**

Parasites observed	Un concentrates		P value	FEA concentrate		P value
	IBS	Controls		IBS	Controls	
<i>Blastocystis</i> spp.	16 (14.7)	3 (3.0)	0.0034 <sup>a</sup>	12 (11.0)	2 (2.0)	0.0112 <sup>a</sup>
<i>Cryptosporidium</i> spp.	10 (9.2)	0 (0.0)	0.0017 <sup>a</sup>	10 (9.2)	0 (0.0)	0.0017 <sup>a</sup>
<i>Giardia lamblia</i>	9 (8.3)	1 (1.0)	0.0197 <sup>a</sup>	12 (11.0)	1 (1.0)	0.0029 <sup>a</sup>
<i>Endolimax nana</i>	3 (2.8)	1 (1.0)	0.6228	3 (2.8)	1 (1.0)	0.6228
<i>Entamoeba hartmanni</i>	3 (2.8)	0 (0.0)	0.2477	5 (4.6)	0 (0.0)	0.2477
<i>Entamoeba histolytica</i> / <i>Entamoeba dipar</i>	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1
Co-infections with 2 parasites	2 (1.8)	1 (1.0)	1	2 (1.8)	1 (1.0)	1

<sup>a</sup>P-value of ≤ 0.05 is statistically significant by Fisher’s test. IBS: Irritable bowel syndrome; FEA: Formalin ethyl acetate concentration technique.

**Table 2 *Blastocystis* and *Cryptosporidium* spp. in irritable bowel syndrome patients and controls as detected by all methods *n* (%)**

Test	<i>Blastocystis</i> spp.			<i>Cryptosporidium</i> spp.		
	IBS	Controls	P value	IBS	Controls	P value
Iodine wet mounts	14 (12.8)	3 (3.0)	0.0106 <sup>a</sup>	10 (9.2)	0 (0.0)	0.0017 <sup>a</sup>
FEA	12 (11.0)	2 (2.0)	0.0112 <sup>a</sup>	12 (11.0)	0 (0.0)	0.0003 <sup>a</sup>
Trichrome stain	14 (12.8)	2 (2.0)	0.0034 <sup>a</sup>	12 (11.0)	0 (0.0)	0.0003 <sup>a</sup>
PCR	28 (25.7)	9 (9.0)	0.0019 <sup>a</sup>	-	-	-
Acid fast stain	-	-	-	14 (12.8)	0 (0.0)	0.0001 <sup>a</sup>
ELISA	ND	ND	-	6 (5.5)	1 (1.0)	0.1211

<sup>a</sup>P-value of ≤ 0.05 was considered statistically significant. FEA: Formalin ethyl acetate concentration technique; IBS: Irritable bowel syndrome; ELISA: Enzyme-linked immunosorbent assay; ND: Not detected.

*limax nana*.

In the 100 control samples, *B. hominis* was observed in 3 (3%) samples, *Cryptosporidium* in 0 (0%) samples, and *Giardia lamblia* cyst in 1 (1%) sample. Nonpathogenic *Endolimax nana* cyst was observed in 1 (1%) sample (Table 2).

**FEA**

In the iodine wet mount preparations of the sediment of IBS patients, *Blastocystis* was observed in 12 (11.0%) samples, *Cryptosporidium* was observed in 10 (9.2%) samples, and *Giardia* cysts in 12 (11.0%) samples. Other nonpathogenic amoebas were also observed (Table 1).

In the trichrome stained smears of the sediment of IBS patients, *Blastocystis* was observed in 14 (12.8%), *Cryptosporidium* oocysts appeared lightly stained or unstained in 12 (11.0%), and *Giardia* cysts were observed in 12 (11%) (Table 2).

In the control subjects, *Blastocystis*, *Cryptosporidium* and *Giardia* were observed in 2 (2%), 0 (0%), and 1 (1%), respectively. In the IBS patients acid fast stain of the sediment, *Cryptosporidium* was observed in 14 (12.8%) compared to none (0%) in the control samples.

**Detection of *Cryptosporidium* antigen by ELISA**

In the IBS samples, 6 (5.5%) were positive for *Cryptosporidium* compared to only 1 (1.0%) positive in the controls (Table 2).

**Detection of *Blastocystis* DNA by PCR**

The specific DNA band was observed in 28 (25.7%) of

the patients, compared to 9 (9%) of the controls (Figure 1). Comparing all methods used for the detection of *Blastocystis* (Table 2), PCR was more sensitive than the other techniques used. There was a significant difference between the cases and controls in all methods used: Iodine wet mount ( $P = 0.0106$ ), FEA ( $P = 0.0112$ ), the trichrome stain ( $P = 0.0034$ ), and PCR ( $P = 0.0019$ ).

Four of five tests used were consistent in the detection of the *Cryptosporidium* spp. and showed significant difference between the IBS patients and the controls ( $P$  values 0.0001 and 0.0017, respectively) (Table 2). Four IBS samples were positive for *Blastocystis* by all methods tested, and one sample was also positive for *Cryptosporidium* by ELISA.

The positive results of both parasites were associated with patients having more episodes of abdominal pain and discomfort or bloating than others. The main IBS subtype based on the symptoms of these patients was constipation-predominant IBS-C (88.7% of patients). Furthermore, stool samples were tested for bacterial pathogens (unpublished data), and patients having positive results were excluded from the study.

**DISCUSSION**

In this study, we investigated the prevalence of protozoan parasites in stool samples of IBS patients and healthy controls. Our results suggest that the occurrence of *Blastocystis*, *Cryptosporidium*, and *Giardia* spp. may have a role in the pathogenesis of IBS in a significant proportion of patients.

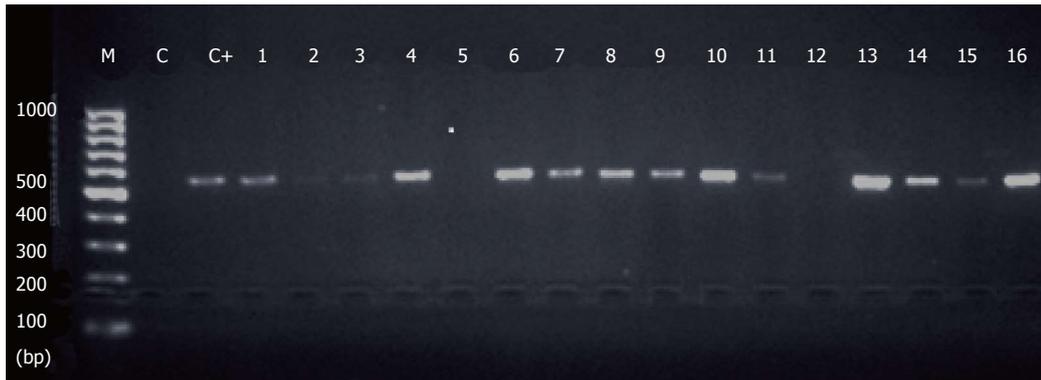


Figure 1 Ethidium bromide stained 2% agarose gel showing polymerase chain reaction products of the small subunit ribosomal RNA of *Blastocystis*.

The presence of *Blastocystis* in 25.6% IBS patients was notably different ( $P = 0.0019$ ) from the controls (9%), as detected by PCR (Table 2). These results are in agreement with previous studies, which reported higher infection rates of *B. hominis* in stool samples of IBS patients compared to controls<sup>[22,23]</sup>. Of note, PCR was reported to be superior over other techniques in the detection of *Blastocystis* in clinical samples<sup>[21]</sup>.

*Blastocystis* has been reported as the most common intestinal parasite among both healthy and immunocompromised individuals<sup>[12]</sup>, and highly prevalent in patients with acute and chronic diarrhea. In addition, a higher prevalence of *Blastocystis* spp. among IBS patients was reported as compared to patients suffering from other gastrointestinal disorders or healthy individuals<sup>[22,24]</sup>. In their epidemiological study, Giacometti *et al.*<sup>[25]</sup> reported that *Blastocystis* was significantly present in IBS patients, suggesting a possible link between this parasite and IBS. Studies from the Middle East, Pakistan, Mexico and Europe<sup>[13,16,22,26,27]</sup> suggested a pathogenic role of *Blastocystis* in the etiology of IBS. However, other studies failed to demonstrate an association between *Blastocystis* and IBS<sup>[24,28]</sup>. A possible explanation for the differences in reporting could be the small number of IBS patients studied or the different diagnostic methods used. The multifactorial etiology of IBS, including microbiological factors, genetic, and environmental factors may also explain this discrepancy<sup>[23]</sup>. *Blastocystis* spp. ability to induce pathophysiological disturbances such as host cell apoptosis, the modulation of host immune response, toxic-allergic reactions leading to a nonspecific inflammation of the colonic mucosa has been linked to the pathogenesis of IBS<sup>[29,30]</sup>. It is well recognized that *B. hominis* is the single species infecting humans. However genetic analysis demonstrated antigenic heterogeneity, with nine different *Blastocystis* types that can infect humans, suggesting that virulent and avirulent species exist<sup>[24]</sup>.

The detection of *Cryptosporidium* in 12.8% of IBS patients by the acid fast staining as compared to none detected in the control samples by any of the four methods used was statistically significant (Table 2). The relative prevalence of *Cryptosporidium* spp. reported in several developed countries in outbreak and non

outbreak settings ranges from 0.1% to 9.1% of clinical cases<sup>[11]</sup>.

Similar to bacterial or other protozoan infections in humans, *Cryptosporidium* may trigger PI-IBS-like symptoms<sup>[19]</sup>. A study of immunocompetent rats infected with *Cryptosporidium parvum* oocysts suggested that cryptosporidiosis results in increased jejunal sensitivity to distension. This infection model mimicked some features of IBS patients, who report visceral pain as a major symptom and increased sensitivity to gut distension. The results of that study allow speculation that cryptosporidiosis may be a potential etiologic factor of IBS and warrant further studies of post cryptosporidiosis bowel disturbances<sup>[30]</sup>. In addition, an association between *C. parvum* with IBS was suggested in a study of immunocompetent suckling rat similar to PI-IBS features supported by the longterm pathological changes triggered by this parasite in the intestine<sup>[31]</sup>. Infections in these rats resulted in jejunal hypersensitivity to distension that was associated with accumulation of activated mast cells at 50 d post-infection<sup>[31]</sup>. These experimental results are consistent with the observation that diarrhea-predominant IBS patients have a marked increase in mast cell numbers and higher tryptase concentrations in jejunal mucosa<sup>[32]</sup>.

Additionally, our results are in agreement with a previous study<sup>[33]</sup> that reported the permanent acid fast staining being more sensitive than iodine-stained wet mounts and ELISA for the detection of *Cryptosporidium*. In fact, acid fast staining is still considered the "gold standard" method routinely used for the detection of this parasite, although it may fail to detect infections in some symptomatic patients.

The presence of *Giardia lamblia* in IBS patients (8.3%) was also statistically significant in both concentrated and unconcentrated stool sediments ( $P = 0.0029$  and  $0.0197$ , respectively) (Table 2). The relative prevalence of *Giardia intestinalis/lamblia* in several developed countries in outbreak and other settings ranges from 0.2% to 29.2%<sup>[12]</sup>. Although most *Giardia* infections are self-limiting, chronic infections and re-infections can occur. A previous study reported *Giardia* in 5%-10% of IBS patients and provided evidence of potential cause of chronic PI-IBS symptoms<sup>[34]</sup>. High frequency of microscopic duodenal inflammation

was found in post-giardiasis patients when the infection lasted 2–4 mo, indicating that longer duration of infection is a risk factor for PI-IBS. However, the cause of the PI-IBS clinical manifestations due to *Giardia*, even after complete elimination of the parasite, remains unexplained.

Parasitic infections, and in particular *Blastocystis hominis* infection have been reported to be common in diarrhea-predominant IBS (IBS-D), suggesting a role for these parasites in the pathogenesis of IBS-D<sup>[1,13,14]</sup>. Of interest, *Blastocystis hominis*, *Cryptosporidium*, and *Giardia spp* were common also in our study patients, composed mainly of IBS-C subtype. Based on our results, we speculate that these parasites may play a role in the pathogenesis of IBS regardless of the subtype.

In conclusion, the significant presence of the protozoan parasites *Blastocystis*, *Cryptosporidium*, and *Giardia spp* in stool samples of IBS patients suggests a pathogenetic link between these parasites and IBS. Therefore, we recommend the inclusion of parasitological tests in the diagnostic work up for presumed IBS patients. Early diagnosis and treatment of these infections may shorten the duration of the infection and help reduce the risk of development of IBS. Further studies with a larger sample size and studies from epidemiologically different countries should provide more insight into the role of parasitic infections in the pathogenesis of IBS.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder of uncertain etiology and multifaceted pathogenesis. Protozoa, such as *Blastocystis*, *Cryptosporidium*, and *Giardia spp*, are common enteric parasites and their carriage is believed to be linked to IBS.

### Research frontiers

There were several early published studies of the possible link between parasitic infections and the development of IBS symptoms. However, many of these studies have yielded inconsistent or even contradictory results.

### Innovations and breakthroughs

The present study provides new pathophysiological insights into the role of protozoan parasites in IBS. Namely, *Blastocystis*, *Cryptosporidium*, and *Giardia spp* have been found to play a role in the pathogenesis of IBS in a significant proportion of patients.

### Applications

Investigating the role of protozoan infections in the pathophysiology of IBS is important in clinical practice, especially in light of the opportunity of developing targeted therapies. Future research should focus on the subset of IBS patients with poor response to conventional therapy, and possibly offer newer, more effective treatment options.

### Terminology

Irritable bowel syndrome is a functional gastrointestinal disorder characterized by a complex pathogenesis, and various clinical manifestations. Protozoan parasites are a group of unicellular, eukaryotic microorganisms, which can infect both humans and animals; they include ciliates, amoebae, and flagellates.

### Peer-review

The authors compared clinical data and test results of protozoan parasites for stool samples between in 109 patients with IBS and in 100 control individuals,

demonstrated that prevalence of Blastocytosis, *Cryptosporidium* and *Giardia* is higher in IBS patients than in controls, and concluded that these parasites were likely to have a role in the pathogenesis of IBS. The paper is well-written and has interesting findings.

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