Blastocystis and Clostridioides difficile: Evidence for a Synergistic Role in Colonization Among IBD Patients with Emphasis on Ulcerative Colitis

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Cite this article as: Azimirad M, Mohammad Ali Gol S, Javanmard E, et al. Blastocystis and clostridioides difficile: Evidence for a synergistic role in colonization among IBD patients with emphasis on ulcerative colitis. *Turk J Gastroenterol.* 2021; 32(6): 500-507.

ABSTRACT

Background: *Regarding the controversial role of Blastocystis in inflammatory bowel diseases (IBD) patients, it seems that this protozoan may lead to an overgrowth of some non-beneficial bacteria. The current study aimed to investigate the co-existence of Blastocystis and Clostridioides difficile in IBD patients.*

Methods: *Stool samples of 102 IBD patients were collected and cultivated for C. difficile and Blastocystis. DNA extraction was performed on positive samples and C. difficile and Blastocystis were toxinotyped and subtyped, respectively. Fisher's exact test and logistic regression were employed to calculate the correlation between the existence of Blastocystis and its subtypes (ST) with C. difficile and its type of toxins. Also, the co-existence of Blastocystis and C. difficile with the frequency of defecations was evaluated.*

Results: *Blastocystis and C. difficile were observed in 17 (16.7%) and 26 (25.5%) of stool samples, respectively. From 26 C. difficile-positive isolates, 24 (92.3%) and 2 (7.7%) were tcdA+/B+ and tcdA+/B-, respectively. Also, 10 (58.8%) and 7 (41.2%) were Blastocystis ST1 and ST3, respectively. Statistically significant correlations between co-existence of Blastocystis and C. difficile and co-existence of these microorganisms and frequency of defecation ($P < .035$) were seen. There was no statistically significant correlation between subtypes of Blastocystis and colonization of C. difficile or its toxinotypes.*

Conclusion: *The co-existence of Blastocystis and C. difficile in IBD patients was observed in the current study. Moreover, it can be proposed that these microorganisms may have synergistic effects on their colonization in the gastrointestinal tract.*

Keywords: *Blastocystis, Clostridioides difficile, co-existence, inflammatory bowel diseases, ulcerative colitis*

INTRODUCTION

Inflammatory bowel diseases (IBD) is a chronic multifactorial disorder of the gastrointestinal tract that its main causative agent has not yet been specified. Crohn's disease (CD) and ulcerative colitis (UC) are relapsing inflammatory conditions responsible for most IBD cases.[1,2,3] The increased number of IBD patients during the last 2 decades[4] has grouped this disease as the recent global trends. According to available data, genetics, environmental, diet, and microbial agents have been signified in the occurrence and flaring up of IBD.[5,6] However, the role of some eukaryotic and prokaryotic microorganisms through dysbiosis of the gut microbiota have been discussed to be important in the deterioration of the clinical symptoms in these patients.[7,8,9,10]

Blastocystis is a eukaryotic intestinal parasite that is mostly reported from a broad range of animals and human subjects.[11,12] This parasite is transmitted via either fecal contamination of food and water resources or close contact with animals.[12,13,14,15] Many reports have indicated the low frequency of *Blastocystis* in IBD patients compared to healthy controls.[8,16,17] It was shown that *Blastocystis* is able to alter both the composition and diversity of the gut microbiota. In other words, this parasite decreases the protective bacteria by changing the composition

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Received: **August 28, 2020** Accepted: **September 8, 2020** Available Online Date: **July 30, 2021**

DOI: **10.5152/tjg.2021.19644**

and increasing the diversity of the microbial community in the human gut.[18,19] Moreover, it was hypothesized that *Blastocystis* prefers a specific composition of the microbiota to favor its colonization in the intestinal tract; therefore, in IBD patients who mostly represent dysbiosis, *Blastocystis* is not able to be colonized in the gut lumen.[16]

On the other hand, it was proven that this protist could increase the permeability of the cell tight-junctions throughout the host intestine via the secretion of proteolytic enzymes such as metalloproteases.[20,21,22,23] It was also suggested that increasing the permeability of the gut barrier can lead to the subsequent induction of inflammatory cascades like what is seen in IBD.[24,25]

One of the most important outcomes of dysbiosis, particularly in IBD patients, is the overgrowth of *Clostridioides difficile*.[26,27,28] *C. difficile* is a spore-forming anaerobic bacterium that can cause *C. difficile* infection (CDI) in IBD patients.[26,29,30,31] However, the increasing rate of morbidity and mortality is the most important concern of physicians accounting with IBD patients who suffered from CDI.[7]

A couple of studies suggested the bilateral effects of *Blastocystis* and the gut microbiota.[18,32,33] However, the main hypothesis here was the possible correlation between co-existence of *Blastocystis* and CDI in IBD patients. Moreover, it was worthy of studying whether *Blastocystis* alters the gut microbiota composition toward overgrowth of *C. difficile*, or lack of *Blastocystis* as one indicator of the healthy gut microbiota composition lead to providing a suitable niche for developing CDI. Therefore, the current study aimed to investigate the possible co-existence of *Blastocystis* and CDI in IBD patients in Iran.

MATERIALS AND METHODS

Patients and Fecal Samples

A total of 102 stool samples were collected from August 2016 to December 2017 from IBD patients who were admitted to the IBD clinic. Informed consent was taken from all participants. All IBD patients were diagnosed based on the clinical criteria and confirmed by colonoscopy. The patients were excluded from the study if they had a previous history of cancer, liver, and autoimmune diseases. Additionally, the presence of other enteric infections was also considered as exclusion criteria. A well-trained interviewer filled a questionnaire consisted of demographic and clinical data. All stool samples were immediately transferred to the anaerobic bacteriology and the parasitology laboratories to cultivate and isolate *C. difficile* and *Blastocystis*, respectively. A portion of stool samples was kept out at -20°C for DNA extraction.

Cultivation and Isolation of C. difficile

All samples were cultivated on cycloserine cefoxitin fructose agar medium (CCFA) (Mast, London, United Kingdom). Briefly, a portion of samples was homogenized with 1 mL of 5% yeast extract broth and directly inoculated onto *C. difficile* medium supplemented with 7% horse blood. The same volume of samples was also treated with 1 mL of methanol for 1-2 min before inoculation on the CCFA. All the cultivated plates were incubated at 37°C for at least 48-72 h under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) using anaerobic generation system (Anoxomat-Mart, Microbiology, Holland). The suspected colonies were characterized based on the colony morphology and Gram staining.

Cultivation of Blastocystis

In order to cultivate and purify *Blastocystis*, approximately 200 mg of stool samples was inoculated into Dulbecco's modified egg medium supplemented with 20% fetal bovine serum and penicillin/streptomycin (1000-unit penicillin and 4 mg/mL streptomycin). All samples were incubated at 37°C and anaerobic conditions. The samples were parasitologically examined for growth of the parasite every 48 h for 10 days—the samples with no growth for *Blastocystis* after 10 days are considered as negative.

DNA Extraction and Polymerase Chain Reaction

DNA extraction from *C. difficile*

DNA was extracted from pure colonies of *C. difficile* by boiling method as mentioned elsewhere.[34,35] Briefly, a loop full of each suspicious colony was resolved in 500 µL of distilled water and centrifuged for 10 min at 13 000 × g. Then, the supernatant was discarded, the pellets were mixed with 100 µL of distilled water, and boiled in a water bath for 10 min. Finally, the tubes were centrifuged for 10 min at 13 000 × g, and the supernatant containing bacterial DNA was stored at –20°C for further investigations.

DNA extraction for Blastocystis

DNA extraction was carried out for all culture-positive samples. Briefly, 250 µL of cultured samples were transferred to a new 1.5-mL tube. After centrifuging at

2500 × g for 5 min, the supernatant was discarded, and the pellet was washed 3 times with sterile phosphate-buffered saline. Finally, the pellet was treated with a stool DNA extraction kit (Yekta Tajhiz Azma, Tehran). Purified DNA was kept out at –20°C until use.

PCR Amplification

C. difficile

For species detection and checking the presence of enterotoxigenic genes, specific primer pairs for *cdd3* and *tcdA*, *tcdB*, respectively, described by Persson et al.,[36] were employed. *C. difficile* strain RIGLD-141 was used as the positive control during microbiological and molecular experiments.

Blastocystis

To determine Blastocystis subtypes, discriminative fragments of the small subunit ribosomal RNA (SSU rRNA) gene were amplified using specific primers mentioned previously.[16,37] The PCR products were electrophoresed in 1.2% agarose gel and stained by 0.5 µg/mL ethidium bromide. The amplicons were visualized using UV Transilluminator.

All the *Blastocystis*-PCR products were sequenced using ABI sequencer 3130, and the sequences were aligned using software alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with sequences that were already deposited in the GenBank database.

Statistical Analysis

To determine the potential correlations between the presence of *Blastocystis* and *C. difficile*, *Blastocystis* subtypes with a type of *C. difficile* toxins, and co-existence of *Blastocystis* and *C. difficile* with the frequency of defecations, Fisher's exact test was employed. To analyze the crude and adjusted odds ratio (OR) for age, gender, frequency of defecation, and antibiotic usage, logistic regression was employed. SPSS v.22 for Windows (Chicago, IL, USA) was used for statistical analyses. The results were considered to be significant at a *P*-value of ≤ 0.05.

RESULTS

Totally, 102 IBD patients, including 3 (2.9%) CD and 99 (97.1%) UC with an average age of 36.79 + 23.12, were involved in this study. Among these patients, 42 (41.2%) and 60 (58.8%) were male and female, respectively. *Blastocystis* and *C. difficile* were observed in 17 (16.7%) and 26 (25.5%) of stool samples, respectively. All demographic and clinical data were summarized in Table 1.

Table 1. Demographic and Clinical Data of the Study Population

Patient's Characteristics	Total (n = 102), %	Infected (n = 43)*		Non-infected (n = 67), %	Co-existence of Clostridioides difficile and Blastocystis (n = 8), %	P†
		C. difficile (n = 26), %	Blastocystis (n = 17), %			
Age (years)						
11-20	5 (4.9)	2 (7.7)	2 (11.8)	2 (3)	1 (12.5)	.458
21-30	41 (40.2)	9 (34.6)	8 (47)	28 (41.8)	4 (50)	
31-40	30 (29.4)	7 (27)	5 (29.4)	19 (28.3)	1 (12.5)	
41-50	15 (14.7)	3 (11.5)	2 (11.8)	12 (17.9)	2 (25)	
>50	11 (10.8)	5 (19.2)	0	6 (8.9)	0	
Gender						
Female	60 (58.8)	15 (57.7)	8 (47)	42 (59.2)	5 (62.5)	.295
Male	42 (41.2)	11 (42.3)	9 (53)	25 (40.8)	3 (37.5)	
Type of disease						
Ulcerative colitis	99 (97.1)	25 (96.1)	16 (94.1)	65 (52.6)	7 (87.5)	.425
Crohn Disease	3 (2.9)	1 (3.84)	1 (5.9)	2 (47.4)	1 (12.5)	
Defecation (times/day)						
2-5	51 (50)	15 (57.7)	7 (41.1)	33 (43.3)	4 (50)	.035
5-8	36 (35.3)	6 (23)	6 (35.3)	27 (39.4)	3 (37.5)	
>10	15 (14.7)	5 (19.23)	4 (23.5)	7 (13.15)	1 (12.5)	

*The infected column indicates separated data attributed to the patients with either *Blastocystis* or *C. difficile*.
†*P* values are attributed to co-existence of *Blastocystis* and *C. difficile* with patient's data.

Blastocystis Subtypes and C. difficile Toxinotyping

The expected fragments were successfully sequenced. Comparison of the generated sequences in the NCBI database (https://www.ncbi.nlm.nih.gov/) showed that 10 (58.8%) and 7 (41.2%) were characterized as ST1 and ST3, respectively. The results of PCR amplifications revealed that from 26 *C. difficile*-positive isolates, 24 (92.3%) strains were *tcdA*$^+$/B$^+$ and 2 (7.7%) were *tcdA*$^+$/B$^-$.

Correlation Between the Presence of Blastocystis and C. difficile

In the current study, the Fisher's exact test showed a statistically significant correlation between co-existence of *Blastocystis* and *C. difficile* ($P < .035$). In other words, 8/17 (47.06%) of the cases who carried *Blastocystis* were positive for *C. difficile,* while from the other 85 *Blastocystis*-negative subjects, 18/85 (21.17%) were positive for *C. difficile* (Fig 1). Furthermore, a statistically significant correlation was observed between co-existence of these microorganisms and the frequency of defecation ($P < .035$). There was no significant correlation between *Blastocystis* subtypes and *C. difficile* colonization, and its toxinotypes (Table 2). The regression analysis demonstrated that the presence of *Blastocystis* significantly ($P < .05$) increased the risk of *C. difficile* colonization 3.3-4.44 folds (Table 3).

DISCUSSION

IBD is a disabling disease leading to unpleasant situations for the normal microbial composition through the gastrointestinal tract, known as dysbiosis.[38,39] Recent studies support the fact that IBD reduces the diversity of the protective bacteria and alters the microbial community toward increasing the pathogenic species.[40,41,42] Indeed, this was suggested that the risk of occurrence of CDI could be elevated to 2.5- to 7-fold in IBD patients compared to healthy controls,[43,44] which can be problematic in treatment and management of IBD.[45] In Iran, the prevalence rate of *C. difficile* in UC patients was reported to be 27/85 (31.8%), of which 15/85 (17.6%) had CDI.[46] In another study conducted by Azimirad et al.,[47] 5.7% of patients suffering from IBD flare were infected with *C. difficile*. It has also been hypothesized that alteration in the gut microbiota composition in IBD patients makes these subjects more susceptible to CDI.[27,29,31,45,48,49] Also, the prevalence rate of *Blastocystis* in Iran has been reported to be up to 30%.[50,51,52,53,54,55,56]

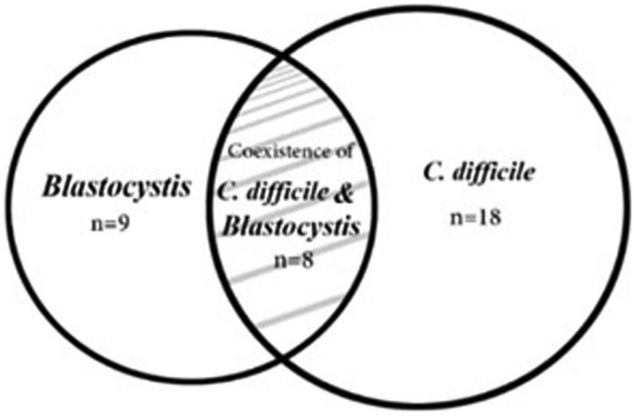

Figure 1. Schematic overview of co-existence of Blastocystis and Clostridioides difficile.

In the current study, a significant correlation was found between the presence of *Blastocystis* and CDI. It is well established that *C. difficile* is the major causative agent for the development of antibiotic-associated diarrhea. The pivotal role of antimicrobial agents in the disruption of the homeostasis of gut microbiota and initiating CDI was previously highlighted.[57,58,59] Notably, routine antibiotics used during CDI therapy can alter the normal flora of the intestine. Nonetheless, antibiotics such as metronidazole and vancomycin, commonly used for CDI treatment, are also widely used in IBD patients to ameliorate clinical manifestations, particularly diarrhea. Therefore, prescribed/unprescribed consumption of these antibiotics in IBD patients could enhance the chance of CDI. Furthermore, studies are indicating a high frequency of metronidazole-resistant isolates of *Blastocystis*.[60,61,62,63] The scenario explaining the significant co-existence of *C. difficile* and *Blastocystis* in studied patients probably is the presence of metronidazole-resistant isolates of *Blastocystis.*

Immunomodulatory drugs such as corticosteroids and biological agents are commonly prescribed in IBD patients who suffer from flare phase. Issa et al.[48] claimed that only immunosuppressant drugs increased the risk of CDI and resulted in diarrhea in IBD patients. Nonetheless, the synergistic effect of immunosuppressant drugs and corticosteroids in enhancing CDI risk in IBD patients was not illustrated. In a meta-analysis performed by D'Aoust et al.[7] synthesized data demonstrated that immunosuppressant drugs could increase the risk of CDI in the general population. Still, there is no strong evidence of the role of these drugs in the increasing risk of CDI in IBD patients. The role of immunosuppressant drugs in the emergence of *Blastocystis* infection is also unclear. According to the results of the studies on intestinal parasites in immunocompromised patients, in contrast

Table 2. Antibiotics and Immunomodulatory Drugs Consumed in the Study Population

Drug Consumption		Infected-Patients (n = 35)		
		Clostridioides difficile (n = 18) %	Blastocystis (n = 9) %	Co-existence of C. difficile and Blastocystis (n = 8) %
Antibiotics	Metronidazole	2 (11.1)	0	4 (50)
	Metronidazole + Ciprofloxacin	1 (5.5)	0	0
	Ciprofloxacin	1 (5.5)	0	0
	Vancomycin	1 (5.5)	0	0
	Metronidazole + Vancomycin	1 (5.5)	0	0
	Un-usage	12 (66.7)	9 (100)	4 (50)
Immunomodulatory drugs	Asacol + pentasa	1 (5.5)	0	0
	Asacloe	1 (5.5)	0	1 (12.5)
	Infleximab	1 (5.5)	0	0
	Pentasa	1 (5.5)	0	0
	Prednizolone	4 (22.2)	3 (33.3)	1 (12.5)

with coccidian parasites and microsporidia as opportunistic parasites,[64,65,66,67] it seems that there is no clear association between the presence of *Blastocystis* and consumption of immunosuppressant drugs. Therefore, consumption of immunomodulators and corticosteroids more likely did not significantly affect the prevalence of both *C. difficile* and *Blastocystis* in the current study.

Several studies have investigated the prevalence of *Blastocystis* in IBD patients. Although almost all of them concluded that the prevalence of this parasite in IBD patients is significantly lower than healthy controls,[16,17,68] there are studies reflecting results contrariwise.[69,70] Interestingly, recent studies investigated the role of *Blastocystis* in gut microbiota dysbiosis and suggested that this protozoan could change the microbial composition of the gastrointestinal tract. Audebert et al.,[18] assessed the microbial diversity of both *Blastocystis*-infected and *Blastocystis*-free subjects using the next-generation sequencing approach. It demonstrated that this parasite could increase the diversity of the gut microbiome. They confirmed the role of *Blastocystis* in the alteration of gut microbiota. Accordingly, Nourrisson et al.[19] showed that *Blastocystis* was also associated with decreasing the population of the protective bacteria. Notably, Nagel et al.[71] studied the association between the presence of *Blastocystis* and fecal microbial diversity in 2 IBS and healthy human subjects. It claimed that although *Blastocystis* could not lead to a significant change in the gut microbiota composition of IBS patients compared to healthy controls, this protozoan may lead to the clinical manifestations in the IBS group.

Table 3. The Correlation Between the Presence of Blastocystis and the Risk of Clostridioides difficile Colonization.

Odds Ratio	CI	*P*
3.3[a]	1.11-9.79	.031
4.441[b]	1.35-14.58	.014

[a]Crude OR value.
[b]Adjusted for age, gender, frequency of defecation, and antibiotic usage.

Apart from the indirect effect of *Blastocystis* on the colonization of *C. difficile* via alteration of the gut microbiota, there is a report of severe blastocystosis similar to CDI.[72] Although bilateral effects of *Blastocystis* and *C. difficile* were still not established, the results of the current study suggest 2 most probable scenarios: (1) *Blastocystis* altered gut microbiota composition toward optimum conditions for overgrowth of *C. difficile* via decreasing both the number and diversity of protective bacteria and (2) IBD provides an unpleasant environment for useful bacteria that this phenomenon provides a suitable niche for co-colonization of *Blastocystis* and *C. difficile.* However, it seems that *Blastocystis* can probably change gut microbial composition toward pleasant conditions for the overgrowth of *C. difficile*.

From the immunological point of view, it was established that *Blastocystis* is able to neutralize the mucosal immunity via either cleaving secretory immunoglobulin A (sIgA)[73] or disruption of the intestinal barrier and tight-junctions between the epithelial cells.[21,74,75] On the other hand, it was shown that together with the normal gut

microbiota composition, a healthy intestinal barrier plays a crucial role against CDI.[76,77,78] *TcdA* and *tcdB* are the 2 most important toxins of *C. difficile*.[79,80,81] The central role of *tcdA* in the pathogenesis of *C. difficile* was known, but the importance of *tcdB* during the early stage of the infection was recently discussed.[78] Islam et al.[78] assessed the susceptibility of patients to CDI and showed that CDI patients had lower IgA levels against tcdB than healthy controls. They concluded that apart from a healthy intestinal epithelial barrier, mucosal immune response via secretory IgA plays a key role during CDI. Therefore, regarding the ability of the protozoan in the destruction of mucosal immune response of the intestine, and also the central role of sIgA in defense against *C. difficile*[82], it seems that the presence of either *Blastocystis* or *C. difficile* could facilitate their successful colonization in the gut.

CONCLUSION

This study indicated the co-existence of *Blastocystis* and *C. difficile* in IBD patients. Furthermore, co-existence of *Blastocystis* and *C. difficile* had a statistically significant effect on the frequency of defecation in IBD patients. The results of our study suggested that the presence of either *Blastocystis* or *C. difficile* probably facilitate their successful colonization in the gut. The bilateral effect of these microorganisms on each other probably happens through attenuation of mucosal immune response by cleavage of sIgA or altering the microbiota composition of the gut and decreasing the number of protective bacteria.

Ethical Committee Approval: All procedures performed in this study were in accordance with the ethical standards (IR.SBMU.RIGLD.REC. 1396.165) released at October 18, 2017 by the ethical review committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Informed Consent: Informed consent was taken from participants.

Peer Review: Externally peer reviewed.

Author Contributions: Conceived and designed the experiments: HM AY. Performed the experiments: MA SMAG EJ HB. Analyzed the data: HM AY. Clinical Experiments: HAA AS SS. Contributed reagents/materials/analysis tools/positive samples: MRZ. Wrote the paper: HM AY MA.

Acknowledgments: The authors would like to thank all the staff of the Foodborne and Waterborne Diseases Research Center for their collaboration.

Conflict of Interest: The authors declare that they have no conflict of interest.

Financial Disclosure: This article has been financially supported by the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grants no. RIGLD 972 and RIGLD A-954).

Consent for Publication: None.

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