ORIGINAL ARTICLE

Frequency, subtypes distribution, and risk factors of *Blastocystis* spp. in COVID-19 patients in Tehran, capital of Iran: A case-control study

Ali Taghipour[a], Majid Pirestani[b], Ramin Hamidi Farahani[a], Mohammad Barati[a] and Esfandiar Asadipoor[a]

a *Infectious Diseases Research Center, AJA University of Medical Sciences, Tehran, Iran and* b *Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

Abstract

Background: Recent theories on the possible interactions between the intestinal parasites and COVID-19 have stated that these co-infections may cause immune imbalance and further complications in the affected patients. Until now, there is no data about *Blastocystis* subtypes as an intestinal parasite in COVID-19 patients. Therefore, the present work was done to evaluate the molecular prevalence of *Blastocystis* spp. and related risk factors in Iranian patients with COVID-19.

Method: Stool samples were gathered from 200 COVID-19 patients and 200 control, being matched regarding age, gender and residence. Then, stool samples were surveyed by parasitological methods, including direct slide smear and formalin-ether concentration. In the following, PCR and sequencing were used to detect *Blastocystis* spp. and their subtypes.

Results: The frequency of *Blastocystis* spp. in patients with COVID-19 (7.5%; 15/200 by molecular method *vs.* 6%; 12/200 by microscopy method) was slightly higher than in individuals without COVID-19 (4.5%; 9/200 by molecular method *vs.* 4%; 8/200 by microscopy method), this difference was not statistically significant (*P* value = 0.57 for molecular method vs. *P* value = 0.81 for microscopy method). Regarding associated factors for *Blastocystis* spp., we found significant differences regarding the residence (rural), loose and watery stool with diarrhea, and duration of treatment (6 weeks <) in the COVID-19 group. *Blastocystis* ST3 was the most common subtype in the patients with COVID-19 and control group.

Conclusions: Based on this results, health education, improved sanitation and good personal hygiene are highly recommended to prevent *Blastocystis* in COVID-19 patients.

Keywords: *Blastocystis*, COVID-19, Iran, Subtyping

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Corresponding author. Infectious Diseases Research Center, AJA University of Medical Sciences, Tehran, Iran.

E-mails: alitaghipor71@yahoo.com **(A. Taghipour),** pirestani@modares.ac.ir **(M. Pirestani),** rgsramin@yahoo.com **(R. Hamidi Farahani),** mbaratim@gmail.com **(M. Barati),** es1355@ajaums.ac.ir **(E. Asadipoor)**

1. Introduction

In late December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic was first reported in Wuhan, China [1–3]. COVID-19 is frequently asymptomatic or it is slightly manifested in immunocompetent individuals, while multi-organ dysfunction mediated by endothelial damage and a severe acute respiratory distress syndrome (ARDS) are the major consequences in at-risk groups [3–6]. A meta-analysis study showed that the case fatality rate due to COVID-19 in general population, hospitalized patients, patients older than 50 years, and patients admitted in intensive care unit (ICU) was 1.0% (95% confidence interval (CI): 1.0-3.0), 13.0% (95% CI: 9.0-17.0), 19.0% (95% CI: 13.0-24.0), and 37.0% (95% CI: 24.0-51.0), respectively [7]. Multiple risk factors for mortality due to COVID-19 include older age, diabetes, asthma or chronic lung disease, sickle cell disease, hypertension, cardiovascular disease, and immune compromise [8–12]. Moreover, a meta-analysis study showed that 19% of patients with COVID-19 have co-infections and 24% have superinfections, leading to poor outcomes and increased mortality [13].

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Blastocystis spp. is an anaerobic protozoan that is often isolated in the digestive tract of humans and a wide range of animals [14–19]. Until now, 17 different subtypes of *Blastocystis* spp. have been characterized in human and animal hosts [20,21]. Nevertheless, the pathogenic potential of *Blastocystis* spp. subtypes is still debatable, since some researchers have demonstrated its association in gastrointestinal disorders, while others have rejected such involvement [22–24]. Therefore, there are no substantial evidences for association between presence of *Blastocystis* subtype and clinical manifestations.

Currently, it was recommended that the status of public health and education affect the incidence rates of *Blastocystis* and COVID-19 [25–27]. Therefore, the prevalence rates of *Blastocystis* and COVID-19 shows that these infections is most common in those areas that suffer from undernourishment, poverty, and low level of sanitation [26–30]. Until now, there is no data about *Blastocystis* subtypes in COVID-19 patients. Therefore, the present study was done to evaluate the frequency of *Blastocystis* spp. and related risk factors in the COVID-19 patients and control groups in Iran.

2. Methodology

2.1. Study population

This study was obtained ethical approval from the Ethics Committee of the Aja University of Medical Science (AJAUMS), Tehran, Iran (no. IR.AJAUMS.REC.1401.044). Stool samples were collected from April 2021 to May 2022 in health care centers, Tehran, Iran. The inclusion criteria were a confirmed COVID-19 history and consent for participation, while the presence of immunodeficiency and administration of antiparasitic drugs during last three months before sampling were set as exclusion criteria. Considering WHO recommendations for the diagnosis of COVID-19, nasopharyngeal/oropharyngeal swabs samples were gathered from each individual and transferred to sterile bottles of viral transport medium, which were sent immediately to the laboratory of COVID-19. The RNA was extracted from the samples using Viral Nucleic Acid Kit and the viral RNA was detected using the real time reverse-transcription polymerase chain reaction (rRT-PCR) assay [31]. Stool samples were taken from 200 confirmed patients with COVID-19 undergoing treatment. The control group comprised 200 non-COVID-19 individuals (negative for COVID-19 test) and without any history of COVID-19, as approved by a clinician. To improve the accuracy of the case-control design of the study, age, gender and the place of residence were matched between both groups. A standard questionnaire was filled by each participant, which included sociodemographic conditions and risk factors related to *Blastocystis* spp., upon giving a consent.

2.2. Fecal sample collection and parasitological analysis

Stool samples were gathered in sterile containers tagged with patient's name and identification number, then immediately sent to the parasitology laboratory at the Tarbiat Modares University, and Jahrom University of Medical sciences under aseptic conditions. Then, stool samples were examined using parasitological methods, including direct slide smear and formalin-ether concentration under light microscopy (Zeiss, Germany) with 10X, 40X and 100X objective magnification. Also, stool samples were preserved in 70% alcohol at 4°C for the DNA extraction.

2.3. DNA extraction PCR and subtyping

The genomic DNA was extracted from 200 mg of stool samples in both case and control groups using a DNA purification mini kit (Yekta Tajhiz Azma Co., Iran), based on the manufacturer's instructions. The extracted DNA was preserved at -20°C until PCR.

A PCR assay was done using primer pairs RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) [23,32]. For this purpose, the following condition was performed for amplification of targeted fragment: denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 30 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final extension step at 72 °C for 5 minutes [23].

Subsequently, PCR products were observed by ultraviolet illumination after electrophoresis on 1.5% agarose gel stained with ethidium bromide. The PCR products of the positive samples were sequenced using Applied Biosystems 3730/3730xl DNA Analyzers (Bioneer, Korea) and the results were compared with those deposited in the GenBank using BLAST software.

2.4. Statistical analysis

The data analysis was used to evaluate the molecular prevalence of *Blastocystis* spp., while other descriptive data were estimated by binomial distribution. The Chi-square and Fisher's exact tests were applied to compare the prevalence of this parasite among cases and controls using SPSS software version 16 (SPSS, Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

3. Results

Among 400 included participants, 200 subjects were confirmed patients with COVID-19 (53.5% male; mean age of

47.14 ± 12.29 years) and 200 participants were subjects without COVID-19 (51% male; mean age 47.77 ± 11.57 years). The frequency of *Blastocystis* spp. in patients with COVID-19 (7.5%; 15/200 by molecular method *vs.* 6%; 12/200 by microscopy method) was slightly higher than in individuals without COVID-19 (4.5%; 9/200 by molecular method *vs.* 4%; 8/200 by microscopy method), this difference was not statistically significant (*P* value = 0.57 for molecular method vs. *P* value = 0.81 for microscopy method). Regarding associated factors for *Blastocystis* spp., we found significant differences regarding the residence (rural), loose and watery stool with diarrhea, and duration of treatment (6 weeks <) in the patients with COVID-19 group (Table 1).

All 24 positive samples were successfully sequenced in both case and control groups. In COVID-19 group, the results of sequences in BLAST presented that subtype 3 (9/15; 60% with accession numbers OP456400– OP456408) was the most common followed by subtype 2 (4/15; 26.66% with accession numbers OP456409-OP456412) and subtype 1 (2/15, 13.33% with accession numbers OP456413-OP456414) (Fig. 1), while in control group, subtype 3 (5/9; 55.55% with accession numbers OP456433-OP456437) was the most common followed by subtype 2 (3/9; 33.33% with accession numbers OP456438-OP456440) and subtype 1 (1/9, 11.11% with accession number OP456441) (Fig. 2).

4. Discussion

The prevalence of *Blastocystis* differs from county to county, different counties of a country as well as studied hosts. In this regard, it appears that the frequency of this protozoan is high in developing region with low-level of public health [33]. However, high prevalence of *Blastocystis* spp. has been reported in different host in some European countries [34–38]. Internationally, very few studies have been performed on the prevalence of *Blastocystis* spp. among patients with COVID-19. In this regard, a prospective cohort study in Ethiopia found that 37.81% (284 of 751) patients with COVID-19 were infected with intestinal parasites [39], although they do not have a specific report on the abundance of *Blastocystis* spp. Different prevalence has been reported for intestinal parasites in both developing and developed countries and numerous parameters may influence such distribution, including the Human Development Index (HDI), geographical, and demographic factors may increase prevalence of intestinal parasites [40–45].

TABLE 1. Age, gender, type of stool, diarrhea status, residence, type of patients, lung complications, and duration of treatment of patients with COVID-19 and healthy group, according to the presence or absence of *Blastocystis* sp. using PCR

	COVID-19 patients (n = 200)				Healthy group (n = 200)			
Variables	**No. Examined**	**No. Positive (%)**	**No. Negative (%)**	***P*-value**	**No. Examined**	**No. Positive (%)**	**No. Negative (%)**	***P*-value**
Age group (year)								
20-30	20	0 (0%)	20 (100%)	0.44	18	0 (0%)	18 (100%)	0.98
31-40	44	2 (4.55%)	42 (95.45%)		44	2 (4.54%)	42 (95.46%)	
41-50	52	3 (5.77%)	49 (94.23%)		48	2 (4.16%)	46 (95.84%)	
51-60	51	7 (13.73%)	44 (86.27%)		64	4 (6.25%)	60 (93.75%)	
61-70	33	3 (9.09%)	30 (90.91%)		26	1 (3.84%)	25 (96.16%)	
Sex								
Male	107	10 (9.35%)	97 (90.65%)	0.28	102	5 (4.90%)	97 (95.10%)	0.77
Female	93	5 (5.38%)	88 (94.62%)		98	4 (4.08%)	94 (95.92%)	
Residence								
Urban	151	8 (5.30%)	143 (94.70%)	**0.03***	145	7 (4.82%)	138 (95.18%)	0.85
Rural	49	7 (14.29%)	42 (85.71%)		55	2 (3.63%)	53 (96.37%)	
Type of stool								
Formed	61	0 (0%)	61 (100%)	**< 0.00005***	71	2 (2.82%)	69 (97.18%)	0.82
Soft	101	5 (4.95%)	96 (95.05%)		99	5 (5.05%)	94 (94.95%)	
Loose	18	5 (27.78%)	13 (72.22%)		17	1 (5.88%)	16 (94.12%)	
Watery	20	5 (25%)	15 (75%)		13	1 (7.69%)	12 (92.31%)	
Diarrhea								
Yes	38	9 (23.69%)	29 (76.31%)	**< 0.00002***	30	2 (6.66%)	28 (93.34%)	0.53
No	162	6 (3.70%)	156 (96.30%)		170	7 (4.12%)	163 (95.88%)	
Duration of treatment of patients with COVID-19								
2 weeks	61	2 (3.27%)	59 (96.73%)	**0.007***	—	—	—	—
2-4 weeks	43	1 (2.33%)	42 (97.67%)		—	—	—	—
4-6 weeks	25	0 (0%)	25 (100%)		—	—	—	—
6 weeks<	71	12 (16.90%)	59 (83.10%)		—	—	—	—
Type of patients								
Inpatients	109	10 (9.17%)	99 (90.83%)	0.32	—	—	—	—
Outpatients	91	5 (5.50%)	86 (94.50%)		—	—	—	—
Lung complications								
25%>	49	3 (6.12%)	46 (93.88%)	0.24	—	—	—	—
25-49%	42	1 (2.38%)	41 (97.62%)		—	—	—	—
50%≤	109	11 (10.10%)	98 (89.90%)		—	—	—	—

*p-value<0.05; Statistically significant.

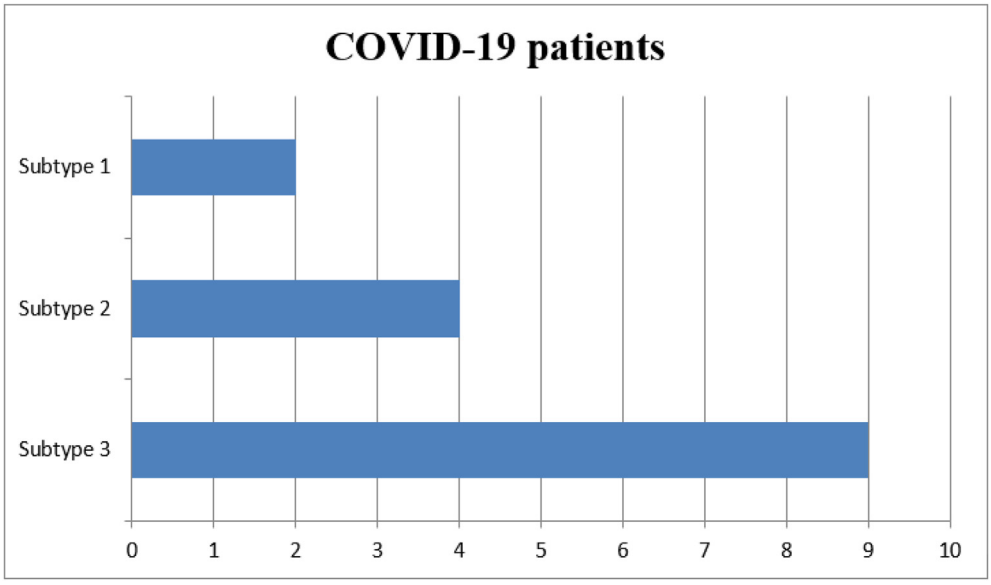

FIG. 1. Distribution of *Blastocystis* sp. subtypes in patients with COVID-19.

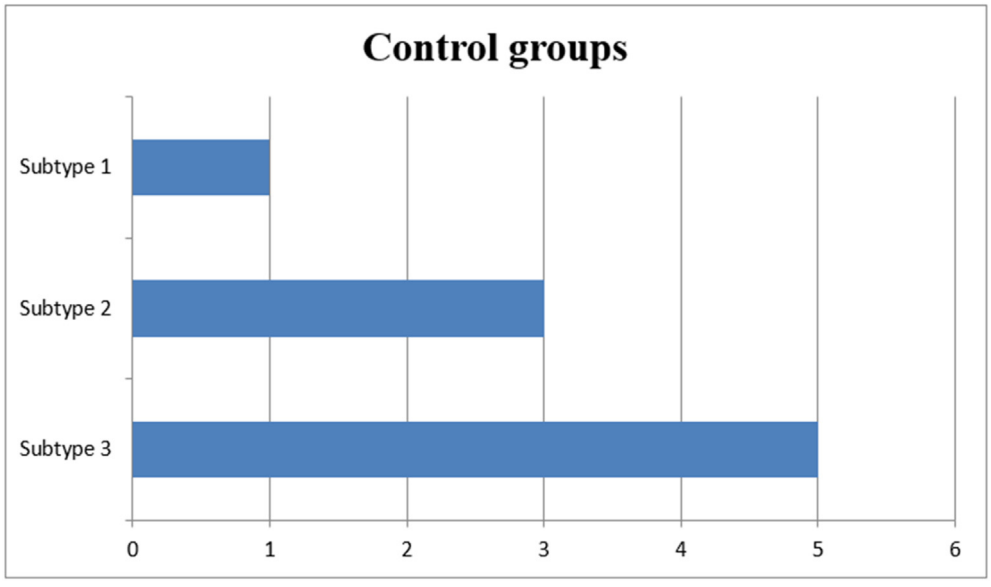

FIG. 2. Distribution of *Blastocystis* sp. subtypes in control groups.

Although the results of molecular and microscopy methods were approximately in the same range, but these methods have its own features. As the current study has shown, the results of the microscopy prevalence are slightly lower than the molecular method, which may be due to misdiagnosis with other elements and microorganisms. In this regard, *Blastocystis* spp. life-cycle is not fully understood and highly polymorphic, this forms include cystic forms, granular, multivacuolar, vacuolar, avacuolar and amoeboid structures [46]. While PCR has higher sensitivity and easier interpretation than the microscopy method [23,47]. Also, PCR is capable of directly differentiating the derived subtypes of *Blastocystis* spp. between different hosts and reservoirs [23].

In this study, we surveyed frequency and *Blastocystis* subtype among COVID-19 patients. Also, ST3 was the most common subtype, while ST1 was only reported from two patients with COVID-19. In Iran, some studies surveyed *Blastocystis* spp. subtypes in healthy groups (without clinical manifestations) [48–50], individuals who suffer from inflammatory bowel syndrome (IBS) [51] and patients with inflammatory bowel

diseases (IBD) [52]. Nevertheless, in Iran, it suggests that ST3 is the most common subtype, followed by ST2 and ST1 [25]. Some studies have showed that ST1 in diarrhea, IBS, and tuberculosis patients is higher than other subtypes and therefore, this subtype was considered that as a pathogenic subtype [23,53]. More recently, a meta-analysis study showed the association of *Blastocystis* spp. with colorectal cancer, and subtypes 1 and 3 had the highest rates in these patients [54]. However, a strong association between *Blastocystis* subtypes and clinical manifestations has not still been recognized. In the current study, *Blastocystis* ST1 was isolated from two diarrhea subjects. Considering the diarrhea status, we found a significant association between *Blastocystis* spp. and diarrhea in COVID-19 patients, which may be associated to the high prevalence of *Blastocystis* spp. in the participants. Based on the literature, intestinal protozoa can potentially polarize the helper T cells towards type 1 (Th1) [44,55–58]. Also, co-infections of intestinal protozoa and some intracellular pathogens, such as *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) may substantially cause imbalances in the host and further pathological consequences [55–58]. Since the emergence of the SARS-CoV-2, there have been some hypotheses on the likely interaction between the intestinal parasite and COVID-19 [59,60]. Nevertheless, diarrhea reported in the present study cannot be reliably attributed to the *Blastocystis* spp., because other infectious (bacterial, fungal and viral agents) and/or non-infectious diseases may have had a role in the initiation and progression of diarrhea. Thus, the likely association between the *Blastocystis* spp. and diarrhea cannot be reliably surmised and requires future extensive studies. Regarding the risk factors, we found that the living in the rural and duration of treatment (6 weeks <) were more likely to be infected with *Blastocystis* spp. These risk factors could be explained by the fact that poor hygiene conditions and low levels of socio-economic factor in living areas of carriers as a potential risk factor of *Blastocystis* spp., therefore, they are more exposed to *Blastocystis* spp.

There were some limitations to the present study. At first, stool samples were gathered only once; it would have been better to collect three samples to increase the sensitivity and accuracy of detection. Second, due to our limited facilities and funds, we did not examine some clinical features such as hematological parameters in the study population. Also, recent studies have shown the potential ability of *Blastocystis* spp. and COVID-19 to alter the gut microbiota ecosystem, which may lead to beneficial or harmful functions in the digestive system [61–64]. Therefore, it is suggested to measure the status of *Blastocystis* spp. in COVID-19 patients by considering the gut microbiota in future studies.

5. Conclusions

Blastocystis ST3 was the most common subtype in both groups. Based on this, health education, improved sanitation and good personal hygiene are highly recommended to prevent *Blastocystis* spp. in COVID-19 patients. Moreover, it is highly suggested to perform more comprehensive epidemiological studies to better recognize the interaction between *Blastocystis* spp. and COVID-19 in Iran and other countries.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study received ethical approval from the Ethics Committee of AJA University of Medical Sciences, Tehran, Iran (Code: IR.AJAUMS.REC.1401.044).

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ali Taghipour: Formal analysis, Writing – review & editing, contributed to all parts of the study, contributed to study implementation, collaborated in the analysis and interpretation of data. collaborated in the manuscript writing and revision. All the authors commented on the drafts of the manuscript and

approved the final version of the article, All authors contributed to study design and. **Majid Pirestani:** contributed to study implementation, All the authors commented on the drafts of the manuscript, and, approved the final version of the article, All authors contributed to study design, and. **Ramin Hamidi Farahani:** Formal analysis, collaborated in the analysis and interpretation of data. All the authors commented on the drafts of the manuscript, and, approved the final version of the article, All authors contributed to study design, and. **Mohammad Barati:** Writing – original draft, Writing – review & editing, contributed to all parts of the study, collaborated in the manuscript writing and revision. All the authors commented on the drafts of the manuscript and approved the final version of the article, All authors contributed to study design, and. **Esfandiar Asadipoor:** Writing – review & editing, Writing – original draft, collaborated in the manuscript writing and revision. All the authors commented on the drafts of the manuscript and approved the final version of the article, All authors contributed to study design.

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