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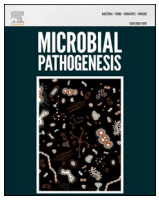

Prevalence and genetic diversity of *Blastocystis* sp. among autochthonous and immigrant patients in Italy

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ABSTRACT

The prevalence of *Blastocystis* sp., its genetic diversity and the distribution of circulating subtypes (STs) were molecularly investigated in a cohort of autochthonous and immigrant patients with gastrointestinal symptoms hospitalized over the period February 2022–June 2023 at the Policlinico Ospedaliero-Universitario "Riuniti", Foggia, in Southern Italy. The population variables, including patient geographical origin, gender and age classes were reported. Out of the 927 investigated patients, 36 (3.9%) were positive for *Blastocystis* sp. A statistically significant association with African origin and age classes >18 years old was found. ST1 (allele 4), ST2 (alleles 9, 13), ST3 (alleles 34, 36) and ST4 (allele 92) were the subtypes detected with a different distribution between autochthonous and immigrant patients. Co-infections with enteric protozoa such as *Giardia duodenalis* and *Dientamoeba fragilis*, pathogenic bacteria as *Clostridioides difficile*, *Campylobacter jejuni* and *Aeromonas* sp. and viral infections such as Norovirus were found in 33% of cases. This is the first study of *Blastocystis* sp., its circulating subtypes and allele variability among patients with different geographical origin in an area of Southern Italy, in the Central Mediterranean, characterized by high immigrant pressure. These results provide baseline data to better investigate a potential interaction between *Blastocystis* sp. and other risk factors in patients with gastrointestinal symptoms.

1. Introduction

Blastocystis sp. (Stramenopiles) is a common enteric protozoan microorganism found in the intestinal tract of humans and several animals, including non-human primates and birds [1]. It is transmitted through faecal-oral route, and with contaminated water and food [2–4]. It has been estimated that over one billion people are infected with *Blastocystis* sp. worldwide [5] with prevalence in human faecal samples ranging from 0.5% to 100% depending on country, hygiene conditions and sanitary practices [6,7]. Indeed, within low-income countries, the prevalence ranges between 50% and 100%, while in high-income countries it is significantly lower, with a 10–15% rate, approximately [5].

Based on the analysis of the polymorphisms within the small subunit ribosomal RNA-encoding SSU-rDNA gene, at least 44 subtypes (STs) of *Blastocystis* sp. have been classified [8–10]. Subtypes ST1 to ST9 and ST12 have been mainly reported in human samples, with ST3 being the most frequent subtype followed by ST1, ST2 and ST4 [8,11]. Worldwide, STs have also a different geographical distribution and allele frequency [6,12,13].

Although this microorganism has been known for over one hundred years, its pathogenic role remains debated. Several studies report an

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association of *Blastocystis* sp. with gastrointestinal symptoms (GI) (i.e., diarrhoea, abdominal pain, nausea, vomiting, fatigue, flatulence, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), etc.) with a higher prevalence of the parasite within IBS patients [14]. Nevertheless, there are also studies reporting that *Blastocystis* sp. has been found in faeces from asymptomatic individuals [15,16]. Moreover, whether pathogenicity is linked to a particular subtype remains still debated. Because of this, testing for *Blastocystis* sp. is not routinely performed by most laboratories and hospitals, which leads to an underestimated *Blastocystis* sp. prevalence. Multiple risk factors (i.e., poor hygiene condition, contact with animals, low socioeconomic status, low education, age, travelling abroad or infections with other enteric pathogens) have been associated with a higher risk of being infected with *Blastocystis* sp [5].

Among Mediterranean countries, Italy has a high circulation and flow of immigrant populations, notably from African countries. In Italy, data on *Blastocystis* sp. prevalence and its genetic variability are still limited and mostly focused on the autochthonous population. Therefore, the aim of this study was to evaluate *Blastocystis* sp. prevalence and to characterize the circulating subtypes and the genetic variability in a cohort of autochthonous and immigrant patients with GI symptoms hospitalized at the Policlinico Ospedaliero-Universitario "Riuniti", Foggia (Southern Italy), which is an area under high migration pressure, over the period February 2022–June 2023. Demographic and clinical data were analysed as factors potentially correlated with *Blastocystis* sp. infection. In addition, co-infection of *Blastocystis* sp. with enteric pathogenic virus, bacteria and protozoa and concomitant infection with other microorganisms in other body sites are also discussed.

2. Materials and methods

2.1. Sample collection and population description

From February 1st 2022 to June 31st 2023, n. 927, consecutive, non-replicated faecal samples from patients with GI symptoms admitted to the Policlinico Ospedaliero-Universitario "Riuniti", Foggia, in Southern Italy, were collected and sent to the Microbiology and Virology Unit of the hospital. The Policlinico Ospedaliero-Universitario "Riuniti" is a public, academic hospital that cares for a combined urban and suburban population of more than 594,000 individuals. Increasing immigration rates have been reported in this area in recent years [17]. Individuals born in foreign countries, mainly from African countries, and those that have access to the Italian universal health coverage were also included in this study.

Overall, n. 878 patients (n. 476 females and n. 402 males) were autochthonous (Caucasian Italians) and n. 49 patients (n. 17 females and n. 32 males) were African immigrants. Patients were also grouped in two age classes as <18 years old (n. 296) and >18 years old (n. 631). The mean age of the patients was 52.5 ± 34.2 years (range: 1–98 years).

Patients were defined as cases (positive result for *Blastocystis* sp. by molecular analysis) and controls (negative result for *Blastocystis* sp. by molecular analysis).

Demographic data (origin, gender and age) were obtained from the Hospital Information Management System. Clinical data were obtained from medical records (presence of gastrointestinal symptoms; eosinophilia; immunodeficiency and antibiotic treatments). Data on intestinal co-infection with pathogenic bacteria, virus or other enteric protozoa and concomitant infection with other microorganisms such as bacteria and viruses in other body sites were obtained from the Laboratory Data Collection System. For the research of pathogens other than *Blastocystis* sp., standard laboratory protocols were adopted.

2.2. Research of Blastocystis sp. from faecal samples

The faecal samples were subjected to DNA extraction by using MagCore® Nucleic Acid Extraction Kit (RBC Bioscience, Taiwan) and subsequently processed with the Allplex™ GI-Parasite Assay Real Time (Seegene Inc. Seoul, Korea) in order to detect and identify six causative protozoan parasites in gastrointestinal disease, i.e. *Blastocystis* sp., *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Dientamoeba fragilis*, *Entamoeba histolytica* and *Giardia duodenalis*, in accordance with the manufacturer's protocol. All the DNA samples were stored at −20 °C until further molecular analysis.

2.3. Blastocystis subtype sequencing and analysis

All the DNA samples found positive to *Blastocystis* sp. by Allplex™ GI-Parasite Assay Real Time were subjected to *Blastocystis*-PCR and sequencing in order to identify *Blastocystis* subtypes. Primers RD5 (5′-ATC TGG TTG ATC CTG CCA GT-3′) and BhRDr (5′-GAG CTT TTT AAC TGC AAC AACG-3′) were used to amplify approximately a fragment of 600 base pairs within the 1800 bp of *Blastocystis* SSU-rDNA gene [18] following the PCR and purification protocols previously described [19].

Purified amplicons were directly sequenced in both directions using the ABI PRIMS Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the same primers as the respective PCR reaction, in accordance with the manufacturer's instructions. Obtained sequences were determined on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) and the chromatograms were inspected by eye using the FinchTV software v1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Primer regions plus bad-quality regions were trimmed. Once the sequences had been cleaned up, each sequence was compared with all the *Blastocystis* homologous nucleotide sequences and isolates available in GenBank databases using the Blast tool (https://blast.ncbi.nlm.nih.gov). Then, the obtained sequences corresponding to *Blastocystis* SSU-rDNA gene portion were gathered in a "fasta" file and aligned with reference *Blastocystis* sp. subtypes sequences (http://entamoeba.lshtm.ac.uk/blastorefseqs.htm) using MAAFT software [20]. Maximum Likelihood Phylogenetic Analysis was achieved according partitions and optimal substitution models identified by the BIC and AIC metrics with 1000 bootstrap replicates as implemented in MEGA X v10.0.5 [21].

Moreover, in order to assign the subtype alleles, each sequence has been submitted to a *Blastocystis* public databases for molecular typing and microbial genome diversity (https://pubmlst.org/bigsdb?db=pubmlst_blastocystis_seqdef&page=sequenceQuery). The STs sequences generated within this study were submitted to GenBank under Accession Numbers OR230201-OR230236.

2.4. Statistical analysis

Blastocystis sp. positivity, geographical origin (Caucasian Italians vs Africans), gender (male vs female) and age classes (young, <18-year old vs adult, >18-year old) were considered as primary variables and subjected to statistical analysis. The Chi-square (X^2) test was used to evaluate the association between *Blastocystis* sp. positivity, geographical origin, gender and age classes. A p value < 0.05 was considered as being statistically significant. *Blastocystis* subtypes, allele diversity, presence of gastrointestinal symptoms, eosinophilia, immunodeficiency, antibiotic treatments, data on intestinal co-infection with pathogenic virus, bacteria or other enteric protozoa and concomitant infection with other microorganisms such as bacteria and viruses in other body sites were considered as secondary variables. All statistical analysis was performed using the QuickCalcs GraphPad online tool available at https://www.graphpad.com/quickcalcs/.

3. Results

3.1. Blastocystis assay real time PCR and subtype analysis

Overall, 36 of 927 (3.9%) samples were positive to *Blastocystis* sp. by Allplex™ GI-Parasite Real Time Assay with the following distribution: n.

27 (17 males and 10 females) were autochthonous (Caucasian Italians) and n. 9 (5 males and 4 females) were African immigrants. Taking geographic provenance into account, *Blastocystis* prevalence was of 3.1% (27/878) in Caucasian Italians patients and of 18.4% (9/49) in African patients. Regarding gender, *Blastocystis* prevalence was of 5.1% (22/434) in male patients and of 2.9% (14/493) in female patients. Among age classes, *Blastocystis* prevalence was of 1.3% (4/296) in age classes <18 and of 5.1% (32/631) in age classes >18 years of age. The positivity was significantly associated with African origin (*p value* = 0.0001) and age classes >18 years old (*p value* = 0.0063). No significant statistical correlation with gender was found (*p value* = 0.0796). The distribution of *Blastocystis* sp. positive patients according to geographical origin, gender and age classes is given in Table 1.

The 36 positive samples were successfully PCR-amplified and unambiguously sequenced with ~600 bp sequences obtained for all of the samples. The phylogenetic analysis enabled the clustering of our isolates with their associated subtypes (Fig. 1). Four subtypes were recorded with ST1 in nine samples (25%), all of which are allele 4; ST2 was recorded in eight samples (22.2%) with one sample identified as allele 9 and seven samples identified as allele 13; ST3 in thirteen samples (36.1%) regrouping seven samples with allele 34 and six samples with allele 36; and ST4 in six samples (16.6%) with five identified as allele 92 and one with closest match allele 92. No mixed subtypes were found. The STs distribution relative to geographical origin and gender was: ST1 in nine Caucasian Italian patients (4 males and 5 females), ST2 in six African patients (4 males and 2 females) and in two Caucasian Italian patients (1 male and 1 female), ST3 in thirteen Caucasian Italian patients (9 males and 4 females) and ST4 in three African patients (1 male and 2 females) and three Caucasian Italian male patients. The distribution of *Blastocystis* subtypes and its alleles according to geographical origin, gender and age classes is given in Table 2.

Out of the 36 positive patients, n. 18 (50%) were positive for *Blastocystis* sp. only and 18 (50%) were concomitantly positive for one or more microorganisms (Table 3). Among the latter 18 patients, n. 12 (66.6%) had a concomitant presence of others enteric protozoa as *D. fragilis* (n. 4), *G. duodenalis* (n. 2) and n. 1 with a double infection with *G. duodenalis* and *D. fragilis*. Moreover, concomitant presence of bacterial pathogens such as *Clostridioides difficile* (n. 2), *Campylobacter jejuni* (n.1), *Aeromonas* sp. (n. 1) and the viral pathogen Norovirus (n. 1) was found; these results are shown in Table 3. There were similar results for infections with others microorganisms in other body sites, i.e. *Staphylococcus capitis* (n. 1, blood), *Enterococcus faecalis* (n. 3, urine and blood), *Enterococcus faecium* (n. 1, urine), *Proteus mirabilis* (n. 1, blood), *Escherichia coli* (n. 3, urine and blood), *Klebsiella pneumoniae* (n. 2, urine), *Acinetobacter baumannii* (n. 1, urine), *Mycobacterium tuberculosis* (n. 1, sputum), Adenovirus (n. 1, rectal swab) and SARS-CoV-2 (n. 5,

Table 1
Social demographic characteristic of enrolled population and of *Blastocystis* sp. positive patients stratified according to geographical origin, gender and age classes.

Social demographic variables	Total cases (n = 927), (n, %)	*Blastocystis* + (n = 36), (n, %)	*p value*
Age (mean + −SD)	52.5 +-34.2	64.5+-30.5	
Ethnicity			
Italians	878 (94.7%)	27 (3.1%)	0.0001*
Africans	49 (5.3%)	9 (18.4%)	
Gender			
Male	434 (46.8%)	22 (5.1%)	0.0796
Female	493 (53.2%)	14 (2.9%)	
Age classes			
<18 years old	296 (31.9%)	4 (1.3%)	0.0063*
>18 years old	631 (68.5%)	32 (5.1%)	

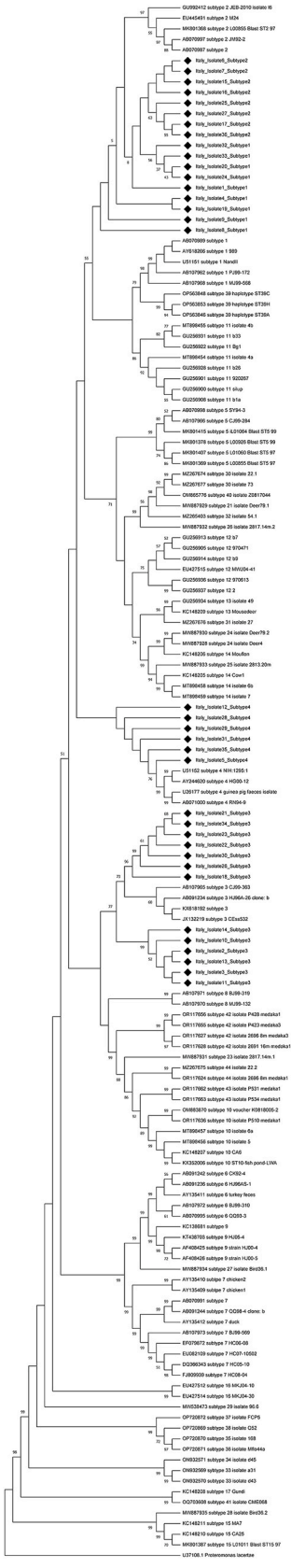

Fig. 1. The Maximum Likelihood (HKY + G + I substitution model) phylogenetic tree based on the analysis of the partial SSU-rDNA gene of *Blastocystis* isolates. Numbers next to the nodes represent posterior probabilities. Probabilities <50% are not shown. Thirty-six sequences from the present study (black triangle) and 110 reference sequences representing *Blastocystis* subtypes (ST1-ST44) from literatures were included in the analysis for comparative purposes. *Proteromonas lacertae* was used as outgroup. Accession numbers of publicly available *Blastocystis* reference sequences are indicated.

Table 2
Distribution of *Blastocystis* subtypes stratified according to geographical origin, gender and age classes.

Subtype	IT male (n, %)	IT female (n, %)	AF male (n, %)	AF female (n, %)	TOT (n, %)
ST1	4 (44.4%)	5 (55.6%)	0	0	9 (25%)
ST2	1 (12.5%)	1 (12.5%)	4 (50%)	2 (25%)	8 (22.2%)
ST3	9 (69.2%)	4 (30.8%)	0	0	13 (36.1%)
ST4	3 (50%)	0	1 (16.7%)	2 (33.3%)	6 (16.6%)
Age classes					
<18	1 (25%)	0	2 (50%)	1(25%)	4 (11.1%)
>18	16 (50%)	10 (31.2%)	3 (9.4%)	3 (9.4%)	32 (88.8%)

nasopharyngeal swab), as summarized also in Table 3.

Notably, out of the 36 *Blastocystis* sp. positive patients, one patient presented immunodeficiency and ten patients were subjected to antibiotic therapy (Table 3). All the *Blastocystis* sp. positive samples and their correlations with geographical origin, gender, age, subtypes, alleles, accession number, coinfections, infection with other microorganisms, GI, eosinophilia, immunodeficiency and antibiotic therapy have been reported in Table 3.

4. Discussion

An ever-increasing number of studies on *Blastocystis* sp. prevalence, subtype distribution and genetic diversity has been produced in the last ten years [22,23]. Moreover, although several studies report the association of *Blastocystis* sp. in patients with gastrointestinal illness, it is still unclear whether the *Blastocystis* is a pathogen microorganism, commensal or beneficial member of the gut microbiota [24,25]. Thus, not always being part of the microbiological diagnostic investigations in many clinical laboratories and hospitals, its prevalence is probably still underestimated.

In this study, the prevalence of *Blastocystis* sp. infection in autochthonous and immigrant patients with gastrointestinal symptoms was of 3.9% (36/927), providing a picture of *Blastocystis* sp. presence and circulation of this microorganism within an area of the Mediterranean (Southern Italy) for which data are still lacking and which is characterized by high immigration flows, mainly from African countries. However, in our study *Blastocystis* sp. prevalence was lower when compared with prevalence data (up to 20%) of a similar study conducted in patients at a referral centre for tropical diseases in Northern Italy [26].

Previous studies have shown that geographical origin influences the prevalence of *Blastocystis* sp. infection [5]. In the current work, *Blastocystis* sp. prevalence was of 3.1% (27/878) in Caucasian Italian patients and of 18.4% (9/49) in patients from African countries with an association statistically significant in the latter (p *value* = 0.0001). *Blastocystis* sp. is mainly transmitted through the faecal-oral route and by means of contaminated food or water. This could explain the higher prevalence of this protozoan in patients from low-income countries, characterized by i.e., poor hygiene conditions, contact with animals, low socioeconomic status and low education [1]. The area surveyed for this study is particularly interesting, within the broader international context, due to high rates of immigrants from African countries contributing to the potential spread of *Blastocystis* infection and transmission of the microorganism.

Within the current work, the presence of *Blastocystis* sp. was statistically correlated with age classes >18 years old (p *value* = 0.0063) but was not correlated with either male or female gender (p *value* = 0.0796). Worldwide, several studies have investigated the association between *Blastocystis* sp. infection rate and the host gender. Although in some works a higher prevalence in either female [27] or male [28] has been reported, data from the majority of previous studies shows that prevalence was not significantly related to gender [29–31]. As regards the association with host age, some studies reported no significant statistical relationship [28,32], while others reported higher *Blastocystis* sp. infection rates among younger adult age groups [33,34] adults aged over 18 years [35], or even older than 60 years of age [36]. Moreover, younger patients (below 30 years of age or children in the 5–15 age group) can potentially have a higher risk of being infected with *Blastocystis* sp [37,38].

By using the DNA sequencing of "barcoding region", four subtypes [ST1 (25%), ST2 (22.2%), ST3 (36.1%) and ST4 (16.6%)] have been found in enrolled patients, with ST3 as the most prevalent subtype, followed by ST1, which is consistent with previous studies conducted in different regions and populations. Indeed, epidemiological studies have shown that the most common subtypes worldwide are ST3, ST1, ST2 [11,22] and that ST4 is the most common mainly in European countries, with a prevalence of ~20% [2,39,40].

By analysing the distribution of the four subtypes and allele variability detected in the current work according to the geographical origin of patients, the results show that Caucasian Italian patients harbour ST1 allele 4 (33.3%), ST2 allele 13 (7.4%), ST3 alleles 34 and 36 (48.1%) and ST4 allele 92 (11.1%), while African patients harbour ST2 alleles 9 and 13 (66.6%) and ST4 allele 92 (33.3%). As expected, ST3 was the most occurring subtype identified in Caucasian Italian patients followed by ST1; these results are consistent with other studies conducted in Italy reporting rates of 23.7% for ST3 followed by 21.5% for ST1 in Northern part of Italy [26] and a prevalence of about 45% and 15% for ST3 and ST1 respectively in other part of Italy [41–43]. So far, ST1-ST4 and ST6, ST7 and ST8 are currently the observed subtypes circulating in Italy, including those detecting in our study.

When investigating the alleles distribution of the different subtypes observed within our current analysis versus the previous isolates from other Italian studies [26,41–43], comparison was possible only with the data reported in two previous studies [41,42]. In detail, a wide diversity of alleles were observed with report of alleles 4 and 77 within ST1, 12 and 9 within ST2, 34 within ST3 and 42 within ST4 [42] as well as extra alleles within the common subtypes ST1 (a75), ST2 (a: 67, 65, 70, 66, 61), ST3 (a:128, 53, 37, 54, 36, 46, 47, 55, 48, 49) and ST4 (a: 89) [41]. It is noteworthy that subtypes ST1 and ST3 [22,44] were not detected within our African patients. Moreover, within ST2 subtype, two alleles were reported as allele 9 and allele 13. According to the literature, ST2 subtype shows evidence of some geographic variation in frequency of detection [6] with allele 9 identified widely within human [45] and animal [46] isolates. The second identified allele 13 was reported in two Caucasian and five Africans patients. However, despite its frequency within our cohort, no correlations can be suggested due the scarce available data about this allele. Indeed, allele 13 is one of the rarest one within ST2, since it has been identified so far from Japanese monkey (Accession number: AB070997) and subjects from Colombia [47].

Interestingly the ST4 subtype was detected in three African patients. In a recent work studying *Blastocystis* subtypes in patients from Guinea, ST4 was found in only two patients, confirming its infrequency in Africa [38]. Unfortunately, we are not in a position to retrieve details of the time interval between the patients' arrival in Europe and the detection of subtype ST4 in them, and therefore we cannot state whether the infection occurred in Africa or in Italy. However, when comparing the allele sequences of the reported ST4 from Africa (Accession number: OM038868.1 from Guinea; Accession number: KF848537.1 from Senegal and Accession number: KX378183.1 from Tunisia), none of them matched with the identified allele 92 in our current study. Indeed, the ST4/allele 92 has been previously reported in rats from France [48], Spain [49], Iran [50] and Japan [51] extending to some other rodent hosts like the squirrels and the *Dolichotis (*Accession number MN123557, MK940493 respectively). More recently, the same allele 92 is being reported from pigeons (MN227363), sheep (MW850521), rabbits

Table 3
Demographic parameters (origin, gender, and age), subtypes (STs), alleles variability, Accession Number, clinical data (enteropathogens coinfections, other infections, GI, eosinophilia, immunodeficiency and antibiotic therapy) of the thirty-six *Blastocystis* positive samples.

ID	Origin	Gender	Age	ST	Allele	Accession number	Coinfections with others enteric pathogens		Other infections (other body sites)		GI	Eosinophilia	Immunodeficiency	Antibiotic treatment
							Bacteria	Protozoa	Bacteria	Virus				
1	IT	M	67	ST1	1	OR230201			*E. coli* (urine)		+	0	no	yes
2	IT	M	63	ST3	36	OR230202					++	0.19	no	no
3	IT	F	87	ST3	36	OR230203	*C. difficile*		*E. faecalis* (urine) *A. baumannii* (urine)	SARS-CoV-2	++	2.9	no	yes
4	IT	M	14	ST1	4	OR230204		*D. fragilis*			++	1.9	nd	nd
5	AF	M	48	ST4	92*	OR230205			*M. tuberculosis* (sputum)	SARS-CoV-2	++	2.3	no	yes
6	AF	M	8	ST2	13	OR230206		*D. fragilis*		SARS-CoV-2	++	3.7	nd	nd
7	AF	M	10	ST2	13	OR230207					+	2.1	nd	nd
8	IT	F	85	ST1	4	OR230208					+	0	no	yes
9	IT	F	64	ST1	4	OR230209					+	0.5	no	yes
10	IT	F	65	ST3	36	OR2302010		*D. fragilis*	*E. coli* (blood)	Adenovirus	++	0.8	yes	yes
11	IT	M	77	ST3	36	OR2302011	*C. difficile*			SARS-CoV-2	++	4.1	no	yes
12	IT	M	47	ST4	92	OR2302012			*E. coli* (blood) *K. pneumoniae* (urine)		++	0.4	no	yes
13	IT	M	61	ST3	36	OR2302013			*E. faecalis* (blood) *S. capitis* (blood)		+	0	no	yes
14	IT	M	84	ST3	36	OR2302014			*E. faecalis* (blood) *P. mirabilis* (blood)		+	0	nd	nd
15	AF	M	22	ST2	13	OR2302015	*C. jejuni*			SARS-CoV-2	+	1.2	nd	nd
16	AF	F	48	ST2	9	OR2302016		*D. fragilis* *G. duodenalis*			+	2.6	no	yes
17	AF	M	29	ST2	13	OR2302017					+	1.29	nd	nd
18	IT	M	89	ST3	34	OR2302018			*K. pneumoniae* (urine) *E. faecium* (urine)		+	0	nd	nd
19	IT	F	74	ST1	4	OR2302019					+	0	nd	nd
20	IT	M	64	ST1	4	OR2302020					+	0	nd	nd
21	IT	M	76	ST3	34	OR2302021					+	0	nd	nd
22	IT	M	80	ST3	34	OR2302022					+	0	nd	nd
23	IT	F	74	ST3	34	OR2302023				Norovirus	+	0	nd	nd
24	IT	F	90	ST1	4	OR2302024					+	0	nd	nd
25	IT	F	26	ST2	13	OR2302025					+	0	nd	nd
26	IT	M	81	ST3	34	OR2302026					+	0	nd	nd
27	AF	F	35	ST2	13	OR2302027					+	1.6	nd	nd
28	AF	F	8	ST4	92	OR2302028		*D. fragilis*			+	2.4	nd	nd
29	AF	M	82	ST4	92	OR2302029		*G.duodenalis*			+	1.2	nd	nd
30	IT	F	55	ST3	34	OR2302030					+	0	nd	nd
31	IT	M	79	ST4	92	OR2302031		*G. duodenalis*			+	0	nd	nd
32	IT	F	86	ST1	4	OR2302032					+	0	nd	nd
33	IT	M	83	ST1	4	OR2302033	*Aeromonas* sp.				+	0	nd	nd
34	IT	M	77	ST3	34	OR2302034					+	0	nd	nd
35	AF	F	48	ST4	92	OR2302035					+	0.8	nd	nd
36	IT	M	86	ST2	13	OR230236					+	0	nd	nd

IT: Italy; AF: Africa; M: male; F: female; ST: subtype; GI: gastrointestinal symptoms; +: diarrhoea; ++: other GI; n.d: not determinated; *: closest match.

(MW785827) from China and cats (MW040154) from South Korea which may suggest its underestimated spread and its potential zoonotic role and transmission.It is also noteworthy that, although the association between subtypes and GI symptoms remains a controversial question, a recent review [2] reported that ST4 is significantly associated with symptomatic cases. The association of this subtype and symptoms was previously reported also elsewhere [52] with ST4 associated with acute diarrhoea in patients from Denmark. In this study ST4 subtype has been detected in both Italian and African patients. In particular, two of them (1 Italian and 1 African, both male, mean age 47.5 years old) showed diarrhoea and asthenia, abdominal pain, loss of appetite, productive cough and higher eosinophilia. Although the number of ST4 samples is too limited to support any statistical evaluation, the result obtained here should not be overlooked and provides the basis to better investigate the potential role that ST4 may have in GI symptoms.

In this study, 50% of patients (18/36) showed a single infection from *Blastocystis* sp. and (50%) (18/36) of patients were concomitantly positive for one or more microorganisms, including co-infections with pathogenic bacteria, viruses or other enteric protozoa. In particular, four patients showed a co-infection with *D. fragilis,* two patients with *G. duodenalis* and one a double infection with *D. fragilis* and *G. duodenalis.* The co-infection between *Blastocystis* and other protozoa has already been reported (e.g., with *G. duodenalis* [53], and with *D. fragilis* [54] although without any statistical correlation with symptoms. Like *Blastocystis*, *D. fragilis* is a common human intestinal protozoan parasite with a still controversial pathogenic role [55]. The coinfection between *Blastocystis* and *D. fragilis* could suggest a cooperative interaction between the two protozoa. Moreover, two patients showed a co-infection with *C. difficile* and one patient with *C. jejuni.* A *Blastocystis* and *C. difficile* co-infection has been recently reported in patients with IBD from Singapore [56], Turkey [57] and Colombia [58]. Very interestingly, previous work [59] reported within their cohort that subtype analysis showed one patient with ST3 subtype, as reported also for two patients in this study. Co-infection between *Blastocystis* and *C. jejuni* has been reported in children with gastrointestinal disorder in Teheran, Iran [60]. Although still uncertain, these studies suggest that other enteric pathogenic bacteria or protozoa could be associated with *Blastocystis* sp. presence and serve as potential risk factors for the infection.

Within this work, high eosinophilia rate has been associated with *Blastocystis* sp. infection in some patients. Usually, it is considered uncommon for eosinophilia to be produced by protozoan infections [61] since in parasitic diseases, blood eosinophilia is associated with helminth infections, especially coinciding with the larval migration through tissues. However, it is interesting to note that some studies have reported a high proportion of eosinophils in the peripheral blood of symptomatic patients infected with *Blastocystis* sp [62]. Moreover, travellers/immigrants from resource-limited countries are those that most likely could acquire these infections. Our results showed that the *Blastocystis* positive patients with higher eosinophilia were also those with coinfection and with African origin, suggesting that eosinophilia should be taken into consideration in the diagnosis of *Blastocystis* sp. infection.

The present study has some limitations, mainly due to its retrospective design. In particular, we were not in a position to accurately retrieve the medical history for each patient (i.e., the time interval between arrival in Italy and testing for *Blastocystis*) and evaluate such clinical features as the patients' immunological conditions or the haematological parameters in the study population. Moreover, recent studies have shown the potential ability of *Blastocystis* sp. to alter the gut microbiota ecosystem, which may lead to beneficial or harmful functions in the digestive system [56]. In particular, an association between ST3 and healthy gut microbiome in a study conducted on a small group of Italian patients has been reported by Gabrielli et al. [43]. Based on concept that the subtypes identification and allelic variability seems to be essential for assessing the relationship between *Blastocystis*, microbiome profile and human disease, our further prospective studies on the human gut microbiota composition in patients with different subtypes/alleles and with different geographical origin will be designed for a better understanding of its potential pathogenic role.

5. Conclusion

In the current study, the prevalence of *Blastocystis* sp. infection was 3.9% in a cohort of hospitalized autochthonous and immigrant patients with gastrointestinal symptoms, giving new insight into a geographical area with high immigration rate for which there is scarce epidemiological data. *Blastocystis* sp. positivity was significant associated with African origin and age >18 years, with ST3/alleles 34 and 36, ST1/allele 4, ST2/allele 13 and ST4/allele 92 found among Italian patients and ST2/alleles 9 and 13 and ST4/allele 92 among African patients. Eosinophilia and gastroenteric symptomatology, such as abdominal pain, loss of appetite and productive cough are associated with ST4 subtype in two patients. Co-infections with pathogenic viruses, bacteria or protozoa were also correlated with *Blastocystis* sp. presence. This is the first study of *Blastocystis* sp., its circulating subtypes and allele variability in this part of the world, an area of Southern Italy, in the Central Mediterranean, characterized by high immigrant pressure. Further research with a larger number of samples needs to be conducted in order to gain further *Blastocystis* epidemiological data. In addition, gut microbiome studies on *Blastocystis* positive samples with different subtypes/alleles will contribute to better understand the potential pathogenic role of this microorganism.

Ethics approval and consent to participate

The present study was performed following the guidelines of the Declaration of Helsinki in 1975, revised in 2013 and all the procedures performed in this study meet the national and international guidelines. A written informed consent was obtained from every patient before the study and patients were completely anonymized by the researchers. Ethical approval was not sought for the present study because of the nature of the study (*in vitro* only). Included samples were obtained according to standard diagnostic and therapeutic protocols for the management of gastrointestinal infections. All the authors ensure that this study is HIPAA (Health Insurance Portability and Accountability Act, 1996) compliant. The researchers followed every mandatory (health and safety) procedure.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

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CRediT authorship contribution statement

Marianna Marangi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Visualization, Conceptualization. **Sonia Boughattas:** Writing – review & editing, Visualization, Methodology. **Rosella De Nittis:** Formal analysis. **Daniela Pisanelli:** Formal analysis. **Valeria delli Carri:** Formal analysis. **Maria Rosaria Lipsi:** Formal analysis. **Gianfranco La Bella:** Writing – review & editing, Visualization. **Gaetano Serviddio:** Visualization. **Mariangela Niglio:** Visualization. **Sergio Lo Caputo:** Visualization. **Maurizio Margaglione:** Visualization. **Fabio Arena:** Writing – review & editing,

Visualization.

Declaration of competing interest

The authors declare that they have no conflict of interest and that they have no actual or potential competing financial interests.

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