Microbes and Infection 27 (2025) 105422

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original Article

# Intragenomic diversity of the small subunit rDNA gene shows limited impact on the pathogenicity of *Blastocystis* infection in clinical patients

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ARTICLE INFO

*Keywords:*
Gastrointestinal symptoms
Clinical patients
Pathogenicity
Virulence
Co-infection
Spain

ABSTRACT

The clinical significance of *Blastocystis* sp. remains to be fully elucidated. This study assesses whether *Blastocystis* subtype diversity can affect the outcome of the infection and the occurrence of clinical manifestations in infected individuals. Stool samples from 219 *Blastocystis*-positive patients by PCR targeting the *ssu* rDNA gene were fully genotyped by Sanger sequencing analyses. Co-infections by other parasitic, viral, and bacterial enteropathogens were identified by molecular and culture methods. Sequence analyses revealed the presence of six *Blastocystis* subtypes including ST1 (21.5 %), ST2 (17.8 %), ST3 (29.7 %), ST4 (22.8 %), ST6 (5.5 %), and ST7 (2.3 %), with a single sample harbouring a ST1+ST3 co-infection (0.5 %). Multivariate risk factor analyses using logistic regression models indicated that neither *Blastocystis* subtypes nor patient-associated variables including sex, country of origin, travelling history, and presence of nonspecific symptoms were positively associated with a higher likelihood of developing gastrointestinal symptoms (abdominal pain and diarrhoea). However, being of a young age (p-value: 0.003) and experiencing skin pruritus (p-value $< 0.001$) and eosinophilia (p-value: 0.016) were found to increase the odds of presenting gastrointestinal symptoms. *Blastocystis* subtypes based on variability within the *ssu* rDNA gene do not seem to be the main drivers of clinical manifestations in the surveyed clinical population.

## 1. Introduction

*Blastocystis* sp. is an anaerobic protist capable of infecting or colonizing the gastrointestinal tracts of humans and a wide range of animals, spanning from large mammals to insects [1]. Its transmission is through the faecal-oral route, either indirectly via ingestion of contaminated water or food or directly via contact with infected individuals/animals [2,3]. Epidemiological investigations frequently identify *Blastocystis* sp. as the most prevalent unicellular eukaryote found in human faeces, reflecting its ubiquitous presence [4]. However, prevalence exhibits significant variation between countries and within smaller geographical areas, ranging from 5 % to over 50 % [4,5]. Typically, low- and middle-income countries exhibit higher parasite infection rates compared with industrialized nations [4,5]. Differences in the presence of *Blastocystis* sp. are often linked to socioeconomic (*e.g.*, consumption of unsafe water, poor hygiene practices, close contact with domestic animals) and methodological (*e.g.*, population under study, detection method used) factors [2,6].

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https://doi.org/10.1016/j.micinf.2024.105422
Received 5 January 2024; Received in revised form 7 August 2024; Accepted 7 September 2024
Available online 11 September 2024

*Blastocystis* sp. can be diagnosed through microscopy examination of stool samples but isolates from humans and other animals are morphologically indistinguishable [6]. Molecular DNA-based methods (including PCR and Sanger sequencing) have been increasingly used to improve diagnostic capabilities and assess genetic diversity. *Blastocystis* sp. exhibits extensive genetic heterogeneity in conserved markers, such as the small subunit rDNA gene (*ssu* rDNA). Based on the analysis of full-length (or near full-length) sequences of the *ssu* rDNA gene [7], currently 40 *Blastocystis* subtypes (ST) are considered valid, including ST1-ST17, ST21, and ST23-ST44 [8–12]. Among them, ST1-ST10, ST12, ST14, ST16, ST23, and ST35 have been found in humans (and, excepting ST35, in other non-human hosts) [9,10,13–15], with ST1-ST4 causing near 90 % of the human cases characterized globally [3–5]. *Blastocystis* ST3 emerges as the predominant subtype in human infections (primarily of anthroponotic origin), while other STs have also been frequently identified in various mammal species (ST1), primates (ST2), rodents (ST4), pigs (ST5), and avian hosts (ST6, ST7) [4].

The clinical significance of *Blastocystis* infection remains elusive due to its universality and presence in both symptomatic and asymptomatic individuals [3,6,16,17]. Some authors have proposed *Blastocystis* sp. as an opportunistic pathogen in immunocompromised populations and its prevalence through age is unclear [3,17]. Nevertheless, *Blastocystis* infection has been linked with a range of nonspecific gastrointestinal signs and symptoms (both acute and chronic) including abdominal pain, diarrhoea, flatulence, vomiting, nausea, and weight loss. Recent studies have suggested that *Blastocystis* pathogenesis could be subtype dependent, but this possibility is still under investigation [18]. When *Blastocystis* sp. is identified in clinical samples, treatment is recommended only when patients exhibit related symptoms, and no other primary causative agent is found. In these cases, the drug of choice is metronidazole [3,6].

Increasing research and knowledge on *Blastocystis* epidemiology has prompted the World Health Organization to issue warnings regarding its potential waterborne transmission [19]. Understanding the clinical implications of this protist is relevant for developing appropriate management strategies. Therefore, our study aims to establish meaningful associations between the presence and molecular diversity of *Blastocystis* sp. and the occurrence of clinical symptoms and disease outcomes in clinical patients.

## 2. Methods

### 2.1. Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Foundation Jiménez Díaz (protocol code PIC227-20_FJD and date of approval November 07, 2022).

### 2.2. Study design and setting

This was a retrospective study involving patients of all ages groups whose stool samples were submitted to the Microbiology Laboratory of the Principe de Asturias University Hospital (Alcalá de Henares, Spain) for intestinal protist testing between November 2020 and March 2022. All positive stool samples for *Blastocystis* sp. received during this period were included in the study without restrictions.

### 2.3. Molecular testing of intestinal protists

The stool samples were initially processed using the Microlab STARTlet Hamilton platform (Werfen, Barcelona, Spain), an automated system for stool DNA extraction and purification. Subsequently, the genetic material extracted from the samples was screened using the Allplex™ Gastrointestinal (GI) Parasite Assay (Seegene, Seoul, South Korea). This commercially available multiplex real-time PCR assay enables the simultaneous detection of six intestinal protist pathogens, namely *Blastocystis* sp., *Cryptosporidium* spp., *Cyclospora cayetanensis, Dientamoeba fragilis, Entamoeba histolytica,* and *Giardia duodenalis*.

### 2.4. Molecular characterisation of Blastocystis sp.

DNA samples that tested positive for *Blastocystis* sp. in the Allplex™ GI Parasite Assay were submitted to the Parasitology Reference and Research Laboratory, National Centre for Microbiology (Majadahonda, Spain) for genotyping purposes. To maximize the chances of successful identification of subtypes, only samples with cycle threshold ($C_T$) values $\leq 30$ were considered. To do so, a direct PCR protocol targeting a 600-bp fragment of the *ssu* rDNA gene of the parasite used [20]. The assay uses the pan-*Blastocystis*, barcode primer pair BhRDr (5′–GAGCTTTT TAACTGCAACAACG–3′) and RD5 (5′–ATCTGGTTGATCCTGCCAGT–3′). Amplification reactions (25 μl) included 5 μl of template DNA and 0.5 μM of each primer. Amplification conditions consisted of one-step of 95 °C for 3 min, followed by 30 cycles of 1 min each at 94 °C, 59 °C and 72 °C, with an additional 2 min final extension at 72 °C.

Amplicons of the expected size were sequenced in both directions by capillary DNA sequencing electrophoresis using BigDye® Terminator chemistry on an ABI PRISM 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Obtained consensus sequences were analysed using the Basic Local Alignment Search Tool (BLAST) for *Blastocystis* confirmation and subtype calling.

### 2.5. Clinical data collection

Relevant clinical information was retrieved from the medical records of the selected patients with *Blastocystis* sp. infection. Data included the date of diagnosis, age, sex, nationality, comorbidities, presence of concurrent infections, and associated signs and symptoms. Notable symptoms such as abdominal pain, diarrhoea, asthenia, flatulence, pruritus, vomiting and ‘others’ were examined. ‘Others’ included weight loss, fever, and constipation. Eosinophilia measures were collected when haematological analyses were available. Additionally, details about prescribed antibiotics and antiparasitic medications related to the illness were documented. Information regarding the resolution of the episode was also documented when possible.

### 2.6. Co-infection analysis

In available samples, additional molecular testing was undertaken to establish the clinical significance of the *Blastocystis* sp. infection. This involved performing PCR utilizing the Allplex™ GI Bacteria I-II and GI Virus Assay (Seegene, Seoul, South Korea), aimed to assess the potential occurrence of co-infections involving gastrointestinal viruses and bacteria that could potentially account for the symptoms experienced by the patient. Pathogens included in the GI Bacteria I-II panel were *Aeromonas* sp., *Campylobacter* sp., *Clostridium difficile* toxin B, *Salmonella* sp., enteroinvasive *Escherichia coli/Shigella* sp., *Vibrio* sp., *Yersinia enterocolitica*, enteroaggregative *E. coli*, enteropathogenic *E. coli, E. coli* O157, enterotoxigenic *E. coli*, hyper-virulent *C. difficile*, and enterohemorrhagic *E. coli.* Pathogens included in the GI Virus panel were adenovirus, norovirus (GI y GII), rotavirus, sapovirus, and astrovirus. Furthermore, upon specific requests by physician/general practitioners, stool cultures were conducted on selected samples to provide a comprehensive diagnostic assessment.

### 2.7. Statistical analysis

Statistical analysis was conducted using STATA/MP 17.0 (StataCorp, Texas, USA). Continuous variables were presented as median and interquartile ranges (IQR), while categorical variables were presented as proportions unless otherwise specified. Group differences were assessed using appropriate statistical tests, including the Mann-Whitney *U* test, $\chi^2$

test, or Fisher's exact test. All p-values were calculated in a two-tailed manner, and significance was defined as p-value $\leq 0.05$.

To concurrently assess the association between various *Blastocystis* genotypes and a single variable of interest, such as abdominal pain, we performed a series of statistical analyses (including contingency tables, ANOVAs, and other relevant tests), to identify any statistically significant differences among the genotype groups.

To investigate the factors contributing to gastrointestinal symptoms in patients diagnosed with *Blastocystis* sp., we conducted both univariate and multivariate logistic regression models using the available dataset. In the univariate analysis, we included all variables and identified significant associations with the occurrence of *Blastocystis* related gastrointestinal symptoms (abdominal pain and diarrhoea). The multivariate analysis aimed to identify variables that independently remained associated with gastrointestinal symptoms in *Blastocystis* sp. infections after adjusting for potential confounding factors. For this second analysis, we considered only covariant variables with $p \leq 0.09$ in the univariate analysis and less than 35 % of missing values. Results were presented as estimated odds ratios (OR) for univariate analysis and adjusted odds ratios (aOR) for the multivariate analysis, along with their corresponding 95 % confidence intervals (CI) for continuous and categorical variables. P-values were calculated using the Wald test for continuous variables and the likelihood ratio test for categorical variables, with statistical significance set at p-value $\leq 0.05$.

This integrated methodology offers a more comprehensive insight into the relationships among *Blastocystis* infections, its genotypes, and various clinical and epidemiological factors. It sheds light on important insights into this complex protozoan infection.

## 3. Results

A total of 219 patients with *Blastocystis* DNA detected in their stool samples using a commercial multiplex real-time PCR assay were included in this study. Generated cycle threshold (CT) values ranged from 14 to 31 (median: 24.5). After genotyping and Sanger sequencing analyses, a six different STs were detected. These included ST1 (21.5 %, 47/219), ST2 (17.8 %, 39/219), ST3 (29.7 %, 65/219), ST4 (22.8 %, 50/219), ST6 (5.5 %, 12/219), and ST7 (2.3 %, 5/219), with a single sample harbouring a ST1+ST3 co-infection (0.5 %, 1/219). Representative nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers OR752318–OR752329.

### 3.1. Epidemiological characteristics

Table 1 summarizes the main epidemiological characteristics and immune status of the recruited patient population categorized by *Blastocystis* subtype. Among the 219 *Blastocystis*-positive patients investigated, 100 were males and 119 females (male/female ratio: 0.84). *Blastocystis* subtypes were similarly distributed between sexes, excepting ST6/ST7 that were more prevalently found in females than in males (82.4 % *vs*. 17.6 %) but without reaching statistical significance (p-value: 0.122). Notably, 27.9 % (61/219) of the study participants were children up to 14 years old, and 5.0 % (11/219) were children under 5 years of age. Nationals comprised 82.7 % (181/219) of the patient population. Only 5.5 % (12/219) of the patients had a documented history of recent international travel to African (n = 3), European (n = 2), American (n = 4) or unspecified (n = 3) countries. Overall, 1.8 % (4/219) of the patients were immunocompromised (one infected by human immunodeficiency virus and three oncologic patients undergoing treatment).

In all 17 patients carrying avian-adapted *Blastocystis* ST6 and ST7, we conducted inquiries into the potential contact of these patients with avian species. Remarkably, two of them were bird owners, and nine reported occasional close contact with these animals.

### 3.2. Signs and symptoms

Table 2 summarizes the main clinical manifestations of the recruited patient population categorized by *Blastocystis* subtype, regardless of their co-infection status with other gastrointestinal pathogens of parasitic, viral, or bacterial nature. Overall, 81.7 % (179/219) of the patients presented with gastrointestinal symptoms including abdominal pain,

**Table 1**
Epidemiological characteristics and immune status of the *Blastocystis*-positive patients according to the subtype of the protist.

| Variable | ST1 (N = 47) | ST2 (N = 39) | ST3 (N = 65) | ST4 (N = 50) | ST6/7 (N = 17) |
|---|---|---|---|---|---|
| **Sex (male, %)** | 54.2 | 46.2 | 47.0 | 40.0 | 17.6 |
| **Age (IQR, yr.)** | 29 (10–48) | 28 (9–60) | 39 (23–53) | 48 (15–59) | 51 (27–55) |
| **Spanish nationality (%)** | 81.2 | 84.6 | 69.7 | 94.0 | 94.1 |
| **Travel abroad (%)** | 6.3 | 5.1 | 6.1 | 4.0 | 5.9 |
| **Immunosuppression (%)** | 2.1 | 2.6 | 1.5 | 2.0 | 0.0 |

IQR: Interquartile range. ST6 and ST7 were combined due to their potential zoonotic (avian) origin. The single sample with a ST1+ST3 co-infection was excluded from analysis.

**Table 2**
Frequency of signs and symptoms in the *Blastocystis*-positive patients according to the subtype of the protist.

| Variable (%) | ST1 (N = 47) | ST2 (N = 39) | ST3 (N = 65) | ST4 (N = 50) | ST6/7 (N = 17) |
|---|---|---|---|---|---|
| **Any gastrointestinal symptom** | 85.4 | 79.5 | 77.3 | 88.0 | 70.6 |
| **Abdominal pain** | 62.5 | 59.0 | 57.6 | 62.0 | 58.8 |
| **Asthenia** | 16.7 | 20.5 | 24.2 | 16.0 | 35.3 |
| **Diarrhoea** | 43.8 | 48.7 | 43.9 | 58.0 | 58.9 |
| **Flatulence** | 16.7 | 7.7 | 9.1 | 24.0 | 35.3 |
| **Vomiting** | 8.3 | 5.1 | 15.2 | 6.0 | 23.5 |
| **Pruritus/urticaria** | 10.4 | 23.1 | 22.7 | 10.0 | 23.5 |
| **Eosinophilia** | 32.1[a] | 40.6[b] | 19.6[c] | 39.4[d] | 50.0[e] |
| **Others** | 35.4 | 30.8 | 33.3 | 28.0 | 58.8 |

The single sample with a ST1+ST3 co-infection was excluded from analysis.
[a] Over 28 patients.
[b] Over 32 patients.
[c] Over 46 patients.
[d] Over 33 patients.
[e] Over 14 patients.

asthenia, diarrhoea, flatulence, and vomiting. Additionally, 17.4 % (38/219) of them reported cutaneous disorders such as pruritus or urticaria, 33.6 % (51/152) had eosinophilia, and 34.2 % (75/219) experienced other related symptoms such as constipation, fever, and weight loss. Notably, most patients showed more than one sign or symptom.

### 3.3. Co-infection analysis

Additional molecular testing was conducted to ascertain the occurrence and frequency of concomitant infection by intestinal pathogens of parasitic, bacterial, and viral nature that can potentially mask the relationship of *Blastocystis* with the occurrence of clinical manifestations.

#### 3.3.1. Gastrointestinal parasite panel

Through the same multiplex real-time PCR assay used for the detection of *Blastocystis* sp., we also identified the presence of pathogenic *Giardia duodenalis* (2.3 %, 5/219) and mostly commensal *Dientamoeba fragilis* (37.9 %, 83/219). Overall, 38.8 % (85/219) of patients were infected by two or more pathogens of parasitic origin. Generated $C_T$ values ranged from 24 to 35 (median: 29.8) for *G. duodenalis*, and from 21 to 38 (median: 29.0) for *D. fragilis*, respectively.

#### 3.3.2. Gastrointestinal virus panel

Molecular testing for enteric viruses was available for 68.5 % (150/219) of patients. We identified the presence of adenovirus (2.0 %, 3/150), astrovirus (3.3 %, 5/150), norovirus (5.3 %, 8/150) including three norovirus GI cases, and sapovirus (4.0 %, 6/150). Overall, 2.0 % (3/150) of patients were infected by two or more pathogens of viral origin. Generated $C_T$ values ranged from 22 to 35 (median: 28.4) for adenovirus, from 31 to 38 (median: 34.2) for astrovirus, from 20 to 35 (median: 29.8) for norovirus, and from 21 to 38 (median: 29.5) for sapovirus, respectively.

#### 3.3.3. Gastrointestinal bacteria panel

Molecular testing for enteric bacteria was available for 32.9 % (72/219) of patients. We identified the presence of *Aeromonas* sp. (1.4 %, 1/72), *Campylobacter* sp. (2.8 %, 2/72), enteroaggregative *Escherichia coli* (5.6 %, 4/72), enteropathogenic *E. coli* (13.9 %, 10/72), enterotoxigenic *E. coli* (1.4 %, 1/72), and *Yersinia enterocolitica* (1.4 %, 1/72). Overall, 25.0 % (18/72) of patients were infected by two or more pathogens of bacterial origin. Generated $C_T$ values were of 37.7 for *Aeromonas* sp., from 25 to 35 (median: 30.1) for *Campylobacter* sp., from 20 to 29 (median: 25.1) for enteroaggregative *E. coli*, from 19 to 38 (median: 32.3) for enteropathogenic *E. coli*, of 27.2 for enterotoxigenic *E. coli*, and of 36.3 for *Y. enterocolitica*, respectively.

#### 3.3.4. Stool culture

Overall, 60.7 % (133/219) of stool samples were cultured. Culture analyses allowed the identification of *Bacillus cereus* (4.5 %, 6/133), *Campylobacter jejuni* (0.8 %, 1/133), *C. difficile* toxin B (1.5 %, 2/133), and *Salmonella* group B (0.8 %, 1/133). Notably, only one of the two cultured stool samples that tested positive for *C. difficile* underwent molecular testing using real-time PCR, which produced a negative result.

#### 3.3.5. Other findings

As a result of complementary analyses and infection history obtained from medical records, we identified other pathogens within our patient's population. These included *Enterobius vermicularis* (0.9 %, 2/219), *Helicobacter pylori* (1.4 %, 3/219), hepatitis C virus (0.5 %, 1/219), human immunodeficiency virus (0.5 %, 1/219), SARS-CoV-2 virus (2.7 %, 6/219), and *Treponema pallidum* (0.9 %, 2/219).

Overall, 38.8 % (85/219) of our patients were infected by intestinal parasites (protists and helminths) other than *Blastocystis*. Viral and bacterial infections were identified in 12.7 % (19/150) and 15.5 % (30/193) of patients, respectively, by any diagnostic method or procedure. Besides *Blastocystis* sp., co-infections by two or more pathogens were observed in 11.0 % (24/219) of patients.

### 3.4. Statistical analysis

To avoid bias caused by potential confounding factors, we excluded from our statistical analysis all patients co-infected with parasitic, viral, or bacterial gastrointestinal pathogens other than *Blastocystis* sp., as determined by any molecular or culture method. The only exception was *D. fragilis*, which in our study, and based on the $C_T$ values obtained by real-time PCR, should be regarded as a commensal organism rather than a pathogen. This resulted in a study population of 170 patients who were solely infected with *Blastocystis* sp. Among them, 38.8 % (66/170) also carried *D. fragilis*. Our analysis focused on abdominal pain and diarrhoea, as these are the most common clinical manifestations associated with *Blastocystis* infections reported in the literature [3,6]. Univariate and multivariate logistic regression tests were employed to assess the prevalence of abdominal pain and diarrhoea in these 170 selected patients based on the considered risk factors (Table 3).

Our univariate regression analysis revealed that neither sex (p-value: 0.995), age (p-value: 0.083), *Blastocystis* ST (p-values: 0.051–0.942), country of origin (p-value: 0.664), travelling history (p-value: 0.984), eosinophilia (p-value: 0.058), nor *D. fragilis* carriage (p-value: 0.307) were independently associated with a higher likelihood of having abdominal pain or diarrhoea among *Blastocystis*-positive patients. In contrast, having cutaneous disorders (p-value $< 0.001$) and nonspecific

**Table 3**
Univariate and multivariate analyses of the distribution of clinical manifestations (encompassing abdominal pain and diarrhoea) among *Blastocystis*-positive patients by risk factor. Bold values indicate statistical significance at the $p < 0.05$ level.

| Variable | Univariate OR (IC 95 %) | p-value | Multivariate aOR (IC 95 %) | p-value |
|---|---|---|---|---|
| **Sex** | 1.00 (050–2.00) | 0.995 | – | – |
| **Age** | 0.99 (0.97–1.00) | 0.083 | 0.96 (0.93–0.99) | **0.003** |
| **ST1** | 1.67 (0.67–4.10) | 0.272 | – | – |
| **ST2** | 0.97 (0.40–2.36) | 0.942 | – | – |
| **ST3** | 0.77 (0.37–1.60) | 0.480 | – | – |
| **ST4** | 1.67 (0.67–4.11) | 0.272 | – | – |
| **ST6/7** | 0.31 (0.09–1.01) | 0.051 | – | – |
| **Country of origin** | 1.03 (0.90–1.17) | 0.664 | – | – |
| **Travelling history** | 1.02 (0.18–5.23) | 0.984 | – | – |
| **Oncology (actual/historic)** | 1.20 (0.24–5.99) | 0.828 | – | – |
| **Pruritus/urticaria** | 0.12 (0.05–0.28) | **< 0.001** | 0.07 (0.02–0.26) | **< 0.001** |
| **Eosinophilia** | 0.42 (0.17–1.03) | 0.058 | 0.24 (0.07–0.76) | **0.016** |
| **Other[a] gastrointestinal symptoms** | 2.83 (1.35–5.93) | **0.006** | 2.10 (0.70–6.31) | 0.185 |
| ***D. fragilis* co-infection** | 0.69 (0.34–1.40) | 0.307 | – | – |

[a] Non-specific symptoms including asthenia, fever, flatulence, vomiting, and weight loss.

gastrointestinal symptoms (p-value: 0.029) favoured the occurrence of abdominal pain and diarrhoea in *Blastocystis*-positive patients (Table 3).

In our multivariate regression analysis, we retained all the variables with p-value $< 0.09$ and less than 35 % missing values. Consequently, we observed that younger age (p-value: 0.003), cutaneous disorders (p-value $< 0.001$), and eosinophilia (p-value: 0.016) were statistically associated with the presence of gastrointestinal symptoms (abdominal pain and diarrhoea) in *Blastocystis*-positive patients.

### 3.5. Treatment

We also monitored the antibiotic/antiparasitic prescriptions for patients diagnosed with *Blastocystis* infections. Overall, 43.4 % (95/219) of patients received chemotherapy for their clinical episodes regardless they were co-infected with other gastrointestinal pathogen(s) or not. Of the treated patients, 69.5 % (66/95) were co-infected with at least one gastrointestinal pathogen, including 37 patients (38.9 %) carrying the mostly commensal protozoan *D. fragilis*. Co-infections in treated patients were of parasitic (52.6 %, 50/95), bacterial (22.1 %, 21/95) or viral (10.5 %, 10/95) nature, respectively. Among treated patients, 89.5 % (85/95) exhibited gastrointestinal symptoms including eosinophilia (33.3 %, 19/57), cutaneous disorders (15.8 %, 15/95), and nonspecific symptoms (36.8 %, 35/95). Additionally, 6.3 % (6/95) of treated patients did not display any sign or symptom that we could track.

Despite the 219 patients included in this study had a *Blastocystis* diagnosis, metronidazole was administered only in 35.2 % (77/219) of cases. In this group, 89.6 % (69/77) of patients had gastrointestinal symptoms, 13.0 % (10/77) reported pruritus/urticaria, 34.1 % (15/44) had eosinophilia, and 35.1 % (27/77) reported other collected symptoms. Remarkably, 67.5 % (52/77) of patients had some gastrointestinal co-infection by parasitic (including *D. fragilis*), viral, or bacterial pathogens.

### 3.6. Follow up

In total, 43.8 % (96/219) of the patients underwent subsequent PCR testing after their initial diagnosis, primarily to monitor the status of the *Blastocystis* infection. Among this group, 24.0 % (23/96) yielded a negative PCR result for *Blastocystis* sp., meaning a successful resolution of the infection. Remarkably, 30.4 % (7/23) of the patients that resolved the infection did so without receiving any treatment after their initial diagnosis. In contrast, 76.0 % (73/96) of patients continued to test positive to the protist, despite having received some form of treatment in 48.0 % of the cases (35/73). Notably, among the patients treated with metronidazole, the drug of choice for *Blastocystis*, 41 underwent follow-up testing, and only 31.7 % (13/41) successfully resolved the infection.

## 4. Discussion

This study aimed at establishing meaningful associations between the occurrence of *Blastocystis* subtypes in clinical patients and available epidemiological variables including clinical manifestations and management of the disease. Strengths of the study are i) the use of a highly sensitive real-time PCR-based assay for the accurate identification of *Blastocystis*-positive patients, ii) the genotyping at the subtype level of all *Blastocystis*-positive cases included in the survey, and iii) the identification, to the maximum extent possible, of co-infections with other gastrointestinal pathogens that can act as confounders in our analyses. Overall, 92.7 % (203/219) of our *Blastocystis*-positive cases were due to ST1-ST4, a finding consistent with the previous published literature in humans globally [3,5]. Among them, ST3 (29.7 %) was the most prevalent *Blastocystis* ST found in our patient population. It should be noted that ST3 is regarded as a primarily anthroponotic *Blastocystis* ST [21–23]. This does not seem to be the case in other Spanish populations, where ST2 was the predominant genetic variant identified. For instance, ST2 has been reported at frequency rates of 36–46 % in mostly asymptomatic toddlers and schoolchildren in the Madrid area [24–26], and of 62.3 % in a community survey in northern Spain [27]. In contrast, ST1 (70.6 %) was the predominant *Blastocystis* ST found in HIV + patients in Madrid [28].

The finding of *Blastocystis* ST6 or ST7 (subtypes typically adapted to infect avian hosts) in 7.8 % (17/219) of our patient population was also interesting. This infection rate is in the higher range of those reported for ST6/ST7 in clinical populations from other European countries such as France (2.1 %) [23], Poland (3.3–8.7 %) [29,30] and Sweden (1.6 %) [31]. Sporadic cases of ST6 have also been identified in studies conducted in France and Spain [32,33]. Remarkably, 64.7 % (11/17) of our ST6/ST7 patients reported contact with pet birds, suggesting that these infections were zoonotic in nature. Pet ownership can be an overlooked source of human infections by *Blastocystis* sp.

When assessing clinical manifestations potentially associated with *Blastocystis* infections, it is essential to note that stool samples analysed in hospital-based clinical microbiology laboratories are received on physician/general practitioner requests for referral testing, implying that patients are expected to exhibit clinical manifestations. However, a wide range of signs and symptoms caused by both infectious and non-infectious agents can lead to a stool testing request [34]. Indeed, co-infections by parasitic, viral, and bacterial pathogens were detected in 38.8 % (85/219) of our *Blastocystis*-positive patients. Among protozoa, diarrhoea-causing *G. duodenalis* (present in 2.3 % of our patient population) is widely known to have clinical relevance [34], whereas *D. fragilis* (present in 37.9 % of our patient population) is a common finding in asymptomatic individuals and its presence is not clearly associated with clinical outcomes [35]. This is the case of the present study, where *D. fragilis* was detected by real-time PCR with a mean $C_T$ value of 29.0, indicative of moderate-to-low parasite burden and, therefore, limited clinical significance.

Viral co-infection data was available from 68.5 % (150/219) of our patient population, with adenovirus, astrovirus, norovirus, and sapovirus being present at low (2.0–5.3 %) infection rates. All of them are well-recognized sources of diarrhoeal illness and other gastrointestinal manifestations [34]. Similarly, bacterial co-infection data was available for 88.1 % (193/219) of the investigated patients, with *Aeromonas* sp., *Campylobacter* sp., enteroaggregative and enterotoxigenic *E. coli*, and *Y. enterocolitica* being present in $\leq$ 2.1 % of the patients. Only enteropathogenic *E. coli* was found at a low-to-moderate frequency (13.7 %), although the exact clinical relevance of these findings may require further evaluation [34].

One of the main goals of this survey was to assess whether the enormous genetic variations between different subtypes of *Blastocystis* could result in differences in pathogenic potential (defined here as the capability of causing abdominal pain and/or diarrhoea). Several previous clinical, epidemiological, and experimental studies have pro-posed a link between *Blastocystis* STs and the occurrence of clinical manifestations. Indeed, ST4 has been identified as the predominant generic variant of *Blastocystis* in individuals with acute or chronic diarrhoea in Denmark [36] and Spain [37]. In addition, ST7 has been suggested to be a pathogenic subtype based on *in vitro* and *in vivo* mouse studies [38]. Confirming its potential pathogenicity to humans, ST7 was the predominant *Blastocystis* subtype found in patients presenting with diarrhoea and suspected *C. difficile* infection (known to be associated with gut dysbiosis) in Singapore [39]. In that study, *Blastocystis* and *C. difficile* co-infections were identified in 2.0 % (5/248) of patients, a rate similar to that (1.5 %, 2/133) found in the present survey. Additionally, 27.2 % (61/220) of Colombian patients with *C. difficile* infection carried also *Blastocystis* [40], suggesting that *Blastocystis* has the ability to thrive under dysbiotic conditions.

It should be also noted that recent population metagenomics studies have considerably challenged the pathogenic nature of *Blastocystis*. Indeed, *Blastocystis* colonization has been i) regarded as a common constituent of the healthy gut microbiota [41], ii) inversely associated with body mass index and obesity [42,43], and iii) linked to healthier

diets and more favourable cardiometabolic outcomes [43]. Taken together, these findings have lead some authors to propose a paradigm shift for *Blastocystis* from parasite to protector of human health [44].

To correctly assess the pathogenic potential of *Blastocystis* STs, we devoted a large effort to detect, to the best of our available resources, all co-infections by parasitic, viral, and bacterial intestinal pathogens that could be acting as confounders, biasing the obtained result and the conclusions reached. Therefore, *Blastocystis*-positive samples co-infected with other gastrointestinal pathogens known to cause clinical manifestations were removed from our analyses. The exception to this general rule was *D. fragilis* that, as discussed before, was regarded as a commensal protozoan rather than a pathogen. Under these circumstances, 77.6 % (170/219) of the recruited patients were considered in our statistical analyses. Of them, 74.7 % (127/170) presented with abdominal pain and/or diarrhoea.

Our univariate regression analysis showed that none of the STs identified in the present study were associated to a clear outcome of the *Blastocystis* infection. Only ST6/ST7 were close to reach statistical significance (p-value: 0.051). Similarly, occurrence of abdominal pain and/or diarrhoea was independent of the sex, age, country of origin, travelling history, eosinophilia level or *D. fragilis* carriage rate of the *Blastocystis*-positive patients. However, having pruritus/urticaria or nonspecific symptoms (*e.g.*, asthenia, fever, flatulence, vomiting, and weight loss) were risk factor for presenting abdominal pain and/or diarrhoea. Indeed, cutaneous disorders are common extraintestinal manifestations reported in *Blastocystis*-positive patients [16]. The observed correlation between the occurrence of intestinal and extraintestinal symptoms underscores the systemic implications of *Blastocystis* infection and the non-specific nature of some related signs and symptoms that seem to manifest in multiples forms [45].

In addition, multivariate regression analysis revealed that occurrence of gastrointestinal (abdominal pain and/or diarrhoea) followed an age-related pattern, with younger patients seeking medical assistance for gastrointestinal symptoms being more likely to have *Blastocystis* sp. as the underlying cause of their health issues compared to other age groups. This finding holds significant importance in terms of disease management, particularly for paediatric populations. Given the increasing concerns regarding antimicrobial resistance and the potential economic impact of choosing the wrong antibiotics, proper infection management in younger age groups becomes imperative [46]. Despite many studies have indicated higher infection rated of *Blastocystis* sp. in older age groups (as it is also the case of the present survey), information on the frequency of the parasite in symptomatic and asymptomatic individuals according to their age group is far scarcer [47].

Regarding treatment, we observed that less than half (43.4 %) of our *Blastocystis*-positive patients received some form of it. Metronidazole is the drug of choice for *Blastocystis* sp. infections [3,48], although its effectiveness against this protist has not been fully proven [48]. However, metronidazole was administered to just 35.2 % of our *Blastocystis*-positive patients. This suggests a lack of awareness regarding the importance of the correct detection and management of this infection, emphasizing the need for more research on this protist. It's worth noting that among the patients treated with metronidazole, 41 underwent follow-up testing, and only 13 showed a negative result for *Blastocystis* sp. infection, which aligns with previous studies that have reported the frequent inefficacy of this drug and its potential contribution to the development of resistances [49]. This raises important questions about the effectiveness and necessity of the treatment protocol and whether an alternative antibiotic, such as paromomycin or trimethoprim-sulfamethoxazole, might be more appropriate [50].

This study has some limitations that might have biased some of the obtained results and the conclusions reached. These include i) its retrospective nature and reliance on clinical and epidemiological data collected from patient' medical records, which can vary in detail and quality, and even being absent in some instances, ii) lack of a control group including *Blastocystis*-positive individuals without clinical manifestations, iii) our analysis of viral and bacterial co-infections did not cover the whole recruited patient population, and iv) the follow-up of the *Blastocystis* infection status was dependent on the clinician's request.

## 5. Conclusions

Our study provides novel data on the molecular diversity and clinical significance of *Blastocystis* sp. in a hospital-related setting. In addition, it also examines the current management practices in place. Key findings of this study include:

- Within our study population, the main *Blastocystis* subtypes identified were ST1-4, with ST3 as the predominant subtype.
- The occurrence of clinical manifestations (abdominal pain and/or diarrhoea) is independent on the *Blastocystis* subtype involved in the infection.
- The occurrence of *Blastocystis*-associated clinical manifestations followed an age-related pattern, with younger patients being more likely to exhibiting them.
- Symptomatic *Blastocystis*-positive patients often present with multiple unspecific manifestations, such as skin pruritus.
- Treatment for *Blastocystis* infection was administered in a limited number of cases, and the recommended drug of choice, metronidazole, was underutilized.
- Metronidazole treatment did not exhibit a high efficacy rate.
- Further research is necessary to comprehend the clinical significance of *Blastocystis* in affected populations and their impact in the healthcare system's workload, including proper management and treatment.

## Funding

This research was partially funded by the Health Institute Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness [grant number PI19CIII/00029].

## CRediT authorship contribution statement

**Laura Seijas-Pereda:** Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Pamela C. Köster:** Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alejandro Dashti:** Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Begoña Bailo:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Isabel Guadano-Procesi:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Rescalvo-Casas:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marcos Hernando-Gozalo:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juan Cuadros-González:** Visualization, Validation, Supervision, Resources, Project administration. **David Carmena:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition. **Ramón Pérez-Tanoira:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration.

## Declaration of competing interest

We declare no competing interests.

## Acknowledgments

This article is based upon work from COST Action *Blastocystis* under One Health, CA21105, supported by COST (European Cooperation in

Science and Technology). We thank the University Hospital Príncipe de Asturias and the University of Alcala for technical and logistic support.

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