Molecular characterization of Blastocystis subtypes in HIV-positive patients and evaluation of risk factors for colonization

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Abstract

Background Blastocystis is one of the most common intestinal protozoa in human faecal samples with uncertain impact on public health. Studies on the prevalence of Blastocystis in HIV-positive patients are limited and dated. Methods A cross-sectional study was carried out involving 156 HIV-positive patients to evaluate the prevalence of Blastocystis-subtypes by molecular amplification and sequencing of the SSU rDNA gene, to identify the risk factors for its transmission, to examine the relationship between the presence of the protist and gastrointestinal disorders. Furthermore, the evaluation of the faecal calprotectin by immunoassay from a sample of subjects was performed to evaluate the gut inflammation in Blastocystis-carriers. Results Blastocystis-subtypes ST1, ST2, ST3, ST4 were identified in 39 HIV-positive patients (25%). No correlation was found between the presence of the protist and virological or epidemiological risk factors. Blastocystis was more frequently detected in homosexual subjects (p=0.037) infected by other enteric protozoa (p=0.0001) and with flatulence (p=0.024). No significant differences in calprotectin level was found between Blastocystis-carriers and free ones. Conclusions Blastocystis is quite common in HIV-positive patients on ART showing in examined patients 25% prevalence. Homosexual behaviour may represent a risk factor for its transmission, while CD4 count and viremia didn’t correlate with the presence of the protist. The pathogenetic role of Blastocystis remains unclear and no gut inflammation status was detected in Blastocystis-carriers. The only symptom associated with Blastocystis was the flatulence, evidencing a link between the presence of the protist and the composition and stability of gut microbiota.

Background

Blastocystis spp. is a common intestinal protist distributed worldwide infecting humans
and animals, with a prevalence from 0.5%-30% and 30-76% in industrialized and developing countries, respectively [1]. This difference can be explained by poor hygiene practices and consumption of contaminated food or water [2, 3, 4, 5] since the faecal-oral route is considered to be the main mode of transmission of this protist [6]. A remarkable genetic diversity has been revealed among Blastocystis spp. isolates from humans and animals based on the comparison of the small-subunit (SSU) rRNA gene sequences [7]. Currently at least 26 subtypes of Blastocystis have been described among mammalian and avian isolates [8, 9], nine of them (ST-1 to ST-8 and ST-12) detected in human population and are potentially zoonotic [10]. Because several STs are shared between humans and animals it has been proposed that a proportion of human infections may result from zoonotic transmission. Indeed, a higher risk of Blastocystis infection was found in people with close animal contact, including zookeepers [11]. Despite several studies reported Blastocystis implicated in different intestinal diseases, potential pathogenetic factors have been described and its presence is frequently associated with symptoms in humans [12], its pathogenetic role is so far under debated and several variables, as well as the Blastocystis subtype and load, host’s immune status and dysbiosis, could affect the occurrence of the disease [13][14].

Although the protist was never considered as an opportunistic protozoon, it has also been frequently found in immunocompromised individuals presenting diarrhoea, with prevalence value ranging from 15% to 72.4% [15]. In addition, the sexual practices among men who have sex with men (MSM) have been reported to increase the transmission of Blastocystis (as for as other enteric organisms) [16]. Finally, recent data suggest that the protozoon is associated with certain gut microbiota profiles and health indices.

Accordingly, a positive correlation between high bacterial richness and the presence of Blastocystis has been reported [17].
Notwithstanding it is commonly accepted that *Blastocystis* is a non-invasive organism, its vacuolar form is able to colonize the lamina propria, the submucosa and even the muscle layers leading to inflammation and active colitis in experimentally infected mice [18]. Currently, it is possible to perform a non-invasive evaluation of gut inflammation using biomarkers as the fecal calprotectin (FC), which is considered a surrogate marker of intestinal inflammation with good diagnostic performance for separating organic and functional intestinal disorders [19]. The increase of FC is due to faecal excretion of neutrophils and macrophages migrants from the bloodstream into the intestinal lumen, which occurs during the intestinal inflammation. The value of FC as a laboratory marker has been shown in inflammatory bowel disease (IBD) [20] but its significance in other gastrointestinal diseases remains unclear. Studies evaluating the correlation between intestinal inflammatory markers and parasitic infections are scant [21, 22, 23, 24]. However, a positive correlation between the level of FC and severe infections due to intestinal parasites such as *Giardia intestinalis* and *Schistosoma mansoni* has been reported [21, 22]. Conversely, lower concentrations of FC have been reported in *Blastocystis* colonized individuals compared to non-colonized subjects [25].

This study aimed to evaluate the prevalence of *Blastocystis* subtypes in HIV-positive patients, to evaluate the potential the risk factors for its transmission and the relationship between the presence of the protozoon and gastrointestinal symptoms and, finally, to assess the level of FC in *Blastocystis*-colonized subjects.

**Methods**

**Study population**

A cross-sectional study was carried out from January 2016 to September 2017 involving HIV-positive patients followed at the Department of Translation and Precision Medicine, Umberto I Academic Hospital, Rome. A standardized questionnaire was designed to collect
information about each participating subject (gender, age, profession, country of origin, recent travels and destinations, exposure to domestic animals, diet, sexual behaviour, comorbidity) in addition to clinical data regarding the presence of gastrointestinal symptoms (diarrhoea, abdominal pain, nausea, anorexia, weight loss, weakness, flatulence). According to World Health Organization definitions, diarrhoea was defined as the passage of three or more loose or liquid stools per day.

Written informed consent was obtained from every participant. The study was approved with respect to the Helsinki Declaration by the Ethical Committee of the Umberto I Academic Hospital (licence n. 4836).

**Laboratory analyses**

Blood and faecal samples were collected from each subject included in the study and submitted to the quantitative measurements of CD4+ T-lymphocytes and viral load [26], and to the microscopic observation of the wet smears stained with Lugol, directly or after Ridley concentration [27]. Genomic DNA was then extracted from stool samples and submitted to PCR amplification using primers previously described [28], which target a fragment of about 600 bp from the *Blastocystis*-SSU rDNA gene, following PCR protocol and conditions described in Mattiucci et al., 2016 [13]. The sequences obtained were compared to those of *Blastocystis* spp. deposited in GenBank using the BLAST application (www.ncbi.nlm.nih.gov/BLAST). The STs were identified by determining the exact match or closest identity (99%), according to the classification given by Stensvold et al., 2007 [29]. Furthermore, the evaluation of the FC from a sample of subjects was performed using a commercial immunoassay (Calprest, EUROSPITAL, Italy), following the manufacturer’s instructions. Samples giving values above 50mg/kg were regarded as having a positive Calprest test, as reported by the manufacturer and by previous published studies [30].

**Statistical analysis**
Continuous variables were summarized as mean ± standard deviation or median ± interquartile range (IQR) and categorical data as counts and percentages. Comparisons between groups were performed using $\chi^2$ test or Fisher’s exact test for categorical variables, and t-test or Mann-Whitney test for continuous variables. Multiple logistic regression was used to identify the predictors of *Blastocystis* colonization. The significance level for all analyses was set at $p < 0.05$. Data were analysed using IBM SPSS, version 21.0 (SPSS Inc, Chicago, USA).

**Results**

A total of 156 consecutive HIV-positive patients were enrolled in the study between January 2016 and September 2017. Demographic and clinical data of the study population are described in Table 1. The majority of subjects were males (75%), mean age was 47.05 ± 12.38 (range 22 to 71 years), 39 participants (25%) were coming from non-EU countries and 50 ones (32%) were MSM (*men who have sex with men*). Moreover 47.4% were owner of pets and 42.9% travelled outside Europe in the last 6 months. Twenty-three patients (14.7%) were naïve and 133 (85.3%) were on ART treatment. Thirty-five subjects (26.3%) were on ART based on non-nucleoside reverse-transcriptase inhibitors (NNRTI), 34 (25.6%) on protease inhibitors (PI/r), 36 (27.1%) on integrase inhibitors and 28 (21%) on dual therapy. Three of the enrolled patients were on prophylaxis treatment with sulfamethoxazol-trimethoprim. The mean CD4 cell count was 655.04 ± 381.56 cells/mm$^3$. Most of patients (62.2%) showed a CD4 count higher than 500 cells/ml, 26.3% patients had a CD4 count between 200 and 500 cells/ml and only 11.5% patients showed a CD4 count lower than 200 cells/ml. The median HIV-RNA level of naive patients was 40010 (7093-824500) -copies/mL. Among patients on ART treatment, HIV-RNA was detectable (>37 cp/mL) in 15.8% of subjects (21/133) with a median HIV-RNA level of
116 (67-991) cp/mL.

**Study population**

| Characteristic                      | N (%)          |
|-------------------------------------|----------------|
| Gender, male                        | 117 (75%)      |
| Age, years (mean ± SD)              | 47.05 ± 12.38  |
| Foreign origin                      | 39 (25%)       |
| Domestic animals                    | 50 (32.0%)     |
| Travels                             | 74 (47.4%)     |
| CD4 count, cells/ml, mean ± SD     | 67 (42.9%)     |
| HIV-RNA (<37 copies/ml)            | 655.04 ± 381.56|
| Naïve subjects                      | 112 (71.8%)    |
| 2NRTI + NNRTI                       | 23 (14.74%)    |
| 2NRTI + IP/r                        | 35 (26.3%)     |
| 2NRTI + INI                         | 34 (25.6%)     |
| Dual therapy                        | 36 (27.1%)     |

Table 1. Epidemiological, demographic, immunologic and virological characteristics of the enrolled patients (N=156). A total of N=23 were naïve and N=133 on ART.

Microscopic examination revealed the presence of *Blastocystis* spp. in 34 patients (21.8%). Molecular and sequence analysis identified the protist in 39 individuals (25%), allowing the characterization of 4 different STs. ST3 was the most common subtype found in 51.3% of the subjects, followed by ST1 (30.8%), ST4 (10.2%) and ST2 (7.7%). As expected, ST4 was identified only in European participants. In 30.7% of the subjects, *Blastocystis* was found in coinfection with other enteric protozoa as *Entamoeba coli* (15%), *Endolimax nana* (8%), *Giardia intestinalis* (6.1%) and *Iodamoeba butschlii* (1.6%). The detection of such intestinal parasites was more frequent in *Blastocystis*-carriers compared to *Blastocystis*-free ones (p<0.0001) and in MSM compared to heterosexual subjects (p<0.0001).

Neither demographic characteristics such as gender, age and nationality nor other epidemiological risk factors such as travel history or, presence of domestic animals differed significantly between the *Blastocystis* positive and *Blastocystis* negative patients (Table 2). In addition, no significant differences were found between the two groups regarding CD4 T cell counts, HIV-RNA undetectability and type of ART regimen.

*Blastocystis* positive subjects reported more frequently homosexual behavior practices compared to negative ones (48.71% vs 26.49%, p=0.037). After adjusting for age, the
relationship between homosexual behavior practices and the presence of *Blastocystis*

remained significant (p=0.01).

|                           | Blastocystis-carriers | Blastocystis-free | p-value |
|---------------------------|-----------------------|-------------------|---------|
| Gender, male              | 33 (82.0%)            | 84 (72.6%)        | 0.289   |
| Age, years (mean ± SD)    | 44.71 ± 11.33         | 47.05 ± 12.37     | 0.06    |
| Foreign origin            | 15 (38.46%)           | 25 (21.36%)       | 0.138   |
| MSM                       | 19 (48.71%)           | 31 (26.49%)       | 0.037*  |
| Domestic animals          | 18 (46.15%)           | 56 (47.86%)       | 0.721   |
| Travels                   | 20 (51.28%)           | 47 (40.17%)       | 0.296   |
| CD4 count, cells/ml, mean ± SD | 637.43 ± 263.32   | 660.90 ± 414.35   | 0.823   |
| CD4 count > 500 cells/ml  | 28 (71.79%)           | 69 (58.97%)       | 0.484   |
| CD4 count 200–500 cells/ml| 8 (20.51%)            | 33 (28.20%)       | 0.257   |
| HIV-RNA (<37 copies/ml)   | 32 (82.05%)           | 80 (68.37%)       | 0.037*  |
| CD4 count <200 cells/ml   | 7 (17.94%)            | 27 (23.07%)       | 0.765   |
| 2NRTI + NNRTI             | 7 (17.94%)            | 26 (22.22%)       | 0.836   |
| 2NRTI + IP/r              | 10 (25.64%)           | 20 (17.09%)       | 0.283   |
| 2NRTI + INI               | 12 (30.76%)           | 17 (14.52%)       | 0.024*  |
| Dual therapy              | 3 (7.69%)             | 6 (5.12%)         | 0.887   |
| Naive subjects            | 3 (7.69%)             | 6 (5.12%)         | 0.658   |

Table 2. Comparison of epidemiological, demographic, immunologic and virological characteristics between *Blastocystis*-carrier (N=39) and *Blastocystis*-free (N=117) patients. Significant results were marked with *.

Most of the *Blastocystis* positive subjects were symptomatic (53.8%), while 33.6% *Blastocystis*-free ones referred gastrointestinal disorders (p=0.029). Among the gastrointestinal symptoms analyzed, flatulence was more frequently observed in *Blastocystis* carriers (30.7%) compared to *Blastocystis* negative ones (14.5%) (p=0.024) (Table 3).

| Symptoms         | Blastocystis-carriers | Blastocystis-free | p-value |
|------------------|-----------------------|-------------------|---------|
| Abdominal pain   | 7 (17.94%)            | 26 (22.22%)       | 0.836   |
| Diarrhoea        | 10 (25.64%)           | 20 (17.09%)       | 0.283   |
| Flatulence       | 12 (30.76%)           | 17 (14.52%)       | 0.024*  |
| Nausea           | 3 (7.69%)             | 6 (5.12%)         | 0.887   |
| Poor appetite    | 5 (12.82%)            | 10 (8.54%)        | 0.180   |
| Weight loss      | 3 (7.69%)             | 10 (8.54%)        | 0.658   |

Table 3. Gastrointestinal symptoms observed in *Blastocystis*-carrier (N=39) and *Blastocystis*-free (N=117) patients. Significant results were marked with *.

To determine whether *Blastocystis* colonization was associated with intestinal inflammation (as inferred by the Rome III criteria questionnaires), we measured FC in a
subset of samples divided in *Blastocystis*-carrier (mono-infected) (N=23) and *Blastocystis*-free (N=36) subjects from all 59 fecal samples (*Blastocystis*-carrier and *Blastocystis*-free), 83% had a value in the standard range (lower than 50 mg/kg) and 11 (18.6%) had an increased value (median 115.7; 95% CI=71.82-303.78). However, no significant differences in calprotectin values was found between *Blastocystis*-carriers (median 15, 95% CI= 18.93-148.53) and non-carriers (median 13.35, 13.93-31.74) (p=0.31).

Discussion

Studies on the prevalence of *Blastocystis* spp. in HIV-positive subjects are relatively scant. The only study conducted on Italian HIV-positive patients date back to 1999, through the microscopic method, showed a *Blastocystis* prevalence of 10.3% [31]. The current study is, to our knowledge, the first to assess the prevalence of *Blastocystis*, through molecular methods in Europe and reported a prevalence of about 25%, in line with that reported in previous studies conducted in Mexico (30%) [32], Malaysia (19.8%) [15] and Iran (19%) [33]. The molecular characterization of *Blastocystis* positive isolates evidenced the circulation of 4 subtypes (ST1, ST2, ST3, ST4) already described in Italy in previous surveys [13, 34], being ST3 the most common one with a prevalence of 51%. In our cohort of HIV-positive patients, the presence of *Blastocystis* resulted no correlated to viro-immunological factors (p>0.05) confirming therefore the non-opportunistic behavior of the protist. No demographic characteristic or epidemiological drivers (presence of domestic animals, dietary habits, travels abroad) were found to be associated with *Blastocystis* colonization. A higher prevalence of *Blastocystis* was reported in MSM compared to heterosexuals as well as of the other intestinal parasites, underlying the faecal-oral contact as the main route of transmission in this group of subjects and the sexual practice and lifestyle linked to the presence of the protist more than the immunological status [16].
Despite the unresolved controversy over its pathogenicity, *Blastocystis* was also been frequently found in immunocompromised individuals presenting diarrhea [15]. In our study, among gastrointestinal symptoms, a positive association was found only for the flatulence. This socially disabling symptom is determined by two main factors: the diet, particularly the quantity of fermentable residues, and the composition and metabolic activity of colonic microbiota [35]. Since we evidenced any differences in the diet between *Blastocystis*-carriers and *Blastocystis*-free subjects, we hypothesized that this symptom was linked to the presence of the protist and the gut microbiota composition. Several Authors evaluated the intestinal microbiome in HIV-positive subjects, with somewhat inconsistent or controversial results [36]. This may be because of small sample sizes, lack of appropriate controls or regional differences in dietary and environmental factors. Regardless, many authors evidenced a change in the Bacteroides:Prevotella ratio, which they suggested to be linked to the antiretroviral therapy [37, 38] or to the sexual practice and lifestyle [39]. Similarly, several studies reported *Blastocystis* colonization associated with higher bacterial richness and Prevotella enterotype [40]. Therefore, investigations on the gut microbiota from HIV-positive subjects *Blastocystis*-carriers are needed to complete and to better understand the preliminary results obtained in this study.

Concerning FC, we found the median of both *Blastocystis*-carrier and free subjects within the normal range, supporting the non-pathogenic role of *Blastocystis* in inducing intestinal inflammation [25].

**Conclusions**

Despite some critical points of this study concerning the lack of data about the faecal microbiota from enrolled subject, to our knowledge this is the first large survey to molecular characterize *Blastocystis* subtypes in HIV-positive patients in Europe. Our results suggest that *Blastocystis* is quite common in such patients and homosexual
behavior resulted a risk factor for its transmission and confirm the presence of the protist not associated with pathological gut inflammation. The role of Blastocystis as intestinal pathogen remains unclear as the only symptom associated with its presence was flatulence. This result is intriguing since flatulence could be linked to the gut microbial communities, therefore the impact of the protist on the faecal microbiota and its possible role to maintain the gut homeostasis could be interesting prospects for further studies.

List Of Abbreviations

ART: antiretroviral therapy; CI: Confidence interval; FC: fecal calprotectin; HIV: Human Immunodeficiency Virus; MSM: men who have sex with men; NNRTI: non-nucleoside reverse-transcriptase inhibitors.

Declarations

Authors’ contributions

LFS and SG designed the study with input from SM and GT; conducted the data analysis, interpretation, and manuscript preparation. FF performed the laboratory analyses and molecular identification and contributed to the manuscript preparation. EB, GI, CT and MM enrolled the patients, performed the sample collection and contributed to the data analysis, data interpretation, and manuscript preparation. SM and GT interpreted the results and revised the manuscript. All authors critically reviewed and approved the final version of this paper for publication.

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Availability of data and materials
The dataset of this article will not be available publicly, to ensure the patient’s privacy, but are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The study protocol was approved by the ethics committees of Umberto I Academic Hospital (licence n. 4836) in accordance with the ethical guidelines of the Declaration of Helsinki. Written informed consents were obtained from the patients after declaring the objectives of the present study

**Competing interest**

The authors declare that they have no competing interests

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