

Subtypes of *Blastocystis* sp. isolated in fecal samples from transplant candidates in São Paulo, Brazil

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ABSTRACT

Blastocystis sp. is an intestinal protozoan commonly found in fecal samples of many animal species, including humans, but poorly studied in transplant candidates. The aim of this study was to evaluate the occurrence and molecular identification of *Blastocystis* sp. in fecal samples from transplant candidates. A polymerase chain reaction was performed using specific primers for *Blastocystis* ribosomal DNA. The DNA sequences obtained were aligned and compared with other sequences from the GenBank and MLST databases. The analyzed samples showed a positivity of 16% (24 of 150) for *Blastocystis* sp. The highest occurrence was observed in renal transplant candidates (31.4%), followed by hepatic transplant candidates (10.4%) and candidates for bone marrow transplantation (5.9%). Subtype (ST) 3 (45.8%) was the most prevalent among the isolates, followed by ST1 (37.5%), ST2 (12.5%), and ST7 (4.2%). This is the first study of molecular identification *Blastocystis* sp. in transplant candidates. Our results confirmed that ST3 was the most common subtype in transplant candidates and reinforce the importance of new studies to investigate of *Blastocystis* sp. in these patients.

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1. Introduction

One of the major challenges facing transplant candidates is the clinical complications that can occur after organ transplantation (Rao et al., 2003). Intestinal infections may occur after transplantation, leading to important clinical manifestations such as diarrhea or dysentery, increasing the morbidity and mortality rates in this population (Azami et al., 2010; Rao et al., 2003).

Blastocystis sp. is an intestinal protozoan found in fecal samples from many animal species, including humans (Stensvold and Clark, 2016). This organism is present worldwide, with a prevalence ranging from 30% to 60% in developing countries (Tan, 2008). In Brazil, its occurrence ranges from 1.4% to 57.8% (Borges et al., 2009; Carvalho-Costa et al., 2007). Some studies have associated the presence of *Blastocystis* sp. with non-specific gastrointestinal symptoms such as nausea, abdominal pain, and chronic diarrhea, which may be severe in immunocompromised patients (Zhang et al., 2017; Kulik et al., 2008; Rao et al., 2003; Garavelli et al.,

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1991). In recent studies, the occurrence of *Blastocystis* sp. has been found to be high in fecal samples of immunocompromised patients (Zhang et al., 2017; Rasti et al., 2017).

Several studies have evaluated the occurrence of *Blastocystis* sp. subtypes in fecal isolates (Melo et al., 2017; Ramírez et al., 2016; Malheiros et al., 2011), but to date there are no reports on the subtypes found in transplant candidates. Thus, the aim of this study was to evaluate the occurrence and molecular identification of *Blastocystis* sp. subtypes in fecal samples from transplant candidates.

2. Materials and methods

2.1. Study population

This retrospective study was conducted on stool specimens from transplant candidates and was approved by the Research Ethics Committee of Hospital das Clínicas of Faculdade de Medicina, Universidade de São Paulo, São Paulo State (HC/FMUSP) from 2011 to 2013 (protocol no. 0123/10). A total of 150 fecal samples from transplant candidates were analyzed: 51 renal transplant candidates (RT), 48 hepatic transplant candidates (HT), and 51 bone marrow transplant candidates (BMT). Of the 150 patients, 56 (37.3%) were females and 94 (62.7%) were males aged from 19 to 83 years. The indications for the transplants were diabetic nephropathy, chronic renal failure, lupus nephritis, and post-sepsis nephropathy for RT; cirrhosis due to hepatitis B and C virus, alcoholic, or cryptogenic, non-alcoholic steatosis, and autoimmune hepatitis for HT; multiple myeloma, acute myeloid leukemia, non-Hodgkin lymphoma, Hodgkin disease, severe aplastic anemia, and myelofibrosis for BMT. Many of these underlying diseases do confer some degree of immune dysfunction.

At the time of collection, the fecal samples were analyzed by spontaneous sedimentation, modified Baermann, and agar plate culture techniques (Garcia, 2001). The remainder of the fecal samples were divided into aliquots and stored at $-20\text{ }^{\circ}\text{C}$ for subsequent molecular analysis.

2.2. Molecular analysis

Approximately 200 mg of fecal sample was used for extraction of DNA with a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The DNA extracted was amplified by conventional polymerase chain reaction (PCR), using specific primers: BhRDr (R: 5'-GAG CTT TTA ACT GCA ACA ACG-3') and RD5 (F: 5'-ATC TGG TTG ATC CTG CCA GT-3') located in *SSU-rDNA* of *Blastocystis* sp. that amplify a fragment of ~600 bp (Sciicluna et al., 2006). The amplification reaction conditions were as described by Melo et al. (2017) in a final volume of 10 μL containing $\sim 50\text{ ng }\mu\text{L}^{-1}$ DNA, 2.0 μg of BSA, 0.2 mM each dNTP, 1.5 mM MgCl_2 , 2 pM each primer (forward and reverse), 1 \times PCR buffer, and 1.25 U of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA). PCR amplification was conducted using a Master cycler Ep gradient S thermocycler (Eppendorf, Hamburg, Germany) and the following conditions: initial denaturation step at $94\text{ }^{\circ}\text{C}$ for 2 min; 30 cycles at $94\text{ }^{\circ}\text{C}$ (denaturation) for 1 min, $61\text{ }^{\circ}\text{C}$ (annealing) for 1 min, and $72\text{ }^{\circ}\text{C}$ for 1 min (extension); and a final extension step of $72\text{ }^{\circ}\text{C}$ for 2 min. Negative (PCR mix with water) and positive (PCR mix with *Blastocystis* sp. culture samples) controls were included in all molecular analysis. Controls for the DNA extraction were the following universal primers: 18SEUDIR (F: 5'-TCT GCC CTA TCA ACT TTC GAT GG-3') and 18SEUINV (R: 5'-TAA TTT GCG CGC CTG CTG-3') located in the eukaryotic *SSU-rDNA* region (Wang et al., 2013).

PCR-positive products were initially purified by the enzyme ExoSAP (GE Healthcare, Piscataway, NJ, USA), and then sequenced by the Sanger method (Sanger et al., 1977). The sequences were evaluated for quality and to determine the subtype (ST) and allele (a) of each sample; the sequences were aligned with other *Blastocystis* sequences present in GenBank using the BLAST tool and the *Blastocystis* Multilocus Sequence Typing online database. The sequences were submitted to GenBank under number: MN658570-MN658583, MN718159, MN686280-MN686283 and MN658567-MN658569.

Table 1
Distribution of subtypes of *Blastocystis* sp. (ST) in fecal samples of transplant candidates from the HC/FMUSP.

	Transplant candidates					
	RT (n = 51)		HT (n = 48)		BMT (n = 51)	
	No.	%	No.	%	No.	%
ST1	8	50.0	–	–	1	33.3
ST2	1	6.25	2	40.0	–	–
ST3	6	37.5	3	60.0	2	66.7
ST7	1	6.25	–	–	–	–
Total	16	100.0	5	100.0	3	100.0

RT, renal transplant; HT, hepatic transplant; BMT, bone marrow transplant.

3. Results

The analysis of the parasitology showed that 12.7% (19 of 150) patients were positive, 11 RT patients (9 for *Strongyloides stercoralis* and 2 for *Entamoeba coli*), 4 HT patients (3 for *S. stercoralis* and 1 for *Endolimax nana*), and 4 BMT patients (2 for *S. stercoralis* and 2 for *E. coli*).

Amplification of *Blastocystis* DNA was observed in 16% (24 of 150) of samples, 31.4% (16 of 51) from RT patients, 10.4% (5 of 48) from HT patients, and 5.9% (3 of 51) from BMT patients. After sequences analysis against the databases, all 24 sequences showed good quality and confirmed *Blastocystis* sp. DNA, with identity ranging from 87% to 100%.

Of the samples confirmed by sequencing as *Blastocystis* sp., the following subtypes were identified: ST1 in 9 (37.5%) samples, ST2 in 3 samples (12.5%), ST3 in 11 samples (45.8%), and ST7 in 1 sample (4.2%). The following subtypes were identified in RT candidates: ST1 (n = 8), ST2 (n = 1), ST3 (n = 6), and ST7 (n = 1). For HT candidates, the subtypes identified were ST2 (n = 2) and ST3 (n = 3); and for BMT candidates, the subtypes were ST1 (n = 1) and ST3 (n = 2) (Table 1).

MLST analysis of the sequences showed alleles 4 (8 of 9) and 78/81 (1 of 9) among ST1; alleles 11 (1 of 3) and 12 (2 of 3) among ST2; alleles 34 (4 of 11), 36 (4 of 11), 37 (2 of 11) and 54 (1 of 11) among ST3; and allele 96 in ST7 isolate (Fig. 1).

4. Discussion

Epidemiological studies show that the occurrence of *Blastocystis* sp., a gastrointestinal protozoan, is high (Piubelli et al., 2019; Azami et al., 2010; Kulik et al., 2008). In Brazil, molecular studies have reported positivity ranging from 40.7% to 71% (Oliveira-Arbex et al., 2018; David et al., 2015). However, this is the first study to investigate the occurrence and molecular characterization of *Blastocystis* sp. in transplant candidates. Our results showed a positivity of 16%, which is lower than that in other studies in Brazil (Oliveira-Arbex et al., 2018; David et al., 2015).

There is a lack of reports about *Blastocystis* sp. in the study population. Considering chronic renal failure as a possible indication for RT, parasitology studies in Brazil showed a positivity of 20.1% (Kulik et al., 2008) and 24.5% (Gil et al., 2013) for *Blastocystis* sp. In addition, Naeini et al. (2012) reported 4.7% positivity for *Blastocystis* sp. in RT patients. Rao et al. (2003) described these protozoa as an important agent responsible for diarrhea in this group. In the present study, we observed positivity of 31.4% for *Blastocystis* sp. in RT patients by molecular analysis. Different results were demonstrated recently by Zhang et al. (2017) who reported occurrence of 4.4% for *Blastocystis* sp. in hemodialysis patients using molecular analysis.

To date, there have been no previous studies investigating colonization by *Blastocystis* sp. in transplant candidates, especially HT and BMT candidates; it was therefore not possible to compare our findings with any other studies. The present study showed *Blastocystis* sp. DNA in 10.4% and 5.9% of the samples from HT and BMT candidates, respectively. A previous analysis of samples from patients with hematologic malignancies demonstrated that 16% were positive for *Blastocystis* sp., which was less than for BMT patients (Poirier et al., 2011). One possible explanation for the variation in positivity may be the molecular technique used; in the present study, we used conventional PCR, whereas Poirier et al. (2011) used real-time PCR. In addition, the different epidemiologic conditions in the region where the samples were obtained may explain the differences in positivity for *Blastocystis* sp.

In this study, the subtypes identified were ST3 (45.8%), ST1 (37.5%), ST2 (12.5%), and ST7 (4.2%), similar to the reports in the literature (Ramírez et al., 2016; Malheiros et al., 2011). According to Alfellani et al. (2013), most human isolates correspond to ST3, followed by ST1, ST2, and ST4. However, ST4 was not isolated in the present study, which can be explained by the geographic distribution of this subtype. It has been proposed that only 2% of isolates in South America correspond to ST4 (Ramírez et al., 2016). ST1–3 have been reported to have the highest occurrence in several countries (Jiménez et al., 2019; Alfellani et al., 2013; Meloni et al., 2011) and in Brazil (Seguí et al., 2018; Melo et al., 2017; David et al., 2015). ST5–9 are rarely found in humans; ST6 and ST7 are commonly detected in birds (Jiménez et al., 2019; Ramírez et al., 2016; Alfellani et al., 2013). On other hand, Brazilian studies conducted in the state of São Paulo detected ST6 in human samples (Melo et al., 2017; David et al., 2015). In the current study, ST6 was not observed, but ST7 was isolated in 4.2%, similar to the findings reported recently (Oliveira-Arbex

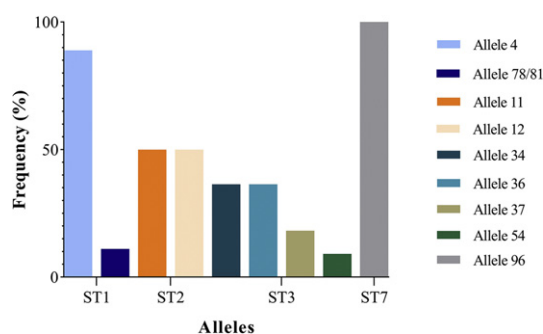


Fig. 1. Frequency of *Blastocystis* spp. subtypes/alleles in transplant candidates from the HC/FMUSP.

et al., 2018). Meloni et al. (2011) found ST7 and ST8 in 2.9% of human isolates in Italy. Of note all isolates had single ST infections and none evidence of mixed infections was found.

The genetic diversity of *Blastocystis* sp. may be related to the possible pathogenic potential of this organism (Stensvold and Clark, 2016). In the current study, ST1, ST2, ST3, and ST7 were observed in RT candidates, ST2 and ST3 in HT candidates, and ST1 and ST3 in BMT candidates. ST3 was found in all 3 groups, whereas ST1 was not detected in HT candidates, and ST2 was not isolated from BMT candidates. No relationship was found between the subtypes and the indications for the transplant. In the present study, only 2 subtypes, ST1 and ST3, were found in BMT candidates, which may be due to the low occurrence of *Blastocystis* sp. in this group.

Different regions of *Blastocystis* DNA have recently been proposed for subtype differentiation, such as rDNA internal spacers (Villalobos et al., 2014), mitochondrial markers (Poirier et al., 2014), or the pyruvate ferredoxin oxidoreductase gene (Alarcon-Valdes et al., 2018). However, analysis of the multilocus gene sequences (MLST) has been shown to be an important tool for distinguishing taxa, especially from problematic and enigmatic species such as *Blastocystis* sp. (Ramírez et al., 2016).

In our findings, alleles 34, 36, and 37 were identified within ST3, reinforcing the greater genetic variability within this subtype. Although there are few MLST studies, some authors point to the importance of sequence analysis methods to improve the understanding of the possible pathogenicity of *Blastocystis* sp. subtypes/alleles (Stensvold and Clark, 2016).

The present study has limitations; the main one is related to the techniques used for parasitologic diagnosis, which may make it impossible to detect *Blastocystis* forms in fecal samples and, consequently, give negative parasitologic results. In addition, the study was retrospective; when the samples were collected, the main objective was to search for *S. stercoralis* larvae. Moreover, knowing the difficulty of identifying *Blastocystis* forms, positivity is operator dependent. This may explain the absence of positive parasitology for *Blastocystis*. Thus, this study focused on identifying *Blastocystis* using only molecular tools rather than parasitology techniques.

5. Conclusion

This is the first study on molecular identification of *Blastocystis* sp. in transplant candidates. Our molecular analysis describes a higher occurrence of ST3, followed by ST1, ST2, and ST7, in fecal samples of transplant candidates, and reinforces the importance of new studies to investigate *Blastocystis* sp. among this population.

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Declaration of competing interest

The authors declare no conflicts of interest.

References

- Alarcon-Valdes, P., Villalobos, G., Martinez-Flores, et al., 2018. Can the pyruvate: ferredoxin oxidoreductase (PFOR) gene be used as an additional marker to discriminate among *Blastocystis* strains or subtypes? *Parasit. Vectors* 11 (564).
- Alfellani, M.A., Stensvold, C.R., Vidal-Lapiedra, A., et al., 2013. Variable geographic distribution of *Blastocystis* subtypes and its potential implications. *Acta Trop.* 126, 11–18.
- Azami, M., Sharifi, M., Hejazi, S.H., et al., 2010. Intestinal parasitic infections in renal transplant recipients. *Braz. J. Infect. Dis.* 14, 15–18.
- Borges, J.D., Alarcón, R.S.R., Amato Neto, V., et al., 2009. Parasitoses intestinais de indígenas da comunidade Mapuera (Oriximiná, Estado do Pará, Brasil): elevada prevalência de *Blastocystis hominis* e encontro de *Cryptosporidium* sp. e *Cyclospora cayetanensis*. *Rev. Soc. Bras. Med. Trop.* 42, 348–350.
- Carvalho-Costa, F.A., Gonçalves, A.Q., Lassance, S.L., et al., 2007. Detection of *Cryptosporidium* spp. and other intestinal parasites in children with acute diarrhea and severe dehydration in Rio de Janeiro. *Rev. Soc. Bras. Med. Trop.* 40, 346–348.
- David, É.B., Guimarães, S., de Oliveira, A.P., et al., 2015. Molecular characterization of intestinal protozoa in two poor communities in the State of São Paulo, Brazil. *Parasit. Vectors* 8, 1–12.
- Garavelli, P.L., Scaglione, L., Biccocchi, R., et al., 1991. Pathogenicity of *Blastocystis hominis*. *Infection* 19, 185.
- Garcia, L.S., 2001. *Diagnostic Medical Parasitology*. fourth ed. American Society for Microbiology, Washington, DC.
- Gil, F.F., Barros, M.J., Macedo, N.A., et al., 2013. Prevalence of intestinal parasitism and associated symptomatology among hemodialysis patients. *Rev. Inst. Med. Trop. Sao Paulo* 55, 69–74.
- Jiménez, P.A., Jaimes, J.E., Ramírez, J.D., 2019. A summary of *Blastocystis* subtypes in North and South America. *Parasit. Vectors* 12, 376.
- Kulik, R.A., Falavigna, D.L.M., Nishi, L., et al., 2008. *Blastocystis* sp. and other intestinal parasites in hemodialysis patients. *Braz. J. Infect. Dis.* 21, 338–341.
- Malheiros, A.F., Stensvold, C.R., Clark, C.G., et al., 2011. Molecular characterization of *Blastocystis* obtained from members of the indigenous Tapirapé ethnic group from the Brazilian Amazon region, Brazil. *Am. J. Trop. Med. Hyg.* 85, 1050–1053.
- Melo, G.B., Paula, F.M., Malta, F.M., et al., 2017. Identification of *Blastocystis* subtypes in clinical stool samples from Sao Paulo City, Brazil. *Parasitol. Open* e3, 3.
- Meloni, D., Sanci, G., Poirier, P., et al., 2011. Molecular subtyping of *Blastocystis* sp. isolates from symptomatic patients in Italy. *Parasitol. Res.* 109, 613–619.
- Naeni, A.E., Sharifi, M., Shahidi, S., et al., 2012. Intestinal fungal and parasitic infections in kidney transplant recipients: a multi-center study. *Saudi J. Kidney Dis. Transpl.* 23, 677–683.
- Oliveira-Arbex, A.P., David, É.B., Guimarães, S., 2018. *Blastocystis* genetic diversity among children of low-income daycare center in Southeastern Brazil. *Infect. Genet. Evol.* 57, 59–63.
- Piubelli, C., Soleymanpoor, H., Giorli, G., et al., 2019. *Blastocystis* prevalence and subtypes in autochthonous and immigrant patients in a referral center for parasitic infections in Italy. *PLoS One* 14, 1–9.

- Poirier, P., Wawrzyniak, I., Albert, A., et al., 2011. Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies. *J. Clin. Microbiol.* 49, 975–983.
- Poirier, P., Meloni, D., Nourrisson, C., et al., 2014. Molecular subtyping of *Blastocystis* spp. using a new rDNA marker from the mitochondria-like organelle genome. *Parasitology* 141, 670–681.
- Ramírez, J.D., Sánchez, A., Hernández, C., et al., 2016. Geographic distribution of human *Blastocystis* subtypes in South America. *Infect. Genet. Evol.* 41, 32–35.
- Rao, K., Sekar, U., Iraivan, K.T., et al., 2003. *Blastocystis hominis* - an emerging cause of diarrhea in renal transplant recipients. *J. Assoc. Physicians India* 51, 719–721.
- Rasti, S., Hassanzadeh, M., Hooshyar, H., et al., 2017. Intestinal parasitic infections in different groups of immunocompromised patients in Kashan and Qom cities, central Iran. *Scand. J. Gastroenterol.* 52, 738–741.
- Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467.
- Scicluna, S.M., Tawari, B., Clark, C.G., 2006. DNA barcoding of *Blastocystis*. *Protist* 157, 77–85.
- Seguí, R., Muñoz-Antoli, C., Klisiowicz, D.R., et al., 2018. Prevalence of intestinal parasites, with emphasis on the molecular epidemiology of *Giardia duodenalis* and *Blastocystis* sp., in the Paranaguá Bay, Brazil: a community survey. *Parasit. Vectors* 11, 490.
- Stensvold, C.R., Clark, C.G., 2016. Current status of *Blastocystis*: a personal view. *Parasitol. Int.* 65, 763–771.
- Tan, K.S., 2008. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin. Microbiol. Rev.* 21, 639–665.
- Villalobos, G., Orozco-Mosqueda, G.E., Lopez-Perez, M., et al., 2014. Suitability of internal transcribed spacers (ITS) as markers for the population genetic structure of *Blastocystis* spp. *Parasit. Vectors* 7, 461.
- Wang, W., Cuttill, L., Bielefeldt-Ohmann, H., et al., 2013. Diversity of *Blastocystis* subtypes in dogs in different geographical settings. *Parasit. Vectors* 6, 215–219.
- Zhang, W., Ren, G., Zhao, W., et al., 2017. Genotyping of *Enterocytozoon bieneusi* and subtyping of *Blastocystis* in cancer patients: relationship to diarrhea and assessment of zoonotic transmission. *Front. Microbiol.* 8, 1835.