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Characterization of subtypes of *Blastocystis* sp. isolated from patients with urticaria, São Paulo, Brazil

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ABSTRACT

Blastocystis sp. is described as an enteric protist prevalent in fecal samples from humans and animals; its pathogenicity and epidemiology are still controversial. Currently, it has been associated with intestinal diseases such as irritable bowel syndrome and clinical manifestations of allergic skin, such as chronic urticaria. In the context of urticaria, it is still uncertain whether this organism is directly related to the allergic manifestation or just a common component of the intestinal microbiota. This study aimed to evaluate the occurrence and molecular diversity of *Blastocystis* sp. in individuals with urticaria from a dermatology outpatient clinic, São Paulo, Brazil. Fecal samples of 58 patients with urticaria were examined using parasitological methods; and subsequently tested by polymerase chain reaction using *Blastocystis*-specific primers. The subtypes (STs) and alleles (a) were determined using BLASTn and MLST tools. ST1, ST2, ST3, ST4, ST6 and mixed infection (ST1 + ST3) were identified in the patients with urticaria; ST1 (a4), ST3 (a34 and a36) and ST4 (a42) were the most prevalent. Our molecular analyses allowed an initial description of *Blastocystis* subtypes in patients with urticaria from São Paulo city, Brazil.

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1. Introduction

Blastocystis sp. is described as one of the most common enteric protists that inhabits the gastrointestinal tract of humans and animals (Kurt et al., 2016; Scanlan, 2012; Cassano et al., 2005). Currently, at least 17 different subtypes (STs) have been recognized, ten of which are found in human samples (ST1-9 and ST12) (Ramírez et al., 2016; Alfellani et al., 2013). Despite recent progress in the number of studies, biological and pathogenic aspects remain controversial. Recent studies described *Blastocystis* sp. as a possible common member of the intestinal microbiota, which may develop clinical manifestations depending on the interaction with the host's intestinal microbiota components (Nieves-Ramírez et al., 2018; Kurt et al., 2016; Scanlan, 2012).

Some studies have indicated that the pathogenic potential of *Blastocystis* sp. can occur through the presence of certain STs or host immunological conditions (Mattiucci et al., 2016; Kurt et al., 2016). In recent years, associations based on case reports have suggested *Blastocystis* sp. as a possible causative agent of clinical manifestations, gastrointestinal and extraintestinal, such as the pathogenesis of urticaria (Casero et al., 2015; Vogelberg et al., 2010; Katsarou-Katsari et al., 2008; Gupta and Parsi, 2006; Valsecchi et al., 2004; Cassano et al., 2005; Barbosa et al., 2018). Parasitic helminths have been considered capable of causing urticaria manifestations by inducing the production of immunoglobulin E (Bakiri and Mingomataj, 2010; De Gentile et al., 1999; Lynch et al., 1998). However, it is unclear if an intestinal protist can act as a causative agent of cutaneous manifestations (Lepczyńska et al., 2016; Bakiri and Mingomataj, 2010).

In Brazil, there are few studies on the molecular characterization of *Blastocystis* sp., none of which are in the context of urticaria (Seguí et al., 2018; Oliveira-Arbex et al., 2018; Melo et al., 2017; David et al., 2015; Malheiros et al., 2011). Thus, the purpose of studying the diversity STs and alleles is mainly to obtain greater knowledge about the diversification of *Blastocystis* STs, as well as to compare different alleles belonging to the same ST and investigate whether certain alleles are responsible for certain clinical manifestations. Therefore, there is a need for comparative investigations between patients with urticaria and *Blastocystis* sp. This study aimed to evaluate the occurrence of *Blastocystis* sp. and characterization of genetic diversity in individuals with urticaria from a dermatology outpatient clinic (Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo [HC/FMUSP]), São Paulo, Brazil.

2. Materials and methods

2.1. Study population and collection of faecal samples

The local ethical committee from HC/FMUSP approved this study (protocol no. 488—701). This study was carried out from January 2017 to December 2018 in the dermatology outpatient clinic at HC/FMUSP. A total of 58 individuals (49 females and 9 males, age range from 13 to 73 years) were included. The inclusion criteria were patients with urticaria with at least 6 weeks of evolution and with a parasitological diagnosis. The exclusion criteria were patients with urticaria with a triggering factor defined as persistent bacterial or viral infections (hepatitis B, hepatitis C, AIDS or presence of positive serology for HIV), pregnant women, lactating women, patients on immunosuppressive drugs, and those with dialytic chronic renal failure. All patients were informed about the research and invited to participate. After written consent for research purposes was obtained, routine parasitological examination was requested.

Socio-demographic (age, sex, education level) and clinical (medication used at time of collection and D-dimer plasma levels) data were considered. Patients with active chronic urticaria have D-dimer serum levels greater than 500 ng/mL fibrinogen equivalent unit (FEU) (Criado et al., 2011).

The fecal samples investigated were assessed by conventional microscopy and polymerase chain reaction (PCR). The parasitological diagnosis was made at the Section of Parasitology, Central Laboratory Division (HC/FMUSP) using the Faust, Lutz, and permanent-stained smears methods. Parasitological results for *Blastocystis* sp. followed the criteria mentioned by Tan (2008) based on the intensity of infection by quantified parasite cells in the fecal samples: rare (one to two cells in every 10 high-power fields), few to moderate (one cell in five high-power fields) or several (five or more cells per high-power field).

Blastocystis-positive samples were sent to the Medical Research Laboratory (LIM06/FMUSP) for molecular analysis.

2.2. Extraction of genomic DNA and PCR amplification

Genomic DNA was extracted from approximately 200 mg of frozen fecal samples using a commercial QIAamp Stool Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

PCR amplification was performed using *Blastocystis*-specific *SSU*-rDNA primers RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGC AACAACG-3′) described by Scicluna et al. (2006). PCR reactions were performed in a 10 μL volume containing ~50 ng μL^{-1} of DNA, 2.0 μg BSA, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 2pM of each primer, 1 × PCR buffer and 1.25 U GoTaq® DNA Polymerase (Promega Corporation, Madison, USA). PCR conditions consisted of an initial denaturation cycle at 94 °C for 2 min, 30 cycles including denaturation at 94 °C for 1 min, annealing 61.8 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 2 min. PCR amplification was conducted with a Mastercycler EP Gradient S Thermocycler (Eppendorf, Hamburg, Germany). The products were separated by electrophoresis in 2% agarose gel containing Sybr Safe (Invitrogen™, Thermo Fisher Scientific Corporation, Waltham, MA, USA) with a size marker of 100bp.

2.3. Subtyping and phylogenetic analysis

Amplicons of the expected size were purified using ExoSAP enzyme (GE Healthcare, Piscataway, NJ, USA) and sequenced by both strands using an ABI PRISM® BigDye™ Terminator kit (Applied Biosystems, Thermo Fisher Scientific), following the Sanger et al. (1977) sequencing methodology. DNA sequencing was performed using an automated ABI 3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

The consensus sequences in the samples were aligned using BioEdit software and analyzed for the presence of double peaks. For subtype and allele assignment the sequences were compared with *Blastocystis* sequences in GenBank using the BLAST tool and *Blastocystis* 18S database available at https://pubmlst.org/blatocystis/. Sequence data were deposited in the GenBank database under the accession numbers: GenBank: MK782495-MK782527.

3. Results

At the time of collection, 22.4% (13 of 58) of patients had no active lesions and were controlled with antihistamines (loratadine and hydroxyzine), 63.8% (37 of 58) had active lesions and were treated with antihistamines (loratadine and hydroxyzine) and 13.8% (8 of 58) patients had no activity data. The values of D-dimer plasma levels ranged from 190 to >10,000 ng/mL FEU. The associated clinical diagnoses were glucose-6-phosphate dehydrogenase deficiency, diabetes mellitus, systemic arterial hypertension, dyslipidemia, epilepsy, subclinical hypothyroidism, multiple sclerosis, Barrett esophagus, fibromyalgia, rhinitis, and gastroesophageal reflux disease. Only one patient reported diarrhea during the visit.

With regard to the parasitology results, 53.5% (31 of 58) of patients were positive for *Blastocystis* sp., three (5.2%) samples presented numerous forms of *Blastocystis* sp. and, rare forms were found in the others. Of these samples, 13.8% (8 of 58) were positive for other enteroparasites: *Endolimax nana* (n = 2), *Entamoeba coli* (n = 1), *E. nana* and *E. coli* (n = 3), *Taenia* spp. (n = 1), and *Giardia lamblia* (n = 1). The expected fragment of *Blastocystis*-specific *SSU*-rDNA was found in 53.5% (31 of 58) of the patients. These positive samples were submitted to sequencing, and some isolates showed low-quality sequences. Comparison of the results obtained with microscopic and PCR methods for the detection of *Blastocystis* sp. are shown in Table 1.

Different subtypes were identified: ST1 in seven isolates (25%), ST2 in five isolates (17.8%), ST3 in eight isolates (28.5%), ST4 in six isolates (21.4%), ST6 in one isolate (3.6%), and in a single isolate was observed a mixed infection ST1 + ST3 (Fig. 1). Allele sequence analyses showed a4 in ST1; a9 and a12 within ST2; a34, a36 and a37 in ST3; a42 and a94 within ST4 and a122 in ST6; a4 and a37 were observed in the sample with mixed infection (ST1 + ST3). The subtypes most frequently identified in patients with active lesions were ST3, ST1, ST2, and ST4 (Table 2).

Socio-demographic (age, sex, education level), clinical (clinical diagnosis, D-dimer plasma levels) data for 28 patients with urticaria and a positive molecular diagnosis of *Blastocystis* sp. are shown in Table 3.

4. Discussion

The factors involved in the possible human infection by *Blastocystis* sp. and the reason for its high frequency worldwide has resulted in increasing numbers of studies (Oliveira-Arbex et al., 2018; Kurt et al., 2016; Alfellani et al., 2013; Scanlan, 2012; Malheiros et al., 2011). Some authors describe the host's immune status, the number or type of intestinal forms, the components of the intestinal microbiota, and the presence of particular subtypes as possible concomitant conditions that may contribute to the high occurrence of *Blastocystis* infection (Nieves-Ramírez et al., 2018; Kurt et al., 2016; Arik Yilmaz et al., 2016).

In the context of urticaria, it is still not known whether this organism has a direct relationship with allergic manifestations (Arik Yilmaz et al., 2016; Hameed et al., 2011; Bakiri and Mingomataj, 2010; Katsarou-Katsari et al., 2008). Zaglool et al. (2012), evaluating patients with allergic skin symptoms, showed that 13.7% were infected with *Blastocystis* sp. In our study, it was shown that 53.5% of the patients with urticaria presented a positive parasitological diagnosis for *Blastocystis* sp. Similarly, Hameed et al. (2011) demonstrated that 61% of patients with urticaria had a positive diagnosis of *Blastocystis* sp.

Table 1
Comparison of the results obtained with microscopic (MC) and PCR methods for the detection of *Blastocystis* sp. in faecal samples from patients with urticaria.

Method	Patients with urticaria (N = 58)	
	N	%
MC + PCR+	21	36.2
MC + PCR−	10	17.2
MC− PCR+	10	17.2
MC− PCR−	17	29.3

+, positive samples; −, negative samples.

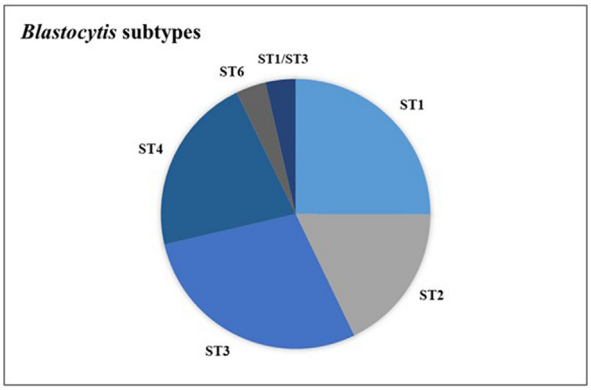

Fig. 1. *Blastocystis* subtypes identified from patients with urticaria.

Table 2
Clinical data and subtypes/alleles identified in fecal samples sequenced from patients with urticaria.

Subtypes	Alleles	AL	NAL	ND
ST1	Allele 4	6	0	1
ST2	Allele 9	1	0	1
	Allele 12	3	0	0
ST3	Allele 34	3	1	0
	Allele 36	3	0	0
	Allele 37	1	0	0
ST4	Allele 42	4	0	1
	Allele 92	0	0	1
ST6	Allele 122	1	0	0
ST1/ST3	Allele 4/37	1	0	0

AL, active lesions; NAL, no active lesions; ND, no data.

Table 3
Socio-demographic (age, sex, education level), clinical (clinical diagnosis, D-dimer plasma levels) data for 28 patients with urticaria and a positive molecular diagnosis of *Blastocystis* sp.

Isolate	Gender	Age (years)	Education level[a]	D-Dimer (ng/mL)	Clinical diagnosis	ST/allele
1UC	F	26	High	ND	ND	ST1/4
2UC	F	49	Low	720	G6PD deficiency	ST1/4
5UC	F	32	Low	690	From another clinic	ST3/36
6UC	F	40	Low	1360	Sinusitis	ST1 and ST3/4 and 37
7UC	F	73	Low	1140	AH	ST2/12
9UC	F	60	Low	230	GRD, DM, depression	ST4/42
10UC	M	45	High	ND	Dyslipidemia, DM, AH	ST2/12
12UC	M	52	High	ND	From another clinic	ST3/34
13UC	F	38	Low	550	From another clinic	ST3/34
15UC	F	50	Low	615	AH, DM, asthma	ST1/4
18UC	F	42	High	230	Epilepsy	ST1/4
20UC	F	40	High	ND	From another clinic	ST1/4
21UC	F	57	High	ND	ND	ST4/94
23UC	F	26	Low	2140	From another clinic	ST1/4
25UC	F	33	High	ND	ND	ST4/42
26UC	F	32	High	ND	Multiple sclerosis	ST4/42
28UC	F	59	High	ND	ND	ST2/9
33UC	F	49	Nd	ND	Epilepsy, DM, AH	ST2/9
40UC	M	51	Low	3940	From another clinic	ST6/122
41UC	F	59	Nd	748	From another clinic	ST3/36
43UC	F	57	Low	ND	DM, AH, dyslipidemia	ST3/34
46UC	F	30	High	ND	From another clinic	ST4/42
47UC	F	57	Low	ND	Rhinitis, fibromyalgia, DM, dyslipidemia, gastritis	ST4/42
48UC	M	49	High	630	From another clinic	ST3/34
53UC	F	35	Low	276	Obesity	ST3/36
54UC	M	56	Low	ND	From another clinic	ST1/4
55UC	F	52	High	250	DM, AH, dyslipidemia, hypothyroidism	ST2/12
56UC	F	54	Low	1.747	Barrett esophagus	ST3/37

AH, arterial hypertension; DM, diabetes mellitus; F, female; G6PD, glucose-6-phosphate dehydrogenase; GRD, gastroesophageal reflux disease; M, male; ND, no data.

[a] Low, primary school or less; high, secondary school or more.

Most of our patients were female. Similarly, Bálint et al. (2014) noticed a greater proportion of female patients in relation to the males with skin manifestations. With regard to the educational background of the study population, we found that most of the individuals has a low educational level; the same picture was shown by Abdulsalam et al. (2013).

Amplification of specific DNA from fecal samples has allowed new perspectives on the laboratory diagnosis of *Blastocystis* sp., especially in view of the difficulty with morphological differentiation (Stensvold, 2013). In our study, we verified the specific target was amplified in 54.4% of the fecal samples. However, some isolates with positive parasitology were negative by molecular diagnosis. PCR inhibitors in fecal samples or isolates that are not amplifiable by primers can explain non-amplification of *Blastocystis* DNA (Stensvold et al., 2006; Abdulsalam et al., 2013). In contrast, 37% of the negative parasitological isolates showed positive amplification of *Blastocystis* DNA, reinforcing the usefulness of PCR as a sensitive diagnostic tool (Stensvold et al., 2006).

Based on analysis of the *SSU*-rDNA, at least 17 subtypes of *Blastocystis* sp. have been detected to date, colonizing humans, other mammals, birds, reptiles, and insects (Lepczyńska et al., 2016; Alfellani et al., 2013; Scanlan, 2012). Analyzing the overall prevalence of *Blastocystis* subtypes, the highest occurrence of ST1 and ST2 has been reported in South and North America; ST1 and ST3 in Australia, Europe, and Southeast Asia, and ST4 in Europe (Alfellani et al., 2013; Abdulsalam et al., 2013). Our molecular analysis revealed the presence of five subtypes: ST1, ST2, ST3, ST4, and ST6. A higher frequency of ST1, ST2 and ST3 has been reported in the literature, and our results confirm these findings (Ramírez et al., 2016; Alfellani et al., 2013). Other studies conducted in Brazil reported the highest frequency of the same subtypes in different populations (Barbosa et al., 2018; Valença-Barbosa et al., 2017). On the other hand, two other studies demonstrated a higher occurrence of ST1 in relation to ST3 (Oliveira-Arbex et al., 2018; Malheiros et al., 2011).

There is evidence that ST2, ST3 and ST4 are etiologically associated with urticaria (Lepczyńska et al., 2016; Vogelberg et al., 2010; Katsarou-Katsari et al., 2008). Until recently, ST4 was considered to be restricted to the European continent, and was had not been identified in isolates from regions of Brazil (Melo et al., 2017; Ramírez et al., 2016; David et al., 2015; Alfellani et al., 2013). However, the ST4 was identified in this study and in other recent studies conducted in Rio de Janeiro (Seguí et al., 2018; Barbosa et al., 2018; Valença-Barbosa et al., 2017). ST4 was isolated from a patient without active lesions and with no reports of gastrointestinal symptoms. Domínguez-Márquez et al. (2009) reported the presence of ST4 in 94.1% of samples from symptomatic patients in Spain.

ST6 was identified in our isolates; this is found sporadically in humans and is described as a circulating avian subtype (Ramírez et al., 2016; Wawrzyniak et al., 2013). This subtype were detected in isolates found in Europe and Brazil (Seguí et al., 2018; Alfellani et al., 2013).

The detection of *Blastocystis* sp. in a large proportion of healthy individuals has suggested that this organism is a normal component of the intestinal microbiota (Kurt et al., 2016; Scanlan, 2012). On the other hand, it has been reported in the literature that the pathogenic expression of enteric protozoa such as *Blastocystis* sp., *Entamoeba histolytica*, species of *Eimeria* and *Giardia* may be related to the type of organisms present in the intestinal microbiota (Lepczyńska et al., 2016). The present results demonstrate that the most frequent protozoan in association with *Blastocystis* was *E. nana* in five isolates (ST1 and ST3).

It has been reported that certain subtypes may promote urticaria in only some patient groups (Lepczyńska et al., 2016). These findings may be due to the presence of high intra-subtype diversity (Stensvold, 2013). Our molecular analyses confirmed the high diversity within the subtypes, particularly the highest number of alleles within ST3, followed by ST1. Similar frequencies of alleles within ST3 have recently been reported in isolates from Brazil (Oliveira-Arbex et al., 2018; Seguí et al., 2018; David et al., 2015). Considering allele diversity, a4 (ST1), a12 (ST2), a34 and a36 (ST3), a42 (ST4) and a122 (ST6) were the most frequent in our study, especially in individuals with active lesions. Our results were similar to those reported by Casero et al. (2015) showing a34 (ST3) as the predominant genetic variant in patients with urticaria.

It has been reported that depending on the method used for subtyping, the occurrence of mixed infections may be underestimated (Scanlan et al., 2015; Stensvold, 2013). Our findings showed that 3.6% of the isolates were composed of mixed infections (ST1/ST3). A similar frequency was described by Abdulsalam et al. (2013) in isolates from patients in the Libyan region. Different studies carried out in Brazil point to the presence of mixed infections (David et al., 2015; Malheiros et al., 2011).

A potential limitation of this study may be the sample size of the urticaria population and the absence of a control group, which may have contributed to the difficulty in making robust conclusions. However, the identification of *Blastocystis* sp. in a large number of samples from patients with urticaria confirms the need for further studies, especially to understand the possible relationship between this protist and urticaria.

5. Conclusion

Our molecular analyses allowed us to make an initial assessment of the presence of *Blastocystis* STs/alleles from patients with urticaria in Brazil. In the present study, we found ST1 (a4), ST 2 (a12), ST3 (a34 and a36), ST4 (a42) and ST6 (a122) in the isolates from patients with urticaria who had active lesions. Thus, more studies focusing on *Blastocystis* and urticaria are encouraged.

Conflicts of interest

The authors declare that has no conflict of interest.

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