Identification of *Blastocystis* subtypes in clinical stool samples from Sao Paulo City, Brazil

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SUMMARY

Blastocystis sp. is a protozoan commonly found in human and animal stool samples. Several pathogenic and zoonotic aspects of this organism are still unknown. The aim of the present study was to investigate *Blastocystis* subtypes (STs) in samples from patients of the Hospital das Clínicas of the Faculdade de Medicina at the Universidade de São Paulo (HC-FMUSP), Brazil. *Blastocystis* sp.-positive stool samples diagnosed at the Section of Parasitology of the Central Laboratory (HC-FMUSP) were used for DNA isolation. Polymerase chain reaction (PCR) was performed using specific primers targeting the small-subunit rRNA gene. Direct DNA sequencing of the PCR products was performed and the DNA sequences were then aligned and compared with other sequences obtained from the GenBank database. Phylogenetic analysis was used to identify STs and determine the phylogenetic relationships between the sequences. Four STs were identified: ST1 (22·5%), ST2 (12·5%), ST3 (60%) and ST6 (5%). In conclusion, ST3 was the most prevalent ST among the human isolates followed by ST1. The present study is one of the few providing STs data from the human population in South America. Determining ST prevalence in human samples may contribute to the monitoring of *Blastocystis* sp. infection transmission in endemic regions.

Key words: *Blastocystis*, subtypes, Brazil.

INTRODUCTION

The high number of samples positive for *Blastocystis* sp., an organism that was rarely reported in routine parasitological diagnosis until three decades ago, has received increasing attention in recent years. Although, the pathogenic role of *Blastocystis* sp. is controversial, accumulating evidence from recent studies supports the idea that they constitute an emerging pathogen (Tan, 2008; Tan *et al.* 2010). This enigmatic organism has also been identified in a wide range of animals. The taxonomic status of *Blastocystis* sp. has remained elusive until recently; phylogenetic studies based on small-subunit ribosomal DNA (SSU rRNA) have demonstrated that it is a member of phylum Stramenopiles (Silberman *et al.* 1996; Santín *et al.* 2011).

Blastocystis sp. is usually detected using conventional parasitological methods such as the direct method, concentration method with formalin–ether and stained smears (Stensvold *et al.* 2008; Tan, 2008). Culturing methods have been regarded as the gold standard for the detection of *Blastocystis* sp., because although these methods show high sensitivity (Yoshikawa *et al.* 2011; Zhang *et al.* 2012). Polymerase chain reaction (PCR) amplification of *Blastocystis* DNA from cultures or feces is thought to be the most sensitive detection method (Tan, 2008). DNA-based methods have been developed to identify genetic variation between *Blastocystis* sp. forms that are morphologically indistinguishable using a microscope (Alfellani *et al.* 2013). Molecular studies have focused predominantly on determining the prevalence of *Blastocystis* subtypes (STs), as well as possible factors associated with pathogenicity (Tan *et al.* 2010). Studies using the sequence analysis of *Blastocystis* sp. small subunit ribosomal RNA genes (SSU rDNA) have demonstrated that there are approximately 17 different lineages, termed subtypes (ST1–ST17), isolates from mammals and birds

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(Wawrzyniak *et al.* 2013). Human isolates have been shown to have a higher occurrence of STs 1–4, with limited presence of STs 5–9 (Stensvold *et al.* 2007; Scanlan, 2012; Alfellani *et al.* 2013).

The molecular epidemiology of *Blastocystis* sp. infections remains unknown in many parts of the world. Recent studies have provided further information regarding ST distribution among human populations (Tan, 2008; Alfellani *et al.* 2013); however, very few studies have been conducted in Latin America (Santín *et al.* 2011). To date, a small number of studies subtyping *Blastocystis* (Malheiros *et al.* 2011; David *et al.* 2015; Ramírez *et al.* 2016) have been conducted in Brazil. The aim of this study was to investigate the prevalence of *Blastocystis* STs in clinical stool samples from the Hospital das Clínicas of the Faculdade de Medicina de São Paulo (HC-FMUSP), Brazil.

MATERIALS AND METHODS

Study population

This study was approved by the local Research Ethics Committee of the Hospital das Clínicas of the Faculdade de Medicina de São Paulo under protocol n°. 488·701. Sixty *Blastocystis* sp.-positive stool samples were selected based on the positivity in routine parasitological examinations conducted at the Section of Parasitology, Central Laboratory, HC-FMUSP, Brazil. The techniques employed at this laboratory include the Faust method, Lutz method and permanent-stained smears (Garcia, 2001), considering positivity in at least one of the parasitological methods. Fifteen *Blastocystis* sp.-negative stool samples, determined by the parasitological methods, and 15 stool samples positive for other parasite infections (*Ascaris lumbricoides*, *Entamoeba histolytica*, *Giardia lamblia*, *Endolimax nana* and *Entamoeba coli*) were used as controls. Once the parasitological techniques were performed, the stool samples were aliquoted and stored at −20 °C for subsequent DNA isolation.

DNA extraction

Approximately 200 mg of stool sample were washed twice with phosphate-buffered saline ($0\cdot01$ M L^{-1}, pH 7·2). DNA was extracted from the pellet using the commercial QIAamp® DNA stool MiniKit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in 100 μL of elution buffer and quantified using a NanoDrop ND-1000 UV–VIS spectrophotometer v.3.2.1 (NanoDrop Technologies, Wilmington, DE, USA).

DNA amplification and sequencing

DNA amplification was performed by PCR using the primers RD5 (5′-ATC TGG TTG ATC CTG CCAG T-3′) and BhRDr (5′-GAG CTT TTT AAC TGC AAC AAC G-3′), located on SSU rRNA, as described by Scicluna *et al.* (2006); these primers amplify a ∼600 bp fragment. PCR 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$_2$, 2 pM of each primer, 1× PCR buffer and 1·25 U GoTaq® DNA Polymerase (Promega Corporation, Madison, USA). PCR amplification was conducted with a Master cycler ep gradient S thermocycler (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation step at 94 °C for 2 min; 30 cycles of 94 °C (denaturation) for 1 min, 61 °C (annealing) for 1 min and 72 °C for 1 min (extension); and a final extension step of 72 °C for 2 min. The PCR products were loaded on a 2% agarose gel containing Syber safe (Invitrogen™, Thermo Fisher Scientific Corporation, Waltham, USA) and subjected to electrophoresis in 1× TAE (Tris-acetate-EDTA) buffer. Positive and negative controls (DNA from positive cultures and PCR mix without DNA template, respectively) were included in each round of amplification.

PCR products were purified using of the ExoSAP enzyme (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions, and then submitted to a sequencing reaction for incorporation of labelled ddNTPs (dideoxynucleotides) using the ABI PRISM® BigDyeTM Terminator kit (Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, USA). Direct DNA sequencing was performed using an automated ABI 3500 sequencer (Applied Biosystems).

Positive controls

The positive controls consisted of approximately 200 mg of *Blastocystis* sp.-positive stool samples cultured as described by Zerpa *et al.* (2000). The cultures were maintained at 37 °C and the pellets obtained by centrifugation at 12, 400 ***g*** for 1 min were examined using Lugol staining smear with optical microscopy (Olympus CX 41) after 24 and 48 h.

For control testing of extracted DNA, all samples without amplification were tested using universal primers (forward: 18SEUDIR 5′-TCTGCCCTAA CTACTTTCGATGG-3′ and reverse: 18SEUINV 5′-TAATTTGGCCTGCGCCTG-3′) that amplify a 140-bp region of the eukaryotic 18S ribosomal RNA gene, as described by Wang *et al.* (2013).

Subtyping analysis

Sequence analysis was performed using BioEdit software (Biological Sequence Alignment Editor http://www.mbio.ncsu.edu/bioedit/page2.html). Sequence alignments were conducted using CLUSTAL W and aligned with *Blastocystis*

Table 1. Comparison of Parasitological methods and conventional PCR (cPCR) for detection of *Blastocytis* in the stool samples

cPCR	Parasitological methods								
	Faust			Lutz			Permanent-stained smears		
	(+)	(−)	Total	(+)	(−)	Total	(+)	(−)	Total
(+)	5	42	47	17	30	47	43	4	47
(−)	0	13	13	4	9	13	9	4	13
Total	5	55	60	21	39	60	52	8	60

sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/tbl2asn2). Phylogenetic analyses were performed using MEGA 5 and the neighbour-joining method with 1000 bootstrap replication for phylogenetic tree construction. The *Proteromonas lacertae* sequence (accession number U37108) was used as the outgroup. Sequences were compared with *Blastocystis* SSU rDNA sequences available from the National Center for Biotechnology Information (NCBI) using the BLAST program (Basic Local Alignment Search Tool). Additionally, *Blastocystis* STs were identified by determining the exact match or closest similarity against all known *Blastocystis* STs using www.pubmlst.org/blastocystis.

RESULTS

Of the 60 clinical stool samples positive for *Blastocystis* sp. by parasitological methods, 47 were positive by PCR (78% positivity). PCR positivity was higher compared with other parasitological methods: 8% positivity by Faust, 35% by Lutz and lower than permanent-stained smears (87%) (Table 1). The expected DNA fragment (~600 bp) was detected in all PCR-positive samples and no DNA amplification of the target fragment was observed in the negative samples and stool samples positive for other parasite infections.

All PCR positive samples were sequenced and those that obtained good quality ($n = 40$) were compared with *Blastocystis* sequences deposited in the GenBank database (BLAST) and exhibited 98–100% similarity. Seven sequences were excluded from the analysis, since they presented low-quality possibly due to poor amplification of DNA.

The phylogenetic analysis was performed and four different STs were identified (Fig. 1). ST1 was found in nine samples (22·5%), ST2 was present in five samples (12·5%), ST3, the most common ST, was found in 24 samples (60%) and ST6 was present in two samples (5%). Based on information available at www.pubmlst.org/blastocystis, three distinct alleles were identified within ST3 (alleles 34, 36 and 37), two in ST2 (alleles 12 and 71) and one in ST1 and ST6, corresponding to alleles 4 and 134, respectively (Table 2).

DISCUSSION

Blastocystis sp. is the most common intestinal parasites found in human feces and are considered an emerging parasite with a worldwide distribution (Tan, 2008). The clinical features associated with blastocystosis range from non-specific intestinal symptoms to cutaneous disorders and the severity of these diseases varies from acute to chronic infections, a possible reason for these differences is genetic diversity (Tan, 2008; Tan *et al.* 2010; Scanlan, 2012). Although to date, no specific association has been observed between clinical findings and *Blastocystis* STs (Özyurt *et al.* 2008).

A variety of parasitological techniques can be used to detect *Blastocystis* sp. in clinical samples (Tan, 2008). However, this technique has low sensitivity, leading to misdiagnosis or underestimation of the true prevalence of *Blastocystis* sp. (Scanlan, 2012; Alfellani *et al.* 2013). Moreover, this parasite appears in polymorphic forms and variable size in stool samples, further hindering its diagnosis. PCR is an alternative method used to detect *Blastocystis* sp. in epidemiological studies, offering increased sensitivity and specificity as well as rapid diagnosis (Parkar *et al.* 2007). The results of the present study demonstrate that PCR was more efficient than the Faust and Lutz methods in the detection of *Blastocystis* sp. Another advantage of using molecular techniques compared with conventional methods is that they can provide more information in terms of genetic variability and zoonotic relationship (Parkar *et al.* 2007).

In this study, 60 samples were diagnosed as *Blastocystis* sp. positive using parasitological technical (considered the Faust method, Lutz method and permanent-stained smears), while the PCR technique confirmed the presence of the DNA parasite in only 47 stool samples. Possible reasons for this difference are PCR inhibition stool samples, degradation of DNA due to extended storage time, or misidentification by light microscopy (Forsell *et al.* 2012; Mattiucci *et al.* 2016). However, negative PCR results were not caused by inhibition of the amplification by fecal components, because amplification using universal primers was observed in all samples.
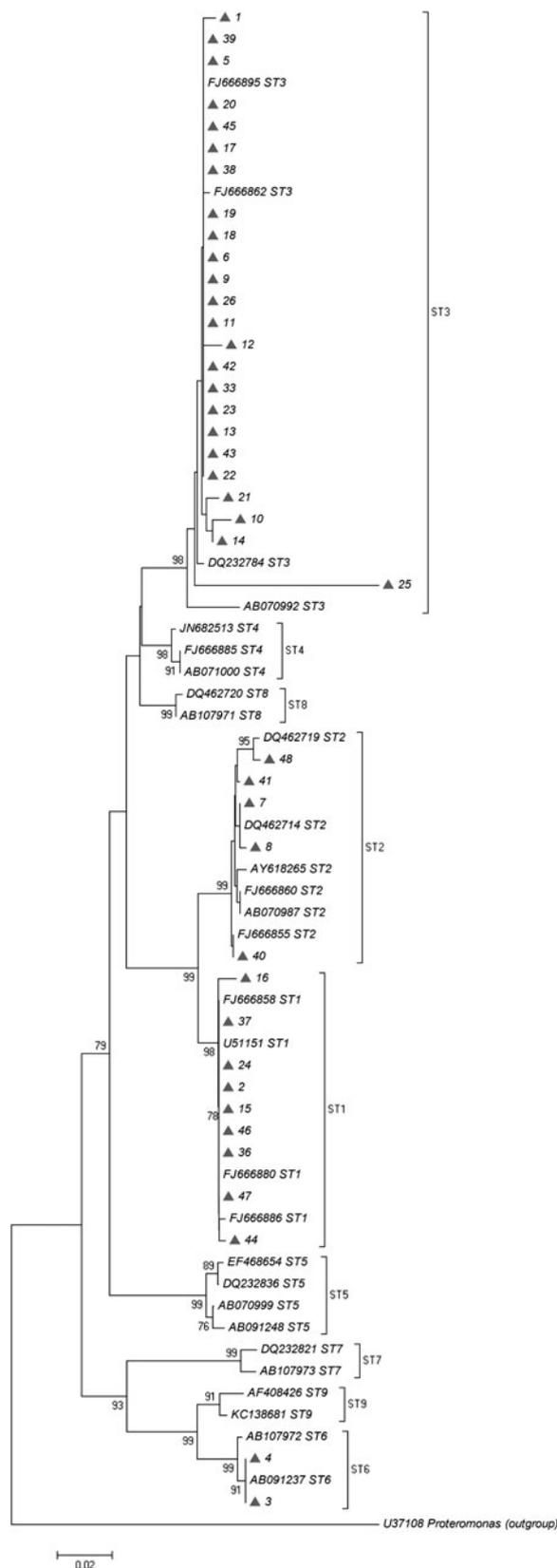
Fig. 1. Phylogenetic analysis of *Blastocystis* sp. SSU rDNA sequences (~600 bp) generated in this study (identified by triangles) and reference sequences from GenBank (identified by accession number and subtypes). The phylogenetic tree was constructed using the neighbour-joining method. Bootstrap values are based on 1000 replicates. Bootstrap values of <70% are not shown.

The suggested criteria for reporting infection intensity by parasitological methods are the observation of five or more parasites per high-powered field (×400) for wet mounts or under oil immersion (×1000) using permanent-stained smears (Tan, 2008). Other studies have included more detailed reports of parasite abundance (Leder *et al.* 2005; Özyurt *et al.* 2008) and quantified parasite abundance using definitions such as rare (one to two parasites in every 10 high-power fields), few to moderate (one parasite in every one to five high-power fields), and abundant (five or more parasites per high-power field) (Leder *et al.* 2005). In the present study, 13 of the 60 *Blastocystis* sp. positive samples identified using parasitological methods failed to produce *Blastocystis*-specific bands by PCR, five of which were reported as rare, four as few to moderate and four as suggestive forms by parasitological analyses. Mattiucci *et al.* (2016) have suggested that negative PCR results may be due to the amount of *Blastocystis* DNA being lower than the detection level.

Blastocystis in mammals and birds are subdivided into 17 STs, nine of which (ST1–ST9) have been found in humans (Stensvold and Clark, 2016). In 2007, a consensus of *Blastocystis* terminology was proposed, based in comparison with representative sequences of all known STs (Stensvold *et al.* 2007). The ST distribution in the present study was quite similar to that found in other countries. Indeed, the studies reported thus far indicate that the majority of human infections with *Blastocystis* sp. are attributable to ST3 isolates (Özyurt *et al.* 2008; Meloni *et al.* 2011; Forsell *et al.* 2012; Ramírez *et al.* 2016), as was the case in the present study (60%). It is suggested that dominance of ST3 may be related to its human origin (Özyurt *et al.* 2008; Meloni *et al.* 2011; Yoshikawa *et al.* 2016).

An analysis of samples from indigenous regions of Brazil conducted by Malheiros *et al.* (2011) showed different results from those of the current study; ST1 was the dominant ST identified (41%). It is possible that ST distribution is affected by the ethnic origin of the population and the limited contact between indigenous groups and people in other communities. ST1 and ST2 are also common in different regions in human isolates, while the other STs are found only sporadically (Alfellani *et al.* 2013). It is important to note the absence of ST4 in our study, similar to the results of other studies conducted in South America (Malheiros *et al.* 2011; David *et al.* 2015). In contrast, ST4 is observed with high frequency in continental Europe and the UK (Forsell *et al.* 2012; Alfellani *et al.* 2013; Mattiucci *et al.* 2016).

Another important finding of this study is the presence of ST6, rarely detected in human isolates. ST6 is considered as an avian ST, which points to a possible zoonotic origin (Mattiucci *et al.* 2016).

Table 2. Distribution of *Blastocystis* SSU rDNA alleles retrieved from the samples on each subtytpes

Subtypes	*n* (%)	Alleles	Sample[a]
ST1	9/40 (22·5)	4 (9/9)	2,15,16,24,36,37,44,46,47
ST2	5/40 (12·5)	12 (4/5)	7, 8, 40, 41
		71 (1/5)	48
ST3	24/40 (60·0)	34 (11/24)	5,6,10,11,12,17,23,26,33,42,45
		36 (11/24)	1,9,13,14,18,19,20,21,22,25,38
		37 (2/24)	39,43
ST6	2/40 (5·0)	134 (2/2)	3,4

[a] Corresponds to the number of samples identified on the phylogenetic tree.

The reported differences in the number of STs identified in human populations as well as their relative abundance might indicate different reservoirs and transmission routes (Noël *et al*. 2005; Meloni *et al*. 2011).

In the present study, alleles 34, 36 and 37 were detected in ST3. This is in agreement with observations reported by David *et al*. (2015) and Mattiucci *et al*. (2016). In contrast to our findings, Ramírez *et al*. (2016) showed a high diversity of alleles from ST1, ST2 and ST3. It is important to highlight that the comparison SSU rDNA alleles within the same ST can help determining the differences between the strains, which may possibly contribute to the identification of the potential for zoonotic transmission and pathogenic strains.

The present study is one of the few to analyse the incidence of *Blastocystis* STs in South America. Thus, our results are very important for understanding the geographic distribution of STs in Latin America. Moreover, these findings provide an initial molecular analysis that will enable future examination of unknown epidemiological aspects.

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