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Blastocystis genetic diversity among children of low-income daycare center in Southeastern Brazil

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ABSTRACT

Blastocystis, an unicellular anaerobic eukaryote, is known to be a very common intestinal parasite found in humans and animals fecal samples worldwide. Currently, there is an increasing interest to yield insights into its prevalence and diversity in human populations living in poor and deprived areas. In this study, we describe the prevalence and genetic variability of *Blastocystis* isolates obtained from daycare center attendees aged 0 to 6 years and staff, as well as some children family members and their dogs in a low-income community in São Paulo State, Brazil. A total of 181 stool samples (123 from daycare children, 14 from workers, 44 from household members and 20 from dogs) were submitted to DNA extraction, tested by polymerase chain reaction (PCR) targeting the SSUrDNA gene and the amplicons retrieved were sequenced. The prevalence of *Blastocystis* was 40.7% (50/123) in children, 28.6% (4/14) in workers and 50% (22/44) in household members. No dog was found positive. Of the 76 PCR products generated, 57 were successfully sequenced. Four subtypes were identified and the most common were ST1 (54.4%) and ST3 (33.3%), followed by ST2 (7.0%) and ST7 (5.3%). The intra-subtype analysis revealed a total of 10 different alleles previously reported. No statistically significant correlation was observed between subtypes and sociodemographic variables analyzed. Here, the following findings must be highlighted: (1) predominance of subtypes 1 and 3, a pattern that has been observed in many populations worldwide; (2) absence of ST4, a common subtype in Europe but rarely detected in South America's human populations and, (3) human infection with ST7, a subtype primarily found in birds but occasionally seen in human infections, raising the possibility of zoonotic transmission.

1. Introduction

The enteric protist *Blastocystis*, this enteric protist has been increasingly isolated in human population as well as in a wide range of hosts including non-human mammals, birds, reptilians, amphibians and, to a lesser extent, insects (Yoshikawa et al., 2016a). This ubiquitous parasite has a worldwide distribution and it is one of the most common intestinal protists found in parasitological surveys carried out in both developed and developing countries. Although widespread, its real geographic distribution is not well known and high prevalence rates have been reported in economically disadvantaged areas where inadequate access to safe water and sanitation services ensures conditions for the parasite transmission (Alfellani et al., 2013a; Tan, 2008).

Despite its early discovery, *Blastocystis* has come into focus only recently and a number of outstanding issues regarding epidemiology and pathogenicity still need investigation (Clark et al., 2013). Clinical relevance of *Blastocystis* infection is controversial because this parasite has been found in healthy individuals as well as in patients with gastrointestinal disorders. Interestingly, over the last two decades, studies have assembled evidence of a possible role of *Blastocystis* in irritable bowel syndrome (Clark et al., 2013; Poirier et al., 2012).

On the other hand, considerable progress has been made towards understanding its genetic diversity and host specificity. Based on gene analysis of the small subunit ribosomal RNA (SSUrDNA), *Blastocystis* genus exhibits a marked genetic diversity and it is known to comprise at least 17 distinct ribosomal lineages identified among mammals and birds isolates, the so-called subtypes (Alfellani et al., 2013b; Stensvold, 2013a). To date, nine different *Blastocystis* subtypes (ST1-ST9) have been found in human infections with a predominance of STs 1–4 which can also infect non-human primates, other mammals and even birds (Stensvold and Clark, 2016). Among these human subtypes, ST9 is so far found in humans while STs 5–8 are more frequent in hosts as hoofed animals (ST5), birds (ST6 and ST7) and in non-human primates (ST8) (Stensvold and Clark, 2016). Also, STs 10–17 have only been reported

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in animals.

Notwithstanding the progress made over the last two decades, current interest has focused on the distribution and relevance of *Blastocystis* subtypes in populations living in low-income areas. Those include Asia (Belleza et al., 2015; Nithyamathi et al., 2016; Pandey et al., 2015; Sanpool et al., 2017), Africa (El Safadi et al., 2014; Poulsen et al., 2016), the Middle East (AbuOdeh et al., 2016; Dogan et al., 2017) and also in Latin America (David et al., 2015; Macchioni et al., 2016; Malheiros et al., 2011; Melo et al., 2017; Ramirez et al., 2014, 2016, 2017; Sánchez et al., 2017; Villegas-Gómez et al., 2016). Recently, the limited data from South America was highlighted (Ramirez et al., 2016, 2017). To date, few investigations were performed in Colombia (Ramirez et al., 2014, 2016, 2017; Sánchez et al., 2017), to a lesser extend in Bolivia (Macchioni et al., 2016) and Argentina (Casero et al., 2015). Particularly in Brazil, there have been only three publications of diversity in *Blastocystis*. One focused on members of an Amazon indigenous community (Malheiros et al., 2011), while the two others conducted among fishing village dwellers (David et al., 2015) and patients attending a university hospital (Melo et al., 2017).

Despite current advances, there is still the expectation for further insights into *Blastocystis* epidemiology, especially in endemic foci where conditions ensure the parasite spreading putting the population at risk of infection. The aim of the current study was to evaluate the prevalence and the genetic diversity of *Blastocystis* isolates related to asymptomatic infection in children attending a day-care center in a small low-income community. In addition, *Blastocystis* infection was also assessed in daycare staff and some household members, as well dogs in close contact with children.

2. Materials and methods

2.1. Study population and fecal samples collection

Sample collection was carried out in a day-care center placed in a low-income community of Botucatu town, São Paulo State, Brazil (22°52′20″S, 48°26′38″W). Fecal specimens, sociodemographic data (age, sex, parents'/guardians' education level, household income, water source and sanitation infrastructure) and genomic DNA were obtained through a previous epidemiological survey to assess the occurrence and the frequency of *Giardia* genotypes infecting children and staff members of a daycare center, as well as household members and their dogs (Oliveira-Arbex et al., 2016). A total of 181 fecal samples were assessed, 123 of which were from daycare children, 14 from center employees, 44 from household members and 20 from dogs. All the protocols were previously approved by the Research Ethics Committees of the Botucatu Medical School, UNESP (protocol number 3898/2011 CEP) and Animal Experimentation/Biosciences Institute/UNESP (protocol number 306 CEEA).

2.2. DNA extraction, PCR and sequence analysis

Genomic DNA previously extracted from stool samples using the QIAamp® Stool mini kit (Qiagen, Hilden, Germany) were subjected to PCR and, the amplification of a 600 bp fragment of the *Blastocystis* SSUrDNA was carried out according to standard protocol (Scicluna et al., 2006). For determining the nucleotide sequences, PCR products were purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced on both strands by the Sequencing Service Macrogen, Inc. (Seoul, Korea). Nucleotide sequences generated were aligned with each other and reference sequences (ST1-ST9) downloaded from GenBank using Clustal X (Larkin et al., 2007). *Blastocystis* subtypes were identified by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the alleles were identified at the *Blastocystis* database (http://pubmlst.org/blastocystis). Phylogenetic analysis were performed with MEGA software v. 7.0 (www.megasoftware.net) and a phylogram was constructed using Neighbor-joining algorithm. Bootstrap analysis was applied to evaluate the reliability of clusters by using 1000 replicates and values of < 50% were not shown. The nucleotide sequences obtained in the present study were deposited on GenBank under the accession numbers KY801207 to KY801263.

2.3. Statistical analysis

Data were entered into the Epi Info software v. 7.0 (Centers for Disease Control and Prevention, USA). Chi-square (χ^2) test was applied to verify associations between *Blastocystis* subtypes and some sociodemographic and environmental factors such as age, sex, family size and income, hygiene habits, water supply, source of drinking water, sewage availability, latrine system, type of housing and contact with domestic and companion animals (the level of significance was $P < 0.05$). *Blastocystis* subtypes distribution between daycare children, workers and household members were tested for significance by the adjusted Wald test ($P < 0.0001$) using the statistical software SAS 9.2 (SAS Institute, Cary, NC, USA).

3. Results

All 181 human fecal samples were screened for *Blastocystis* by PCR and 76 of them (41.9%) were identified as positive. None of the dog specimens generated PCR products for *Blastocystis*. The frequency of human infection was 40.7% (50/123) in children, 28.6% (4/14) in workers and 50% (22/44) in household members. At the time of this survey, *Blastocystis*-infected and non-infected individuals reported none gastrointestinal complaints.

Of those 76 PCR products, 57 (38 isolates from children, 4 from workers and 15 from household members) were successfully sequenced for STs and alleles analysis. Nineteen samples with low quality sequences were excluded from further analysis. Among the isolates from family members, four of them were from individuals (HM50C, HM50D, HM59B and HM59C) living with a *Blastocystis*-negative daycare child (Appendix 1). All the 57 isolates had single ST infections and none evidence of mixed infections was found. The overall prevalence of *Blastocystis* subtypes was as follows: ST1 (31/57; 54.4%), ST2 (4/57; 7.0%), ST3 (19/57; 33.3%) and ST7 (3/57; 5.3%). Among the isolates from children, ST1 (57.9%) was the most common, followed by ST3 (26.3%), ST2 (10.5%) and ST7 (5.3%) (Table 1; Supplementary Appendix 1). Four samples from staff members were found positive for two STs: ST1 (n = 2) and ST3 (n = 2). In addition, 15 isolates retrieved

Table 1
Prevalence and subtype distribution of *Blastocystis* isolates infecting daycare children, household members and care workers.

Samples (n)	No.	No. PCR Positive	Subtype (%)				Total
			ST1	ST2	ST3	ST7	
Children	123	50	23 (57.9)	04 (10.5%)	10 (26.3)	02 (5.7%)	38
Household members	44	22	07 (46.7)	–	07 (46.7)	01 (6.7)	15
Staff members	14	04	02 (50.0)	–	02 (50.0)	–	04
Total	181	76	31 (54.4%)	04 (7.0)	19 (33.3)	03 (5.3)	**57**

Blatocystis subtypes distribution between daycare children, workers and household members were tested for significance by the adjusted Wald test ($P < 0.0001$).