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Blastocystis and urticaria: Examination of subtypes and morphotypes in an unusual clinical manifestation

Q1 Rodolfo Daniel Casero [a,*], Florencia Mongi [a], Angie Sánchez [b], Juan David Ramírez [b]

[a] Departamento de Parasitología, Hospital Nacional de Clínicas, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina
[b] Grupo de Investigaciones Microbiológicas – UR (GIMUR), Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia

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ABSTRACT

Blastocystis is a human common enteric protist that may colonize a large variety of non-human hosts linked to symptoms and diseases such as abdominal pain, constipation, diarrhea, urticaria, flatulence and irritable bowel syndrome (IBS). *Blastocystis* exhibits remarkable genetic diversity and multiple subtypes (STs) within the genus with no absolute associations with clinical symptomatology. Here we analyzed fecal samples from Argentinean patients ($n = 270$) belonging to symptomatic (urticaria and non-specific gastrointestinal symptoms, $n = 39$) and asymptomatic control ($n = 28$). Those patients infected with *Blastocystis* ($n = 67$) were submitted for morphological analysis, DNA extraction, 18S PCR, sequencing and STs identification according to DNA barcoding. *Blastocystis* vacuolar forms were the predominant morphotype (75%), ameboid-like forms were evidenced in 1.5% of samples. *Blastocystis* ST3 was detected in 71.6% ($n = 48$), of which 71.4%, ($n = 35$) and 28.6% ($n = 14$) belonged to symptomatic and asymptomatic respectively. Other subtypes identified were ST1 (14.9%), ST6 (7.5%) and ST2 (5.9%). *Blastocystis* 18S barcoding evidenced in non-urticaria symptomatic patients and asymptomatic control group the presence of allele 134 (ST3) ($p < 0.0001$), while allele 34 (ST3) was detected in 85.7% (18/21) of symptomatic uricaria as compared with control group (1/21) ($p < 0.0001$). The presence of a particular allele ($a34$) significantly associated with urticaria patients was detected and the clinical implications of these findings are herein discussed.

1. Introduction

Q3 For a long time, the taxonomy of *Blastocystis* was controversial. Despite the application of molecular phylogenetic approaches, it was only recently that *Blastocystis* sp. was unambiguously classified within the Stramenopiles. This eukaryotic major lineage, also called Heterokonta, encompasses very diverse organisms (unicellular or multicellular, heterotrophic or photosynthetic) such as slime nets, diatoms, water molds and brown algae (Silberman et al., 1996). With a cosmopolitan distribution, *Blastocystis* is often the most common protozoan isolated in parasitological surveys and presents a different number of species living in the gastrointestinal tract of humans, farm animals, birds, rodents, reptiles and others (Yoshikawa et al., 2004). The transmission is thought to occur via fecal–oral route and the cyst is the only form involved; the extent to which human–human, human–animal and animal–human transmission occur remains under debate (Yoshikawa et al., 2004). *Blastocystis* displays a relevant genetic diversity where several distinct ribosomal lineages or subtypes (STs) have been reported. In 2007, a consensus of *Blastocystis* nomenclature was proposed revealing nine subtypes infecting humans, other mammals and birds, based on the polymorphisms of the small subunit (SSU) rRNA gene. So far, no strict associations between the STs and the hosts have been reported, although moderate host specificity is seen (Stensvold et al., 2009; Victory et al., 2009). Today, at least 17 genetically distinct SSU rRNA clusters are known (Alfellani et al., 2013 and Graham et al., 2013). In humans, nine subtypes have been Q4 reported; the subtypes 1, 2, 3 and 4 are common in Europe; while ST1, ST2 and ST3 seem to present equal prevalences in patients with diarrhea and healthy individuals; ST4 is also linked to diarrhea and/or IBS (Ramírez et al., 2014). In America, the information is scarce: in Colombia, ST1 (34%) and ST2 (23%) and lower proportions of ST 3 (11.4%), ST 4 (0.8%), ST 6 (19.8%) and ST 8 (10.5%) have been described. Domesticated mammals shared the same STs as those usually seen in humans (ST1, ST2, ST3), while birds and marsupials had STs, which are usually rare in humans (ST6,

* Corresponding author at: Departamento de Parasitología Hospital, Nacional de Q2 Clínicas, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Santa Rosa 1564, (5008) Córdoba, Argentina. Tel.: +54 3188270427.
E-mail address: rodaca@argentina.com (R.D. Casero).

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ST8) (Ramírez et al., 2014). In Brazil, ST1 (41%), ST2 (32%) and ST3 (17%) were identified in an ethnic group (Malheiros et al., 2011) in Mexico a local survey identified 51% of ST1, 23% of ST2, 19% of ST3 and 2% of ST7 (Villalobos et al., 2014). Despite of the initial descriptions of this protozoan in the early 1900s, there is scarce information and misleading assumptions regarding the pathogenesis of this Stramenophile (Tan, 2008). Similarly, until a few years ago *Blastocystis* was considered as a gut commensal protozoan but later, accumulative epidemiological data suggested not only a close relationship between its presence or changing morphotypes with symptoms but often as a true pathogen (Tan, 2008; Roberts et al., 2013). Thus, the pathogenic role of *Blastocystis* sp. as the primary cause of enteric symptoms is dubious. Clinical features of illness attributed to this pathogen are non-specific (Tamalee et al., 2014) and include nausea, abdominal pain, flatulence, acute or chronic diarrhea, irritable bowel syndrome (IBS), (Yakoob et al., 2010), skin disorders such as allergic cutaneous lesions, urticaria (Ur) and angioedema (Logar et al., 1994; Surangsrirat et al., 2010; Vogelberg et al., 2010; Hameed et al., 2011; Verma and Delfanian, 2013). Ur is a relatively common skin disorder that has been attributed to immunological, non-immunological and idiopathic causes. Only few case reports have been published about the association of *Blastocystis* infection and Ur but it seems to be an indication that pathogenicity might depend on the subtype (Vogelberg et al., 2010; Hameed et al., 2011; Verma and Delfanian, 2013; Katsarou-Katsari et al., 2008). Regarding the associations of subtypes with morphotypes and clinical findings, PCR subtyping indicated not only that diverse STs correlates with different morphotypes but also their close relationship with defined symptoms (Dogruman-Al et al., 2008; Jones et al., 2009). Although ST3 is the most common subtype found in the majority of human epidemiological studies, there has been a lower association reported of ST3 with symptoms as compared with ST1 (Ozyurt et al., 2008; Yan et al., 2006). In Argentina, *Blastocystis* sp. represents one of the most common parasites detected in stools. In a previous report, of 924 asymptomatic and symptomatic patients (diarrhea, abdominal pain, constipation, flatulence, bloating, and urticaria), we detected a *Blastocystis* sp. prevalence of 24% (221/924), where urticaria and non-specific gastrointestinal symptoms were significantly associated with this protist as compared with asymptomatic carriers ($p < 0.03$). According to our previous results, the aim of this study was to determine the morphotypes and subtypes of *Blastocystis* present in stools from a Cordoba city population in Argentina. A further aim was to determine if non-specific gastrointestinal symptoms (NSGI) or urticaria (Ur), were related with any *Blastocystis* subtype or morphotype.

2. Materials and methods

2.1. Patients

A total of 270 patients (141 female, 129 male, ranging from 1 to 57 years old) from the metropolitan area of Cordoba city, in Argentina attended to the University Hospital and the Benito Soria Dispensary during a 6-month period and were subsequently enrolled in the study. After the regular health checks, clinical histories with data of recent travels, clinical examinations (within the last 6 months) were recorded and patients were submitted to the laboratory for a fecal microscopy examination. Patients were grouped in asymptomatic control group and symptomatic group, where individuals with chronic Ur or NSGI (intestinal symptoms like diarrhea, constipation, abdominal pain and bloating) were included. The local study ethics committee approved this study and written informed consent was obtained from all participants.

2.2. Stool specimens and microscopy

Fresh and SAF (saline, sodium acetate, formalin) preserved stool specimens (four different specimens per person, along four days collection) were examined immediately by performing formalin–ethyl acetate and Hoffman sedimentation concentrations techniques and iodine wet mounts, Gram-chromotrope, trichrome-stained smears, modified acid-fast and modified-trichrome stains were performed in parallel (Garcia, 2007). Positive stools for *Blastocystis* and *Blastocystis* co-parasitized with other intestinal parasites were selected for culturing. Duplicate fresh samples containing *Blastocystis* from symptomatic and asymptomatic patients were immediately cultivated in Jones's medium at 37 °C (Suresh and Smith, 2004) and checked by light microscopy to record parasite growth and morphologic features on days 1, 2 and 7 as described previously (Tan, 2008). *Blastocystis* morphotype prevalence was estimated by counting 100 parasites in 50 fields (light microscopy, magnification 400×). *Blastocystis* forms were classified as vacuolar (V): round or spherical trophozoites with the presence of an evident central body surrounded by a cytoplasm with peripheral nucleus; granular (Gr): trophozoites with central body and cytoplasm covered with granules; ameboid-like forms (Am): irregular ameboid-shaped trophozoites with absent central body in the cytoplasm but with peripheral nucleus as described before (Tan and Suresh, 2006; Zhang et al., 2012). The percentage and type of parasite (morphotype) in co-parasitized samples in symptomatic and asymptomatic control group was also estimated.

2.3. DNA extraction, PCR and sequence analysis

Fresh portion of stool (500 mg) was stored in 70% ethylic alcohol after collection and frozen at −20 °C for DNA extraction. From each sample, 250 mg were submitted to DNA extraction using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Genomic DNA was preserved until analysis. The samples were verified for the presence of *Blastocystis* via PCR amplification of *Blastocystis*-specific SSU rDNA 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′) as recently recommended (Scicluna et al., 2006; Stensvold, 2013). PCR products were purified and sequenced by both strands using de dideoxy-terminal method (Macrogen, Korea). Sequences were edited in MEGA 4.0 (Tamura et al., 2007) and compared with reference sequences representing each ST in GenBank by BLAST queries. Additionally, sequences were submitted to sequence queries at the *Blastocystis* 18S database available at http://pubmlst.org/blastocystis/ for *Blastocystis* 18S allele calling and confirmation of ST.

2.4. Statistical analysis

Blastocystis morphotypes and STs in asymptomatic (control group) and symptomatic patients (Ur and NSGI) were analyzed statistically using the chi-square test from the program MedCal Version 10.0.2. $p < 0.05$ was considered statistically significant.

3. Results

The partial results of this research were presented in the International Conference of Parasitology held in Mexico city in 2014 (Casero DR, Mongi F, Ramírez JD. 2014). *Blastocystis* subtype 3 (Allele 34) is associated with urticaria in Argentinean patients. ICOPA XIII. P#2212.

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