

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

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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 urticaria as compared with control group (1/21) ($p < 0.0001$). The presence of a particular allele ($\alpha 34$) significantly associated with urticaria patients was detected and the clinical implications of these findings are herein discussed.

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1. Introduction

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 Clark et al., 2013). In humans, nine subtypes have been 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,

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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 (García, 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.

Table 1Gender, age, and co-parasitism distribution of *Blastocystis* isolates from asymptomatic and symptomatic patients.

	Asymptomatic (n = 28)	Symptomatic (n = 39)			Only <i>Blastocystis</i> (n = 50)	Co-parasitism (n = 17)
		Ur (n = 18)	NSGI (n = 18)	UR + NSGI (n = 3)		
Adults	10	18	16	2	34	12
Children	18	0	2	1	16	5
Men	15	8	6	1	24	9
Women	13	10	11	3	26	8

§ *Endolimax nana* (5/17), *Dientamoeba fragilis* (5/17), *Giardia intestinalis* (5/17), *Entamoeba coli* (3/17), *Entamoeba hartmanni* (3/17), *Enterobius vermicularis* (3/17), *Cystoisospora belli* (1/17), *Chilomastix mesnili* (1/17), *Iodamoeba butschlii* (1/17).

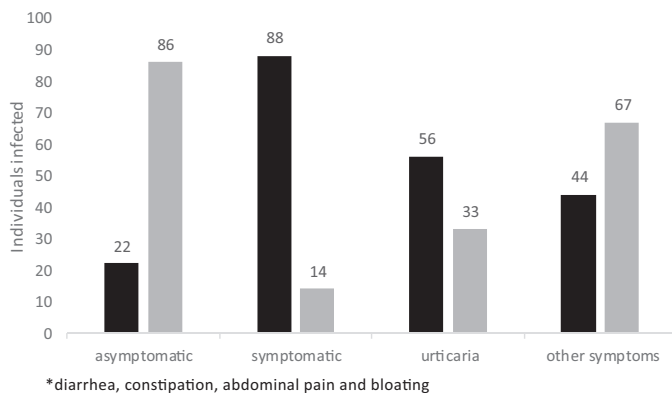


Fig. 1. Percentage of *Blastocystis* in asymptomatic and symptomatic patients: adults (black bars) and children (gray bars) with urticaria and other gastrointestinal symptoms*.

3.1. Morphotypes distribution

Sixty-seven patients out of the 270 included in the study were positive for *Blastocystis* (24.8%). From the positive patients 39 out of 67 (58.2%) and 28 out of 67 (41.8%) were symptomatic and asymptomatic respectively (Fig. 1). In the symptomatic patients, the distribution of *Blastocystis* infection was as follows NSGI (48.7%), Ur (46.6%), and Ur + NSGI (7.7%) (Table 1). Co-infections with other intestinal parasites (direct-mount examination and permanent staining) were detected in 25.4% (17/67) of the total of patients harboring *Blastocystis* infection.

The predominant morphotypes observed in fresh stools were V and G forms in 75% and 23.5% respectively. Only one adult with Ur revealed Am-like forms (1.5%) (Fig. 2). Under Jones' culture media after 7 days culture follow-up, V forms were observed in 97% of cultures followed by G forms (3%), no Am-like forms were detected. No statistical association was observed when *Blastocystis*



Fig. 2. Ameboid-like form detected in a *Blastocystis* infected patient.

morphotype and symptoms were compared ($p > 0.05$). *Blastocystis* alone or co-parasitized revealed no significant association in both asymptomatic and symptomatic groups ($p = 0.5$).

3.2. *Blastocystis* subtypes detected

Blastocystis subtypes demonstrated that 71.6% ($n = 48$) showed a ST3 profile, of which 71.4%, ($n = 35$) and 28.6% ($n = 14$), belonged to symptomatic and asymptomatic respectively. Other subtypes identified were ST1 (14.9%, $n = 10$), ST6 (7.5%, $n = 5$) and ST2 (5.9%, $n = 4$) (Fig. 3). Alleles retrieved from the *Blastocystis* 18S database evidenced that, while $\alpha 34$ and $\alpha 134$ represented 72% (49/68) of all alleles retrieved; $\alpha 34$ was detected in 85.7% (18/21) of patients with Ur as compared with asymptomatic and NSGI cohorts ($p < 0.0001$) (Table 2). On the other hand, $\alpha 134$ predominated in 46.4% of asymptomatic (control group) ($p < 0.0001$) and in 42.8% of patients with NSGI symptoms ($p < 0.0001$).

4. Discussion

Blastocystis is a protist able to colonize the intestinal tract of a diverse range of hosts and the fact *Blastocystis* is detected among healthy and diseased individuals complicates efforts to reach a consensus regarding its clinical significance. The increasing interest in deciphering *Blastocystis* biology has been driven by the unresolved controversy concerning its role in pathogenicity. This microorganism appears to exist in different morphological forms and whether the morphotypes observed are truly linked to gastrointestinal or extraintestinal disorders is still under revision and controversy. *Blastocystis* pathogenic potential (if any) is still puzzling. Recently there is increasing evidence that *Blastocystis* is a proxy for gut health (Stensvold, 2014).

Our findings regarding the prevalent morphotypes detected in feces and culture are in agreement with previous reports where V and Gr forms are described as the predominant ones (Tan and Suresh, 2006; Zhang et al., 2012). The fact that the Am-like parasites were detected only in a low percentage of fresh feces (1.5%) but not in culture, leads us to consider they are rare and probably their presence are likely to be the result of changes in biological–physical–chemical conditions rather than particular morphotypes (Alfellani et al., 2013). Similarly, the fact that 95% of V forms were associated with Ur as to NSGS, might reflect that *Blastocystis* pathogenic potential could be triggered whether in its amoeboid form or not. Amoeboid *Blastocystis* relevance with gastrointestinal symptoms and hives could possibly be interpreted as a collateral outcome (and not a cause) of gut microbiota imbalance as reported elsewhere (Vassalos et al., 2010; Katsarou-Katsari et al., 2008; Valsecchi et al., 2004). Nevertheless, samples from new surveys have to be higher and statistically validated in order to corroborate these findings in our populations. On the other hand, we compared *Blastocystis* morphotypes from asymptomatic and symptomatic groups and found not to be significantly different ($p = 0.5$). Presumably, different morphologies linked to symptoms reported

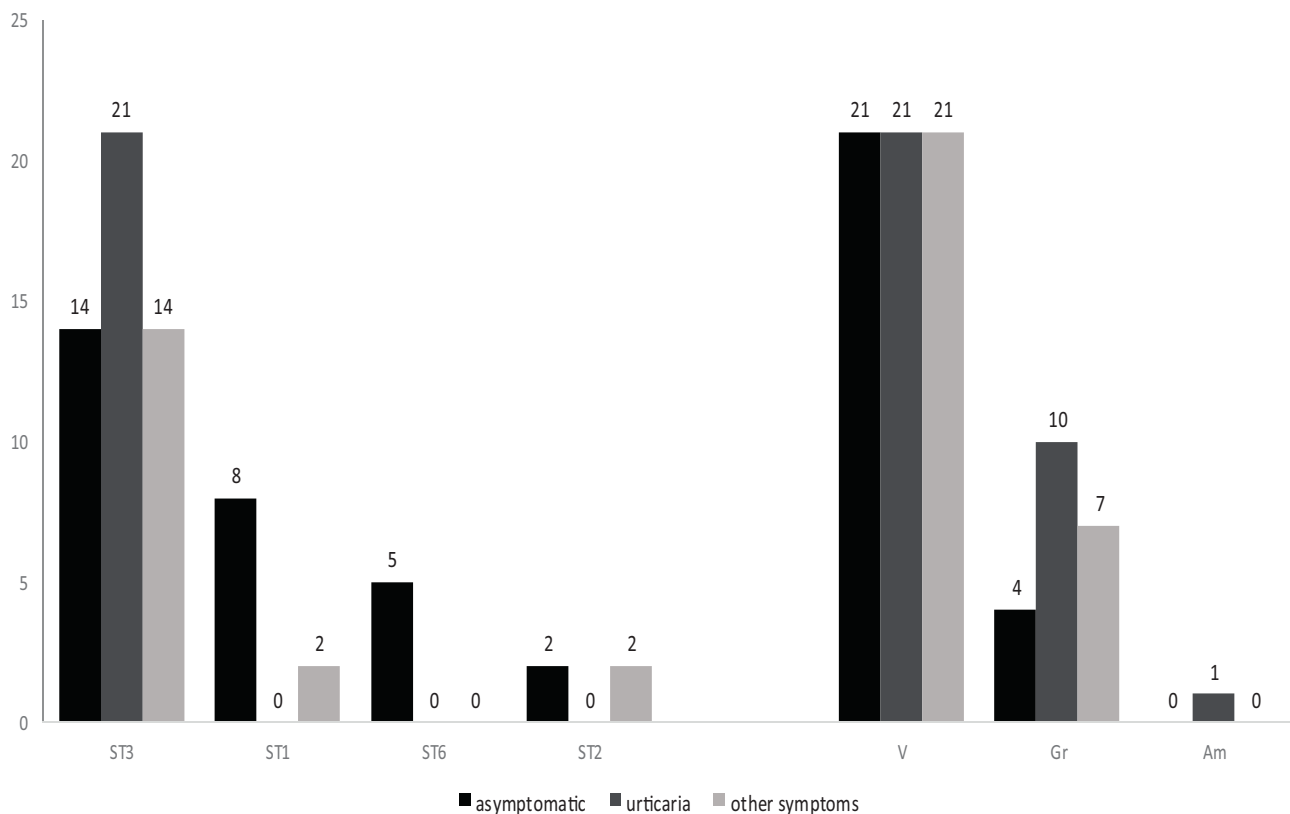


Fig. 3. *Blastocystis* subtypes and morphotypes distribution. Asymptomatic (black bars), urticaria (dark gray bars) and other symptoms study subjects (light gray bars). Vacuolar forms (V), granular forms (Gr), ameboid-like forms (Am).

Table 2

Blastocystis and alleles (a) distribution among asymptomatic carriers and symptomatic subjects with (Ur) urticaria and NSGS*.

	ST3 (n = 49)		ST1 (n = 10)		ST6 (n = 5)	ST2 (n = 4)
	a34	a134	a2	a4	a122	a9
Ur	18** (p < 0.00001)	3	0	0	0	0
NSGS*	1**	13 (p < 0.00001)	1	1	0	2
Asymptomatic	2	12 (p < 0.00001)	5	3	5	2
Total	21	28	6	4	5	4

* Diarrhea, constipation, abdominal pain, bloating.

** Includes one patient with Ur and diarrhea (NSGS) who presented both a34 and a134.

§ Non significant, p > 0.05.

in different regions of the world, not seen in our isolates, might reflect geographic variations of this protist (Tan, 2008; Malatyali and Özçelik, 2011; Ragavan et al., 2014).

The genus *Blastocystis* comprises a variety of lineages or so-called STs where more than 90% of human isolates are grouped into STs 1–4. Although dramatic differences are seen across regions (Ramírez et al., 2014; Rivera, 2008), the distribution of STs in our samples demonstrated that ST3 was the most frequently detected ST, followed by STs 1, 6 and 2; these findings are similar to those previously reported in the Americas (Alfellani et al., 2013). The presence of ST6 is of interest, because particularly this subtype has been only detected in wild animals but not in humans across South America (Ramírez et al., 2014; Malheiros et al., 2011; Villalobos et al., 2014) and the fact that ST6 in Europe represents one of the STs identified in human samples might reflect the European genetic background present in Argentinean populations or vast migrant flow among these areas suggesting that host-factors might play a relevant role on the pathogenesis adaptation of *Blastocystis*

infection. An interesting point to address is the absence of ST4; this ST appears relatively common in *Blastocystis*-positive European human patients with diarrhea. In South-America, only in Colombia, ST4 was detected in two specimens of *Alouatta* spp., which are considered primates linked to wild-life habitats and not presenting any or little synanthropic activities relation which does not associate this ST with human infection. The fact that ST4 was not identified in our patients, might indicate not only the relative diversity and prevalence of STs isolated from humans, but the different epidemiological/geographical/demographic patterns of this parasitic colonization and/or infection.

The development and implementation of molecular tools together with the characterization of STs/alleles allowed us exploring possible associations between *Blastocystis* genetic diversity and certain disease/health aspects. Although our results reflect a clear excess of ST3 in Ur and NSGI samples, the distribution of this ST within symptomatic groups and asymptomatic controls did not show any overall significant difference ($p = 0.05$) but it is

of interest the detection of $\alpha 34$ in 85.7% (18/21) of patients with Ur ($p < 0.0001$). Although no previous reports describe the likely pathogenic relationship of a particular allele and the fact that we cannot conclude that clones of *Blastocystis* carrying this allele could trigger this condition, our findings lead us to report the presence of a predominant allele significantly associated in patients with Ur.

It has been suggested that within ST3, the amoeboid form is the prevalent one may be due to the fact that parasites might adhere better to the epithelial cells lining the gut affecting gut immune homeostasis and resulting in an immune response against the parasite surface antigens, with consequent inflammatory-cell recruitment, release of histamine factors consequently activating mast cells and basophils leading to Ur (Valsecchi et al., 2004). On the other hand, $\alpha 134$ significantly present in NSGI and in the asymptomatic control group ($p < 0.0001$), might indicate a link between this allele with gastrointestinal symptoms other than skin pathologies in our patients. However, these associations are not merely absolute because for the case of Colombia, $\alpha 34$ was present in patients with IBS and in Argentina this allele was associated with Ur as compared with NSGI (Ramírez et al., 2014). These analyses suggest that a spatiotemporal sampling is required in South-America to understand *Blastocystis* pathogenic potential in different genetic background populations. Another aspect to be considered is the related with the frequent associations in literature between *Blastocystis* and the irritable bowel syndrome (IBS) because symptoms referred as NSGS may resemble IBS (not previously diagnosed in our cohort after patient clinical examinations), the presence of *Blastocystis*/IBS association, as previously reported in other countries deserves to be investigated in our geographical area (Poirier et al., 2012; Yakoob et al., 2010). In Colombia, there was described a strict association between IBS and ST3 in humans (Ramírez et al., 2014) but other groups have not detected this association at all. Moreover, the fact that *Blastocystis* is detected in both healthy and diseased individuals, its implication with gastrointestinal symptoms and the few reports dealing with *Blastocystis*/urticaria relationship complicates a consensus regarding a clinical significance. Our results not only represent the first report regarding *Blastocystis* genetic diversity in Argentina that contributes to the understanding of the molecular epidemiology of *Blastocystis* in South-America but also represent a good approach to understand the plausible association between *Blastocystis* STs and clinical manifestations probably caused by this protozoan. Our attempt clearly demonstrates the need to pursue investigations with the aim of establishing the real role of this protozoan in skin disorders and other types of symptomatology. The advent of new studies will undoubtedly expand the knowledge of human prevalent *Blastocystis* subtypes related to pathology/colonization.

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