

RESEARCH ARTICLE

Intestinal parasite infections in a rural community of Rio de Janeiro (Brazil): Prevalence and genetic diversity of *Blastocystis* subtypes

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OPEN ACCESS

Citation: Barbosa CV, Barreto MM, Andrade RdJ, Sodré F, d'Avila-Levy CM, Peralta JM, et al. (2018) Intestinal parasite infections in a rural community of Rio de Janeiro (Brazil): Prevalence and genetic diversity of *Blastocystis* subtypes. PLoS ONE 13 (3): e0193860. <https://doi.org/10.1371/journal.pone.0193860>

Editor: Maria Victoria Periago, Consejo Nacional de Investigaciones Científicas y Técnicas, Fundación Mundo Sano, ARGENTINA

Received: November 6, 2017

Accepted: February 20, 2018

Published: March 9, 2018

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. In this study, SSU rDNA partial gene sequences were deposited in GenBank under accession numbers KX523972 to KX524055 and MG309719.

Funding: Financial support was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Oswaldo Cruz

Abstract

Background

Intestinal parasitic infections are considered a serious public health problem and widely distributed worldwide, mainly in urban and rural environments of tropical and subtropical countries. Globally, soil-transmitted helminths and protozoa are the most common intestinal parasites. *Blastocystis* sp. is a highly prevalent suspected pathogenic protozoan, and considered an unusual protist due to its significant genetic diversity and host plasticity.

Methodology/main findings

A total of 294 stool samples were collected from inhabitants of three rural valleys in Rio de Janeiro, Brazil. The stool samples were evaluated by parasitological methods, fecal culture, nested PCR and PCR/Sequencing. Overall prevalence by parasitological analyses was 64.3% (189 out of 294 cases). *Blastocystis* sp. (55.8%) was the most prevalent, followed by *Endolimax nana* (18.7%), *Entamoeba histolytica* complex (7.1%), hookworm infection (7.1%), *Entamoeba coli* (5.8%), *Giardia intestinalis* (4.1%), *Iodamoeba butchillii* (1.0%), *Trichuris trichiura* (1.0%), *Pentatrichomonas hominis* (0.7%), *Enterobius vermicularis* (0.7%), *Ascaris lumbricoides* (0.7%) and *Strongyloides stercoralis* (0.7%). Prevalence of IPIs was significantly different by gender. Phylogenetic analysis of *Blastocystis* sp. and BLAST search revealed five different subtypes: ST3 (34.0%), ST1 (27.0%), ST2 (27.0%), ST4 (3.5%), ST8 (7.0%) and a non-identified subtype.

(PAPES-VI/FIOCRUZ) number (407768/2012-2) to HLCS and MMB and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro to CMd. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusions/significance

Our findings demonstrate that intestinal parasite infection rates in rural areas of the Sumidouro municipality of Rio de Janeiro, Brazil are still high and remain a challenge to public health. Moreover, our data reveals significant genetic heterogeneity of *Blastocystis* sp. subtypes and a possible novel subtype, whose confirmation will require additional data. Our study contributes to the understanding of potential routes of transmission, epidemiology, and genetic diversity of *Blastocystis* sp. in rural areas both at a regional and global scale.

Introduction

Intestinal parasitic infections (IPIs) are ubiquitous in humans, both in urban and rural environments from tropical and subtropical countries. The poorest and most deprived communities are at increased risk of intestinal parasitic infections, which are present in more than a quarter of the world's population [1–3]. The frequency of intestinal parasites is an indicator of low socioeconomic development of a population, which is directly associated with educational deficits and poor sanitary conditions [4]. However, in tropical developing countries, rural life is by itself associated with a high risk of infections due to negligible health knowledge, lower socio-economic conditions, inadequate environmental sanitation, insufficient water supply [5, 6] and higher contact rates with wildlife and domestic reservoirs of infection [7–9]. Rural populations experience a vicious cycle of malnutrition and re-infections leading to continuous morbidity and perpetuation of poverty cycles. IPIs are a public health problem caused by helminths and intestinal protozoa [3, 10, 11]. Globally, the soil-transmitted helminthes *Ascaris lumbricoides*, hookworm and *Trichuris trichiura* and the protozoan *Entamoeba histolytica*, *Giardia intestinalis* and *Cryptosporidium* sp. are the most common intestinal parasites [12]. Except for *E. histolytica*, *Cryptosporidium* spp and *Balantidium coli*, they are unable to invade the mucosal tissues or other organs. In the recent years, there has been a growing recognition that *Blastocystis* sp. presents pathogenic potential, although its virulence mechanisms are not understood. They infect a wide range of animals including birds, amphibians, reptiles, insects and mammals, including humans [13–18]. Although the current knowledge of reservoirs for human infection is limited, it is known that a vehicle for *Blastocystis* sp. transmission is close contact with animals [15, 16, 19]. *Blastocystis* sp. has potential pandemic distribution, possibly reaching 30% in industrialized countries and up to 76% in developing nations [12, 20, 21].

Analysis of the small-subunit ribosomal RNA of *Blastocystis* sp. isolates revealed substantial genetic diversity, represented by 17 genetically distinct ribosomal lineages (subtypes/ST1–ST17) [18, 22]. Ten subtypes (ST1–ST9, and ST12) have been isolated from human and animal fecal samples (except ST9) [7, 16, 23–25], whereas others were exclusively found in non-human hosts [14, 26]. The significance of *Blastocystis* sp. to public health, potential zoonotic differences and clinical outcomes may be related to specific subtype, a hypothesis that has been the topic of recent debate and requires further examination [15, 27].

The molecular epidemiology of *Blastocystis* sp. infections is still unknown in many parts of the world. Brazil is a continental country and information on the distribution of STs in the country is still incipient, but a high prevalence of *Blastocystis* sp. has been reported both in urban and rural environments. Moreover, IPIs are still a major public health problem, especially among the impoverished and underprivileged communities living in rural and remote areas, which represent an important target of public health interventions. To address this gap,

we investigated the prevalence of intestinal parasites and distribution of *Blastocystis* sp. STs in inhabitants of rural areas of Rio de Janeiro, Brazil. We report a high prevalence of intestinal parasitic infections and a wide range of *Blastocystis* sp. STs in the area.

Materials and methods

Study population

A cross-sectional study was carried out from October to December of 2013 in Pamparrão, Porteira Verde and Porteira Verde Alta valleys, in rural area of Sumidouro, State of Rio de Janeiro, Brazil (22° 02' 46" S; 42° 41' 21" W). The valleys are populated by small rural properties characterized by vegetable crops and pasture. Total population is around 14,900 inhabitants, and 87.7% of people work in agriculture [28]. The Sumidouro municipality is located in the central mountains bordering the Serra do Mar in Rio de Janeiro, at altitudes varying between 264 and 1,300 meters. The region exhibits marked climatic contrasts due to its peculiar geographical position and humid-mesothermic climate [29], with average rainfall between 153.5 mm and 269.4 mm.

Participants were informed about the study aims, potential risks and benefits, and provided written informed consent followed by signatures of two external witnesses. Relatives or legal tutors provided informed consent for children. Participation was voluntary and people could withdraw from the study at any time without further obligation. To preserve anonymity, each study participant was given a unique identification number. At the end of the study, all participants diagnosed with enteric parasitic pathogens received proper therapy. Treatment was not offered to individuals infected with *Blastocystis* sp., because its pathogenic potential is still a topic of debate. The study protocol was reviewed and approved by the Human Ethics Committee of the Faculdade Medicina from Universidade Federal Fluminense (CAAE 35028314.2.0000.5 243).

Parasitological assays

A total of 294 unpreserved stool samples were collected from participants at home, kept at 4°C and transported to the laboratory at the Fundação Oswaldo Cruz in ice packs. Fresh unpreserved stool samples were fractionated into two aliquots upon receipt. Parasitological examination was performed by spontaneous sedimentation technique (also known as Lutz technique or Hoffman, Pons and Janer technique)[30] and flotation in saturated sodium chloride solution density 1.2 g/mL[31]. Direct *in vitro* xenic cultivation of *Blastocystis* sp. from an aliquot of stool samples was performed using Pavlova's medium, supplemented with 10% heat inactivated adult bovine serum and penicillin-streptomycin antibiotics and incubated at 37°C [32]. Screening of *Blastocystis* sp. cultures was performed using standard light microscopy during the first three days of incubation. When typical forms of the parasite (vacuolar, granular and amoeboid forms) were observed, *Blastocystis* sp. culture aliquots were frozen at -20°C for subsequent DNA extraction. An aliquot of fresh unpreserved stool samples was stored at -20°C prior to analysis. Statistical analyses were carried out with Epi-Info 3.5.1. Relationships between variables were examined by chi-square tests with significance levels set at 5%.

Molecular analysis

DNA extraction from cultured samples, positive for *Blastocystis* sp. and from human stools, positive for *E. histolytica* complex was carried out using the Qiamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. Samples exhibiting

structures of the *E. histolytica* complex under microscopic examination were subjected to DNA amplification.

Nested PCR targeting 18S-like ribosomal RNA gene was applied to genetically identify the *E. histolytica*, *E. dispar* and *E. moshkovskii* complex [33]. First-round PCR for detection of *Entamoeba* genus used the forward primer E-1 (5' TAAGATGCACGAG AGCGAAA3') and reverse primer E-2 (5' GTACAAAG GGCAGGGACGTA 3'). PCR reaction was performed in a 50 μ L volume, with the final mix containing 10 \times PCR buffer, 1.25 mM of dNTPs, 2.5 mM MgCl₂, 10 pmoles of each primer, 2.5 U of Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 2.5 μ L of DNA template. PCR cycles consisted of 5 min at 95°C and 40 cycles of 30 s at 95°C, 60 s at 56°C, and 60 s at 72°C, with a final step of 2 min at 72°C. Afterwards, the primary PCR products were subjected to second-round PCRs for Entamoeba species-specific characterization, which had multiple primers sets in the same tube. Amplification was achieved using the following primer sets EH-1 (5'-AAGCATTGTTTCTAGA TCTGAG-3') and EH-2 (5'-AAGAG GTCTAACCGAAATTAG-3') to detect *E. histolytica* (439 bp); ED-1 (5'-TCTAATTTTCGATTA GAACTCT-3') and ED-2 (5'-TCCCTACCTATTAGACATAGC-3') to detect *E. dispar* (174 bp); Mos-1 (5'-GAAACCAAGAGTTTCACAAC-3') and Mos-2 (5'-CAATATAAGGCTTGGATGA T-3') to detect *E. moshkovskii* (553 bp) [33]. For the second PCR round, only the annealing temperature was changed to 48°C, while all other amplification cycle parameters were unchanged. Amplified products were visualized with stained with Gelred (Biotium Inc., Hayward, CA, USA) under UV transillumination after electrophoresis on 1.5% agarose gels. PCR amplification of partial SSU rDNA to identify subtypes of *Blastocystis* sp. was performed as protocol previously described [17], which amplifies a fragment approximately 500 bp long.

Genomic DNA was subjected to PCR analysis using primer pair Blast 505–532 (5' GGA GGT AGT GAC AAT AAATC 3') and Blast 998–1017 (5'TGC TTT CGC ACT TGT TCAT C 3'). The PCR reaction was performed in a final volume of 50 μ L and each reaction contained 100 mM of TrisHCl (pH 9.0), 500 mM of KCl, 1.5 mM of MgCl₂, 200 μ M of dATP, dGTP, dCTP, and dTTP each, 0.2 μ M of primer, 1.5 U of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.05% of bovine serum albumin (BSA), and 5 μ L of DNA sample. PCR conditions consisted of an initial cycle at 95°C for 5 min, 35 cycles including denaturing at 95°C for 30 s, annealing at 55°C for 30 s, extending at 72°C for 2 min, with a final step of 2 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel in Tris-borate ethylenediamine tetraacetic acid (EDTA) buffer, stained with Gelred (Biotium Inc., Hayward, CA, USA) and visualized under UV transillumination. Amplicons were purified using the Wizard[®] SV gel and PCR clean up system kit (Promega, Madison, WI, USA) and sequenced in both directions using Big Dye chemistries in an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Our SSU rDNA partial gene sequences were deposited in GenBank under accession numbers KX523972 to KX524055. They were compared to available GenBank sequences using the BLASTN program on the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov/BLAST>). In addition, DNA sequences of ST1 to ST9 of *Blastocystis* sp. were downloaded from Genbank, and multiple sequence alignment was performed using the ClustalW algorithm of the MEGA software version 6.0 [34].

Phylogenetic analysis

To identify the phylogenetic position of isolates, phylogenetic trees were created using *Proteromonas lacertaeas*, as the outgroup [35, 36]. Phylogenetic trees were constructed using two probabilistic methods: Maximum Likelihood (ML) and Bayesian Inference (BI), which are based on HKY + G (gamma distribution of rates with four rate categories) model [37]. The

best model of nucleotide substitutions was selected based on the Akaike Information Criterion using Jmodeltest [38] for ML, and Mrmodeltest for BI [39]. The ML tree was created using PhyML 3.1 [40] and BI in MrBayes software version 3.2 [41]. Bootstrap values were calculated through the analysis of 1000 replicates for the ML analysis and through Bayesian posterior probability analysis using the MCMC algorithm, with four chains. Chains were run for 10^7 generations and sampled every 100 generations. Convergence was assessed by evaluating the average standard deviation of split frequencies, which were well below the recommended values (< 0.01). The first 25% of the sampled trees were discarded as burn-in for each data set, and the consensus tree topology and nodal support were estimated from the remaining samples as posterior probability values.

Results

A total of 294 individuals participated in this study, 145 (49.3%) males and 149 (50.7%) females (age range = 2–87 years, mean age = 38.3 years). Participants were stratified by age and gender (Table 1). There were 21 (7.1%) participants aged 1–5 years, 24 (8.2%) aged 6–14 years, 190 (64.6%) aged 15–55 years and 59 (20.1%) participants aged 55 years and over. A total of 126 individuals (42.8%) provided three stool samples, 99 (33.6%) two samples and 69 (23.4%) one sample. The study population was characterized predominantly by adults (81.2%). Overall, 189 of participants out of 294 (64.3%) were infected with at least one enteric parasite, with gender specific prevalence being 54.8% in males and 45.2% females. Prevalence was significantly different between the genders ($\chi^2 = 6.82$; $p = 0.009$). The total prevalence of protozoan infections was 60.2%. The most predominant species was *Blastocystis* sp., followed by *Endolimax nana*, *E. histolytica* complex, *Entamoeba coli*, and *Giardia intestinalis*. The total prevalence of helminth infections was 13.7%. Hookworm was the most prevalent helminth species, followed by *Trichuris trichiura* (Table 1).

All 21 microscopically samples positive for *E. histolytica* complex were successfully amplified by nested PCR. Of those samples, 20 (95%) were positive for *E. dispar* and one (5.0%) had a mixed infection of *E. histolytica* and *E. dispar*. Culture and Hoffman techniques, both used for diagnosis of *Blastocystis* sp., exhibited very similar sensibility: 118 of 164 (71.9%) samples were diagnosed as positive by both techniques, 24 only by the use of culture and 22 only by the Hoffman technique. There was no statistical difference between techniques ($p = 0.888$). Of the 164 samples positive for *Blastocystis* sp., a subset of 85 (51.8%) was successfully sequenced. The sequencing of partial SSU rDNA gene indicated the presence of subtypes: ST3 (34.0%), ST1 (27.0%), ST2 (27.0%), ST4 (3.5%), ST8 (7.0%) and a non-identified subtype (ID 210). Sequences showed high identity (98–100%) to sequences available at the NCBI database, while sample ID 210 returned a BLAST identity of 93%. Topology of the constructed phylogenetic tree revealed that ST1, ST2, ST3, ST4, ST8 and the non-identified ST clustered into well-supported clades (with grouped bootstrap values over 70.0% and Bayesian posterior probabilities) (Fig 1). The non-identified *Blastocystis* sp. ST is evolutionarily closer to ST7. Intra-ST diversity was observed in ST1, ST2 and ST3 as indicated by their small clades.

Discussion

We assessed the prevalence of IPIs and identity of *Blastocystis* sp. subtypes in a rural community of Rio de Janeiro, Brazil. IPIs have been described as the greatest worldwide cause of diseases [42]. Current epidemiological evaluations suggest that 450 million people, especially in developing countries, may host multiple parasite species [1]. Our results showed that 64.3% of participants presented IPIs, with the most common human parasite being *Blastocystis* sp., in accordance to previous work across Brazilian regions [12, 43–46]. Although IPIs had received

Table 1. Distribution of intestinal parasites according to age groups and gender.

	Age group, in years				Gender*		Total (%)
	1–5	6–14	15–55	55 y plus	M	F	
N° (%)	21(7.1)	24(8.2)	190(64.6)	59(20.1)	145(49.3)	149(50.7)	294(100)
Pos	8 (38.0)	12 (50.0)	128 (67.3)	41 (69.5)	103 (71.0)	86 (57.7)	189 (64.3)
Neg	13 (62.0)	12 (50.0)	62 (32.7)	18 (30.5)	42 (29.0)	63 (42.3)	105 (35.7)
Blast	7 (33.3)	10 (41.7)	110 (57.9)	37 (62.7)	92 (63.4)	72 (48.3)	164 (55.8)
Ec	2 (9.5)	1 (4.2)	9 (4.7)	5 (8.5)	10 (6.9)	7 (4.7)	17 (5.8)
Eh complex	2 (9.5)	1 (4.2)	14 (7.4)	4 (6.8)	12 (8.3)	9 (6.0)	21 (7.1)
En	2 (9.5)	0 (0.0)	44 (23.2)	9 (15.3)	33 (22.8)	22 (14.8)	55 (18.7)
Gi	2 (9.5)	3 (12.5)	5 (2.6)	2 (3.4)	9 (6.2)	3 (2.0)	12 (4.1)
Ib	0 (0.0)	0 (0.0)	1 (0.5)	2 (3.4)	2 (1.4)	1 (0.7)	3 (1.0)
Hook	0 (0.0)	0 (0.0)	19 (10.0)	2 (3.4)	13 (9.0)	8 (5.4)	21 (7.1)
Ss	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.7)	0 (0.0)	1 (0.3)
Ph	0 (0.0)	1 (4.2)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.3)	2 (0.7)
Al	1 (4.8)	0 (0.0)	0 (0.0)	1 (1.7)	1 (0.7)	1 (0.7)	2 (0.7)
Tt	0 (0.0)	0 (0.0)	3 (1.6)	0 (0.0)	1 (0.7)	2 (1.3)	3 (1.0)
Ev	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.4)	1 (0.7)	1 (0.7)	2 (0.7)

Pos = positive samples; neg = negative samples; Blast = *Blastocystis* sp; Ec = *Entamoeba coli*; En = *Endolimax nana*; Eh complex = *E. histolytica*, *E. dispar*, or *E. moshkovskii* Gi = *Giardia intestinalis*; Ib = *Iodamoeba butschlii*; Hook = hookworm; Ss: *Strongyloides stercoralis*; Tt = *Trichiuris trichiura*; Ph = *Pentatrichomonas hominis*; Al = *Ascaris lumbricoides*; EV = *Enterobius vermicularis*. F = female; M = male; y = years.

*(p = 0.009)

<https://doi.org/10.1371/journal.pone.0193860.t001>

attention in Sumidouro as early as 1995, most of these efforts targeted Schistosomiasis. Gonçalves et al. [47] previously reported a prevalence of 13% of hookworm, 5% of *Ascaris lumbricoides*, 20% of *Entamoeba coli* and 10% of *Giardia intestinalis* in the Sumidouro population. The prevalence of protist parasites that we are reporting is much higher than of helminths, which may be explained by self-medication (antihelminths) practiced by the population, or by the use of pesticides in agriculture above recommended doses, which may have a significant effect on soil-transmitted helminths survival. However, these hypotheses still require investigation.

We also identified statistically significant differences in infection rates between men and women. Several investigations have reported a higher prevalence of parasitic infection in males than females [48, 49]. A possible explanation is the higher engagement of males in farming activities. Most of the Sumidouro population is rural and their most important economic activities are cattle breeding and vegetable cultivation. People from rural communities usually eat fresh food, drink untreated water and disposal of sewage is generally inadequate [50], resulting in increased infection risk. The diversity of protist parasites revealed by our analyses suggest that their distribution depends on factors such as environmental conditions, lack of sanitation and health education, confirming previous studies in rural communities [51, 52]. It was shown that short-term xenic *in vitro* culture (XIVC) is a more sensitive diagnostic method than parasitological examination [53]. Contrary to the expectations, our findings showed that the proportion of specimens negative by parasitological examination but positive by xenic *in vitro* culture, and vice-versa, were similar. The likely explanation is that the fecal samples did not contain viable *Blastocystis* sp. or alternatively cultured organisms were scant and imperceptible thereby avoiding detection by microscopy. Moreover, the screening of *Blastocystis* sp. cultures was performed in the first three days of incubation, when parasite count was low due to slow growth rates.

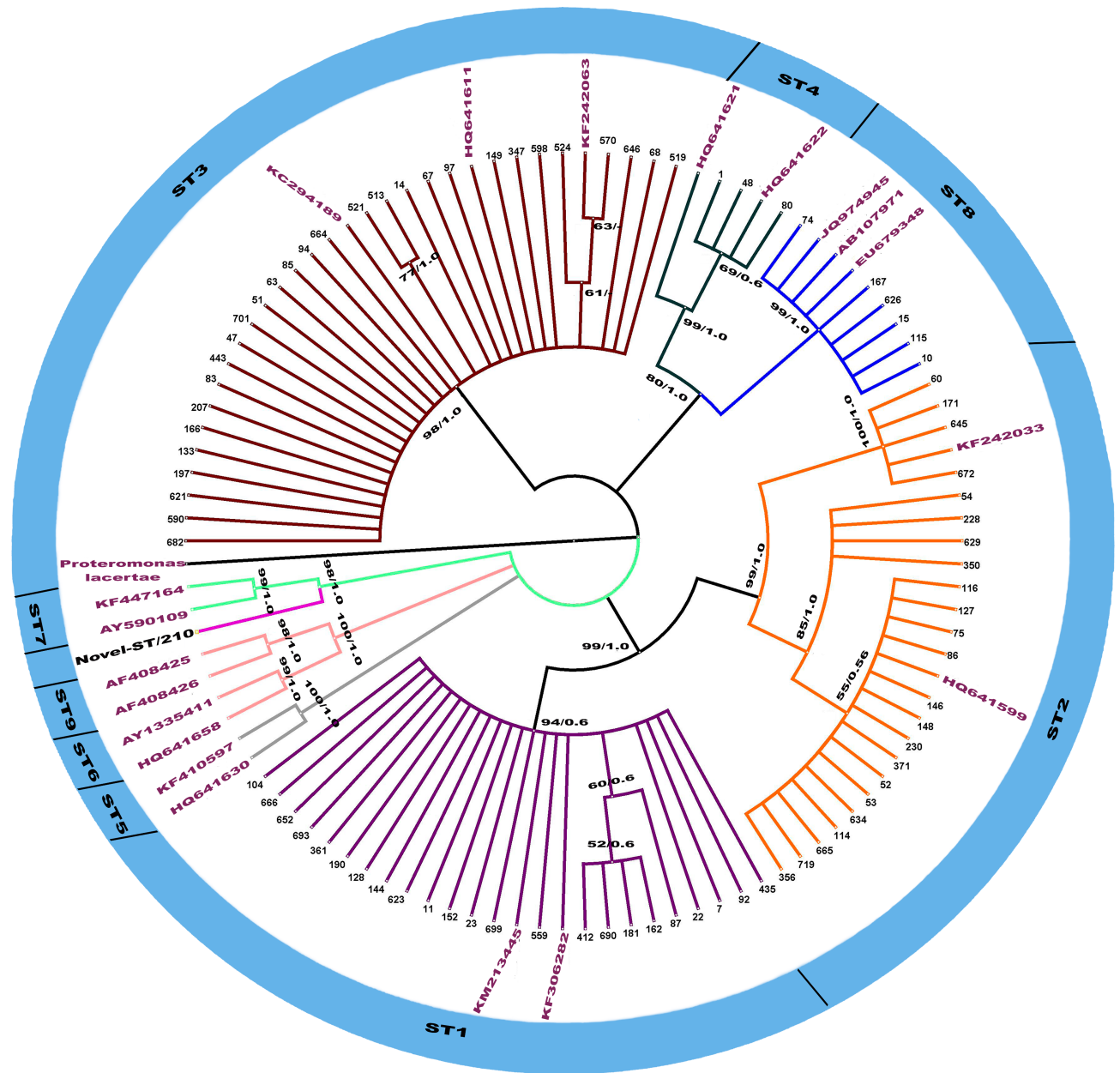


Fig 1. Maximum likelihood (ML) and Bayesian inference (BI) trees based on the partial sequences of the partial SSU rDNA (437 bp) of *Blastocystis* sp. isolates of the present study. The high genetic divergence of rDNA gene revealed six clusters (denoted by ST1, 2, 3, 4, 8 and Novel-ST). The numbers along branches correspond to bootstrap values and Bayesian posterior probability values (values above 50% are shown) in the order: MP/BI.

<https://doi.org/10.1371/journal.pone.0193860.g001>

Screening after a week of cultivation for example might reveal additional positives missed by the analyses performed herein.

The predominant *Blastocystis* sp. subtype was ST3, ST1 and ST2. STs 1, 2 and 3 had already been identified as the most abundant subtypes in previous studies [53–56]. Recently, Ramirez et al. [21] have reported percentages ranging from 15 to 92% regarding these subtypes. Other investigations have singled out ST3 as the most frequent subtype [18, 57–64]. Subtypes 1 and 2 have been identified both in humans and a wide range of synanthropic animals such as pigs, rats, dogs, horses, monkeys, cattle and chickens [15, 16, 65]. A recent study using animal

models revealed that various human *Blastocystis* sp. subtypes, including ST1 and ST2, could infect chickens and rats [66, 67], suggesting the zoonotic potential of human *Blastocystis* sp. isolates. We also observed the occurrence of ST4 in only three residents, although this subtype is the second most common in the UK and commonly found across Europe [15, 27, 68–70]. In Colombia, ST4 was identified in a few humans and non-human primates [21]. Rodents were also shown to be reservoir hosts of ST4 [23, 71]. Brazil was the only country in South America where ST8 has been identified [21], although it was also found in Denmark, the UK, Italy and Australia [62, 65, 72, 73]. Large American opossums and non-human primates can be infected by ST8, which is rarely found in other hosts [18]. We could not identify the subtype from sample 210. However, our phylogenetic tree shows that the sample is closely related to ST7. A similar case was reported by Ramirez et al. [21] who reported a novel ST closely related to ST1 and found in six South American countries including Brazil. Further analyses and full-length sequencing of the SSU-rDNA gene are required to truly confirm the existence of the novel ST.

Conclusion

Our study revealed the presence of IPIs in a rural community in Brazil, with high prevalence and a wide diversity of *Blastocystis* sp. subtypes, and possibly a new subtype of the protozoan. Our findings reinforce the need for additional studies of *Blastocystis* sp. subtype diversity, which may contribute to more efficient identification of infection sources and potential transmission routes under various ecological conditions, as well as a re-evaluation of control strategies by public health services.

Supporting information

S1 Table. New sequences generated as a result of this study.
(DOCX)

Acknowledgments

The authors thank Marisa Soares for her significant contributions given to the study and the staff from the DNA sequencing platform/PDTIS Fiocruz.

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References

1. Robertson LJ, van der Giessen JW, Batz MB, Kojima M, Cahill S. Have foodborne parasites finally become a global concern? *Trends Parasitol.* 2013; 29(3):101–3. Epub 2013/02/05. <https://doi.org/10.1016/j.pt.2012.12.004> PMID: 23375923.
2. de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, Savioli L. Soil-transmitted helminth infections: updating the global picture. *Trends Parasitol.* 2003; 19(12):547–51. Epub 2003/12/03. PMID: 14642761.
3. Alum A, Rubino JR, Ijaz MK. The global war against intestinal parasites—should we use a holistic approach? *Int J Infect Dis.* 2010; 14(9):e732–8. Epub 2010/04/20. <https://doi.org/10.1016/j.ijid.2009.11.036> PMID: 20399129.
4. Agudelo-Lopez S, Gomez-Rodriguez L, Coronado X, Orozco A, Valencia-Gutierrez CA, Restrepo-Betancur LF, et al. Prevalence of intestinal parasitism and associated factors in a village on the Colombian Atlantic Coast. *Rev Salud Publica (Bogota).* 2008; 10(4):633–42. Epub 2009/04/11. PMID: 19360213.
5. Abdulsalam AM, Ithoi I, Al-Mekhlafi HM, Ahmed A, Surin J, Mak JW. Drinking water is a significant predictor of *Blastocystis* infection among rural Malaysian primary schoolchildren. *Parasitology.* 2012; 139(8):1014–20. Epub 2012/03/27. <https://doi.org/10.1017/S0031182012000340> PMID: 22444778.
6. Speich B, Croll D, Furst T, Utzinger J, Keiser J. Effect of sanitation and water treatment on intestinal protozoa infection: a systematic review and meta-analysis. *Lancet Infect Dis.* 2016; 16(1):87–99. Epub 2015/09/26. [https://doi.org/10.1016/S1473-3099\(15\)00349-7](https://doi.org/10.1016/S1473-3099(15)00349-7) PMID: 26404667.
7. Yoshikawa H, Wu Z, Pandey K, Pandey BD, Sherchand JB, Yanagi T, et al. Molecular characterization of *Blastocystis* isolates from children and rhesus monkeys in Kathmandu, Nepal. *Vet Parasitol.* 2009; 160(3–4):295–300. Epub 2009/01/13. <https://doi.org/10.1016/j.vetpar.2008.11.029> PMID: 19136214.
8. Helenbrook WD, Shields WM, Whipps CM. Characterization of *Blastocystis* species infection in humans and mantled howler monkeys, *Alouatta palliata aequatorialis*, living in close proximity to one another. *Parasitol Res.* 2015; 114(7):2517–25. Epub 2015/04/11. <https://doi.org/10.1007/s00436-015-4451-x> PMID: 25859926.
9. Yoshikawa H, Tokoro M, Nagamoto T, Arayama S, Asih PB, Rozi IE, et al. Molecular survey of *Blastocystis* sp. from humans and associated animals in an Indonesian community with poor hygiene. *Parasitol Int.* 2016; 65(6 Pt B):780–4. Epub 2016/04/16. <https://doi.org/10.1016/j.parint.2016.03.010> PMID: 27080248.
10. Turkeltaub JA, McCarty TR, 3rd, Hotez PJ. The intestinal protozoa: emerging impact on global health and development. *Curr Opin Gastroenterol.* 2015; 31(1):38–44. Epub 2014/11/14. <https://doi.org/10.1097/MOG.000000000000135> PMID: 25394233.
11. Hotez PJ, Bottazzi ME, Strych U, Chang LY, Lim YA, Goodenow MM, et al. Neglected tropical diseases among the Association of Southeast Asian Nations (ASEAN): overview and update. *PLoS Negl Trop Dis.* 2015; 9(4):e0003575. Epub 2015/04/17. <https://doi.org/10.1371/journal.pntd.0003575> PMID: 25880767; PubMed Central PMCID: PMC4400050.
12. David EB, Guimaraes S, de Oliveira AP, Goulart de Oliveira-Sequeira TC, Nogueira Bittencourt G, Moraes Nardi AR, et al. Molecular characterization of intestinal protozoa in two poor communities in the State of Sao Paulo, Brazil. *Parasit Vectors.* 2015; 8:103. Epub 2015/04/19. <https://doi.org/10.1186/s13071-015-0714-8> PMID: 25889093; PubMed Central PMCID: PMC4335703.
13. Abe N. Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. *Vet Parasitol.* 2004; 120(3):235–42. Epub 2004/03/26. <https://doi.org/10.1016/j.vetpar.2004.01.003> PMID: 15041098.
14. Yoshikawa H, Abe N, Wu Z. PCR-based identification of zoonotic isolates of *Blastocystis* from mammals and birds. *Microbiology.* 2004; 150(Pt 5):1147–51. Epub 2004/05/11. <https://doi.org/10.1099/mic.0.26899-0> PMID: 15133074.
15. Stensvold CR, Alfellani MA, Norskov-Lauritsen S, Prip K, Victory EL, Maddox C, et al. Subtype distribution of *Blastocystis* isolates from synanthropic and zoo animals and identification of a new subtype. *Int J Parasitol.* 2009; 39(4):473–9. Epub 2008/08/30. <https://doi.org/10.1016/j.ijpara.2008.07.006> PMID: 18755193.
16. Parkar U, Traub RJ, Vitali S, Elliot A, Levecke B, Robertson I, et al. Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers. *Vet Parasitol.* 2010; 169(1–2):8–17. Epub 2010/01/22. <https://doi.org/10.1016/j.vetpar.2009.12.032> PMID: 20089360.
17. Santin M, Gomez-Munoz MT, Solano-Aguilar G, Fayer R. Development of a new PCR protocol to detect and subtype *Blastocystis* spp. from humans and animals. *Parasitol Res.* 2011; 109(1):205–12. Epub 2011/01/07. <https://doi.org/10.1007/s00436-010-2244-9> PMID: 21210149.

18. Alfellani MA, Taner-Mulla D, Jacob AS, Imeede CA, Yoshikawa H, Stensvold CR, et al. Genetic diversity of *blastocystis* in livestock and zoo animals. *Protist*. 2013; 164(4):497–509. Epub 2013/06/19. <https://doi.org/10.1016/j.protis.2013.05.003> PMID: 23770574.
19. Rajah Salim H, Suresh Kumar G, Vellayan S, Mak JW, Khairul Anuar A, Init I, et al. *Blastocystis* in animal handlers. *Parasitol Res*. 1999; 85(12):1032–3. Epub 1999/12/22. PMID: 10599928.
20. Balint A, Doczi I, Bereczki L, Gyulai R, Szucs M, Farkas K, et al. Do not forget the stool examination!—cutaneous and gastrointestinal manifestations of *Blastocystis* sp. infection. *Parasitol Res*. 2014; 113(4):1585–90. Epub 2014/02/21. <https://doi.org/10.1007/s00436-014-3805-0> PMID: 24553977.
21. Ramirez JD, Sanchez LV, Bautista DC, Corredor AF, Florez AC, Stensvold CR. *Blastocystis* subtypes detected in humans and animals from Colombia. *Infect Genet Evol*. 2014; 22:223–8. Epub 2013/07/28. <https://doi.org/10.1016/j.meegid.2013.07.020> PMID: 23886615.
22. Stensvold CR, Alfellani M, Clark CG. Levels of genetic diversity vary dramatically between *Blastocystis* subtypes. *Infect Genet Evol*. 2012; 12(2):263–73. Epub 2011/11/26. <https://doi.org/10.1016/j.meegid.2011.11.002> PMID: 22116021.
23. Noel C, Dufernez F, Gerbod D, Edgcomb VP, Delgado-Viscogliosi P, Ho LC, et al. Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *J Clin Microbiol*. 2005; 43(1):348–55. Epub 2005/01/07. <https://doi.org/10.1128/JCM.43.1.348-355.2005> PMID: 15634993; PubMed Central PMCID: PMC540115.
24. Parkar U, Traub RJ, Kumar S, Mungthin M, Vitali S, Leelayoova S, et al. Direct characterization of *Blastocystis* from faeces by PCR and evidence of zoonotic potential. *Parasitology*. 2007; 134(Pt 3):359–67. Epub 2006/10/21. <https://doi.org/10.1017/S0031182006001582> PMID: 17052374.
25. Yan Y, Su S, Ye J, Lai X, Lai R, Liao H, et al. *Blastocystis* sp. subtype 5: a possibly zoonotic genotype. *Parasitol Res*. 2007; 101(6):1527–32. Epub 2007/08/01. <https://doi.org/10.1007/s00436-007-0672-y> PMID: 17665214.
26. Cian A, El Safadi D, Osman M, Moriniere R, Gantois N, Benamrouz-Vanneste S, et al. Molecular Epidemiology of *Blastocystis* sp. in Various Animal Groups from Two French Zoos and Evaluation of Potential Zoonotic Risk. *PLoS One*. 2017; 12(1):e0169659. Epub 2017/01/07. <https://doi.org/10.1371/journal.pone.0169659> PMID: 28060901;
27. Stensvold CR, Christiansen DB, Olsen KE, Nielsen HV. *Blastocystis* sp. subtype 4 is common in Danish *Blastocystis*-positive patients presenting with acute diarrhea. *Am J Trop Med Hyg*. 2011; 84(6):883–5. Epub 2011/06/03. <https://doi.org/10.4269/ajtmh.2011.11-0005> PMID: 21633023; PubMed Central PMCID: PMC3110361.
28. BRASIL. Contagem Populacional. Censo Demográfico 2010. Instituto Brasileiro de Geografia e Estatística (IBGE) 2010.
29. Nimer E. Climatologia do Brasil 2.ed. ed: IBGE, Departamento de Recursos Naturais e Estudos Ambientais; 1989.
30. Hoffman WA, Pons JA, Janer JL. The sedimentation concentration method in *schistosomiasis mansoni*. *P R J Publ Health Trop Med*. 1934; 9: 283–91.
31. Willis H H. A simple levitation method for the detection of hook-worm ova. *Medicine Journal of Australia*. 1921; 29: 375–376
32. Zerpa LRH, L.; Naquira C.; Espinoza I. A simplified culture method for *Blastocystis hominis*. *Revista Mexicana de Patología Clínica*. 2000; 47(1):17–9.
33. Khairnar K, Parija SC. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. *BMC Microbiol*. 2007; 7:47. Epub 2007/05/26. <https://doi.org/10.1186/1471-2180-7-47> PMID: 17524135; PubMed Central PMCID: PMC1888694.
34. Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; 30(12):2725–9. Epub 2013/10/18. <https://doi.org/10.1093/molbev/mst197> PMID: 24132122; PubMed Central PMCID: PMC3840312.
35. Leipe DD TS, Goggin CL, Slemenda SB, Pieniazek NJ, Sogin ML. 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacerate* confirm that the stramenopiles are a primarily heterotrophic group. *European Journal of Protistology*. 1991; 32(4):449–58. [https://doi.org/10.1016/S0932-4739\(96\)80004-6](https://doi.org/10.1016/S0932-4739(96)80004-6)
36. Silberman JD, Sogin ML, Leipe DD, Clark CG. Human parasite finds taxonomic home. *Nature*. 1996; 380(6573):398. Epub 1996/04/04. <https://doi.org/10.1038/380398a0> PMID: 8602239.
37. Hasegawa M, Iida Y, Yano T, Takaiwa F, Iwabuchi M. Phylogenetic relationships among eukaryotic kingdoms inferred from ribosomal RNA sequences. *J Mol Evol*. 1985; 22(1):32–8. Epub 1985/01/01. PMID: 3932662.

38. Posada D, Buckley TR. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst Biol.* 2004; 53(5):793–808. Epub 2004/11/17. <https://doi.org/10.1080/10635150490522304> PMID: 15545256.
39. Nylander JAA. Mrmodeltest 2.3. Program Distributed by the Author. Uppsala University; Uppsala, Switzerland. 2004.
40. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol.* 2003; 52(5):696–704. Epub 2003/10/08. PMID: 14530136.
41. Huelsenbeck JP, Ronquist F. Bayesian Analysis of Molecular Evolution Using MrBayes 2005. 183–226 p.
42. Steketee RW. Pregnancy, nutrition and parasitic diseases. *J Nutr.* 2003; 133(5 Suppl 2):1661S–7S. Epub 2003/05/06. PMID: 12730482.
43. Aguiar JI, Goncalves AQ, Sodre FC, Pereira Sdos R, Boia MN, de Lemos ER, et al. Intestinal protozoa and helminths among Terena Indians in the State of Mato Grosso do Sul: high prevalence of *Blastocystis hominis*. *Rev Soc Bras Med Trop.* 2007; 40(6):631–4. Epub 2008/01/18. PMID: 18200414.
44. Gil FF, Busatti HG, Cruz VL, Santos JF, Gomes MA. High prevalence of enteroparasitosis in urban slums of Belo Horizonte-Brazil. Presence of enteroparasites as a risk factor in the family group. *Pathog Glob Health.* 2013; 107(6):320–4. Epub 2013/10/05. <https://doi.org/10.1179/2047773213Y.000000107> PMID: 24091002; PubMed Central PMCID: PMC4001612.
45. Rebolla MF, Silva EM, Gomes JF, Falcao AX, Rebolla MV, Franco RM. High prevalence of *Blastocystis* spp. infection in children and staff members attending public urban schools in Sao Paulo state, Brazil. *Rev Inst Med Trop Sao Paulo.* 2016; 58:31. Epub 2016/04/14. <https://doi.org/10.1590/S1678-9946201658031> PMID: 27074325; PubMed Central PMCID: PMC4826084.
46. Cabrine-Santos M, Cintra Edo N, do Carmo RA, Nascentes GA, Pedrosa AL, Correia D, et al. Occurrence of *Blastocystis* spp. in Uberaba, Minas Gerais, BRAZIL. *Rev Inst Med Trop Sao Paulo.* 2015; 57(3):211–4. Epub 2015/07/23. <https://doi.org/10.1590/S0036-46652015000300005> PMID: 26200960; PubMed Central PMCID: PMC4544244.
47. Goncalves MM, Barreto MM, Maldonado A, Jr., Maione VR, Rey L, Soares Mda S. [Socio-cultural and ethical factors involved in the diagnosis of *schistosomiasis mansoni* in an area of low endemicity]. *Cad Saude Publica.* 2005; 21(1):92–100. Epub 2005/02/05. doi: /S0102-311X2005000100011. PubMed PMID: 15692642.
48. Goncalves AQ, Junqueira AC, Abellana R, Barrio PC, Terrazas WC, Sodre FC, et al. Prevalence of intestinal parasites and risk factors for specific and multiple helminth infections in a remote city of the Brazilian Amazon. *Rev Soc Bras Med Trop.* 2016; 49(1):119–24. Epub 2016/05/11. <https://doi.org/10.1590/0037-8682-0128-2015> PMID: 27163576.
49. Nasiri V, Esmailnia K, Karim G, Nasir M, Akhavan O. Intestinal parasitic infections among inhabitants of Karaj City, Tehran province, Iran in 2006–2008. *Korean J Parasitol.* 2009; 47(3):265–8. Epub 2009/09/03. <https://doi.org/10.3347/kjp.2009.47.3.265> PMID: 19724700; PubMed Central PMCID: PMC42735692.
50. Boonjaraspinyo S, Boonmars T, Kaewsamut B, Ekobol N, Laummaunwai P, Aukkanimart R, et al. A cross-sectional study on intestinal parasitic infections in rural communities, northeast Thailand. *Korean J Parasitol.* 2013; 51(6):727–34. Epub 2014/02/12. <https://doi.org/10.3347/kjp.2013.51.6.727> PMID: 24516280; PubMed Central PMCID: PMC43916464.
51. Valverde JG, Gomes-Silva A, De Carvalho Moreira CJ, Leles De Souza D, Jaeger LH, Martins PP, et al. Prevalence and epidemiology of intestinal parasitism, as revealed by three distinct techniques in an endemic area in the Brazilian Amazon. *Ann Trop Med Parasitol.* 2011; 105(6):413–24. Epub 2011/11/29. <https://doi.org/10.1179/1364859411Y.0000000034> PMID: 22117850; PubMed Central PMCID: PMC4100303.
52. Damazio SM, Lima Mde S, Soares AR, Souza MA. Intestinal parasites in a quilombola community of the Northern State of Espirito Santo, Brazil. *Rev Inst Med Trop Sao Paulo.* 2013; 55(3). Epub 2013/06/07. <https://doi.org/10.1590/S0036-46652013000300007> PMID: 23740008.
53. Stensvold CR, Arendrup MC, Jespersgaard C, Molbak K, Nielsen HV. Detecting *Blastocystis* using parasitologic and DNA-based methods: a comparative study. *Diagn Microbiol Infect Dis.* 2007; 59(3):303–7. Epub 2007/10/05. <https://doi.org/10.1016/j.diagmicrobio.2007.06.003> PMID: 17913433.
54. Popruk S, Udonsom R, Koombapong K, Mahittikorn A, Kusolsuk T, Ruangsittichai J, et al. Subtype distribution of *Blastocystis* in Thai-Myanmar border, Thailand. *Korean J Parasitol.* 2015; 53(1):13–9. Epub 2015/03/10. <https://doi.org/10.3347/kjp.2015.53.1.13> PMID: 25748704; PubMed Central PMCID: PMC4384802.
55. Malheiros AF, Stensvold CR, Clark CG, Braga GB, Shaw JJ. Short report: Molecular characterization of *Blastocystis* obtained from members of the indigenous Tapirape ethnic group from the Brazilian

- Amazon region, Brazil. *Am J Trop Med Hyg.* 2011; 85(6):1050–3. Epub 2011/12/07. <https://doi.org/10.4269/ajtmh.2011.11-0481> PMID: 22144442; PubMed Central PMCID: PMCPCMC3225150.
56. Rene BA, Stensvold CR, Badsberg JH, Nielsen HV. Subtype analysis of *Blastocystis* isolates from *Blastocystis* cyst excreting patients. *Am J Trop Med Hyg.* 2009; 80(4):588–92. Epub 2009/04/07. PMID: 19346381.
 57. Scicluna SM, Tawari B, Clark CG. DNA barcoding of *blastocystis*. *Protist.* 2006; 157(1):77–85. Epub 2006/01/25. <https://doi.org/10.1016/j.protis.2005.12.001> PMID: 16431158.
 58. Yan Y, Su S, Lai R, Liao H, Ye J, Li X, et al. Genetic variability of *Blastocystis hominis* isolates in China. *Parasitol Res.* 2006; 99(5):597–601. Epub 2006/05/12. <https://doi.org/10.1007/s00436-006-0186-z> PMID: 16688468.
 59. Ozyurt M, Kurt O, Molbak K, Nielsen HV, Haznedaroglu T, Stensvold CR. Molecular epidemiology of *Blastocystis* infections in Turkey. *Parasitol Int.* 2008; 57(3):300–6. Epub 2008/03/14. <https://doi.org/10.1016/j.parint.2008.01.004> PMID: 18337161.
 60. Jones MS, Whipps CM, Ganac RD, Hudson NR, Boorum K. Association of *Blastocystis* subtype 3 and 1 with patients from an Oregon community presenting with chronic gastrointestinal illness. *Parasitol Res.* 2009; 104(2):341–5. Epub 2008/10/17. <https://doi.org/10.1007/s00436-008-1198-7> PMID: 18923844.
 61. Souppart L, Moussa H, Cian A, Sanciu G, Poirier P, El Alaoui H, et al. Subtype analysis of *Blastocystis* isolates from symptomatic patients in Egypt. *Parasitol Res.* 2010; 106(2):505–11. Epub 2009/12/03. <https://doi.org/10.1007/s00436-009-1693-5> PMID: 19953268.
 62. Meloni D, Sanciu G, Poirier P, El Alaoui H, Chabe M, Delhaes L, et al. Molecular subtyping of *Blastocystis* sp. isolates from symptomatic patients in Italy. *Parasitol Res.* 2011; 109(3):613–9. Epub 2011/02/23. <https://doi.org/10.1007/s00436-011-2294-7> PMID: 21340563.
 63. Bart A, Wentink-Bonnema EM, Gilis H, Verhaar N, Wassenaar CJ, van Vugt M, et al. Diagnosis and subtype analysis of *Blastocystis* sp. in 442 patients in a hospital setting in the Netherlands. *BMC Infect Dis.* 2013; 13:389. Epub 2013/08/27. <https://doi.org/10.1186/1471-2334-13-389> PMID: 23972160; PubMed Central PMCID: PMCPCMC3765316.
 64. Pandey PK, Verma P, Marathe N, Shetty S, Bavdekar A, Patole MS, et al. Prevalence and subtype analysis of *Blastocystis* in healthy Indian individuals. *Infect Genet Evol.* 2015; 31:296–9. Epub 2015/02/24. <https://doi.org/10.1016/j.meegid.2015.02.012> PMID: 25701123.
 65. Roberts T, Stark D, Harkness J, Ellis J. Subtype distribution of *Blastocystis* isolates identified in a Sydney population and pathogenic potential of *Blastocystis*. *Eur J Clin Microbiol Infect Dis.* 2013; 32(3):335–43. Epub 2012/09/22. <https://doi.org/10.1007/s10096-012-1746-z> PMID: 22996007.
 66. Iguchi A, Ebisu A, Nagata S, Saitou Y, Yoshikawa H, Iwatani S, et al. Infectivity of different genotypes of human *Blastocystis hominis* isolates in chickens and rats. *Parasitol Int.* 2007; 56(2):107–12. Epub 2007/01/26. <https://doi.org/10.1016/j.parint.2006.12.004> PMID: 17251054.
 67. Hussein EM, Hussein AM, Eida MM, Atwa MM. Pathophysiological variability of different genotypes of human *Blastocystis hominis* Egyptian isolates in experimentally infected rats. *Parasitol Res.* 2008; 102(5):853–60. Epub 2008/01/15. <https://doi.org/10.1007/s00436-007-0833-z> PMID: 18193282.
 68. Forsell J, Granlund M, Stensvold CR, Clark CG, Evengard B. Subtype analysis of *Blastocystis* isolates in Swedish patients. *Eur J Clin Microbiol Infect Dis.* 2012; 31(7):1689–96. Epub 2012/02/22. <https://doi.org/10.1007/s10096-011-1416-6> PMID: 22350386.
 69. Poirier P, Wawrzyniak I, Albert A, El Alaoui H, Delbac F, Livrelli V. Development and evaluation of a real-time PCR assay for detection and quantification of *blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies. *J Clin Microbiol.* 2011; 49(3):975–83. Epub 2010/12/24. <https://doi.org/10.1128/JCM.01392-10> PMID: 21177897; PubMed Central PMCID: PMCPCMC3067686.
 70. Dominguez-Marquez MV, Guna R, Munoz C, Gomez-Munoz MT, Borrás R. High prevalence of subtype 4 among isolates of *Blastocystis hominis* from symptomatic patients of a health district of Valencia (Spain). *Parasitol Res.* 2009; 105(4):949–55. Epub 2009/05/28. <https://doi.org/10.1007/s00436-009-1485-y> PMID: 19471964.
 71. Yoshikawa H, Nagano I, Wu Z, Yap EH, Singh M, Takahashi Y. Genomic polymorphism among *Blastocystis hominis* strains and development of subtype-specific diagnostic primers. *Mol Cell Probes.* 1998; 12(3):153–9. Epub 1998/07/17. <https://doi.org/10.1006/mcpr.1998.0161> PMID: 9664577.
 72. Stensvold R, Brillowska-Dabrowska A, Nielsen HV, Arendrup MC. Detection of *Blastocystis hominis* in unpreserved stool specimens by using polymerase chain reaction. *J Parasitol.* 2006; 92(5):1081–7. Epub 2006/12/13. <https://doi.org/10.1645/GE-840R.1> PMID: 17152954.
 73. Clark CG. Extensive genetic diversity in *Blastocystis hominis*. *Mol Biochem Parasitol.* 1997; 87(1):79–83. Epub 1997/07/01. PMID: 9233675.