REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

BRIEF COMMUNICATION

http://doi.org/10.1590/S1678-9946202062051

Culture isolation and molecular identification of *Blastocystis* sp. in Brazilian human isolates: preliminary results

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ABSTRACT

Blastocystis sp. is a protist commonly found in stool samples of humans and animals. Biological and genetic factors of this organism remain controversial. The present study aimed to develop and implement the *Blastocystis in vitro* culture of Brazilian human isolates for routine use. The fecal isolates (n = 20) were maintained in our laboratory by several passages in Pavlova's medium. Cultures were monitored every 72 h by light microscopy. Genomic DNA was extracted to identify the subtypes (STs). In most isolates, the vacuolar form was prevalent. The amoeboid, granular and cystic forms were observed during *in vitro* cultivation. STs 1, 2, 3, 4 and 7 were identified. Our preliminary results show the generation time and forms present in the *in vitro* culture of *Blastocystis* subtypes isolated from Brazilian human isolates. Therefore, we emphasize the use of *in vitro* culture as a tool in future studies for the better understanding of the biological aspects of *Blastocystis* sp.

KEYWORDS: *Blastocystis sp. In vitro* culture. Subtypes. Brazil.

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Received: 20 December 2019

Accepted: 1 July 2020

Over the years, the number of studies on biological and genetic factors of *Blastocystis* sp. has increased[5-7]. *In vitro* culture has been evaluated as a method for the sensitive diagnosis of *Blastocystis* infections[1,8,9]. *In vitro* culture has been shown to be two to five-fold more sensitive than standard smears and formalin-ethyl acetate concentration[8] that allows the parasite purification from fecal debris. The latter can negatively influence the molecular analysis, preventing its application in the clinical diagnosis of *Blastocystis* sp.[9]. In Brazil, studies have used *in vitro* culture of *Blastocystis* sp. as a parasitological diagnostic method[10,11] and as a method for molecular typing[12-14] but has not been reported as a tool for biological evaluations. The present study aimed to develop and implement in the clinical laboratory routine, the *in vitro* culture of *Blastocystis* from Brazilian human isolates to evaluate the generation time and the forms present in the culture, and to identify the subtypes in the isolates.

Blastocystis sp. is a unicellular protist that colonizes the intestinal tract of humans and is commonly found in stool samples in clinical laboratories[1,2]. Currently, studies based on the phylogenetic analysis of partial sequences of the ribosomal region (*SSU*-rDNA) classify *Blastocystis* sp. as a member of the Stramenopiles phylum[3,4]. The relationship between this organism and clinical manifestations in humans has been controversial until now[1,2].

The isolates were obtained from 20 fecal samples that yield positive results for *Blastocystis* sp. according to microscopic examinations held at the Section of Parasitology, Central Laboratory Division (HC/FMUSP), using the Faust, Lutz

and permanent-stained smears methods[15]. All the isolates were obtained from fresh fecal samples of asymptomatic patients. All the procedures performed in this study were in accordance with the ethical standards (protocol Nº 488-701) of the Ethical Committee of the Hospital das Clinicas da Faculdade de Medicina, Universidade de Sao Paulo, Brazil (HC/FMUSP). The samples were divided into two portions: one portion was cultured on the same day of collection and the other portion was stored at -20 °C for molecular analyses.

For the *in vitro* culture, approximately 200 mg of fecal samples containing a few forms (< 5 cells per field, x 400 magnification) and containing numerous forms (> 5 cells per field, x 400 magnification) were inoculated into 2-mL tubes containing Pavlova's medium[16] with 12 mg/mL ampicillin, 4 mg/mL streptomycin and 10% inactivated (50°C for 50 min) human serum. The culture tubes were incubated at 37 °C under anaerobic conditions. *Blastocystis* cultures were monitored every 72 h by light microscopy. Ten microliters of the culture were collected and *Blastocystis* forms were counted at x 400 magnification. Cultures were considered negative when *Blastocystis* sp. forms were not observed after 72 h. If *Blastocystis* sp. forms were detected, one aliquot of the culture was subcultured in fresh medium and the remaining was stored at -20 °C for molecular analyses. In addition, cell growth and viability, the type and number of *Blastocystis* forms, and the presence or absence of yeasts and bacteria before and during the subculture were evaluated.

For molecular analyses, DNA was extracted from the isolates obtained after 72 h of cultivation (exponential growth period) of fresh fecal samples, and from isolates recovered after more prolonged periods of *in vitro* cultivation, corresponding to the deceleration (reduction of cell division) and decline (initiation of cell lysis) periods. Genomic DNA of *Blastocystis* sp. was obtained using a QIAamp® DNA Stool Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. To determine the subtypes and alleles of *Blastocystis* sp., a fragment of approximately 600 bp, located at the *SSU*-rDNA, was amplified as described by Scicluna *et al.*[17]. PCR was performed following the protocol described by Melo *et al.*[18]. The amplification products were identified in 2% agarose gel electrophoresis with the addition of SYBR safe (Invitrogen™, Thermo Fisher Scientific Corporation, Waltham, MA, USA).

Both strands of the amplicons were sequenced using the ABI PRISM® BigDye™ Terminator kit (Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, USA), according to Sanger *et al.*[19] sequencing method. To determine the subtype and alleles of each sample, *Blastocystis* sp. sequences retrieved from the NCBI website using the BLASTn tool[20] and *Blastocystis* subtypes (18S) sequence typing[21] were aligned and compared with the sequences obtained in this study. The sequences were deposited in the GenBank database under the accession numbers MK511783-MK511793.

The *in vitro* culture can be used to identify *Blastocystis* sp. from fecal samples, mainly to facilitate the morphological discrimination, and has an excellent diagnostic performance compared with conventional microscopy[9]. However, many other biological aspects remain unexplored and a possible explanation is the lack of an adequate experimental model, that has not yet been well established for *Blastocystis.* In this context, the *in vitro* culture can be a promising tool for a better understanding of these aspects, such as antigen and antibody production, host-parasite interactions and strain differences[2,6,22].

The presence of a large number of forms was observed in the growing period of *in vitro* cultivation (exponential growth period), as already described in the literature[7,9]. Of the 20 isolates subjected to culture, 16 were maintained for up to 72 h. In our isolates, vacuolar, granular, amoeboid, cystic, multivacuolar and avacuolar forms were detected. In most isolates, the vacuolar form was the most prevalent form in the exponential growth period (Figure 1). The vacuolar form has been considered the typical *Blastocystis* cell form and the predominant form of the organism in culture[6-8], mainly in short-term *in vitro* cultivation[9]. In this study, the granular form was observed after 72 h but was not observed in the decline period. While the cultures were maintained, the amoeboid, granular and cystic forms were observed. Zhang *et al.*[22] described cysts in long-term cultures. In addition, the amoeboid forms have rarely been reported in cultures, mainly in fecal samples from symptomatic patients[6], however, only one isolate (IS05) was described.

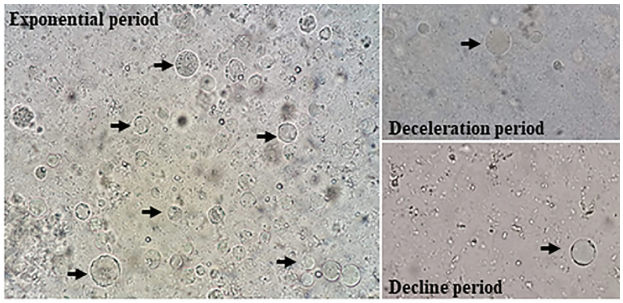

Figure 1 - Light microscopy images of the morphological forms of *Blastocystis* sp. observed in the exponential, deceleration and decline periods of *in vitro* cultivation. High-powered field (× 400 magnification).

In this study, the cultures were monitored for four months. The mean duration of *in vitro* cultures was 31.5 days (median,

17 days). Some studies have reported that the growth time of *Blastocystis* cultures may vary accordimg to the culture conditions[7], and among different subtypes[23]. According to Irikov *et al*.[7], the maximum *Blastocystis* cell count occurs between 3 and 5 days of culture. In the present study, the exponential period occurred between 3 to 6 days of culture, and the deceleration period began after 10 days in most cultures. During the decline period, yeast cells were observed. Some studies suggest the use of antibiotic and anti-fungi cocktails to eliminate potential contamination by bacteria or yeast, but this does not guarantee the successful elimination of contaminants or the culture survival[6].

The cultures with the highest viability were obtained from the following isolates: IS05 (72 days), IS15 (130 days) and IS16 (34 days). They contained the largest number of forms, mainly granular forms. Although the cultivation period in this study was four months, isolate IS15 was still maintained in culture after 130 days.

Of the 16 isolates that were maintained for at least 72 h, only 14 had results confirmed by PCR, reinforcing the applicability of the culture as a diagnostic tool. On the contrary, a study by Roberts *et al*.[24] found a lower sensitivity of culture compared with PCR. In our analysis of the 14 PCR products, 11 were successfully sequenced and three samples were excluded due to the low-quality of sequences These findings are in accordance with the literature[13,14]. Table 1 details the morphological and molecular characterization of isolates cultivated *in vitro*.

Subtypes 1 (27.3%), 2 (9.1%), 3 (27.3%), 4 (27.3%), and 7 (9.1%) were identified by molecular analysis (Table 1).

Table 1 - Morphological characterization during *in vitro* cultivation in the exponential (after 72 h of cultivation), deceleration (reduction of cell division) and decline (initiation of cell lysis) periods, and molecular identification of Brazilian-human isolates of *Blastocystis*.

Isolate (IS)	*Blastocystis* forms inoculated	Type of *Blastocystis* forms			Mean number of *Blastocystis* forms	Total period of *in vitro* maintenance (days)	Similarity (%)	Subtype	Alleles	GenBank Accession number
		Exponential period	Deceleration period	Decline period						
IS1	N	Vacuolar, amoeboid, binary division	Vacuolar, amoeboid, cysts,	Vacuolar, avacuolar, yeast	2×10^6	21	99.14-100	3	37	MK511783
IS2	F	Vacuolar, avacuolar, binary division	Vacuolar, avacuolar, multivacuolar	Vacuolar, avacuolar, yeast	10^6	21	99.48-100	3	36	MK511784
IS3	F	Vacuolar, avacuolar, binary division	Vacuolar	Vacuolar, yeast	6×10^5	17	98.65-100	1	4	MK511785
IS4	F	Vacuolar, avacuolar, binary division	Vacuolar	Vacuolar, yeast	10^6	15	99.00-100	3	34	MK511786
IS5	F	Vacuolar, granular, binary division	Vacuolar, granular, amoeboid, cysts	Vacuolar, yeast	4×10^6	72	98.33-100	4	42	MK511787
IS7	F	Vacuolar	Vacuolar	Vacuolar, yeast	4×10^5	6	99.35	4	42	MK511788
IS8	N	Vacuolar	Vacuolar	Vacuolar, yeast	4×10^5	6	99.35	4	42	MK511789
IS11	F	Vacuolar	Vacuolar	Vacuolar, yeast	6×10^5	16	98.51-100	1	4	MK511790
IS12	N	Vacuolar, granular, binary division	Vacuolar, granular	Vacuolar	2×10^6	9	95.83-100	7	99	MK511791
IS15	N	Vacuolar, granular, binary division	Vacuolar, granular, cysts, schizogony	Vacuolar, granular	2×10^6	130	97.85-99.83	2	12	MK511792
IS16	F	Vacuolar, granular, binary division	Vacuolar, granular	Vacuolar	4×10^5	34	98.65-100	1	4	MK511793

F = few forms (< 5 cells per field; x 400 magnification); N = numerous forms (> 5 cells per field; x 400 magnification).

The comparison of the sequences with reference ones described previously showed a similarity ranging from 95.83% to 100%. The allele assignment was obtained for all the sequences. For ST1 and ST4, all the sequences were classified as alleles 4 and 42, respectively. For ST3, three alleles were detected (34, 36 and 37), for ST2 (allele 12) and ST7 (allele 99). Variable alleles occurring within subtypes of *Blastocystis* in a given population are common[25].

Subtypes 1 (IS16) and 2 (IS15) were successfully maintained *in vitro* for 34 to 130 days, demonstrating that they were adapted to the culture conditions. In addition, we observed a shorter maintenance time of some isolates (IS7, IS8 and IS12) belonging to subtypes 4 and 7, that were maintained *in vitro* for 6 to 9 days. Furthermore, amoeboid forms were observed in IS05 (subtype 4), a characteristic that has been associated with the possible pathogenicity of *Blastocystis* sp.

Our findings show preliminary results involving the generation time and type of forms present *in vitro* during the maintenance of *Blastocystis* subtypes isolated in Brazil. Therefore, *in vitro* culture can be used as a tool in future studies, including a larger number of samples and clinical data, which will help the understanding of biological aspects of the *Blastocystis* sp.

ACKNOWLEDGMENTS

This study was supported by grants from the *Fundação de Amparo* à *Pesquisa do Estado de São Paulo* (FAPESP grant Nº 2015/18213-6) and *Coordenação de Aperfeiçoamento de Pessoa de Nível Superior* (CAPES grant Nº 1749729).

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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