The first molecular detection of *Blastocystis* subtypes in human faecal samples from Ibague, Colombia

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ABSTRACT

Blastocystis spp. has become one of the protozoans arousing the greatest scientific interest because of the controversy surrounding its biology; it is currently considered one of the most prevalent organisms in humans and animals worldwide. Such prevalence increases, especially in tropical countries where infection rates are high, highlighting the need to conduct studies focused on understanding this protozoan's biology. Interestingly, molecular tools are emerging as the best option for diagnosing this infection. This study was thus aimed at conventional PCR molecular detection and characterisation of *Blastocystis* spp. in human faecal samples from Ibagué, Colombia, using primers targeting the small subunit ribosomal ribonucleic acid (rRNA) gene. One hundred human faecal samples with confirmed *Blastocystis* spp. were studied, revealing the following subtype genetic diversity: ST1 50%, ST2 33% and ST3 17%. The results contributed to the limited information available regarding *Blastocystis* spp. in Colombia and created a reference point for further studies in the region.

1. Introduction

Blastocystis spp. is a cosmopolitan protozoan that lives in the intestinal tract of humans and numerous animals, so it is believed to have high zoonotic potential. It has been estimated that it infects more than 1 billion people worldwide, having higher prevalence in developing countries' tropical and subtropical areas (Ramírez et al., 2016). Although its pathogenic potential is still controversial, *Blastocystis* spp. is recognised as a protozoan having wide genetic diversity; it has different subtypes and information concerning at least 17 genetic subtypes (ST1-17) can be found in the pertinent literature (Del Coco et al., 2017; Stensvold, 2013). This study has involved the molecular detection and characterisation of *Blastocystis* spp. to provide preliminary results concerning the parasite's subtypes and their distribution in human faecal samples with confirmed *Blastocystis* spp. in Ibagué, Colombia.

2. Methods

The Naizir Clinical Laboratory delivered one hundred anonymous human faeces samples, collected between March 10th, 2017 and March 16th, 2018, having a positive diagnosis for *Blastocystis* spp. by microscopy to the University of Tolima's Tropical Para-

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sitology Research Laboratory and the samples were stored at 4 °C until further analysis. Faecal examination involved using Lugol's iodine as staining solution for a second microscopic observation to identify parasitic forms. The zinc sulphate flotation technique (Faust concentration method) was used with all samples to obtain the parasitic forms DNA (Faust, 1938; Faust et al., 1939).

Concentration products were washed by centrifuging to remove zinc sulphate from the samples and liquid nitrogen thermal shock and water bath at 56 °C were used for the rupture of cysts, alternating temperatures to facilitate the release of the protozoan's DNA (Polverino et al., 2004). Cellular proteins were digested with proteinase K (200 μg/ml) (Promega), using 1 μl proteinase K per 100 μl of sample at 56 °C for 1 h. DNA was extracted from 300 μl digested sample by the phenol-chloroform-isoamyl alcohol method (PCI) (Restrepo-Cardona, 2010); 50 μg/ml RNase A was used for purification at 37 °C for 1 h and subsequent repetition of the PCI method, for improving DNA quality (Sambrook and Green, 2012).

The extracted DNA was molecularly characterised by amplifying the small subunit ribosomal ribonucleic acid (SSU-rDNA) gene. The primers used for detecting *Blastocystis* spp. were bl1400ForC and bl1710RevC (Stensvold et al., 2006), having an expected 310 base pair (bp) product, while the primers designed by Yoshikawa et al., were used for subtype 1 to 7 differentiation (Yoshikawa et al., 2004). The thermal profiles were the same as those used in the reference (Yoshikawa et al., 2004), while the reaction mixture (20 μl total volume) consisted of 2 μl 10× buffer, 0.25 μl KCl (750 mM), 1.2 μl MgCl2 (25 mM), 1.6 μl dNTPs (2.5 mM), 0.8 μl primers (25 μ M), 0.1 μl Taq polymerase (1 U), 11.25 μl H$_2$O and 2 μl DNA.

Polyacrylamide gel electrophoresis (PAGE) was chosen for its high sensitivity for detecting the samples' PCR products due to the low amount of DNA recovered in the process; 4 μl of the PCR reaction products were taken and mixed with loading buffer for separation in a 1× Tris/Borate/EDTA (TBE) buffer solution at 80 V for 40 min. Silver nitrate was used for staining and a UV/White Light 8-W Transilluminator for products visualisation. Fragment size was determined using a 1 Kb Plus DNA ladder (Invitrogen).

Fifteen positive samples were chosen to confirm PCR accuracy and the amplification products were sent to Macrogen (Korea) for purification and sequencing. The STADEN package (DNA Sequence Read/Assembly Toolkit) was used for manually editing product sequences, MEGA 6.0 was used for aligning them and BLAST queries were used for comparing them to reference sequences retrieved from GenBank.

3. Results

The samples received from the clinical laboratory ready for fresh examination with Lugol's stain revealed the presence of *Blastocystis* spp. none of them diarrhoeal. The parasitic forms found were vacuolar, granular and cystic and, although no specific count was made, 1 to 3 forms were identified per field. Conventional PCR amplification gave a positive result for ninety-four out of the one hundred samples, the expected 310 bp band being observed (Stensvold et al., 2006). The entire treatment was repeated for the six samples that could not be amplified, giving a second negative result. The ninety-four samples proving positive by PCR detection were specifically amplified for characterisation by subtype. They were tested with the first seven subtypes, genetic diversity framed by only the first three subtypes being found and distributed as follows: 50% distribution (47/94) for ST1, 33% (31/94) for ST2 and 17% (16/94) for ST3; no coinfection was detected.

Of the subtypes identified by PCR, 15 samples having amplified intense bands (whose subtypes had been identified by band size) were sent to sequencing to confirm the subtype. The MEGA analysis led to the obtaining six well edited sequences that were used for local alignment with BLAST. Of the six, one was confirmed as ST3, 1 as ST2 and 1 as ST1, with percent identity of 97%, 77% and 81% respectively, with reference sequences in the GenBank database. The other three (which gave the ST3 banding in amplification) had identity with Blastocystis sequences but not with a specific subtype.

14% of the samples (14/100) had other organisms in them besides *Blastocystis* spp.; *Entamoeba coli* (2%), *Iodamoeba butschlii* (4%) and *Endolimax nana* (11%) were reported.

4. Discussion

The visualisation of three of the four main forms of *Blastocystis* spp., in this study was consistent with the information in the pertinent literature that indicates these forms as occurring most in extended human faecal samples (Anuar et al., 2013; Tan, 2008; Wawrzyniak et al., 2013; Zhang et al., 2003). Despite recent efforts focused on research regarding this protozoan, little information is currently available regarding the use of molecular techniques for detecting *Blastocystis* spp. in human faecal samples. Little *Blastocystis* spp. research has been carried out in South America and the few studies have mostly involved conventional diagnosis, such as light microscopy, PCR just now being used as a tool in such studies.

Venezuela, Argentina and Brazil are some of the region's countries that have provided information in the literature regarding this protozoan, Brazil having more frequently complemented its work with molecular techniques. Specific research involving the use of molecular methods concerning *Blastocystis* spp. is being fully developed in Colombia (Ramírez et al., 2014; Ramírez et al., 2017; Sánchez et al., 2017). This investigation can be added to the few studies using molecular tools as a diagnostic method, focusing just on defining the protozoan's current situation in a target city (i.e. in this case, Ibague).

Regarding genetic diversity, ST3 has been recorded internationally as the subtype occurring most frequently in humans, followed by subtypes 1, 2 and, to a lesser extent, by subtypes 4 to 7 (Alfellani et al., 2013). However, the results obtained in Colombia have differed; greater ST1 occurrence (followed by ST2) has been seen in two cases (Ramírez et al., 2016; Ramírez et al., 2014) and higher ST3 prevalence in another two cases (Ramírez et al., 2017; Sánchez et al., 2017) which could be explained by the samples' differing geographical origins.

Only one of the studies carried out in Colombia included samples from the municipalities of Guamo and Coyaima in the Tolima department, ST2 and ST3 being found in Coyaima (ST3 being the most prevalent) while ST1, 2 and 3 were identified in Guamo (ST1 having the highest occurrence) (Ramírez et al., 2017). This study's results coincided with those obtained in previous studies in Colombia (Ramírez et al., 2016; Ramírez et al., 2014) and in the department with results obtained for Guamo (Ramírez et al., 2017) which could be explained by this municipality's proximity to the city of Ibagué. In turn, this could explain the difference with the results obtained for Coyaima, a municipality located in the south of the department (Ramírez et al., 2017).

The association found here between *Blastocystis* spp. and other intestinal parasites coincided with that found in national and international studies (Ramírez et al., 2017; Brito-Núñez and Arocha, 2014; Devera et al., 2014; Navone et al., 2017; Villegas-Gómez et al., 2016) where polyparasitism's relevance has been specified as a factor regarding its implications in boosting intestinal infections' manifestations (Fernández-Niño et al., 2007). The known information about *Blastocystis* spp. concerning several animal species which classifies it as a protozoan having zoonotic potential could infer that parasitic contamination in Ibague could have originated from human-animal contact, more specifically involving cattle, domestic animals and rodents, i.e. species where subtypes 1, 2 and 3 have also been found in Colombia (Ramírez et al., 2014).

This work provides the first results regarding *Blastocystis* spp. molecular detection and genetic diversity in the city of Ibague, contributing to information regarding the region and Colombia, and opening the way forward doors for broader/large-scale studies in the region.

Declaration of competing interest

None.

Acknowledgments

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