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Research article

Blastocystis infection frequency and subtype distribution in university students

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ABSTRACT

Blastocystis is a parasite commonly found in the gut of humans and animals; there are 22 known subtypes (STs). STs 1-9 and 12 have been found in humans. This parasite has a faecal-oral route of transmission; its high infection prevalence in developing countries is due to poor hygiene practices, exposure to infected animals, and intake of contaminated water or food. Its pathogenicity has not been established, because it has been found in symptomatic and asymptomatic patients. The goal of this study was to analyze the frequency of *Blastocystis* and its subtypes (1, 2, 3, 4, 5, and 7), and assess the relationship between these subtypes and abdominal pain and distension. 202 university students participated in this study. A questionnaire was applied to assess the gastrointestinal symptoms, and subsequently the students were asked to provide faecal samples. The presence of parasites was determined by optical microscopy. *Blastocystis*-positive samples had their DNA extracted and end-point PCR was performed to corroborate the presence of *Blastocystis* and determine its subtypes. Among the samples, 47.03% were positive according to PCR analysis. The most prevalent subtypes were ST3 (29.79%), ST4 (16.84%), and ST1 (14.89%). We found a relationship between ST1 and abdominal pain (OR = 0.196; CI = 0.0533–0.7318; $p = 0.015$), and between ST4 and abdominal distension (OR = 0.2928; CI = 0.1017–0.8429; $p = 0.023$). However, the presence of this parasite and the probable relationship with gastrointestinal symptoms suggest the need to determine its role within intestinal microbiota in order to confirm whether its eradication is really necessary or not.

1. Introduction

Blastocystis, a genus of parasites commonly detected in the human gastrointestinal tract, belonging to the Stramenopile Blastocystis sp. group [1]; is characterized by having a cosmopolitan distribution [2]. Its prevalence has reached 0.5–35% in developed countries [3, 4], and 55–100% in developing countries [2, 5, 6, 7]. It is transmitted through faeces and its presence in humans is related to poor conditions of environmental sanitation, overcrowding and poor nutrition. In Mexico, frequencies have ranged from 4.0 to 80.0 %, this depends on the population studied and the methodology used for its detection [3, 8, 9, 10, 11].

Currently 22 subtypes (STs) have been identified, of which the first 17 have been recognized and the rest are still under investigation, and only 10 have been identified in humans (ST1-ST9 and ST12) [12]. Subtypes 1 2, 3, and 4 are have been the most frequent in human infections [13, 14, 15, 16, 17]. According to the geographical distribution of the different subtypes, the information obtained varies from one region to another. In the majority of studies conducted in Europe, the most common STs are1, 2, 3, and 4. In the case of South America, it has been observed that human colonization was commonly related to STs 1, 2, and 3; a smaller proportion of the reports was related to ST4 [18, 19, 20, 21]. The majority of the investigations carried out in the world have identified ST3 as the most predominant agent [22, 23]. Subtype 5 is present in pigs and cattle, and its main hosts are cats and pigs [24]. Subtype 7 is also zoonotic, it has the highest resistance to antibiotics and has been related to irritable bowel syndrome (IBS), since it decreases the richness of the intestinal microbiota [25].

There is great controversy about whether this parasite is a commensal microorganism or a pathogen, given that it has been found in asymptomatic and symptomatic individuals [15, 26, 27].

Previous studies have associated different pathologies with particular subtypes; subtype 4 has been associated to diarrhea. In Spain 94 % of

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identified patients with diarrhea were monoinfected with *Blastocystis* [28]; in Denmark patients with acute diarrhea were associated with this subtype [29]; and patients with IBS and/or diarrhea in Italy were also infected [30]. This same ST was identified at low prevalence rates of 7.5 % in asymptomatic Spanish populations in Northern Spain [4] and in Madrid [31, 32], suggesting that ST4 may be more virulent than other *Blastocystis* STs. Although there are a number of studies in which Blastocystis is showed as an emerging pathogen [33, 34, 35, 36]. It has also been suggested that *Blastocystis* is a member of normal microbiota [1, 36, 37, 38]. The goal of the present study was to analyze the frequency of *Blastocystis*; its subtypes (1, 2, 3, 4, 5, and 7), and the relationship of these with the presence of symptoms, such as abdominal pain and abdominal distension, in a population of university students.

2. Materials and methods

2.1. Sample collection

The study has a cross-sectional design; the sampling was non-probabilistic and was conducted between March and October 2018. Bachelor students of the medical surgeon and nutrition program were invited to participate (Table 1). The study was approved by the Ethics Committee of the Faculty of Health Sciences from Universidad Juárez del Estado de Durango (PI-01-2018). The participants received information about the study and, subsequently, they signed an informed consent form. Exclusion criteria were: the presence of chronic degenerative diseases and have received any pharmacological treatment in the last three months before the study. Later, they answered a gastrointestinal symptoms questionnaire in digital format, consisting of multiple choice questions based on Rome III diagnostic criteria [39, 40].

2.2. Parasitological examination

Samples were collected in containers with 10% formaldehyde for coproparasitoscopic exams in triplicate. Each microscopic identification of *Blastocystis* sp was carried out on a different day of deposition. A modified Ritchie technique was performed for the preparation of the samples to later be observed under the microscope. 10 μl of each stool sample was mixed with 20 μl of Lugol's iodine solution and covered with a 21 × 26 mm cover slip. The slide was examined for *Blastocystis* for 300 optical fields with a magnification of 250× (20× objective and 12.5 eye piece) and, in case of suspected organisms, 500 × (40 × 12.5×). The observation of each slide lasted an average of 5 min. The diagnostic criterion for positivity was at least 2 clear vacuolar forms of the parasite in either of the three samples [3].

2.3. DNA extraction

To confirm the microscopy diagnosis, molecular biology techniques were used to detect *Blastocystis* and its subtypes. A fresh sample was dispensed into a DNAse and RNase free sterile bottle, and kept under refrigeration until transport to the laboratory, where it was stored at -20 °C until use.

DNA was extracted from the faeces samples using a commercial kit (Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions, and preserved at -20 °C until processing.

2.4. Polymerase chain reaction (PCR)

2.4.1. Genus determination

The extracted DNA samples were used to determine the presence of *Blastocystis*. We used 3 μL of each DNA sample and Radiant™ Red 2x Taqman Mastermix (Alkali Scientific Inc.), to a final volume of 13 uL for the PCR. The primers used were: F1- 5′-GGA GGT AGT GAC AATAAA TC-3′ and R1- 5′-CGT TCA TGA TGA ACA ATT AC-3' [5]. The PCR conditions consisted of an initial denaturation step at 94 °C for four minutes, followed by 35 denaturation cycles at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. In addition, the B-globin gene was amplified as an internal amplification control. The PCR products were resolved in a 1.5% agarose gel stained with RedGel™ Nucleic Acid Gel Stain (Biotium).

2.4.2. Subtyping of Blastocystis using sequence-tagged sites (STS) primers

For the genotyping of *Blastocystis*, we used a set of sequence-tagged site primers derived from products of randomly amplified polymorphic DNA (RAPD) sequences [35, 41, 42]. We used 4 uL of each DNA sample that was positive for *Blastocystis* in a Polymerase chain reaction (PCR); with a final volume of 13 uL using Radiant™ Red 2x Taqman Mastermix (Alkali Scientific Inc) with the primers described in Table 2. The PCR conditions were: an initial denaturation step at 94 °C for four minutes; followed by 40 denaturation cycles at 94 °C for 30 s; annealing at 58 °C for 30 s; extension at 72 °C for one minute; and a final extension at 72 °C for seven minutes. The PCR products were resolved in a 1.5% agarose gel stained with RedGel™ Nucleic Acid Gel Stain (Biotium). Additionally, PCRs of the samples were performed in a randomized manner using Sanger sequencing to corroborate both the presence of *Blastocystis* and different genotypes.

2.5. Statistical analysis

Descriptive statistics were performed with measures of central tendency. For the bivariate analysis, we used a chi-square test (x2) or Fisher's exact test. The odds ratio (OR) and 95% confidence interval were estimated to analyze the relationship between *Blastocystis* and gastrointestinal symptoms. A P value < 0.05 was considered significant. The statistical analysis was performed using the Stata® Statistics Package, version 13.0.

3. Results

Of 202 samples studied, 47.03% (n = 95) exhibited carriage with *Blastocystis*, 48.41% of the carriage was detected by microscopy, and

Table 1. General description of the sample.

n: 202	Median	IR
Age (years)	20.05	19–21
BMI, median (IR)	24.29	21.08–28.76
Sex	n	%
Men	68	33.66
Women	134	66.33
Programme		
Medicine	138	68.32
Nutrition	64	31.68
Abdominal distension	131	64.85
Abdominal pain	112	55.45

Note. BMI = body mass index; IR = interquartile range; n = number.

Table 2. Primer sequences for genotyping *Blastocystis spp*.

Subtype	STS	Product(pb)	Primer sequence	GenBank accession	Clade in the SSU rRNA Phylogeny
1	SB82	462	F-GAAGGACTCTCTGACGATGA R-GTCCAAATGAAAGGCAGC	AF166086	I
2	SB155	650	F-ATCAGCCTACAATCTCCTC R-ATCGCCACTTCTCCAAT	AF166087	VII
3	SB227	526	F-AGGATTTGGTGTTTGGAGA R-TTAGAAGTGAAGGAGATGGAAG	AF166088	III
3	SB228	473	F-GAC TCC AGA AAC TCG CA R-TCT TGT TTC CCC AGT TAT CC	AF166089	III
3	SB229	631	F-CACTGTGTCGTCATTGTTTTG R-AGGGCTGCATAATAGAGTGG	AF166090	III
4	SB332	338	F-GCATCCAGACTACTATCAACATT R-CCATTTTCAGACAACCACTTA	AF166091	VI
5	SB340	704	F-TGTTCTTGTGTCTTCTCAGCTC R-TTCTTTCACACTCCCGTCAT	AY048752	II
7	SB337	487	F-GTCTTTCCCTGTCTATTCTGCA R-AATTCGGTCTGCTTCTTCTG	AY048750	IV

(Mohamed et al., 2017).

47.03% by PCR. It was found that the frequency of infection was 46.27% in women and 48.53% in men. The subtype analysis indicated that ST3 had the highest prevalence, with 29.79%, followed by ST4 with 16.84%, and ST1 with 14.89% (Table 3).

The subtype analysis in relation to the host's gender indicated that ST3 is the most frequent in the two groups (women = 13.43%; men = 14.71%), followed by ST1 for women (7.46%), and ST4 for men (10.29%). The assessment of the relationship between *Blastocystis* and abdominal pain indicated an almost significant association, with an OR of 0.5831 (CI = 0.333–1.021; $p = 0.058$). According to the subtype analysis, ST1 had an OR of 0.197 (CI = 0.053–0.731; $p = 0.015$). ST4 showed an OR of 0.452 (CI = 0.158–1.297; $p = 0.14$). ST5, with an OR of 3.29 (CI = 0.361–30.02; $p = 0.29$) (Table 4). There was an inverse relationship between *Blastocystis* and abdominal distension, with an OR of 0.612 (CI = 0.334–1.096; $p = 0.097$). ST4 showed an OR of 0.292 (CI = 0.101–0.842; $p = 0.023$) (Table 5).

4. Discussion

The frequency of *Blastocystis* was high: 47.06%. The most accepted form of *Blastocystis* transmission is the fecal-oral pathway, from carrier animals [43]; suggesting a certain relationship between the cattle raised in this region and ST1. This subtype has been more frequently found in cows [22], although the stockyards are located in the periphery of Comarca Lagunera. However, this is a desert area with whirlwinds, which may be responsible for the dissemination of feces. However, the lack of adequate instruments to monitor animal mobility makes it difficult to study the dynamics of transmission.

It can also be transmitted through the consumption of contaminated water [44]. This situation is common in the region, because in spite of being a developing city, not all areas have proper drainage and piped water. Because of this, the management of excreta is still rudimentary, and water is collected from wells and waterwheels. Transmission may occur too through consumption of contaminated water (drinking or recreational) and food, and from person to person [45, 46].

With respect to the relationship between *Blastocystis* and gastrointestinal symptoms, we analyzed the presence of this parasite and its subtypes as a risk factor. We found that the presence of this parasite differ between patients with symptoms and without symptoms, Therefore, it has been suggested that the pathogenicity of *Blastocystis* depended on subtype. This fact was demonstrated by the presence of beneficial bacteria, such as *Akkermansia*, which represents 1–5% of intestinal bacteria. Its presence is related to an obesity decrease in patients with diabetes (it decreases insulin resistance, lean mass, and causes low degrees of inflammation) [1]. When dysbiosis occurs, the amount of *Akkermansia muciniphila* decreases, and there is an increased risk of infection with ST3, which is closely related to IBS and may explain the presence of gastrointestinal symptoms. In contrast, the increase of these bacteria is related to the presence of ST4. This correlation has been reported in healthy individuals, suggesting that this subtype is associated with diverse microbiota and supports a subtype-dependent pathogenicity. In 2014, Nourrisson *et al.* performed cultures *in vitro* and observed that one of the main pathogenic mechanisms of *Blastocystis* was the capacity of adherence to enterocytes, which caused alterations in the permeability of the epithelial barrier. Their study showed that ST4 did not alter transmembrane proteins and occlusion zones, in addition to the fact that enterocyte adherence was not complete [47]. Therefore, it can be suggested that the synergy between *Akkermansia* and ST4 is beneficial, because it increases mucus production. The relationship we found with gastrointestinal symptoms (abdominal pain) was almost significant (OR = 0.452; CI = 0.0158–1.297; $p = 0.14$) (Table 4), which could also lead us to question whether low pathogenicity of the parasite is counteracted by microbiota diversity, or is part of it.

Our study showed that ST1 has a relationship with abdominal pain (OR = 0.197; CI = 0.053–0.731; $p = 0.015$), and ST4 is not related to this symptom (OR = 0.452; CI = 0.158–1.297; $p = 0.14$) (Table 4). On the other hand, the relationship with distension was significant (OR = 0.292; CI = 0.101–0.842; $p = 0.023$) (Table 5). It has been demonstrated that *Blastocystis* stimulates the production of IL-8 and hyperplasia in goblet cells and caecal mucosa in rats [1, 22].

Table 3. Distribution of *Blastocystis spp.* in the total sample.

Sex	n	%
Men	33	48.53
Women	62	46.27
Subtype		
ST1	14	14.89
ST2	8	8.51
ST3	28	29.79
ST4	16	16.84
ST5	5	5.26
ST7	12	12.63
ST1/ST3	6	6.32

Note. n = number; % = percentage; ST = subtype.

Table 4. Relationship between abdominal pain and *Blastocystis spp*.

	OR	95% CI	*p*
Blastocystis	0.583	(0.333–1.021)	0.058
ST1	**0.196**	**(0.053-0.731)**	**0.015**
ST2	1.35	(0.315–5.829)	NS
ST3	1.08	(0.483–2.425)	NS
ST4	0.452	(0.158–1.297)	0.140
ST5	3.29	(0.361–30.02)	NS
ST7	0.554	(0.169–1.808)	NS
ST1/ST3	0.390	(0.069–2.184)	NS

Note. OR = odds ratio; CI = confidence interval; *p* = *p*-value; ST = subtype. Bold value represents they are statistically significant.

Table 5. Relationship between abdominal distension and *Blastocystis spp*.

	OR	95% CI	*p*
Blastocystis.	0.612	(0.342–1.096)	0.097
ST1	0.704	(0.234–2.117)	NS
ST2	1.656	(0.325–8.427)	NS
ST3	1.168	(0.498–2.738)	NS
ST4	**0.292**	**(0.101-0.842)**	**0.023**
ST5	1	/	/
ST7	0.108	(0.316–3.751)	NS
ST1/ST3	0.108	(0.194–8.083)	NS

Note. OR = odds ratio; CI = confidence interval; *p* = *p* value; ST = subtype; NS = non-significant. Bold value represents they are statistically significant.

The pathogenicity and reproduction of *Blastocystis* may depend on the integrity of the host's microbiota. Dysbiosis (low amount of beneficial microorganisms, such as *B. longum*, *bifidobacteria*, *Lactobacillus*, etc.) increases the intestinal adhesion of the parasite, triggering an inflammatory process, with increased IL-6, necrosis factors, cellular hyperplasia, and eosinophil overproduction. Consequently, it causes immunosuppression that cannot be counteracted, and promotes body adaptation. This fact might explain the chronicity of *Blastocystis*.

It is currently accepted that *Blastocystis* is related to the presence of IBS [48]. In 2017, Khademvatan *et al.* managed to relate ST3 to this pathology, given that they found it in 30–40% of European individuals with IBS [49]. In contrast, studies conducted in the Danish population showed the relationship of this parasite with the syndrome, but without determining any particular subtype [50].

In 2011, Jiménez *et al.* conducted a case-control study (45 cases and 45 controls) in Mexico. They found a high frequency of *Blastocystis* in the group with gastrointestinal symptoms (IBS) (31 *vs.* 13%, respectively; $p = 0.043$). However, STs 1 and 3 were equally frequent in the two groups [50]. In this study, we found a higher frequency of *Blastocystis* in the group without gastrointestinal symptoms than in the group with gastrointestinal symptoms; 51 *vs.* 36%; OR = 0.536; CI = 0.286–1.005; $p = 0.049$ [data not shown]. However, that study found the same results we found with respect to the high frequency of STs 1 and 3 in the two groups. It is worth noting that the design of the two studies was not the same, given that ours is a cross-sectional study that assessed a symptomatic group and an asymptomatic group composed of healthy subjects that had not been hospitalized; as in the study conducted by Jiménez *et al.* previously mentioned.

Regarding the analyzed STs in our study, the classification of STs 1-5 and 7 was carried out in order to analyze their relationship with symptoms, such as distension and abdominal pain. For this, specific identification primers, reported by Yoshikawa *et. al.* (2003), were used. These were developed to evaluate the presence of subtypes in human and animal isolates, and in the aforementioned study they also identified an isolate with mixed ST1/ST3 genotypes [42]. In our study, the direct sequencing by Sanger was carried out to corroborate the identification of the most frequent genotypes. Maloney *et al.* (2019) identified 4% of mixed infections by new generation sequencing (NSG) [51]; in our study we found 6.32%, however it is important to mention that one of the limitations of our work was the use of qualitative PCR, which only identifies the presence or absence of ST. The implementation of a more sensitive molecular technique, as the one used by Maloney (2019), could give us additional information, such as which is the most predominant ST, or the existence of more than two subtypes in a single sample. It could help also with the identification of genotypes that were not detected with the primers used. A more accurate assessment of *Blastocystis* diversity is the key to understanding its transmission mechanism and its pathogenicity in our population.

5. Conclusions

The study reports the distribution of *Blastocystis* in university students. Subtype 4 has been very little reported in Latin populations, as well as nationwide. This may indicate a change in the behavior and frequency of *Blastocystis*; and in its relationship with its host, as a microbiota modulating agent, that could lead to future research in the clinical-epidemiological aspect. The presence of this parasite and the probable relationship with gastrointestinal symptoms raises the need to establish its role in intestinal microbiota. This will determine whether its eradication is necessary or not.

Declarations

Author contribution statement

J.O.G. Gomez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

C.M. Yáñez: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M.R. Perez: Performed the experiments; Wrote the paper.

A.M. Hernandez: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

J.J.D. Sustaita: Performed the experiments.

E.G. Jimenez: Analyzed and interpreted the data.

M.R. Andrade: Analyzed and interpreted the data; Wrote the paper.

G.G.G. Vargas: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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