Occurrence and subtype distribution of *Blastocystis* sp. in humans, dogs and cats sharing household in northern Spain and assessment of zoonotic transmission risk

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Abstract

*Blastocystis* sp. is probably the most common enteric parasite in humans globally. Although the role of *Blastocystis* in human disease is still controversial, epidemiological and experimental evidence suggests that pathogenicity may be associated with certain subtypes of the protist. Since the life cycle of *Blastocystis* is maintained through still elusive pathways, companion animals have attracted the attention of researchers as potential reservoirs of human infections. In order to evaluate the risk of zoonotic transmission of *Blastocystis*, we investigated the occurrence and molecular diversity of this microorganism in human, canine and feline populations sharing temporal and spatial settings in the province of Álava, northern Spain. A total of 268 (including 179 human, 55 canine and 34 feline) faecal specimens were obtained from 63 family households during February–December 2014. Detection of *Blastocystis* was achieved by PCR amplification and sequencing of small subunit rRNA genes. *Blastocystis* was found in 35.2% (95% CI: 0.29%–0.42%) of the human stool samples analysed, but not in any of the canine or feline faecal specimens investigated. Out of the 63 PCR-positive human samples, 84.1% (53/63) were successfully subtyped, allowing the identification of the subtypes ST2 (62.3%), ST3 (17.0%), ST1 (13.2%) and ST4 (7.5%). No mixed subtype infections were identified. *Blastocystis* carriage was independent of the gender and region of origin of the affected individuals, but children in the age groups of >5–10 years and >10–15 years were significantly more affected by the protist. None of the risk factors considered (water-use practices, contact with livestock, contact with individual undergoing diarrhoeal episodes) were associated with increased prevalence of *Blastocystis*. Our data demonstrate that pet dogs and cats play a negligible role as natural reservoirs of human *Blastocystis* infection in this geographic region, although the applicability of these results should be corroborated in future molecular epidemiological studies.

Keywords

Álava, *Blastocystis*, cats, children, dogs, epidemiology, genotyping, humans, pets, Spain, zoonotic transmission
1 | INTRODUCTION

Blastocystis sp. (phylum Stramenopiles, class Blastocystidae) is regarded as the most frequently enteric parasite found in human faecal samples, probably affecting more than 1 billion people globally (Stensvold, 2012). Blastocystis is a highly polymorphic organism for which four major (vacular, granular, amoeboid and cyst) forms have been described. Avacuolar and multivacuolar forms have been less frequently identified during the stages of encystation or excystation (Tan, 2008). Although the life cycle of Blastocystis is not fully understood, most researchers agree that transmission is by the faecal-oral route through ingestion of cyst-contaminated water or food. Animal-to-human transmission has also been suggested in a limited number of molecular surveys (Eroglu & Koltas, 2010; Parkar et al., 2010; Stensvold et al., 2009), although the extent and frequency of zoonotic (or anthroponotic) events remain largely unknown and must be further investigated.

Blastocystis exhibits a high degree of genetic diversity, allowing the recognition of at least 17 genetically distinct small subunit (SSU) ribosomal RNA lineages or subtypes (ST), some of them likely representing distinct Blastocystis species (Alfellani, Taner-Mulla, et al., 2013). Additional extensive genetic diversity has also been identified within STs, particularly for ST1 and ST3 (Stensvold, Alfellani, & Clark, 2012). STs 1-4 account for ~90% of human infections reported globally (Alfellani, Stensvold, et al., 2013). ST5 is commonly isolated from livestock, ST6 and ST7 from birds, and ST8 from arboreal non-human primates (Alfellani, Jacob, et al., 2013; Badparva, Sadraee, & Kheirandish, 2015; Ramirez et al., 2014); so far, ST9 has only been found in humans. The fact that STs 5-8 have been only sporadically found in humans has been interpreted as indicative of zoonotic transmission (Clark, Giezen, Alfellani, & Stensvold, 2013). Finally, STs 10-17 have only been documented in non-human species so far (Alfellani, Taner-Mulla, et al., 2013; Clark et al., 2013).

The role of Blastocystis as a pathogen in humans is still the focus of intense debate. While some studies have found no link between Blastocystis and disease (Leder, Hellard, Sinclair, Fairley, & Wolfe, 2005; Ozyurt et al., 2008), there is some evidence from epidemiological (El Safadi et al., 2016; Mohamed et al., 2017), in vitro (Puthia, Lu, & Tan, 2008) and in vivo (Elwakil & Hewedi, 2010) surveys supporting the pathogenic potential of the parasite. Thus, the presence of Blastocystis has been associated with gastrointestinal disorders (Roberts, Stark, Harkness, & Ellis, 2014), irritable bowel syndrome (Boorom et al., 2008) and cutaneous lesions (Balint et al., 2014). Moreover, recent studies have suggested that the occurrence of clinical signs may be subtype-related. For instance, ST4 has been associated with infectious diarrhea in European countries including Denmark (Stensvold, Christiansen, Olsen, & Nielsen, 2011) and Spain (Domínguez-Márquez, Guna, Muñoz, Gómez-Muñoz, & Borrás, 2009).

Blastocystis sp. has attracted little attention in Spain, where only few epidemiological surveys have been attempted to investigate the epidemiology of this protist in human and animal populations (Table 1). Reported prevalence rates of human Blastocystis carriage in Spain have varied from 3%-7% in symptomatic outpatients to 10% in HIV-infected children. Blastocystis also appears common in school children (8%-23%) and in temporarily hosted children from developing countries (15%-22%). A retrospective cohort study involving a larger series of cases compiled to date in the country revealed that 56% of patients with Blastocystis had no clinical signs (Salvador et al., 2016). In those patients whose symptoms were not attributable to other aetiological agents, the most frequent symptoms associated with Blastocystis infections were diarrhea (66%), abdominal pain (37%) and cutaneous manifestations (10%), with seven per cent of cases requiring specific pharmacological treatment (Salvador et al., 2016). Blastocystis has also been documented at prevalence rates of 8%-67% in captive animals from zoological gardens and of 2%-47% in livestock (Table 1). However, no research has been directed to investigate the presence of this parasite in companion animals in Spain and to elucidate the potential role of domestic dogs and cats as natural reservoirs of human Blastocystis infection. In an attempt to improve our current knowledge on the epidemiology of the disease in Spain, we here provide novel data on the prevalence and molecular diversity of Blastocystis sp. in human, canine and feline populations sharing temporal and spatial conditions. We also assess the risk of zoonotic (or anthroponotic) transmission of the parasite among household members.

2 | MATERIAL AND METHODS

2.1 | Ethical statement

Written informed consent was obtained from all participants, or their parents or legal tutors in the case of children, who volunteered to participate in this study. Socio-demographic or epidemiological data were coded prior to any analysis to protect the identity of the participants. This study and the procedures involved, including the data collection spreadsheets used, were approved by the Research Ethics Committee of the Carlos III Health Institute (reference number: CEI PI 30_2012).

2.2 | Study area and faecal sample collection

This report is a retrospective study based on analysis of genomic DNA extracted from human, canine and feline samples collected in a previous epidemiological survey carried out in the province of
Álava, Northern Spain, between February and December 2014 (de Lucio et al., 2017). In that survey, families with children and pet dogs and cats living in rural (Añana, Ayala, Campezo-Montaña Alavesa, Goerbeialdea and Salvatierra) and urban (Vitoria-Gasteiz) regions of Álava were asked to provide individual faecal samples from each member of the household, including dogs and cats. Consenting participants were provided with a prelabelled sampling kit including sterile polystyrene flasks and instructions on how to take and identify the samples safely. Standardized data collection spreadsheets were also developed and distributed in order to gather socio-demographic data (age, gender, area of residence), water-use practices (source of drinking water, washing hands, raw fruits and vegetables before eating, aquatic sports), contact with livestock, known episodes of diarrhoea affecting any member of the family or classmates during the previous month and travelling abroad during the last 3 months. Collected stool samples and questionnaires were checked for matching and completeness and shipped to the Spanish National Centre for Microbiology (Majadahonda). Stool samples were kept at −20°C without any additive until further laboratory processing.

2.3 DNA extraction and purification

Total DNA was extracted from an aliquot of ~200 mg of fresh faecal material using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. Purified DNA samples (200 μl) were stored at −20°C until downstream PCR-based diagnostic and subtyping analyses were conducted. A water extraction control was routinely included in each sample batch processed.

2.4 Molecular detection and characterization of Blastocystis sp. isolates

Identification of Blastocystis sp. was achieved by a PCR protocol targeting a fragment of the SSU rRNA gene of the parasite (Scicluna, Tawari, & Clark, 2006). This method uses the pan-Blastocystis barcode primers RD5 (5’-ATCTGTTGTGATCCGTCCAGT-3’) and BhRDr (5’-GAGCTTTTTAACTGCAACAACG-3’) to generate a PCR product of ~600 bp. Additionally, the primer set RD5 and RD3 (5’-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3’) was used to amplify a larger fragment (~1,800-bp) of the very same gene (Clark, 1997) in order to further assess the presence of Blastocystis in the canine and feline isolates investigated. In all instances, reaction mixes were conducted in a final volume of 25 μl, including 5 μl of template DNA, 0.5 μM of the primer set RD5/BhRDr (or RD5/RD3), 2.5 units of MyTaq™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and 5× MyTaq™ Reaction Buffer containing 5 mM dNTPs and 15 mM MgCl₂. Amplification conditions consisted of one step of 95°C for 3 min, followed by 30 cycles of 1 min each at 94, 59 and 72°C, with an additional

| Location | Period | Population under study | Diagnostic method | Total no. of individuals | Positive individuals (%) | Reference |
|----------|--------|------------------------|-------------------|--------------------------|--------------------------|-----------|
| Cataluña | 1999-2005 | Symptomatic outpatients | CM | 8,313 | 7.0 | González-Moreno et al. (2011) |
| La Coruña | 2007-2013 | Symptomatic children | CpC, CM | 530 | 4.5 | Méndez-Bustelo et al. (2015) |
| Madrid | 1996-1997 | HIV-positive children | CM | 83 | 9.6 | Del Águila et al. (1997) |
| Salamanca | 1989-1990 | Preschool children School children | CM | 220 | 8.2-13.0 | Martin-Sánchez, Canut-Blasco, Rodríguez-Hernández, Montes-Martínez, and García-Rodríguez (1992) |
| Valencia | 2007 | Hosted Shaharawi children | CM | 270 | 14.8 | Soriano, Domènech, Martínez, Mañes, and Soriano (2011) |
| Various | 1993-1997 | Hosted Shaharawi children | CM | 242 | 22.0 | Paricio Talayero et al. (1998) |
| Granada | 2006-2007 | Zoological animals | CM | 432 | 8.3-66.6 | Pérez Cordón et al. (2008) |
| Granada | 2006-2007 | Zoological birds | CM | 984 | 23.6 | Cordón et al. (2009) |
| Aragón | 1990-1993 | Farmed cattle | CM | 554 | 1.8 | Quiñez, Sánchez-Acedo, Clavel, and Causapé (1995) |
| Aragón | 1992-1993 | Farmed pigs | CM | 360 | 7.5 | Quiñez, Clavel, Sánchez-Acedo, and Causapé (1995) |
| Valencia | 2004-2007 | Farmed pigs | CM, PCR | 395 | 46.8 | Navarro et al. (2008) |

Note. CpC: copro-culture; CM: conventional microscopy; PCR: polymerase chain reaction.
2 min of final extension at 72°C. PCR reactions were carried out on a 2720 Thermal Cycler (Applied Biosystems). Laboratory-confirmed Blastocystis-positive, -negative and no-template controls were included in each run. PCR amplicons were visualized on 1%-2% D agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda). PCR products of the expected size were sequenced directly in both directions using the primer sets RDS/BhRDr or RDS/RD3 described above. DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry (Applied Biosystems) on an on ABI PRISM 3130 automated DNA sequencer.

2.5 | Data analyses

The chi-square test was used to compare Blastocystis sp. infection rates in the surveyed human population according to gender, age group and place of residence. A probability (p) value <0.05 was considered evidence of statistical significance. Prevalence risk ratios (PRR) with 95% confidence intervals (CI) were calculated to assess the association between potential risk factors considered in the individual data collection spreadsheets and Blastocystis infection.

2.6 | Sequence and phylogenetic analyses

Raw sequencing data in both forward and reverse directions were viewed using the Chromas Lite version 2.1 sequence analysis program (https://chromaslite.software.informer.com/2.1/). The blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare nucleotide sequences with sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Generated DNA consensus sequences were aligned to appropriate reference sequences using the MEGA 6 software (https://www.megasoftware.net/) to identify Blastocystis subtypes (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Blastocystis sequences were submitted to the publicly available online Blastocystis 18S database (https://pubmlst.org/blastocystis/) for subtype confirmation and allele identification.

For the estimation of the phylogenetic inferences among the identified Blastocystis-positive samples, a phylogenetic tree was inferred using the Neighbour-Joining (NJ) method in MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method and modelled with a gamma distribution. The reliability of the phylogenetic analyses at each branch node was estimated by the bootstrap method using 1,000 replications. Representative sequences obtained in this study have been deposited in GenBank under accession numbers MF669062–MF669075.

3 | RESULTS

3.1 | Blastocystis infections in human, canine and feline populations

A total of 268 (including 179 human, 55 canine and 34 feline) individual faecal specimens were obtained from 63 family households with domestic dogs and cats during the study period. The average (mean) numbers of people, dogs and cats per household were 2.8 [standard deviation (SD: 1.2)], 0.9 (SD: 0.8) and 0.5 (SD: 0.7), respectively. Four family households provided faecal specimens of animal, but not human, origin, whereas the opposite was true for an additional four family households.

The results of the SSU rDNA PCR revealed the presence of Blastocystis in 35.2% (95% confidence interval: 0.29%–0.42%) of the human stool samples analysed. None of the canine or feline faecal samples tested positive for the parasite using the primer set RDS/BhRDr, but a number of amplicons of the expected size (1,800-bp) varying in band intensity were observed after gel electrophoresis when the primer pair RDS/RD3 was used (Supporting Information Figures S1 and S2). Subsequent sequence analysis revealed the presence of fungi of the genera Thelebolus and Lasiobolidium, bacteria belonging to the family Enterobacteriaceae (Pantoea and Shigella), and coniferous trees of the genus Tetracrinis, confirming the nonspecific affinity of these primers. Table 2 shows the distribution of human Blastocystis infections stratified by gender, group of age, municipality of origin and type of settlement. Blastocystis infections were equally present in males and females, but were significantly more frequent (p < 0.05) in children in the age groups 6-10 and 11-15 years old. Individuals living in the municipalities of Salvatierra and Vitoria-Gasteiz harboured the highest (41%–44%) infection rates detected in the present survey. Blastocystis was more often detected in urban (44%) than in rural (33%) areas. None of the socio-demographic variables considered in the analysis contributed in a significant way to increase the prevalence of the protist.

3.2 | Assessment of risk factors for human Blastocystis infection

A total of 63 data collection spreadsheets (one per family household) were satisfactorily completed and considered in the analysis, although information for some individual variables could not be consigned in a number of cases (Table 3). None of the factors investigated were associated with a higher risk of being diagnosed with Blastocystis infection, although children ≤15 years of age and individuals declaring contact with livestock had a higher prevalence.

3.3 | Subtype analysis of Blastocystis isolates of human origin

Out of the 63 samples of human origin that tested positive for Blastocystis sp. by PCR, 84.1% (53/63) were successfully subtyped by sequence analyses of the SSU rRNA genes (barcode region). BLAST searches allowed identification of Blastocystis subtypes ST1–ST4. The most common subtype was ST2 (62.3%; 33/53), followed by ST3 (17.0%; 9/53), ST1 (13.2%; 7/53) and ST4 (7.5%; 4/53), respectively (Figure 1). Neither mixed infection involving different STs of the parasite nor infections caused by subtypes predominantly (ST5–ST8) or exclusively (ST10–ST17) found so far in non-human animal species were identified. Allele calling using the Blastocystis 18S database allowed the identification of allele 4 within ST1, alleles 10–12
**TABLE 2** Infection rates by *Blastocystis* sp. in household family members owning pet dogs and/or cats, as determined by SSU rRNA-PCR, according to their socio-demographic features. Álava, Northern Spain, 2014. Chi-square statistics are indicated.

| Variable                  | No. | No. positive results | %  | p-Value |
|---------------------------|-----|----------------------|----|---------|
| **Sex**                   |     |                       |    |         |
| Male                      | 100 | 35                   | 35.0 | 0.951   |
| Female                    | 79  | 28                   | 35.4 |          |
| **Age (years)**           |     |                       |    |         |
| 0-5                       | 26  | 5                    | 19.2 | 0.008a  |
| 6-10                      | 33  | 18                   | 54.5 |          |
| 11-15                     | 23  | 12                   | 52.2 |          |
| >15                       | 85  | 26                   | 30.6 |          |
| No data                   | 12  | 2                    | 16.7 |          |
| **Municipality of origin**|     |                       |    |         |
| Añana                     | 28  | 8                    | 28.6 | 0.058   |
| Ayala                     | 14  | 1                    | 7.1  |          |
| Campezo-Montaña Alavesab  | 4   | 0                    | 0.0  |          |
| Gorbeialdeaab             | 3   | 0                    | 0.0  |          |
| Salvatierra               | 91  | 37                   | 40.7 |          |
| Vitoria-Gasteiz           | 39  | 17                   | 43.6 |          |
| **Geographic area**       |     |                       |    |         |
| Urban                     | 39  | 17                   | 43.6 | 0.215   |
| Rural                     | 140 | 46                   | 32.9 |          |
| Total                     | 179 | 63                   | 35.2 |          |

*a* Result statistically significant at *p* < 0.05. *b*Municipalities excluded from the analysis due to small sample size.

within ST2, allele 34 within ST3 and allele 42 within ST4. Alleles 12 (47.2%; 25/53), 34 (17.0%; 9/53) and 4 (13.2%; 7/53) were found as the most represented *Blastocystis* alleles in the human population under study. A number of isolates (two in ST2 and one in ST4) could not be analysed at the allele level due to inaccurate or incomplete sequencing data.

Figure 2 shows the phylogenetic tree constructed using the NJ method with representative, unambiguous (homozygous), sequences from all the *Blastocystis* subtypes ST1-ST4 generated in the present study at the SSU rRNA locus. For reference and comparative purposes, sequences reported in other European countries were retrieved from the *Blastocystis* 18S database and included in the analysis.

Interestingly, close inspection of chromatogram traces corresponding to ST2 allele 12 sequences revealed a number polymorphic (double peaks) sites at positions 161 (A/G), 243 (A/T), 453 (G/C) and 454 (A/T) of reference sequence JF274672 (Supporting Information Figure S1 and Table S1). These findings probably reflect the intrinsic degree of intrastrain variation within ST2. Whereas overlapping nucleotide peaks S (G/C) and W (A/T) observed at positions 453 and 454 appear to be the product of combined allele 12-specific genetic variants of ST2, the double peak A/G detected at position 161 may be indicative of a mixed infection involving alleles 11 and 12.

### 4 | DISCUSSION

Data presented in this survey demonstrate that roughly one in three asymptomatic individuals living in Álava harboured *Blastocystis*, a proportion somehow unexpected considering that this is one of the wealthiest regions in Spain in terms of per capita income (Eurostat, 2016). This rate is considerably higher than those (0.5%–7%) reported in similar community- or hospital-based studies targeting apparently healthy individuals in other developed countries including Japan (Horiki et al., 1997) and United States (Scanlan, Knight, Song, Ackermann, & Cotter, 2016), but lower than that (56%) documented in Ireland (Scanlan et al., 2014). Interestingly, *Blastocystis* carriage was more frequently observed in individuals older than five years of age independently of their sex. This finding appears to indicate that (a) as in other enteric protists, the faecal-oral transmission route should account for most of the paediatric cases identified, and (b) *Blastocystis* colonization acquired during childhood may persist for long periods of time and explain many of the cases observed in adult individuals, although we cannot rule out the possibility that subjects of mature age may become *Blastocystis* carriers by means of other, still unidentified, routes.

There are only two previous published molecular studies attempting to ascertain the subtype diversity within *Blastocystis* in Spain. In a preliminary chromosomal study, a total of eleven karyotypic profiles were detected among 15 isolates from symptomatic patients obtained from axenic and monoxenic cultures (Carbajal, Castillo, Lanuza, Villar, & Borrás, 1997). In an ensuing study by the same research group, a total of 51 cultured isolates from clinical specimens were investigated by SSU rRNA gene-PCR and subsequent restriction fragment length polymorphism analysis (Domínguez-Márquez et al., 2009). In that survey, the vast majority (94%) of the human infections were caused by ST4, a fact that was interpreted as evidence of the pathogenic potential of this particular *Blastocystis* subtype. Similar findings have been reported in patients presenting with acute diarrhoea in Denmark (Stensvold et al., 2011) and in patients suffering from irritable bowel syndrome and chronic diarrhoea in Italy (Mattucci, Crisafi, Gabrielli, Paoletti, & Cancrini, 2016). The results presented here could support this hypothesis, as ST4 was the least common subtype, found in less than 8% of the asymptomatic individuals investigated, with ST2 (62.3%) and ST3 (17.0%) being the most prevalent *Blastocystis* subtypes identified. Because of its clonal structure and its restricted (predominantly European) geographical distribution, ST4 has been envisaged as a lineage with a recent entry into the human population (Stensvold, 2012). However, it is important to bear in mind that ST4 showed no obvious pathogenic effects in surveys carried out in other European regions (Meloni et al., 2011; Seyer et al., 2017). Definitively, more research should be
conducted to conclusively demonstrate the association between ST4 and human disease. The absence in the surveyed human population of *Blastocystis* subtypes typically found in non-human animal hosts (e.g., ST5-8) seems to suggest that the transmission of this protist in the province of Álava should be primarily anthroponotic in origin. This does not preclude that some of the human infections detected may have an animal origin, as livestock and even wildlife have been previously described as carriers of *Blastocystis* ST1-4 (Alfellani, Taner-Mulla, et al., 2013; Ramírez et al., 2014).

Recently, increasing interest has been paid to the potential role of companion animals, particularly dogs and cats, as natural reservoirs of human *Blastocystis* infection. Epidemiological studies conducted to date in this research area have revealed inconsistent, and often conflicting, results. For instance, surveys targeting sheltered canine and feline populations failed to demonstrate the presence of *Blastocystis* in Japan (Abe, Nagoshi, Takami, Sawano, & Yoshikawa, 2002) and Malaysia (Chuong, Suresh, Mak, Init, & Kathijah, 1996), but the protist has been reported at moderate (~12%) to very high (~70%) prevalence rates in the United States (Ruaux & Stang, 2014) and Australia (Duda, Stenzel, & Boreham, 1998), respectively. Infection rates in the range of 14%–37% have also been documented in stray dogs in India (Wang et al., 2013), in stray cats in Iran (Khademvatan et al., 2014), and in symptomatic dogs and cats attending a veterinary clinic in Chile (López, Abarca, Paredes, & Inzunza, 2006), but only 1.3% of semidomesticated Cambodian dogs harboured the protist (Wang et al., 2013). *Blastocystis* carriage has also been reported at low prevalence rates (<4%) or not at all in household dog populations in Australia (Wang et al., 2013), Brazil (David et al., 2015) and France (Osman et al., 2015), indicating that

### Table 3

| Variable (no. of observations missing) | Category     | Cases | Noncases | PRR   | 95% CI    |
|---------------------------------------|--------------|-------|----------|-------|-----------|
| Population (0)                        | Rural        | 46    | 94       | 0.6333| 0.307-1.307|
|                                       | Urban        | 17    | 22       | Ref   |           |
| Gender (0)                            | Male         | 35    | 65       | 0.981 | 0.529-1.819|
|                                       | Female       | 28    | 51       | Ref   |           |
| Age group (12)                        | ≤15 years old| 35    | 47       | 1.690 | 0.895-3.192|
|                                       | >15 years old| 26    | 59       | Ref   |           |
| Relatives with diarrhoea (12)         | Exposed      | 34    | 54       | 1.443 | 0.758-2.746|
|                                       | Unexposed    | 24    | 55       | Ref   |           |
| Classroom mates with diarrhoea (12)   | Exposed      | 23    | 38       | 1.228 | 0.636-2.369|
|                                       | Unexposed    | 35    | 71       | Ref   |           |
| Contact with livestock (12)           | Exposed      | 11    | 10       | 2.246 | 0.892-5.655|
|                                       | Unexposed    | 48    | 98       | Ref   |           |
| Drinking water source (19)            | Bottled      | 16    | 28       | 1.086 | 0.527-2.239|
|                                       | Municipal tap| 40    | 76       | Ref   |           |
| Hand washing (5)                      | Not always   | 48    | 84       | 1.143 | 0.549-2.379|
|                                       | Always       | 14    | 28       | Ref   |           |
| Raw vegetables/fruits washing (5)     | Not always   | 17    | 32       | 0.944 | 0.473-1.888|
|                                       | Always       | 45    | 80       | Ref   |           |
| Swimming (8)                          | Yes          | 26    | 36       | 1.593 | 0.834-3.043|
|                                       | No           | 34    | 75       | Ref   |           |
| Travelling abroad (5)                 | Yes          | 8     | 19       | 0.725 | 0.297-1.769|
|                                       | No           | 54    | 93       | Ref   |           |

*a*Cases: samples that tested positive for *Blastocystis* sp. by SSU rRNA-PCR. *b*Noncases: samples that tested negative for *Blastocystis* sp. by SSU rRNA-PCR.
present in their environment and should not, therefore, be considered opportunistic infections by whatever species causing Blastocystis sp. have led some authors to propose that domestic dogs are transiently infected. This is also the epidemiological scenario depicted in the present study, where DNA in isolates of domestic dog and cat populations. This statement was supported by the negative findings obtained with two independent primer sets targeting different fragments of the SSU rRNA gene of Blastocystis. Failure to amplify primer sets targeting different fragments of the SSU rRNA gene of Blastocystis was apparently absent in the investigated domestic dog and cat populations. This statement was supported by the negative findings obtained with two independent primer sets targeting different fragments of the SSU rRNA gene of Blastocystis. Failure to amplify Blastocystis DNA in isolates of canine or feline origin could be associated with the inefficient removal of the parasite. Failure to amplify Blastocystis DNA in isolates of canine or feline origin could be associated with the inefficient removal of the parasite.

well-cared animals are probably less exposed to the microorganism. Additionally, these studies demonstrated that domestic dogs can carry a wide range of Blastocystis STs including ST1, ST2, ST4, ST5, ST6 and ST10 (Osman et al., 2015; Wang et al., 2013). This combination of low prevalence rates and apparent lack of ST host specificity have led some authors to propose that domestic dogs are transiently and opportunistically infected by whichever Blastocystis subtype is present in their environment and should not, therefore, be considered as natural hosts or primary sources of human infections (Wang et al., 2013). This is also the epidemiological scenario depicted in the present study, where Blastocystis was apparently absent in the investigated domestic dog and cat populations. This statement was supported by the negative findings obtained with two independent primer sets targeting different fragments of the SSU rRNA gene of the parasite. Failure to amplify Blastocystis DNA in isolates of canine or feline origin could be associated with the inefficient removal of the parasite.
of PCR inhibitors during the DNA extraction and purification procedure, or to primer competition during the amplification reaction with homologous (e.g., fungal) DNA sequences. Regarding the first possibility, it should be taken into account that the same set of DNA isolates of animal origin was successfully tested in a preliminary investigation (de Lucio et al., 2017). However, sequence analyses of isolates yielding strongest amplicons with the primer pair RDS5/RD3 allowed the identification of organisms including diverse genera of fungi, bacteria belonging to the family Enterobacteriaceae and coniferous trees, demonstrating the unspecific nature of these PCR reactions. Because no sequencing data could be generated from isolates yielding faint bands after gel electrophoresis, we cannot rule completely out the possibility that some of them correspond indeed to Blastocystis isolates, although this possibility seems unlikely. Of note, direct evidence of zoonotic transmission has been provided by other surveys investigating human and canine/feline populations living in the same spatial and temporal setting. Blastocystis subtypes ST2-5 were simultaneously found in people and domestic dogs living in an urban community in the Philippines (Belleza, Reyes, Tongol-Rivera, & Rivera, 2016), whereas ST concordance was also demonstrated between symptomatic Blastocystis patients undergoing chemotherapy and their pets in Australia (Nagel et al., 2012). Additionally, potentially zoonotic ST1 has been detected in shelter-resident dogs and cats in the Pacific Northwest of the United States, highlighting the potential role of stray dogs and cats as source of human blastocystosis (Ruax & Stang, 2014). Although the present study has only focused on owned companion animals, further molecular studies should be conducted in this geographical area to ascertain whether stray and/or abandoned dogs and cats may carry Blastocystis STs of zoonotic concern.

Another interesting contribution of this study is the demonstration of a relatively large degree of genetic variability at the nucleotide level within Blastocystis ST2 sequences, translating into the identification of a number of polymorphic sites in the form of double peaks at chromatogram inspection. This finding provides molecular evidence in support of the occurrence of mixed infections involving different inter- and intra-allelic combinations of the protist. In this regard, it should be emphasized that Blastocystis subtype-mixed infections have been previously detected in a significant proportion (22%) of apparently healthy individuals in a recent study using Blastocystis subtype-specific PCRs (Scanlan, Stensvold, & Cotter, 2015). High levels of intrasubtype genetic diversity have also been demonstrated between ST3 and ST4 isolates, a fact that may help explaining differences in host specificities and geographical distributions (Stensvold et al., 2012).

5 | CONCLUSIONS

Blastocystis carriage is a frequent event in apparently healthy individuals in northern Spain, independently of gender, and geographic origin. The protist primarily affects children in the group of age of >5–15 years. Failure to detect Blastocystis in domestic dogs and cats suggests that well-cared-for pets play a minor role or no role at all as natural reservoirs of human Blastocystis in this Spanish region. This is also the most comprehensive molecular epidemiological study assessing the diversity and frequency of Blastocystis in isolates of human origin conducted in Spain to date. Our subtyping analyses confirm the predominance of ST2 and ST3 in asymptomatic carriers and might support the suggested clinical relevance of ST4. More research should be conducted in other human and animal populations to establish the elusive transmission dynamics and public health significance of Blastocystis in Spain.

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CONFLICT OF INTEREST

None.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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