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Paulos, S., Köster, P. C., de Lucio, A., Hernández-de-Mingo, M., Cardona, G. A., Fernández-Crespo, J. C., Stensvold, C. R., & Carmena, D. (2018). Occurrence and subtype distribution of Blastocystis sp. in humans, dogs and cats sharing household in northern Spain and assessment of zoonotic transmission risk. Zoonoses and public health, 65(8), 993–1002.

which has been published in final form at:

https://doi.org/10.1111/zph.12522

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 a subject of intense debate and controversy, epidemiological and experimental evidence suggests that pathogenicity may be associated to 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 recent years. In order to evaluate the risk of zoonotic (or anthroponotic) 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 rural and urban areas of 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. Sequence analyses were subsequently conducted for subtype confirmation and allele identification. *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 infections involving different STs 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 to 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.

Key words: *Blastocystis*; Children; Humans; Dogs; Cats; Pets; Epidemiology; Genotyping; Zoonotic transmission; Álava; Spain

Introduction

Blastocystis sp. (phylum Stramenopiles, class Blastocystae, order Blastocystida, and family 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 (vacuolar, granular, amoeboid, and cyst) forms have been described. Avacuolar and multivacuolar forms have also 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 (e.g. Eroglu and 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 et al., 2013c). Additional extensive genetic diversity has also been identified within STs, particularly for ST1 and ST3 (Stensvold et al., 2012). STs 1–4 account for ~90% of human infections reported globally (Alfellani et al., 2013b). ST5 is commonly isolated from livestock, ST6 and ST7 from birds, and ST8 from arboreal non-human primates (Alfellani et al., 2013a; Ramírez et al., 2014; Badparva et al., 2015); so far ST9 has only been found in humans. The fact that STs 5–9 have been only sporadically found in humans has been interpreted as indicative of zoonotic transmission (Clark et

al., 2013). Finally, STs 10–17 have only been documented in non-human species so far (Alfellani et al., 2013c; Clark et al., 2013).

The role of *Blastocystis* as human pathogen is still the focus of intense debate. While some studies have found no link between *Blastocystis* and disease (Leder et al., 2005; Ozyurt et al., 2008), there is increasing evidence from epidemiological (El Safadi et al., 2016; Mohamed et al., 2017), *in vitro* (Puthia et al., 2008), and animal (Elwakil and Hewedi, 2010) surveys supporting the pathogenic potential of the parasite. Thus, the presence of *Blastocystis* has been associated with gastrointestinal disorders (Roberts et al., 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 diarrhoea in European countries including Denmark (Stensvold et al, 2011) and Spain (Domínguez-Márquez et al., 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 to10% in HIV-infected children. *Blastocystis* also appears common in school children (8–23%) and in temporally 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 etiological agents, the most frequent symptoms associated to *Blastocystis* infections were diarrhoea (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.

Material and methods

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).

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–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

134 Salvatierra) and urban (Vitoria-Gasteiz) regions of Álava were asked to provide
135 individual faecal samples from each member of the household, including dogs and cats.
136 Consenting participants were provided with a pre-labelled sampling kit including sterile
137 polystyrene flasks and instructions on how to take and identify the samples safely.
138 Standardised data collection spreadsheets were also developed and distributed in order
139 to gather socio-demographic data (age, gender, area of residence), water-use practices
140 (source of drinking water, washing hands, raw fruits and vegetables before eating,
141 aquatic sports), contact with livestock, known episodes of diarrhoea affecting any
142 member of the family or classmates during the previous month and traveling abroad
143 during the last 3 months. Collected stool samples and questionnaires were checked for
144 matching and completeness and shipped to the Spanish National Centre for
145 Microbiology (Majadahonda). Stool samples were kept at −20 °C without any additive
146 until further laboratory processing.

147 *DNA extraction and purification*

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

153 *Molecular detection and characterization of* Blastocystis *sp. isolates*

154 Identification of *Blastocystis* sp. was achieved by a PCR protocol targeting a
155 fragment of the SSU rRNA gene of the parasite (Scicluna et al. 2006). This method uses
156 the pan-*Blastocystis* barcode primers RD5 (5´-ATCTGGTTGATCCTGCCAGT-3´) and
157 BhRDr (5´-GAGCTTTTTAACTGCAACAACG-3´) to generate a PCR product of

~600-bp. 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, 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5× MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. 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 2 min of final extension at 72 ºC. 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 2% D5 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 set RD5/BhRDr 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.

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.

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

(http://chromaslite.software.informer.com/2.1/). The BLAST tool (http://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 (http://www.megasoftware.net/) to identify *Blastocystis* subtypes (Tamura et al., 2013). *Blastocystis* sequences were submitted to the publicly available online *Blastocystis* 18S database (http://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 Neighbor-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 to MF669075.

Results

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 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. 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 >5–10 and >10–15 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.

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 to a higher risk of *Blastocystis* infection, although children of paediatric (≤15 years old) age and people declaring contact with livestock were more often infected.

Subtype analysis of Blastocystis *isolates of human origin*

Out of the 63 isolates 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 four within ST1, alleles 10–12 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 (Supplementary material Table 1 and Figure 1). These findings probably reflect the intrinsic degree of intra-strain 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.

Discussion

Data presented in this survey demonstrate that roughly one in three asymptomatic individuals living in Álava harboured *Blastocystis*, a proportion somehow

254 unexpected considering that this is one of the wealthiest region in Spain in terms of per
255 capita income (Eurostat, 2016). This rate is considerably higher than those (0.5–7%)
256 reported in similar community- or hospital-based studies targeting apparently healthy
257 individuals in other developed countries including Japan (Horiki et al., 1997) and USA
258 (Scanlan et al., 2016), but lower than that (56%) documented in Ireland (Scanlan et al.,
259 2014). Interestingly, *Blastocystis* carriage was not age-related in both males and
260 females, affecting individuals of all age groups. This finding seems to indicate that the
261 faecal-oral transmission route, expected to account for most of the paediatric cases,
262 cannot satisfactorily explain the presence of the protist in all adult individuals. A
263 significant proportion of the adult subjects may therefore have acquired the infection
264 from yet unidentified sources.

265 There are only two previous published molecular studies attempting to ascertain
266 the subtype diversity within *Blastocystis* in Spain. In a preliminary chromosomal study,
267 a total of eleven karyotypic profiles were detected among 15 isolates from symptomatic
268 patients obtained from axenic and monoxenic cultures (Carbajal et al., 1997). In an
269 ensuing study by the same research group, a total of 51 cultured isolates from clinical
270 specimens were investigated by SSU rRNA gene-PCR and subsequent restriction
271 fragment length polymorphism analysis (Domínguez-Márquez et al., 2009). In that
272 survey, the vast majority (94%) of the human infections were caused by ST4, a fact that
273 was interpreted as evidence of the pathogenic potential of this particular *Blastocystis*
274 subtype. Similar findings have been reported in patients presenting with acute diarrhoea
275 in Denmark (Stensvold et al., 2011) and in patients suffering from irritable bowel
276 syndrome and chronic diarrhoea in Italy (Mattiucci et al., 2016). The results presented
277 in the present study could support this hypothesis, as ST4 the least common subtype,
278 found in less than 8% in this primarily asymptomatic study population, with ST2

279 (62.3%) and ST3 (17.0%) being the most prevalent *Blastocystis* subtypes identified.
280 Because of its clonal structure and its limited distribution (ST4 is predominantly found
281 in Europe), ST4 has been envisaged as a lineage with a recent entry into the human
282 population (Stensvold, 2012). However, it is important to bear in mind that ST4 showed
283 no obvious pathogenic effects in surveys carried out in other European regions (Meloni
284 et al., 2011; Seyer et al., 2017). Definitively, more research should be conducted to
285 conclusively demonstrate the association between ST4 and human disease.
286 Recently, increasing interest has been paid to the potential role of companion
287 animals, particularly dogs and cats, as natural reservoirs of human *Blastocystis*
288 infection. Epidemiological studies conducted to date in this research area have revealed
289 inconsistent, and often conflicting, results. For instance, surveys targeting sheltered
290 canine and feline populations failed to demonstrate the presence of *Blastocystis* in Japan
291 (Abe et al., 2002) and Malaysia (Chuong et al., 1996), but the protist has been reported
292 at moderate (~12%) to very high (~70%) prevalence rates in USA (Ruaux et al., 2014)
293 and Australia (Duda et al., 1998), respectively. Infection rates in the range of 24–37%
294 have also been documented in stray dogs in India (Wang et al., 2013) and in
295 symptomatic dogs and cats attending a veterinary clinic in Chile (López et al., 2006),
296 but only 1.3% of semi-domesticated Cambodian dogs harboured the protist (Wang et
297 al., 2013). *Blastocystis* carriage has also been reported at low prevalence rates (<4%) or
298 not at all in household dog populations in Australia (Wang et al., 2013), Brazil (David
299 et al., 2015), and France (Osman et al., 2015), indicating that well-cared animals are
300 probably less exposed to the microorganism. Additionally, these studies demonstrated
301 that domestic dogs can carry a wide range of *Blastocystis* STs including ST1, ST2, ST4,
302 ST5, ST6, and ST10 (Wang et al., 2013; Osman et al., 2015). This combination of low
303 prevalence rates and apparent lack of ST host specificity have led some authors to

304 propose that domestic dogs are transiently and opportunistically infected by whichever
305 *Blastocystis* subtype is present in their environment and should not, therefore,
306 considered as natural hosts or primary sources of human infections (Wang et al., 2013).
307 This is also the epidemiological scenario depicted in the present study, where
308 *Blastocystis* was apparently absent in the investigated domestic dog and cat populations.
309 Failure to amplify *Blastocystis* DNA in isolates of canine or feline origin could be
310 associated to the inefficient removal of PCR inhibitors during the DNA extraction and
311 purification procedure, or to primer competition during the amplification reaction with
312 homologous (e.g. fungal) DNA sequences. These do not seem to be the case of our
313 study, as the same set of DNA isolates of animal origin was successfully tested in a
314 preliminary investigation (de Lucio et al., 2017), whereas unspecific amplicons were
315 not routinely observed after gel electrophoresis. Of note, direct evidence of zoonotic
316 transmission has been provided by other surveys investigating human and canine/feline
317 populations living in the same spatial and temporal setting. *Blastocystis* subtypes ST2–5
318 were simultaneously found in people and domestic dogs living in an urban community
319 in the Philippines (Belleza et al., 2016), whereas ST concordance was also demonstrated
320 between symptomatic *Blastocystis* patients undergoing chemotherapeutical treatment
321 and their pets in Australia (Nagel et al., 2012).

322 Another interesting contribution of this study is the demonstration of a relatively
323 large degree of genetic variability at the nucleotide level within *Blastocystis* ST2
324 sequences, translating into the identification of a number of polymorphic sites in the
325 form of double peaks at chromatogram inspection. This finding provides molecular
326 evidence in support of the occurrence of mixed infections involving different inter- and
327 intra-allelic combinations of the protist. In this regard, it should be emphasized that
328 *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 et al., 2015). High levels of intra-subtype 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).

Conclusions

Blastocystis carriage is a frequent event in apparently healthy individuals in northern Spain, independently of gender, age group, or geographic origin. Failure to detect the protist in domestic dogs and cats suggests that well-cared pets play a minor role or no role at all as natural reservoirs of human *Blastocystis* in this 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.

Acknowledgements

This study was funded by the Health Institute Carlos III, Ministry of Economy and Competitiveness under project CP12/03081.

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FIGURE CAPTIONS

Figure 1

Diversity and frequency of *Blastocystis* subtypes and 18S alleles identified in the present study. Álava, Northern Spain, 2014.

Figure 2

Phylogenetic tree showing the inferred evolutionary relationships among subtypes and alleles of *Blastocystis* sp. causing human infections in Europe at the *SSU* rRNA marker. The analysis was conducted using the Neighbor-Joining method of the nucleotide sequence covering a 571-bp region (positions 1 to 570 of GenBank accession number JF274672) of the gene. Bootstrap values lower than 50% were not displayed. Red filled circles represent sequences of human origin generated in the present study.

SUPPLEMENTARY MATERIAL

Supplementary material Table 1

Sequence alignment analysis of *Blastocystis* isolates assigned to ST2 at the *SSU* rRNA gene in this study. Reference ST2 sequences (various alleles) gathered from the *Blastocystis* Subtype (18S) database were also included for comparative purposes. Polymorphic (double peaks) positions are highlighted in dark blue.

Supplementary material Figure 1

Partial nucleotide sequences of *Blastocystis* isolates assigned to ST2 at the *SSU* rRNA gene indicating the presence of polymorphic (double peaks) sites during chromatogram inspection. Sequencing data of both (forward and reverse) DNA strands show overlapping nucleotide

peaks A/G (red arrow), G/C (blue arrows), or A/T (orange arrows) at positions 161, 453, and 454, respectively, of reference sequence JF274672 (Allele 12).