The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

SHORT COMMUNICATION

Blastocystis sp. Subtype Diversity in Wild Carnivore Species from Spain

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Keywords
Next generation sequencing; Sanger; wildlife; zoonotic transmission.

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Received: 16 April 2019; revised 14 October 2019; accepted October 26, 2019.
Early View publication November 26, 2019

doi:10.1111/jeu.12772

ABSTRACT

The occurrence and molecular diversity of the stramenopile eukaryote *Blastocystis* sp. was investigated by PCR and sequencing (Sanger and NGS) methods in 380 faecal specimens of free-living carnivores in Spain. *Blastocystis* sp. was confirmed in 1.6% (6/380) of the specimens analysed. Two samples from a common genet and a fox were successfully subtyped as ST7 by Sanger. Using NGS, ST14 was found in a fox and a European polecat, ST7 in a fox, and two additional foxes presented mixed infections of ST1/ST2/ST4 and ST1/ST2/ST7, respectively. Wild carnivore species could act as carriers of zoonotic *Blastocystis* subtypes.

BLASTOCYSTIS sp. (Stramenopiles, Blastocystidae), one of the most common enteric parasites in humans globally (Scanlan and Stensvold 2013), possesses elusive transmission pathways. Although the life cycle of *Blastocystis* is not fully understood, faecal-oral transmission through ingestion of cyst-contaminated water or food is mostly accepted. *Blastocystis* pathogenicity remains controversial. *Blastocystis* infections have been linked with gastrointestinal symptoms, irritable bowel syndrome, and extra-intestinal disorders (Casero et al. 2015; Nourrisson et al. 2014), but in most instances, *Blastocystis* carriage is asymptomatic (Zhang et al. 2016). Indeed, recent metagenomics-based studies seem to suggest that *Blastocystis* colonization is associated with a healthy gut microbiota (Audebert et al. 2016).

Blastocystis is frequently reported in a wide range of vertebrates including human and nonhuman primates, other mammals and birds. Animal-to-human (or vice versa)

transmission has been suggested in several molecular epidemiological surveys (Eroglu and Koltas 2010; Ramírez et al. 2014; Stensvold et al. 2009) but not in others (Paulos et al. 2018). Taken together, these data seem to indicate that zoonotic/anthroponotic transmission of *Blastocystis* sp. likely occurs under appropriate conditions. A high degree of genetic diversity has been found within *Blastocystis* sp. (Alfellani et al. 2013b; Stensvold et al. 2012) leading to the description of at least 26 subtypes (ST), based on polymorphism at the small subunit (*ssu*) rRNA gene, with marked differences in host specificities and even geographical distributions (Alfellani et al. 2013a; Clark et al. 2013; Maloney et al. 2019a). Among them, ST1–9 and ST12 have been reported in humans. Additionally, subtype-dependent variability in *Blastocystis* pathogenicity has been proposed by some authors (Domínguez-Márquez et al. 2009; Ramírez et al. 2014; Stensvold et al. 2011).

Little research has been conducted to investigate the occurrence of this eukaryote in wild animal populations (Alfellani et al. 2013b; Betts et al. 2018; Roberts et al. 2013), and most animal studies focused on livestock species or wild animals kept in zoological gardens (Abe et al. 2002; Alfellani et al. 2013b; Cian et al. 2017; Maloney et al. 2019a; Stensvold et al. 2009). Here, we present novel data on the occurrence and molecular diversity of *Blastocystis* sp. in free-living carnivore populations in Spain.

MATERIALS AND METHODS

Sample collection

A total of 380 faecal specimens from 13 wildlife mammalian species belonging to the Canidae (n = 187), Erinaceidae (n = 2), Felidae (n = 25), Herpestidae (n = 11), Mustelidae (n = 133), Procyonidae (n = 11), and Viverridae (n = 11) families were collected in seven Spanish regions between December 2013 and October 2017 (Table 1 and Data S1). Samples were obtained from road- and hunter-killed animals, from accidentally found carcasses, camera-trap surveys, or animals entering rescue shelters. Hunted animals had been legally shot during official hunting seasons.

DNA extraction

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.

Molecular detection and characterization of *Blastocystis* sp. using Sanger sequencing

Identification of *Blastocystis* sp. was achieved by a PCR protocol targeting a fragment of the *ssu* rRNA gene as previously described (Scicluna et al. 2006). Briefly, this method uses the barcoding primers RD5 and BhRDr to generate a PCR product of ~600-bp. Laboratory-confirmed *Blastocystis* positive, negative, and no-template controls were included as controls in each PCR run. PCR amplicons were visualized on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda). All PCR products were sequenced in both directions with the same PCR primers using Big Dye™ chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA). Generated DNA consensus sequences were aligned to appropriate reference sequences using the MEGA 7 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. These nucleotide sequences were deposited in the GenBank database under accession numbers MK587503 and MK587504.

Molecular detection, next-generation amplicon sequencing library preparation, and bioinformatic analysis

Next-generation amplicon sequencing (NGS) libraries were prepared and analysed as previously described (Maloney et al. 2019b). Briefly, all samples were screened by PCR using primers ILMN_Blast505_532F and ILMN_Blast998_1017R. These primers amplify a fragment of the *ssu* rRNA gene and are identical to Blast505_532F/Blast998_1017R (Santín et al. 2011), with the only exception of containing the Illumina overhang adapter sequences on the 5′ end. Final libraries were quantified using the Quant-iT dsDNA Broad-Range Assay Kit (Thermo Fisher, Waltham, MA) on a SpectraMax iD5 (Molecular devices, San Jose, CA) prior to normalization. A final pooled library concentration of 8 pM with 20% PhiX control was sequenced using Illumina MiSeq 600 cycle v3 chemistry (Illumina, San Diego, CA). Paired-end reads were processed and analysed with an in-house pipeline that uses the BBTools package v38.22 (Bushnell 2014), VSEARCH v2.8.0 (Rognes et al. 2016), and BLAST+ 2.7.1. After removing singletons, clustering and the assignment of centroid sequences to operational taxonomic units (OTU) were performed within each sample at a 98% identity threshold. All raw fastq files were deposited to the NCBI sequence read archive under the accession number PRJNA523831. The nucleotide sequences for unique OTUs obtained in this study have been deposited in GenBank under the accession numbers MK587489–MK587502.

RESULTS AND DISCUSSION

Blastocystis was found and confirmed by Sanger or NGS in nearly 1.6% (6/380) of the faecal samples analysed (Table 1). The protist was identified in four (2.2%) red foxes (*Vulpes vulpes*) from Basque Country, Castile-La

Table 1. Number of wild carnivore species examined, number of positives, and prevalence (%) of *Blastocystis* sp. by host and location

Family	Species (scientific name)	Region	No. of samples examined	No. of *Blastocystis*-positive (%)
Canidae	Red fox (*Vulpes vulpes*)	Andalusia	1	0
		Basque Country	39	1 (2.6)
		Castile and Leon	94	0
		Castile-La Mancha	25	1 (4.0)
		Extremadura	22	2 (9.0)
		Sub-total	181	4 (2.2)
	Iberian wolf (*Canis lupus*)	Basque Country	5	0
		Castile-La Mancha	1	0
		Sub-total	6	0
Felidae	European wildcat (*Felis silvestris*)	Basque Country	1	0
		Castile and Leon	4	0
		Extremadura	1	0
		Sub-total	6	0
	Iberian lynx (*Lynx pardinus*)	Castile-La Mancha	2	0
		Extremadura	17	0
		Sub-total	19	0
Herpestidae	Egyptian mongoose (*Herpestes ichneumon*)	Castile and Leon	5	0
		Extremadura	6	0
		Sub-total	11	0
Mustelidae	Beech marten (*Martes foina*)	Basque Country	2	0
		Castile and Leon	14	0
		Castile-La Mancha	5	0
		Extremadura	2	0
		Sub-total	23	0
	European badger (*Meles meles*)	Asturias	69	0
		Castile and Leon	6	0
		Castile-La Mancha	1	0
		Extremadura	1	0
		Sub-total	77	0
	European polecat (*Mustela putorius*)	Castile-La Mancha	2	0
		Extremadura	2	1 (50.0)
		Sub-total	4	1 (25.0)
	Eurasian otter (*Lutra tutra*)	Castile and Leon	2	0
		Castile-La Mancha	1	0
		Extremadura	1	0
		Sub-total	4	0
	American mink (*Neovison vison*)	Extremadura	25	0
Procyonidae	Raccoon (*Procyon lotor*)	Madrid	11	0
Viverridae	Common genet (*Genetta genetta*)	Basque Country	2	0
		Castile and Leon	5	0
		Castile-La Mancha	1	1 (100)
		Extremadura	3	0
		Sub-total	11	1 (9.0)
Erinaceidae	Western European hedgehog (*Erinaceus europaeus*)	Castile and Leon	2	0
Total			380	6 (1.6)

Mancha, and Extremadura, one (9.0%) common genet (*Genetta genetta*) from Castile-La Mancha, and one (25.0%) European polecat (*Mustela putorius*) from Extremadura (Table 1 and Data S1). In the only previous epidemiological survey assessing the presence of *Blastocystis* sp. in wild carnivore species kept in captivity in Spain, an 8.3% prevalence was found by microscopy in red foxes, Hudson Bay wolves, Iberian wolves, and brown bears (Pérez Cordón et al. 2008). This figure is over five-fold higher than that (~1.6%) reported here in free-living

carnivores. This discrepancy is likely due either to differences in the animal populations compared or to the higher infection pressure expected to occur in animals living in crowded conditions. *Blastocystis* sp. has also been reported at prevalence rates ranging from 0 to 7.5% in captive (Alfellani et al. 2013b; Cian et al. 2017) and free-living (Betts et al. 2018) carnivore species, and from 0 to 2.6% in domestic dogs (Abe et al. 2002; Moura et al. 2018; Paulos et al. 2018) in different geographical areas of the world. In contrast, the occurrence of the parasite has been identified at comparatively higher rates (6.0–54.1%) in domestic ruminants, particularly cattle and sheep (Lee et al. 2018; Li et al. 2018; Masuda et al. 2018). Taken together, these findings seem to indicate that *Blastocystis* colonization/infection is less common in strict carnivore species than in ruminants. These discrepancies may arise from specific differences in gut microbiome profiles between carnivores and herbivores, or to differences in the structure and functionality of the gastrointestinal tract, as obligatory carnivores have a reduced caecum compared to that of herbivores. It is currently unknown if the presence of *Blastocystis* in carnivores represents active infection or is the result of consumption of an infected animal.

Out of the 380 samples tested using primers RD5 and BhRDr 12 samples were PCR positive, but only two specimens, one from a young female genet (Sample #98) and one from an adult vixen (Sample #113), were successfully subtyped by the Sanger method as ST7 allele 140. Both animals were from the province of Ciudad Real in the autonomous regions of Castile-La Mancha (Data S2). The high number of positives by PCR that could not be successfully identified as *Blastocystis* when using primers RD5 and BhRDr could be related to their ability to amplify other eukaryotes (mostly fungi), with no obvious differences in PCR product size, especially when screening DNA extracted directly from faeces (Stensvold 2013). Therefore, barcoding primers are better suited for molecular characterization of already known positive samples and not for screening (Stensvold and Clark 2016). The Santín et al. (2011) primers perform better for screening faecal specimens for *Blastocystis* as they produce fewer false positives without compromising specificity and sensitivity.

Analysis of all samples ($n = 380$) by NGS using the ILMN_Blast505_532F and ILMN_Blast998_1017R primers allowed the identification of five *Blastocystis* positive. A total of 2,611,628 read pairs were generated from those samples. After trimming, quality filtering, and pair merging, 444,911 merged read pairs were retained. 418,784 merged reads remained following chimera filtering. Clustering generated 16 OTUs across those five samples. There were 13 OTUs that were unique *Blastocystis* sequences in the study population (Data S3). NGS confirmed the presence of ST7 in fox #113 and identified and subtyped *Blastocystis* in four additional samples including three foxes and one European polecat (Data S2). NGS also revealed a much higher genetic diversity with identification of three subtypes in two foxes (samples #127 and #156) as well as intrasubtype variability in three foxes (samples #113, #127, and #156) for ST1, ST2, and ST7 (Data S2). The relative abundance of *Blastocystis* subtypes identified in each positive sample is shown in Data S4. ST7 displayed the greatest intrasubtype variability with five unique OTUs, two of which were found in both ST7 positive samples (#113 and #156) (Data S2). ST1 and ST2 also displayed intrasubtype variability in this study with three unique OTUs observed for both subtypes. No intrasubtype variability was observed for ST4 or ST14 (Data S2).

Combining Sanger and NGS sequencing, five *Blastocystis* subtypes were identified in the surveyed population, namely ST1, ST2, ST4, ST7, and ST14. ST14 was found in a fox and a European polecat, ST7 in a fox and a common genet, and the other two foxes presented mixed infections with ST1/ST2/ST4 and ST1/ST2/ST7, respectively. Mixed subtype infections were observed in two of the five (40.0%) *Blastocystis*-positive samples by NGS and included the identification of subtypes present in low abundance (Data S2 and Data S4). No mixed subtype infections were found using the traditional method, barcoding primers coupled with Sanger sequencing, possibly due to the inherent nature of PCR, which preferentially amplifies the predominant subtypes that will be identified by Sanger sequencing. The use of NGS could aid in understanding host-parasite specific epidemiological cycling in nature because of its capacity to resolve a higher level of genetic diversity identifying low abundance of *Blastocystis* STs within the same host (Maloney et al. 2019b). ST7 was the most common ST found causing single or mixed infections in combination with other *Blastocystis* STs in three foxes and a common genet. Of interest, ST7 is a relatively common finding in birds (Stensvold et al. 2009), suggesting that wild carnivores may acquire *Blastocystis* by preying on birds carrying the protist. The fact that ST7 has been reported previously in a variety of human populations in Angola, Colombia, or Thailand (Dacal et al. 2018; Ramírez et al. 2016; Yowang et al. 2018) including Spanish symptomatic individuals (Carmena, unpublished data) indicates that ST7 could be zoonotically transmitted. The finding of ST14 in a fox and a European polecat is interesting, as this *Blastocystis* subtype has so far been primarily found in production animals (Fayer et al. 2012; Li et al. 2018) and wildlife members of the family Artiodactyla (Cian et al. 2017). There is no evidence of zoonotic transmission of ST14 as it has not been yet detected in humans. This is, to the best of our knowledge, the first description of ST14 in a carnivore species.

The identification of ST1, ST2, and ST4 in foxes expands the host range of these potentially zoonotic subtypes and supports the potential role of wild carnivores as carriers of zoonotic *Blastocystis* subtypes. There is limited information of *Blastocystis* subtypes in captive or free-living carnivore species, and only few studies have reported molecular-based surveys. ST2 and ST3 were previously identified in a captive cheetah and a grey wolf in France (Cian et al. 2017), ST4 in a pine marten from a conservation park in the U.K. (Betts et al. 2018), and ST8 in omnivorous common wild opossums in Colombia (Ramírez et al.

2014). Companion animal species, dogs and cats, have also been investigated to assess their potential role as suitable reservoirs of *Blastocystis* transmission to humans. Evidence of transmission between pets and their owners involving ST1, ST2, and/or ST3 (the *Blastocystis* subtypes more prevalently found circulating in humans) has been indicated by some surveys conducted in Australia (Nagel et al. 2012), Philippines (Belleza et al. 2016), and Turkey (Eroglu and Koltas 2010). In contrast, recent studies conducted in France and Spain showed that domestic dogs and cats had a negligible role as source of human infections (Osman et al. 2015; Paulos et al. 2018).

Regarding the diversity and frequency of *Blastocystis* in Spain, ST1, ST2, ST3, and ST4 have been identified in a general, asymptomatic population in the North of the country (Paulos et al. 2018), whereas those same subtypes and to much lower extent ST6 and ST7 have been described in clinical samples (Carmena, unpublished data). Interestingly, ST4 has been previously documented as the most prevalent (94.1%) *Blastocystis* ST in mono-infected patients with acute or chronic diarrhoea (Domínguez-Márquez et al. 2009), but the least frequent ST circulating in asymptomatic subjects in Spain (Paulos et al. 2018), raising the question of whether ST4 is more pathogenic than other *Blastocystis* STs. Our results demonstrate that STs with zoonotic potential are present in carnivores, corroborating their potential role as a source of *Blastocystis* human infection and environmental contamination. In addition, this population was examined for other intestinal parasites with zoonotic potential including several *Cryptosporidium* species and *Enterocytozoon bieneusi* genotypes supporting free-living carnivores as a source of zoonotic parasites (Mateo et al. 2017; Santin et al. 2018).

In conclusion, this is the first study that explored the occurrence and genetic diversity of *Blastocystis* in wild carnivores in Spain. The host range for *Blastocystis* and its subtypes was expanded with the identification of ST1, ST4, and ST2 in foxes; ST14 in a fox and a European polecat; and ST7 in a fox and a common genet. The application of NGS provided higher resolution allowing the identification of mixed infections (representing 40.0% among *Blastocystis* positives) as well as detection of low abundance subtypes. Further studies using high-resolution methods such NGS are necessary to understand the dynamics of *Blastocystis* transmission in wild populations and their role in the zoonotic transmission of this stramenopile.

ACKNOWLEDGMENTS

This study was funded by the Health Institute Carlos III (ISCIII), Ministry of Economy and Competitiveness (Spain), under project PI16CIII/00024. Additional funding was obtained from the Complutense University of Madrid (grant CT65/16), the University of Aveiro (Department of Biology) and FCT/MEC for the financial support to CESAM (UID/AMB/50017/2019) through national funds co-financed by the FEDER (PT2020 Partnership Agreement). The authors thank Aleksey Molokin for technical services in support of this study.

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SUPPORTING INFORMATION

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

Data S1. Map of Spain showing the geographical distribution of the main carnivore families sampled in the present study.
Data S2. *Blastocystis* subtypes identified by Sanger- and NGS-based (including unique OTUs information and percentage of reads) methods in each carnivore-positive sample in Spain are presented.
Data S3. Unique operational taxonomic units (OTUs) obtained for *Blastocystis* subtypes by next-generation amplicon sequencing.
Data S4. Relative abundance of *Blastocystis* sp. subtypes observed in each positive carnivore sample using next-generation amplicon sequencing (NGS).