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PROTOZOOLOGY - ORIGINAL PAPER

Occurrence and genetic diversity of *Enterocytozoon bieneusi* (Microsporidia) in owned and sheltered dogs and cats in Northern Spain

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Abstract

Enterocytozoon bieneusi is an obligate intracellular protist-like fungi parasite that infects numerous mammal hosts including humans, raising concerns of zoonotic transmission. There is little information available on the presence and diversity of *E. bieneusi* genotypes in companion animals. Here, we determined the occurrence and genetic diversity of *E. bieneusi* in domestic dogs and cats from Northern Spain. A total of 336 genomic DNA samples extracted from canine ($n = 237$) and feline ($n = 99$) faecal specimens were retrospectively investigated. The presence of *E. bieneusi* was assessed by PCR of the rRNA internal transcribed spacer (ITS) gene. The parasite was detected in 3.0% (3/99) and 0.8% (2/237) of the cats and dogs examined, respectively. All three feline positive samples were from stray cats living in an urban setting, whereas the two canine samples were from owned dogs living in rural areas. Sequence analysis revealed the presence of two genotypes in dogs, BEB6 and PtEb IX, and two genotypes in cats, D and Peru11. The identification of Peru11 in a cat and BEB6 in a dog constitutes the first report of those genotypes in such hosts as well as first report in Spain. This is also the first evidence of genotype D in cats and PtEb IX in dogs in Spain. Three out of the four genotypes, BEB6, D and Peru11, have been previously reported as human pathogens and are potentially zoonotic indicating that dogs and cats need to be considered potential sources of human infection and environmental contamination.

Keywords Microsporidia · *Enterocytozoon bieneusi* · Dogs · Cats · ITS · Genotyping · Spain

Introduction

Microsporidia is a diverse and ubiquitous phylum of obligate intracellular and spore-forming parasites related to fungi with hosts ranging from protists to mammals (Stentiford et al. 2016). There are approximately 1500 described species of Microsporidia which are distributed into about 200 genera (Weiss and Becnel 2014), among which *Enterocytozoon bieneusi* is the most common species in human gut infections. It is responsible for 90% of the cases of human microsporidiosis reported globally (Santín 2015). Infection with *E. bieneusi* is mainly associated with chronic diarrhoea and wasting syndrome in immunocompromised (e.g. HIV-infected patients and organ transplant recipients) patients, but also with self-limited acute diarrhoea and malabsorption in immunocompetent individuals (Lobo et al. 2012). In developed countries, *E. bieneusi* is considered an emerging opportunistic pathogen with prevalence ranging from 2 to 78% and

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5–15% in HIV-seropositive and HIV-seronegative individuals, respectively (reviewed by Matos et al. 2012). In developing countries, reported infection rates can be as high as 58% in apparently healthy subjects (reviewed by Matos et al. 2012). In addition, *E. bieneusi* has been widely reported in a broad range of animal hosts including wild and domestic animals strongly suggesting the zoonotic potential of this parasite in which animals may act as natural reservoirs of human infections (Santín and Fayer 2011). Transmission is faecal-oral through ingestion of contaminated food or water with spores of the parasite (Izquierdo et al. 2011; Stentiford et al. 2016) as well as direct transmission from infected humans or animals (Mathis et al. 2005).

Several hundred *E. bieneusi* genotypes have been defined based on sequence analysis of the internal transcribed spacer (ITS) of the rRNA gene which constitute 11 phylogenetic groups with distinct differences in their host specificity and zoonotic potential (Santín 2015; Henriques-Gil et al. 2010; Karim et al. 2014b; Li et al. 2019). Group 1 and Group 2 include most of the potentially zoonotic genotypes, whereas the rest of the clusters display genotypes with strong host specificity (Li and Xiao 2019; Li et al. 2019).

The rate of pet ownership is increasing globally as animals enrich the lives of humans. In Spain, it has been estimated that over 49% (a total of 11.6 million) of the households own a companion animal with an estimated number of dogs and cats of 5.4 and 3.8 million, respectively (http://www.anfaac.org/macromagnitudes-del-sector/censo-de-mascotas/). Although there are benefits of animal companionship, pets can carry diseases that may spread between them and humans arising a potential threat to public health. However, in Spain, there is no information of the presence of *E. bieneusi* in cats and information in dogs is still scarce with only one study reporting genotypes (del Águila et al. 1999; Lores et al. 2002a; Dado et al. 2012; Galván-Díaz et al. 2014) (Table 1). Therefore, this study aims to investigate the occurrence and genetic diversity of *E. bieneusi* in dogs and cats, owned and sheltered, in Northern Spain, and to assess zoonotic potential and risk to public health.

Materials and methods

Origin of specimens

A total of 336 genomic DNA samples extracted from dog ($n = 237$) and cat ($n = 99$) faecal specimens collected in the province of Álava (Northern Spain) between 2014 and 2016 were retrospectively investigated. Dogs and cats sampled were owned and sheltered living in rural and urban settings (Fig. 1) and did not present with any clinical manifestation associated to gastrointestinal illness at the time of sample collection. The characteristics of the population studied are summarised in Table 2. Because sex and age were not available for many of the animals surveyed, these variables were not considered in the study. Sample preparation and DNA extraction and purification procedures were as described elsewhere (Gil et al. 2017; de Lucio et al. 2017).

PCR and sequence analysis

Genomic DNA was analysed by nested PCR using primers specific for *E. bieneusi* that amplified the ITS region as well as a portion of the flanking large and small subunit ribosomal RNA genes as previously described (Buckholt et al. 2002). Negative and positive controls were included in all PCR rounds. The PCR products were subjected to electrophoresis in a 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda).

PCR products of the expected size were directly sequenced in both directions with the inner primers in 10 μl reactions using Big Dye™ chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA). Raw sequencing data 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. The established nomenclature system based in ITS nucleotide sequence was used to determine *E. bieneusi* genotypes (Santín and Fayer 2009). Sequences generated in this study were deposited in the GenBank database under accession numbers MK932867–MK932870.

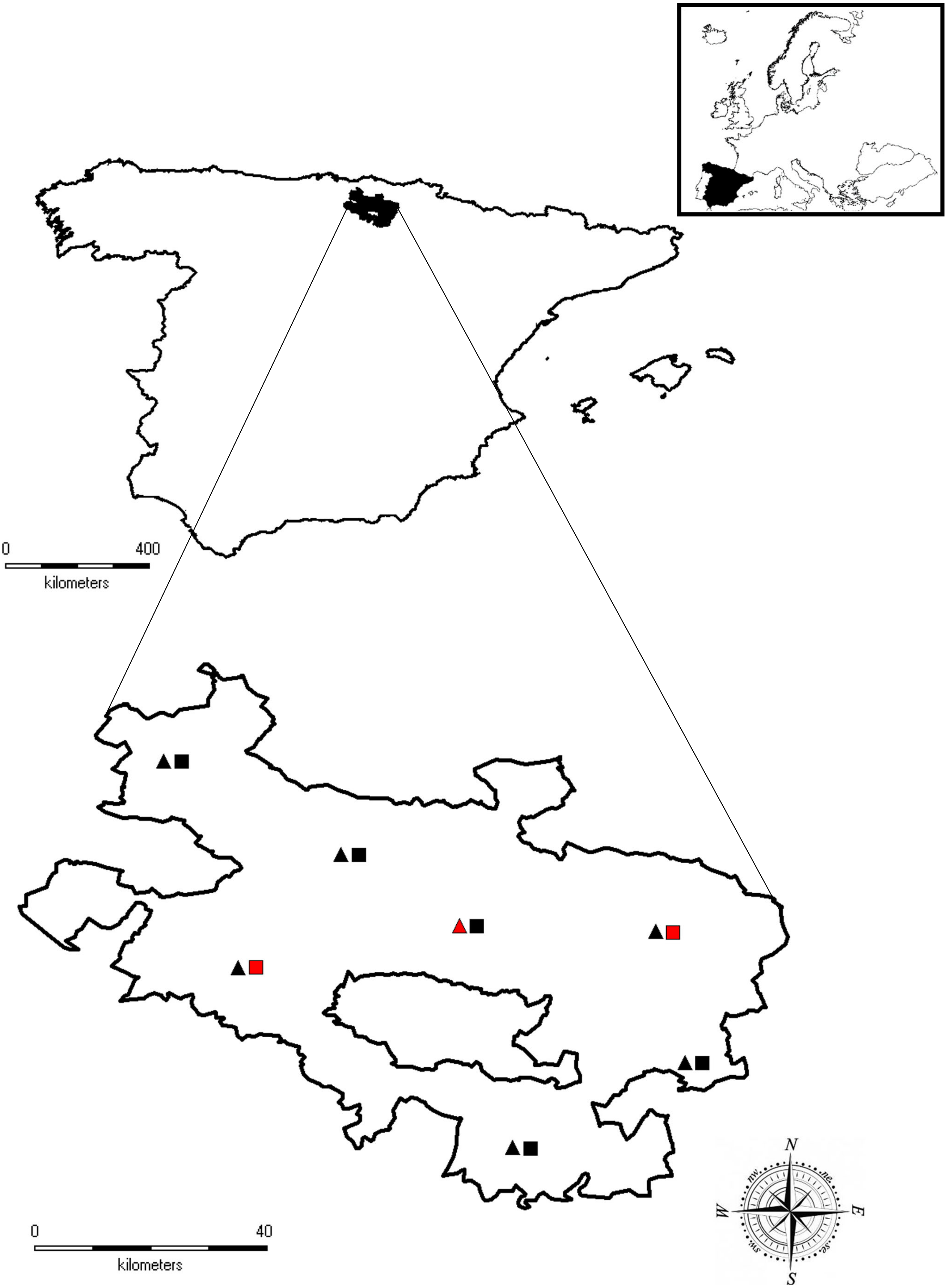
Results

In this study, stray dogs and cats (59.9%) constituted the most common category, followed by owned (26.5%) and surrendered (13.7%). Overall, 56.0% of the dogs and cats examined were from rural areas in the province of Álava, whereas the remaining were from urban settings (Table 2). Of the 237 dogs and 99 cats tested for the presence of *E. bieneusi*, 2 (0.8%) and 3 (3.0%) were found positive, respectively. All positive cats were stray cats living in the capital city Vitoria-Gasteiz, whereas the two positive dogs were rural owned dogs (Fig. 1 and Table 3). Nucleotide sequence analysis of the ITS region revealed the presence of four distinct genotypes, in dogs, BEB6 and PtEb IX, and in cats, D and Peru11 (Table 3). Mixed infections with more than one genotype of *E. bieneusi* were not detected. Both genotypes identified in cats, D and Peru11, belong to Group 1. For genotypes identified in dogs, BEB6 belongs to Group 2 and PtEB IX to Group 2.

Table 1 Main PCR-based studies confirming the presence of *Enterocytozoon bieneusi* in faecal samples from human and animal populations in Spain

Region	Host population/type of sample	No. samples	Diagnostic method	Prevalence (%)	Genotype (no. isolates)	Reference
Madrid	HIV-positive children	158	LM, IFA, PCR	1.2	ND	del Águila et al. (1997a)
Madrid	HIV-positive patients with diarrhoea	50	LM, IFA, PCR	12.0	ND	del Águila et al. (1997b)
Gran Canaria	Renal transplant recipients	2	LM, PCR	100	D (2)	Galván et al. (2011)
Tropics	Travellers with diarrhoea	40	LM and PCR	10.0	ND	López-Vélez et al. (1999)
Vigo	Geriatric outpatients with diarrhoea	47	LM and PCR	17.0	ND	Lores et al. (2002b)
Tenerife	Immunocompetent patients, most with diarrhoea	156	PCR, hybridisation	11.5	Identical to L16868	Abreu-Acosta et al. (2005)
NS	Dogs	46	LM, PCR	10.8	ND	del Águila et al. (1999)
Galicia	Dogs	17	LM, PCR	11.7	ND	Lores et al. (2002b)
Madrid	Dogs	73	LM, PCR	9.6	A (7)	Galván-Díaz et al. (2014)
Various	Rabbits	84	LM, PCR	4.8	ND	del Águila et al. (1999)
Various	Rabbits	19	LM, PCR	21.1	D (1)	Galván-Díaz et al. (2014)
Galicia	Farmed goats	7	LM, PCR	14.2	ND	Lores et al. (2002b)
Various	Farmed pigs	34	LM, PCR	20.6	I (1)	Galván-Díaz et al. (2014)
Castile-La Mancha	Farmed ostriches	17	LM, PCR	5.9	Type IV(1)	Galván-Díaz et al. (2014)
Murcia	Urban pigeons	124	PCR	9.7%	Col01 (1), Col02 (1), Col03 (1), Col04 (1), Col05 (1), Col06 (1), Col07 (1)	Haro et al. (2005, 2006)
Andalusia	Wild red foxes	7	LM, PCR	14.3	D(1)	Galván-Díaz et al. (2014)
Various	Wild red foxes	87	PCR	9.2	Wildboard3 (6), EbCar4 (1), S9(1)	Santín et al. (2018)
Various	Wild beech martens	9	PCR	11.1	EbCar1(1)	Santín et al. (2018)
Various	Wild European badgers	69	PCR	23.2	PtEb IX (1), EbCar2 (5), Wildboard3 (4), EbCar3 (1), S5 (5)	Santín et al. (2018)
Madrid	Soil from public parks	79	LM, PCR	10.1	ND	Dado et al. (2012)

IFA, indirect fluorescence assay; *LM*, light microscopy; *NA*, not applicable; *ND*, not determined; *NS*, not specified; *PCR*, polymerase chain reaction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◀ **Fig. 1** Map of the province of Álava (Northern Spain) showing the geographical location of the canine (identified as filled squares) and feline (identified as filled triangles) sub-populations sampled in the present study. Black and dark red figures represent *Enterocytozoon bieneusi*-negative and *Enterocytozoon bieneusi*-positive results by the ITS-PCR method, respectively

Discussion

In the present study, molecular techniques were used to test for presence of *E. bieneusi* organisms in 234 dogs and 99 cats in Northern Spain. Three cats (3.0%) and two dogs (0.8%) were found positive for *E. bieneusi*. Although *E. bieneusi* has frequently been reported in cats in many other countries including Brazil, China, Colombia, Czech Republic, Egypt, Germany, Iran, Japan, Poland, Portugal, Slovakia, Switzerland, Thailand, and Turkey (Table 4), this constitutes the first report of *E. bieneusi* in cats in Spain. Two previous studies aiming to detect Microsporidia in animals in Spain that included household cats ($n = 9$) from Madrid (Galván-Díaz et al. 2014) and Galicia ($n = 10$) (Lores et al. 2002a) did not find *E. bieneusi*. However, another Microsporidia species, *Encephalitozoon intestinalis*, was identified in one of the cats from Madrid (Galván-Díaz et al. 2014). In dogs, *E. bieneusi* has already been reported not only in Spain (del Águila et al. 1999; Lores et al. 2002a; Galván-Díaz et al. 2014) but also in other counties including China, Colombia, Egypt, Japan, Poland, Portugal, Switzerland, and the USA (Table 4). The prevalence of *E. bieneusi* in dogs (0.8%) and cats (3.0%) was lower than those reported in previous studies that ranged from 1.7–25.8% and 3.3–31.3% in dogs and cats, respectively (Table 4). Differences in prevalence could be related to the detection method (molecular or microscopy), differences in the type of population included in the studies (stray or pet), or others such geographic area or infection pressure. All *E. bieneusi* positive dogs were rural owned dogs suggesting that dogs living in rural areas may be more likely to be exposed to the parasite than their counterparts in urban settings. It could also be associated with differences in health care standards between urban and rural dog populations. However, we need to be cautious because prevalence was low and a higher number of rural than urban dogs were examined. On the other hand, we only found *E. bieneusi* in stray cats living in an urban setting. This is in accordance with observations in other studies in Central Europe that only found *E. bieneusi* in stray cats (8.8%; 12/135) with no positives in any of the 120 owned cats examined (Kvac et al. 2017).

Table 2 Main epidemiological features of the canine and feline populations investigated in the present survey

	Dogs		Cats		Total	
Variable	No.	%[3]	No.	%[3]	No.	%[3]
Category						
Owned	55	23.2	34	34.3	89	26.5
Stray[1]	147	62.0	54	54.5	201	59.8
Surrendered[2]	35	14.8	11	11.1	46	13.7
Setting						
Urban	78	32.9	70	70.7	148	44.0
Rural	159	67.1	29	29.3	188	56.0
Total	237	100	99	100	335	100

[1] Stray animals refer to wandering dogs and cats captured by the personnel of the Animal Rescue Centre, City Council of Vitoria-Gasteiz

[2] Surrendered animals refer to unwanted dogs and cats rendered by their owners to the Animal Rescue Centre, City Council of Vitoria-Gasteiz

[3] Relative frequencies are shown

Table 3 Main epidemiological and molecular features of the canine and feline samples infected with *Enterocytozoon bieneusi* in the province of Álava (Northern Spain), 2014–2016

	Epidemiological data			Genotyping data	
Sample	Host	Status	Setting	Genotype	GenBank accession no.
59_14	Cat	Stray	Urban	D	MK932867
161_14	Cat	Stray	Urban	D	–
151_15	Cat	Stray	Urban	Peru11	MK932868
72_14	Dog	Owned	Rural	BEB6	MK932869
109_14	Dog	Owned	Rural	PtEb IX	MK932870

Our sequencing data analysis revealed the presence of two genotypes in dogs, PtEb IX and BEB6. PtEb IX is the most common genotype reported in dogs (Table 4), but this is the first report of this genotype in dogs in Spain. So far, only genotype A, not found in this study, has been reported in dogs in Spain (Galván-Díaz et al. 2014). However, PtEb IX has been previously identified in Spain in wild European badgers (Santín et al. 2018). PtEb IX is considered a host-adapted genotype and, therefore, poses limited (if any) zoonotic potential (Li et al. 2019). This is the first report in dogs of BEB6 (also reported as SH5). This genotype was initially detected in dairy cattle in the USA (Fayer et al. 2007), and since then it has expanded its host range to include humans, non-human primates, cattle, sheep, alpacas, goats, horses, sika deer, hog deer, and cats (Feng et al. 2011; Wang et al. 2013; Karim et al. 2014b; Stensvold et al. 2014; Zhao et al. 2014; Karim et al. 2015; Li et al. 2016; Qi et al. 2016; Shi et al. 2016). BEB6 has been reported in domestic cats in China, but not in domestic dogs (Karim et al. 2014a). In China, BEB6 has also been identified in raw waste water (Ye et al. 2017) and in a hospitalised child during a *Cryptosporidium* outbreak investigation at a paediatric hospital (Wang et al. 2013), suggesting that this genotype may have some zoonotic potential. BEB6 seems to be rare in Europe, where it has been described so far only in Swedish lambs (Stensvold et al. 2014).

Two genotypes were identified in the three *E. bieneusi* positive cats, D in two cats, and Peru 11 in the third cat. The

Table 4 Summary of studies on *Enterocytozoon bieneusi* in cats and dogs including information of prevalence and genotypes reported. Genotypes in bold denotes those genotypes reported also in humans

Host	Country	Prevalence (no. of positive/no. examined)	Genotype (no.)	References
Cat	Brazil	3.3% (2/60)	**D** (2)	Prado et al. (2019)
	China	11.5% (11/96)	**D** (3), CC2 (1), CC3 (1), CC4 (1), **BEB6** (2), CC1 (1), **I** (1), PtEb IX (1)	Karim et al. (2014a)
		5.8% (3/52)	**D** (2), **Type IV** (1)	Li et al. (2015)
		1.4% (2/143)	**Type IV** (1), **D** (1)	Li et al. (2018)
		5.6% (9/160)	**Type IV** (5), **D** (4)	Xu et al. (2016)
	Colombia	17.4% (8/46)	D-like (4), **Type IV** (2), **Peru10** (1), **WL11** (1)	Santín et al. (2006)
	Czech Republic	2.5% (3/118)	**D** (3)	Kvac et al. (2017)
	Egypt	12.5% (13/104)	N/A	Al-Herrawy et al. (2016)
	Germany	5.0 (3/60)	**Type IV** (2), L (1)	Dengjel et al. (2001)
	Iran	7.5% (3/40)	N/A	Jamshidi et al. (2012)
		11.5% (3/26)	N/A	Askari et al. (2015)
	Japan	14.3% (1/7)	**Type IV** (1)	Abe et al. (2009)
	Poland	9.1% (4/44)	PtEb IX (3), Eb52 (1)	Piekarska et al. (2017)
		6.3% (4/64)	**D** (4)	Kvac et al. (2017)
	Portugal	100 (6/6)#	**Type IV** (4), PtEb IV (1), PtEb VIII (1)	Lobo et al. (2006)
	Slovakia	6.8% (5/73)	**D** (5)	Kvac et al. (2017)
	Spain	0% (0/10)	–	Lores et al. (2002a)
		0% (0/9)	–	Galván-Díaz et al. (2014)
		3.0% (3/99)	**D** (2), **Peru11** (1)	This study
	Switzerland	8.3 (1/12)	EbfelA (1)	Mathis et al. (1999)
	Thailand	31.3% (25/80)	**D** (22), ETMK2 (1), ETMK2 (1), ETMK4 (1)	Mori et al. (2013)
	Turkey	5.5% (4/72)	**Type IV** (2), **D** (2)	Pekmezci et al. (2019)
Dog	China	7.7% (2/26)	CHN5 (1), CHN6 (1)	Zhang et al. (2011)
		15.5% (54/348)	PtEb IX (26), **O** (4), CD8 (4), **D** (3), **EbpA** (2), Macaque3 (2), CD7 (2), **EbpC** (1), **Peru8** (1), **PigEBITS5** (1), **Type IV** (1), CD1 (1), CD2 (1), CD3 (1), CD4 (1), CD6 (1), CD5 (1), WW8 (1)	Karim et al. (2014a)
		6.7% (18/267)	PtEb IX (14), **EbpC** (2), **D** (1), NED1 (1), NED2 (1); NED3 (1), NED4 (1)	Li et al. (2015)
		8.6% (27/315)	PtEb IX (16), **EbpC** (4), CHD3 (3), CHD1 (2), CHD2 (2)	Li et al. (2018)
		6.0% (29/485)	PtEb IX (28), **D** (1)	Xu et al. (2016)
	Colombia	15.0% (18/120)	PtEb IX (16), **Type IV** (1), **WL11** (1)	Santín et al. (2008)
	Egypt	13% (14/108)	N/A	Al-Herrawy and Gad (2016)
	Germany	0% (0/60)	–	Dengjel et al. (2001)
	Iran	25.8% (8/100)	N/A	Jamshidi et al. (2012)
		11.8% (2/17)	N/A	Askari et al. (2015)
	Japan	2.5% (2/79)	PtEb IX (2)	Abe et al. (2009)
		4.4% (26/597)	PtEb IX (26)	Phrompraphai et al. (2019)
	Poland	4.9% (4/82)	**D** (2), PtEb IX (2)	Piekarska et al. (2017)
	Portugal	100% (3/3)#	**D** (1), **Peru6** (1), PtEb IX (1)	Lobo et al. (2006)

Table 4 (continued)

Host	Country	Prevalence (no. of positive/no. examined)	Genotype (no.)	References
	Spain	9.6% (7/73)	**A** (7)	Galván-Díaz et al. (2014)
		8.7% (4/46)	N/A	del Águila et al. (1999)
		11.8% (2/17)	N/A	Lores et al. (2002a)
		0.8% (2/237)	**BEB6** (1), PtEb IX (1)	This study
	Switzerland	8.3% (3/36)	PtEb IX (3)	Mathis et al. 1999
	Thailand	0% (206/80)	–	Mori et al. (2013)
	USA	100%(1/1)#	PtEb IX (1)	Feng et al. (2011)

N/A, not available

Samples used in the study were faecal samples previously identified as *E. bieneusi* positive

presence of both genotypes is not surprising, as these genotypes are known to have a broad host spectrum and have been reported in humans, non-human primates, livestock, poultry, companion animals, and wildlife (Santín 2015; Li et al. 2019). This is the first report in cats of Peru11 (also reported as Peru12) while D (also reported as PigEBITS9, WL8, Peru9, CEbC, PtEb VI) has been reported in cats in Brazil, China, Thailand, and Turkey (Mori et al. 2013; Karim et al. 2014a; Li et al. 2015; Xu et al. 2016; Li et al. 2018; Pekmezci et al. 2019; Prado et al. 2019). Genotypes D and Peru 11 belong to Group 1 that includes *E. bieneusi* genotypes most frequently found not only in humans but also in domestic animals worldwide, suggesting potential for zoonotic transmission. In Spain, genotype D has been described in renal transplant recipients (Galván et al. 2011), domestic rabbits (Galván-Díaz et al. 2014), and wild red foxes (Galván-Díaz et al. 2014) as well as water (Galván et al. 2013), but no previous reports of Peru 11 were found.

In Spain, the epidemiology and transmission dynamics of *E. bieneusi* remains largely unknown. It has been reported in HIV-positive and HIV-negative patient populations, renal transplants recipients, travellers returning from endemic areas and geriatric outpatients (Table 1). In addition, *E. bieneusi* has been identified in companion, farmed and wild animal species, in urban pigeons and in environmental water samples (Table 1).

In summary, this study represents a significant contribution to our understanding on the epidemiology of *E. bieneusi* in Spain. Our data indicate that rural dogs and stray cats are more prone to harbour *E. bieneusi* infections, very likely due to higher exposure to the parasite and worse standards of care compared with owned animals living in urban settings. Our findings expand current knowledge on host and geographical distribution of *E. bieneusi* genotypes. The identification of Peru11 in a cat and BEB6 in a dog constitutes the first report of those genotypes in those hosts as well as first report in Spain. Genotype D has been previously reported in cats in China, but this is the first report in cats in Spain. PtEb IX has been reported in multiple studies in dogs but this will be the first report of this genotype in Spain. The identification of genotypes with zoonotic potential (D, BEB6, Peru11) represents a public health concern that should be further investigated in future molecular epidemiological surveys.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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