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Prevalence and distribution of *Babesia* and *Theileria* species in roe deer from Spain

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ABSTRACT

Babesiosis and Theileriosis are important worldwide-distributed tick-borne diseases for human and animals. Their presence in a particular area depends on the presence of suitable tick-vector and host species as well as competent reservoirs such as roe deer, one of the most abundant wild cervids in Spain.

Spleen samples from 174 roe deer hunted in Spain were analysed to determine the prevalence of *Babesia* and *Theileria* species. DNA of both piroplasms was firstly detected using a commercial qPCR. Then, positive samples were molecularly characterized at the 18S rRNA and ITS1 genes of *Babesia* spp. and *Theileria* spp. The possible influence of some factors such as ecological area, age and sex was also assessed.

Overall, 89.7% of roe deer were positive to any of the two piroplasms. *Theileria* spp. was more prevalent (60.9%) than *Babesia* spp. (19.0%); species identification could not be achieved in 17.3% of positive samples. *Babesia* prevalence was significantly higher in young animals and in roe deer from Oceanic regions, in contrast to *Theileria* spp. Five species were identified: *Theileria* sp. OT3 (60.3%), *Babesia capreoli* (15.5%), *Babesia venatorum* (2.9%), *Theileria* sp. 3185/02 (0.6%) and *Babesia bigemina* (0.6%). The coinfection *B. capreoli/T.* sp. OT3 was the most common (4.6%) followed by *B. venatorum/T.* sp. OT3 (0.6%) and *B. bigemina/T.* sp. OT3 (0.6%).

Our results reveal that *Theileria* spp. and *Babesia* spp. are prevalent piroplasms in roe deer from Spain. These cervids can act as reservoirs for several *Babesia* and *Theileria* species, including the zoonotic *B. venatorum*. This study represents the first description of *B. venatorum* and *B. bigemina* in roe deer from Spain.

1. Introduction

Piroplasmoses are important worldwide-distributed tick-borne diseases of both domestic and wild animals caused by apicomplexan hemoparasites of the genus *Babesia* and *Theileria* (Zanet et al., 2014). The importance of these pathogens in the northern hemisphere has increased in recent years since some of them can cause diseases considered emerging zoonosis (Hildebrandt et al., 2013).

The distribution of both *Babesia* and *Theileria* infections depends on several factors, mainly the presence of proper tick vector species as well as suitable hosts and reservoirs (Estrada-Peña and de la Fuente, 2014). Although *Babesia* spp. can be transmitted by a wide range of tick genera including *Rhipicephalus*, *Haemaphysalis*, *Hyalomma* and *Dermacentor* (Uilenberg, 2006; Requena-García et al., 2017), it is worth noting that the most distributed tick in Europe, *Ixodes ricinus*, has been identified as a vector for some species such as *B. divergens, B. microti, B. ovis* and *B. venatorum* (Pérez et al., 2012; Rizzoli et al., 2014). It has also been reported that some species of *Rhipicephalus*, *Hyalomma*, *Amblyomma* and *Haemaphysalis* can transmit *Theileria* spp. (Viseras et al., 1999; Mans et al., 2015).

Wildlife animals are important hosts for ticks, playing a critical role in their life-cycle as well as in the transmission of several tick-borne pathogens such as piroplasms (Medlock et al., 2013). Thus, a wide variety of free ranging hosts such as roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), Spanish ibex (*Capra pyrenaica*), alpine chamois (*Rupicapra rupicapra*) and wild boar (*Sus*

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scrofa) have been identified as susceptible to *Theileria* and/or *Babesia* infections in Europe (Ferrer et al., 1998; Hoby et al., 2007; Tampieri et al., 2008; Bastian et al., 2012). Since that wildlife rarely develop clinical disease, these wild animals act as asymptomatic carriers or reservoirs for these piroplasm species (Bastian et al., 2012; Zanet et al., 2014). Up-to-now, several *Babesia* and *Theileria* species and genotypes have been identified in wild ungulates, including *Babesia bigemina, Babesia capreoli, Babesia divergens, Babesia microti, Babesia odocoilei, Babesia ovis, Babesia sp. MO1, Babesia venatorum* (formerly *Babesia* sp. EU1), *Theileria* sp. 3185/02, *Theileria* sp. OT3 and *Theileria* sp. ZS TO4 (García-SanMartin et al., 2007; Hoby et al., 2007; Zintl et al., 2011; Fuehrer et al., 2013; Michel et al., 2014; Zanet et al., 2014; Ebani et al., 2016). It is worth noting that some of these piroplasms have zoonotic potential; thus, the common bovine piroplasm *B. divergens* is the most frequent agent of human babesiosis in Europe, and other species such as *B. venatorum* and *B. microti* have also been found infecting humans (Olmeda et al., 1997; Malandrin et al., 2010). In contrast, no zoonotic *Theileria* species have been currently identified (Yabsley and Shock, 2012).

Roe deer is one of the most abundant wild cervids in Spain, especially in northern closed forests and broad meadows. This ungulate may play an important role as a reservoir for several *Babesia* species with negative implications in animal and public health such as *B. capreoli,* which can cause fatal infections in Alpine chamois (Hoby et al., 2007, 2009). In addition, the roe deer-associated *B. venatorum* (Zintl et al., 2011) has been related to human babesiosis cases in Europe (Herwaldt et al., 2003). Despite of the increase of roe deer populations and their distribution over Europe in recent years (Fandos and Buron, 2013; Valente et al., 2014), epidemiological studies about tick-borne pathogens in this cervid host are still limited in Spain. For those reasons, the main objective of this study was to identify the *Babesia* and *Theileria* species in roe deer hunted in Spain using molecular techniques and to determine their prevalence; the possible influence of several intrinsic (age, sex) and extrinsic (ecological area of hunting) factors on the prevalence of the different piroplasm species was also assessed. These results will be useful to unravel the role of roe deer as a reservoir of piroplasm species on human and animal health in Spain.

2. Materials and methods

2.1. Sample collection and preservation

A total number of 174 roe deer hunted in 14 Spanish provinces during five years (2013–2017) were examined in this study. Samples were collected by hunters of the Spanish Roe Deer Association (Asociación del Corzo Español; ACE); scientific collaborators members of ACE supervised the collection of samples in order to ensure a correct sampling technique and avoid cross-contaminations.

Hunting locations were classified in four different ecological areas previously described (Morrondo et al., 2017): Oceanic ($n = 67$), Mountain ($n = 28$), Continental ($n = 34$) and Mediterranean ($n = 45$) (Fig. 1). In addition, most roe deer included in this study were male ($n = 123$) and only 51 were females since the hunting period is longer for males than for females. Age was estimated on the basis of teeth analysis (Høye, 2006); thus, animals were divided in 2 age-groups, younger than 2 years ($n = 40$) and adults older than 2 years ($n = 132$). Information from two animals was incomplete so they were not age-classified.

In order to detect *Babesia* and *Theileria* DNA, the whole spleen of each roe deer was collected during field evisceration, individually bagged up, identified and kept refrigerated. In the laboratory, the spleen was firstly rinsed in sterile PBS, and then dissected with a sterile scalpel; small pieces of spleen were taken and stored at −20 °C until DNA extraction.

2.2. DNA extraction and polimerase chain reaction

DNA was extracted from 200 μg of splenic tissue using a commercial kit (High Pure PCR Template Preparation Kit®, Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer' instructions for DNA extraction from tissue, and then stored at −20 °C until used.

In order to detect *Babesia* and *Theileria* DNA, a commercial real time PCR detecting both piroplasms (EXOone Piroplasm®, Exopol, Zaragoza, Spain) was performed following the manufacturer' instructions. All qPCR positive samples were then selected and molecularly characterized using a conventional PCR assay targeting the 18S rRNA gene of *Babesia* spp. and *Theileria* spp. using previously reported primers (Table 1) and protocols (Zahler et al., 2000; da Silveira et al., 2011). In order to confirm species identification, a subset of samples of each piroplasm species detected at the 18S rRNA gene was selected and tested individually using a second PCR assay targeting the internal transcribed spacer 1 (ITS1) of *Babesia* spp. and *Theileria* spp. as previously described (Blaschitz et al., 2008; Bajer et al., 2014). In order to detect *Babesia-Theileria* coinfections, all positive samples were further analysed using two 18S rRNA PCR specific for *Babesia* and *Theileria*, respectively (Birkenheuer et al., 2003; Heidarpour-Bami et al., 2009). In each amplification reaction, negative controls as well as positive controls obtained from blood of *Babesia*-positive dogs and spleen of *Theileria*-positive roe deer were included.

2.3. Sequence analysis

All products obtained using conventional PCR assays were purified and subsequently sequenced in both senses on an ABI 3730xl® (Applied Biosystems, Foster City, CA, USA) using a Big dye Terminator v3.1 cycle sequencing kit® (Applied Biosystems, Foster City, CA, USA) at the Sequencing and Fragment Analysis Unit of the Santiago de Compostela University (Spain). Sequences were aligned and edited using ChromasPro® (Technelysium, Brisbane, Australia), and consensus sequences were scanned against the GenBank database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Unique partial sequences identified in this study were deposited in GenBank under accession numbers MH522427-MH522439 and MK318268-MK318270.

2.4. Statistical analysis

All statistical analyses were performed using the statistical software R (R Core Team, 2018). The possible influence of intrinsic (age, sex) and extrinsic (ecological area) factors on the prevalence of infection by *Babesia* spp. and *Theileria* spp. was analysed using a logistic regression. Factors were eliminated from the initial model using a backward and forward conditional method based in AIC value (Akaike Information Criterion) until the best model was built. Next, all pairwise interactions were evaluated. Odds ratio were computed by raising "e" to the power of the logistic coefficient over the first category of each factor (reference category), not over the last. The logistic analyses and the AIC selection were performed with glm () and step () functions in the R statistical package (R Core Team, 2018).

3. Results

Using qPCR, 156 out of 174 (89.7%) spleen samples yielded *Babesia* and/or *Theileria* DNA. All qPCR positive samples also amplified at the 18S rRNA gene, but sequencing was only successful in 129 (82.7%) samples; twenty-seven samples (17.3%) had underlying signals in the electropherogram that prevented the accurate readout of sequences.

After sequence analysis, the total prevalence for *Theileria* spp. and *Babesia* spp. Were 60.9% and 19.0%, respectively. Mixed *Babesia/Theileria* infections were identified in 10 (5.7%) animals. When the possible effect of the sex of the animals on the prevalence was analysed

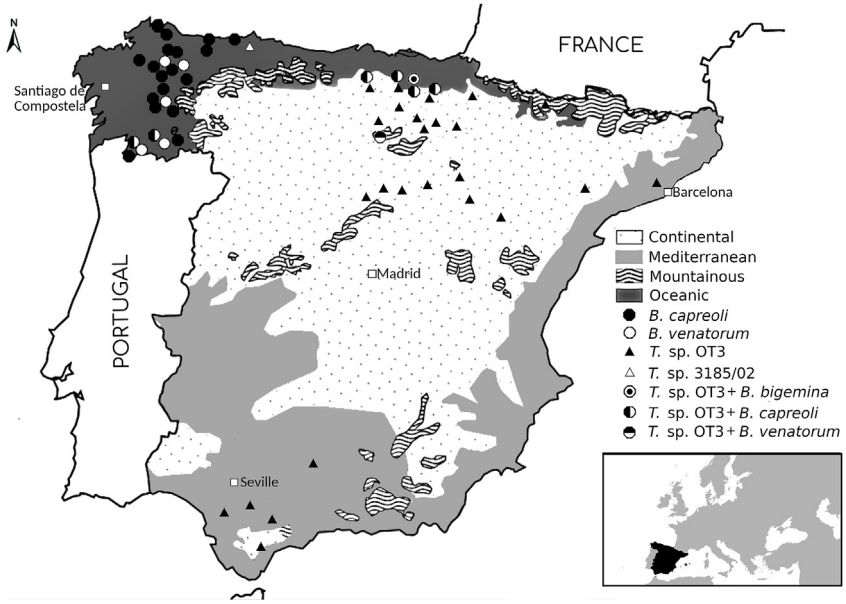

Fig. 1. Map of Spain (modified from Morrondo et al., 2017) showing the four ecological areas. Dots represent the presence of *Babesia* spp. and/or *Theileria* spp. in each region.

Table 1
Primers used in the four conventional PCR assays used for detecting *Babesia* and *Theileria* DNA.

Order	Primer	Nucleotide sequence (5′-3′)	Product size	Reference
First PCR analysis:	RIB-19	CGG GAT CCA ACC TGG TTG ATC CTG C	430 bp	(Zahler et al., 2000; da Silveira et al., 2011)
18S rRNA of *Babesia* and/or *Theileria* spp.	RIB-20	CCG AAT TCC TTG TTA CGA CTT CTC		
	BAB-rumF	ACC TCA CCA GGT CCA GAC AG		
	BAB-rumR	GTA CAA AGG GCA GGG ACG TA		
Second PCR analysis:	BAITS1-F	CGA GTG ATC CGG TGA ATT ATT C	600 bp	(Blaschitz et al., 2008; Bajer et al., 2014)
ITS1 of *Babesia* and/or *Theileria* spp.	BAITS1-R	CCT TCA TCG TTG TGT GAG CC		
Third PCR analysis: *18S rRNA* of *Babesia* spp.	5–22F	GTT GAT CCT GCC AGT AGT	293-338 bp	Birkenheuer et al. (2003)
	1661R	AAC CTT GTT ACG ACT TCT C		
	455–479F	GTC TTG TAA TTG GAA TGA TGG TGA C		
	793-772R	ATG CCC CCA ACC GTT CCT ATT A		
Fourth PCR analysis:	TheiF1	AAC CTG GTT GAT CCT GCC AG	≈1420 bp	Heidarpour-Bami et al. (2009)
18S rRNA of *Theileria* spp.	TheiR 1	AAA CCT TGT TAC GAC TTC TC		
	TheiF2	TGA TGT TCG TTT YTA CAT GG		
	TheiR 2	CTA GGC ATT CCT CGT TCA CG		

(Table 2), both sexes showed similar percentages of infection by *Babesia* spp. In contrast the prevalence of *Theileria* spp. was higher in males than in females. Nevertheless, those differences were not significant in any case (Table 3). In addition, *Babesia* prevalence was significantly higher in young than in adult animals in contrast to that observed for *Theileria* spp. (Table 2); nevertheless, logistic regression results showed that differences in *Theileria* prevalence were not significant (Table 3). The percentage of *Babesia*-infected roe deer was significantly higher in the Oceanic area than in Continental and Mountain areas (Tables 2 and 3). In contrast, the prevalence of *Theileria* spp. was significantly lowest in animals from the Oceanic area when compared to those from the rest of areas (Tables 2 and 3).

Five piroplasms species/genotypes were identified: *Theileria* sp. OT3 was the most frequently identified piroplasm (105/174; 60.3%), followed by *Babesia capreoli* (27/174; 15.5%) and *Babesia venatorum* (5/174; 2.9%); *Theileria* sp. 3185/02 (0.6%) and *Babesia bigemina* (0.6%) were only found in a single animal each. The most common coinfection was *B. capreoli/T.* sp. OT3 (4.6%) followed by *B. venatorum/T.* sp. OT3 (0.6%) and *B. bigemina/T. sp.* OT3 (0.6%).

Most *T.* sp. OT3 sequences at the 18s rRNA were identical or showed a single nucleotide polymorphism (SNP) when compared to the *T.* sp. OT3 reference isolate KF470868 (Supplementary data 1); more than seven nucleotide differences were found with other *Theileria* species such as *T. capreoli* or *Theileria luwenshuni*. At the ITS1, in contrast, our *T.* sp. OT3 sequences only showed a 97% (459/475 base pairs) similarity with the deposited sequences of *T.* sp. OT3 obtained from sheep in China (KF470865-KF470867); it is noteworthy that most differences were due to deletions in poly-adenine repeat regions. Most *B. venatorum* isolates showed one to three SNP's when compared to KM289158 at the 18S rRNA gene, whereas a 100% identity with the reference sequence HM113372 was observed at the ITS1. *Babesia capreoli* sequences at the 18S rRNA gene were identical or showed a single SNP when compared to KX839234; they were also showed a 99% homology with *B. divergens* sequences (KP742785). Although no sequences of *B. capreoli* at the ITS1 gene are currently deposited in GenBank, our *B. capreoli* ITS1 isolates showed a 94% similarity (512/543 bp) with the ITS1 gene of *B. divergens* (LK935835) and overlapped a 23% of a 18S rRNA sequence corresponding to *B. capreoli* (KP742785), showing a 99% identity (127/128).

The *T.* sp. 3185/02 sequence showed a single SNP when compared to the reference sequence DQ866842, and more than 5 nucleotide discrepancies when compared to other *Theileria* species such as *Theileria ovis*, *Theileria parva* or *Theileria annulata*. As for *B. capreoli*, no ITS1 sequences of *T.* sp. 3185/02 are currently deposited in GenBank, and the most similar sequence was a fragment of the ITS1 gene of *T.* sp. OT3 (KF470867) showing an homology of 76% (340/445 bp). Finally, a

Table 2
Prevalence of piroplasm species in roe deer from Spain when considering different intrinsic and extrinsic factors.

	Hunting location				Sex		Age	
	Oceanic (n = 67) (95% CI)	Mountain (n = 28) (95% CI)	Continental (n = 34) (95% CI)	Mediterranean (n = 45) (95% CI)	Female (n = 51) (95% CI)	Male (n = 123) (95% CI)	Young (n = 40) (95% CI)	Adult (n = 132) (95% CI)
B. bigemina	1.49% (0.08–9.14)	0 (0.00–15.02)	0 (0.00–12.64)	0 (0.00–9.80)	0 (0.00–8.73)	0.81% (0.43–5.11)	ND	ND
B. venatorum	5.97% (1.93–15.35)	3.57% (0.19–20.24)	0 (0.00–12.64)	0 (0.00–9.80)	5.88% (1.53–17.23)	1.63% (0.28–6.34)	10.00% (3.25–24.60)	0.76% (0.04–4.77)
B. capreoli	35.82% (24.74–48.53)	0 (0.00–15.02)	8.82% (2.31–24.81)	0 (0.00–9.80)	13.73% (6.15–26.87)	16.26% (10.45–24.24)	27.50% (15.14–44.14)	12.12% (7.30–19.22)
Total Babesia	43.28% (31.42–55.92)	3.57% (0.19–20.24)	8.82% (2.31–24.81)	0 (0.00–9.80)	19.61% (10.29–33.55)	18.70% (12.46–26.95)	37.50% (23.17–54.19)	12.88% (7.90–20.09)
T. sp. 3185/02	1.49% (0.08–9.14)	0 (0.00–15.02)	0 (0.00–12.64)	0 (0.00–9.80)	0 (0.00–8.73)	0.81% (0.43–5.11)	0 (0.00–10.91)	0.76% (0.04–4.77)
T. sp. OT3	25.37% (15.88–37.73)	75.00% (54.78–88.87)	85.29% (68.17–94.46)	84.44% (69.94–93.01)	47.06% (33.16–61.40)	65.84% (56.69–74.02)	50.00% (35.20–64.80)	63.64% (54.77–71.70)
Total Theileria	26.87% (17.10–39.31)	75.00% (54.78–88.87)	85.29% (68.17–94.46)	84.44% (69.94–93.01)	47.06% (33.16–61.40)	66.67% (57.52–74.75)	50.00% (35.20–64.80)	64.39% (55.54–72.40)
Not identified	19.40% (11.13–31.25)	25.00% (11.43–45.22)	5.88% (1.03–21.06)	11.11% (4.16–24.85)	25.49% (14.77–39.91)	11.38% (6.59–18.68)	12.5% (4.70–27.60)	16.67% (10.96–24.37)

ND: No age information was collected from this animal.

single isolate showed a 99% similarity with the *B. bigemina* reference sequence (KU206297) since a single SNP was detected; more than four discrepancies were detected when compared to other *Babesia* species such as *Babesia ovata* (LC125457) and *Babesia* sp. Sichuan (AY603403). Although amplification at the ITS1 was observed, the band was faint and could not be sequenced.

4. Discussion

Our results reveal that piroplasms are prevalent parasites in roe deer from Spain since more than 80% of these wild ungulates tested positive. These results are far higher than those previously reported in roe deer from northern areas of Spain (62.3%) (García-SanMartín et al., 2007) or from other European countries such as Italy or Germany (Tampieri et al., 2008; Bastian et al., 2012; Fuehrer et al., 2013; Kauffmann et al., 2017) where prevalence rates ranged from 12.6% to 62.7%.

Multivariate analysis showed considerable differences in *Babesia* and *Theileria* prevalences when considering the ecological areas. In this way, most of the identified *Babesia* species were found in roe deer from oceanic areas located in the northwest of the country, whereas *Theileria* species were significantly more prevalent in the rest of Spain. These results are consistent with a previous investigation performed in roe deer from north and northeast regions of Spain (García-SanMartín et al., 2007) where 53.62% of samples were positive to *Theileria* spp. and only 8.70% to *Babesia* spp. which is probably related to the presence of suitable tick vectors as well as reservoirs and amplification hosts (Estrada-Peña and de la Fuente, 2014). In this respect, *I. ricinus*, the main vector of *B. venatorum* and a competent vector for *B. capreoli* (Malandrin et al., 2010), is the most common tick in northern Spain, especially in the northwest region, where its presence is favoured by high humidity levels (Barandika et al., 2011; Espí et al., 2017; Remesar et al., 2019). Since *I. ricinus* is also the most common tick in northern and central Europe, our results agree with other studies carried out in roe deer from other European countries showing higher prevalences of *Babesia* spp. than those of *Theileria* spp. (Duh et al., 2005; Tampieri et al., 2008; Michel et al., 2014). In contrast, transmission of *Theileria* spp. is mainly related to ticks species belonging to *Rhipicephalus*, *Dermacentor*, *Hyalomma* and *Haemaphysalis* genera (Viseras et al., 1999; Mans et al., 2015); those ixodids are more prevalent than *I. ricinus* in central and southern Spain (Márquez, 2008; Fernández de Mera et al., 2013; Requena-García et al., 2017), coinciding with the predominance of *Theileria* spp. in roe deer from those areas.

With regard to host-related variables, the age of roe deer only had a significant impact on the prevalence of *Babesia.* Thus, a significant inverse relation between *Babesia* prevalence and the age of animals was found in this study. Since some *Babesia* species, such as *Babesia capreoli*, have been reported as fatal to roe deer (Hinaidy, 1987), our results might be a consequence of pathogenic *Babesia* infections negatively influencing the survival of young roe deer and thus avoiding reaching adulthood. Nevertheless, several investigations report that *Babesia* spp. are low virulent for roe deer, showing little clinical signs of disease (Hinaidy, 1987; García-SanMartín et al., 2007; Bastian et al., 2012; Zanet et al., 2014). These discrepant opinions indicate that this issue should be further investigated, analysing more samples, especially from young roe deer, to obtain more robust conclusions. In addition, irregular sample distribution should be considered, since most young roe deer originated from Oceanic areas.

After sequence analysis, five species/genotypes were identified. *Theileria* sp. OT3, *T.* sp. 3185/02 and *B. capreoli* had been previously reported in Spain (García-SanMartín et al., 2007). However, this is the first report of *B. venatorum* and *B. bigemina* in roe deer from this country. All these species/genotypes are widely distributed in roe deer from Europe, since *B. venatorum* and *B. capreoli* were detected in Germany (Kauffmann et al., 2017), Italy (Tampieri et al., 2008; Zanet et al., 2014) and France (Bastian et al., 2012), *B. bigemina* and *T.* sp. OT3 in Italy (Zanet et al., 2014) and *T.* sp. 3185/02 was also identified in

Table 3
Logistic regression model for the prevalence of *Babesia* spp. and *Theileria* spp. Factors were removed following the Akaike information criterion value until the best model was built.

	Estimate	Z-value	Pr (> |t |)	OR	CI 95%
***Babesia* spp.**					
(Intercept)	−0.7019	−2.298	0.022	0.50	0.27–0.89
Adults	–	–	–	–	–
Young	1.2787	2.630	0.009	3.59	1.40–9.56
Oceanic area	–	–	–	–	–
Continental area	−2.0212	−3.026	0.002	0.13	0.03–0.43
Mountain area	−3.1320	−2.944	0.003	0.04	2.34e-03 - 0.23
Mediterranean area	−19.0800	−0.012	0.990	5.17e-09	6.63e-211- 1.44e25
***Theileria* spp.**					
(Intercept)	−1.1285	−3.253	0.001	0.32	0.16–0.62
Oceanic area	–	–	–	–	–
Mountain area	3.3257	5.271	1.36e-07	27.81	8.87–109.82
Continental area	3.2079	4.558	5.16e-06	24.73	7.03–119.30
Mediterranean area	1.9758	3.300	0.001	7.21	2.33–24.93
Oceanic:Adult	–	–	–	–	–
Mediterranean:Young	−2.1972	−1.944	0.052	0.11	0.01–1.12
Continental:Young	−1.1632	−1.122	0.262	0.31	0.04–2.83
Mountain:Young	1.0986	0.935	0.350	3.00	0.40–62.51
Oceanic:Young	0.2122	0.357	0.721	1.24	0.37–3.91

Hungary (Hornok et al., 2017).

In this study, the most prevalent piroplasm was *T.* sp. OT3, detected in more than a half of the analysed samples, especially in roe deer from central and southern areas of Spain. This species was also found in other wildlife species such as red deer, fallow deer and chamois from Spain and other European countries (García-SanMartín et al., 2007; Pereira et al., 2016). In addition, several investigations have identified this species in sheep from Italy (Giangaspero et al., 2015), Turkey (Bilgic et al., 2017) and China (Tian et al., 2014); in fact, *T.* sp. OT3 was the second more prevalent *Theileria* species/genotype in sheep from northern Spain (42.2%) following *T.* sp. OT1 (48.1%) (Nagore et al., 2004). For those reasons, the possible role of cervids as asymptomatic reservoirs of this species has been suggested (García-SanMartín et al., 2007), although its pathogenicity for livestock has not been completely demonstrated yet (Uilenberg, 2006).

Babesia capreoli was the second most prevalent piroplasm in our study. It is morphological and serologically related to *B. divergens* but showing different host specificity (García-SanMartín et al., 2007); thus, *B. capreoli* has been found in roe deer, sika deer, fallow deer and red deer, although it has been suggested that the former could represent the natural reservoir for this species (Kauffmann et al., 2017). A previous study carried out in roe deer from Spain showed a lower prevalence of *B. capreoli* (8.7%) than that found in our study (García-SanMartín et al., 2007). Nevertheless, it was the main piroplasm species found in chamois, red deer and roe deer from Italy (Tampieri et al., 2008; Zanet et al., 2014), roe deer from Sweden (Andersson et al., 2016) and mouflon and roe deer from Germany (Kauffmann et al., 2017). It seems that this species is unable to infect cattle or sheep erythrocytes (Malandrin et al., 2010), and no human infections were currently described (Andersson et al., 2016).

The percentage of infection by *B. venatorum* was low in roe deer from Spain, being consistent with other investigations on roe deer from Italy, Germany and Slovenia (Duh et al., 2005; Tampieri et al., 2008; Zanet et al., 2014; Kauffmann et al., 2017); it was also found in a low percentage of mouflons from Germany (Kauffmann et al., 2017). Since *B. venatorum* has been implicated in human babesiosis cases in Europe (Herwaldt et al., 2003), its presence in roe deer from Spain may involve zoonotic implications, especially considering that the population and distribution of this wild ungulate has been recently increased in Spain (Fandos and Buron, 2013; Valente et al., 2014); in addition, roe deer has been identified as a reproduction host for tick populations (Kauffmann et al., 2017) which could be potentially infected with this *Babesia* species.

Theileria sp. 3185/02 and *Babesia bigemina* were only occasionally identified. The former has been previously identified in roe deer and red deer from northern Spain with a prevalence of 10.1% and 53.6% respectively (García-SanMartín et al., 2007), which is higher than that found in our study. *Babesia bigemina* is one of the causative agents of bovine babesiosis; some wildlife species may act as reservoirs since it has been previously identified in white-tailed deer in USA and Brazil (da Silveira et al., 2011; Holman et al., 2011).

It should be pointed out that all the piroplasm species detected in this study are not specific for roe deer; thus, it has been demonstrated that roe deer share *T.* sp. OT3, *T.* sp. 3185/02 and *B. capreoli* with red deer; *Babesia capreoli, B. ovis* and *T.* sp. OT3 with chamois; *T.* sp. OT3 with fallow deer and *B. capreoli* and *B. venatorum* with mouflons (García-SanMartín et al., 2007; Tampieri et al., 2008; Zanet et al., 2014; Kauffmann et al., 2017). Consequently, roe deer may play an important role as reservoir of pathogenic *Babesia* and *Theileria* species for wild and domestic animals and even humans.

5. Conclusions

Theileria spp. and *Babesia* spp. are prevalent piroplasms in roe deer from Spain, showing higher percentages of infection than those previously reported in other areas of Europe. Five different species were found, including the first citation of *B. venatorum* and *B. bigemina* in roe deer from Spain. In this regard, these results suggest that roe deer may act as a reservoir of pathogenic *Babesia* species for human and animals, playing a role in the epidemiology of the disease and thus involving public and animal health risk.

Authors' contributions

PDB and PM established the final methods and design. GF, CML and RP assisted with preliminary design of the study. FM managed the collection of samples. SR and GLL extracted the DNA from spleen samples. SR, PDF and AP developed and performed the PCRs. CML and JMD conducted the statistical analysis. SR, PD and RP prepared the first paper draft. All authors read and approved the final manuscript.

Conflicts of interest

All authors declare the absence of any financial or personal interests that could inappropriately influence the current work. The final article has been approved by all authors.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.05.005.

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