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

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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 scrofa*).

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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 odocoiilei, Babesia ovis, Babesia sp. M01, 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 incofections, 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. 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 (Morondo 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 collected was fragmented 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 polymerase 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’s 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’s 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).

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® (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...
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: 18S rRNA of Babesia and/or Theileria spp. | RIB-19 | OGG GAT CCA ACC TGG TTG ATC CTG C | 430 bp | (Zahler et al., 2006; da Silva e et al., 2011) |
| | RIB-20 | OGG AAT TCC TGG TTA CGA CTT GTC | | |
| | BAB-rumF | ACC TCA CGA GGT CCA GAC AG | | |
| | BAB-rumR | GTA CAA AGG GCA GGG ACG TA | | |
| Second PCR analysis: ITS1 of Babesia and/or Theileria spp. | BAITS1-F | CGA GTG ATC CGG TGA ATT ATT C | 600 bp | (Bilschitz et al., 2008; Rajer et al., 2014) |
| | BAITS1-R | CTC TCA TGG TGT TGG GAC GC | | |
| 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 TCA CCA GGT CCA GAC AG | | |
| | 455-479F | GTC TGG TAA TTG GAA TGA TGG TGA C | | |
| | 793-772R | ATG CCC CCA ACC GTT CCT ATT A | | |
| Fourth PCR analysis: 18S rRNA of Theileria spp. | TheiF1 | AAC CTG GTT GAT CCT GCC AG | 1420 bp | Heidarpour-Bami et al. (2009) |
| | TheiR1 | AAA CCT TGT TAC GAC TGC TGC | | |
| | TheiF2 | TGA TGT TCG TTT YTA CAT GG | | |
| | TheiR2 | CTA GGC ATT CCT GGT 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%).

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 laevis. 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

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

| Hunting location | Sex | Age | Adult (n = 123) | Young (n = 40) | Mediterranean (n = 45) | Oceanic (n = 67) |
|------------------|-----|-----|----------------|---------------|----------------------|-----------------|
|                  |     |     | (95% CI)       | (95% CI)      | (95% CI)             | (95% CI)        |
|                  |     |     |                |               |                      |                 |
|                 |     |     | 0.81% (0.42–1.49)| 0 (0.00–0.77) | 13.73% (6.15–24.85) | 19.61% (10.29–33.55) |
|                 |     |     | 5.97% (1.93–15.35) | 3.57% (0.19–8.73) | 10.91% (6.15–15.02) | 16.67% (10.96–24.37) |
|                 |     |     | 1.49% (0.08–9.14) | 0 (0.00–9.80)  | 1.63% (0.28–4.77)  | 47.06% (33.16–61.40) |
|                 |     |     | 43.32% (21.43–74.83) | 75.00% (54.78–94.46) | 47.06% (33.16–61.40) | 66.67% (57.55–75.75) |
|                 |     |     | 6.32% (0.74–35.15) | 23.85% (14.77–39.91) | 47.06% (33.16–61.40) | 65.84% (56.69–71.70) |
|                 |     |     | 19.40% (11.13–31.25) | 25.49% (14.77–45.22) | 25.49% (14.77–45.22) | 11.11% (4.64–23.85) |
|                 |     |     | 42.74% (24.43–68.11) | 75.00% (54.78–94.46) | 42.74% (24.43–68.11) | 84.44% (69.94–93.01) |
|                 |     |     | 5.79% (0.71–15.43) | 4.42% (0.50–7.16)  | 19.40% (11.13–31.25) | 16.67% (10.96–24.37) |
|                 |     |     | 2.82% (0.53–12.39) | 8.70% (1.45–24.24) | 2.82% (0.53–12.39) | 7.69% (1.45–24.24)  |
|                 |     |     | 6.32% (0.74–35.15) | 23.85% (14.77–39.91) | 6.32% (0.74–35.15) | 16.67% (10.96–24.37) |
|                 |     |     | 19.40% (11.13–31.25) | 25.49% (14.77–45.22) | 19.40% (11.13–31.25) | 11.11% (4.64–23.85) |
|                 |     |     | 42.74% (24.43–68.11) | 75.00% (54.78–94.46) | 42.74% (24.43–68.11) | 84.44% (69.94–93.01) |
|                 |     |     | 5.79% (0.71–15.43) | 4.42% (0.50–7.16)  | 19.40% (11.13–31.25) | 16.67% (10.96–24.37) |
|                 |     |     | 2.82% (0.53–12.39) | 8.70% (1.45–24.24) | 2.82% (0.53–12.39) | 7.69% (1.45–24.24)  |

ND: No age information was collected from this animal.

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

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; Kauffman 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; Espi 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 (Hainady, 1987), our results might be a consequence of host-pathogen Babesia interactions 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 (Hainady, 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 a single isolate showed a 99% similarity with the B. bigemina reference sequence (KU206297) since a single SNP was detected; moreover, 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.
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 |

In this study, the most prevalent piroplasm was *B. 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, *B. sp. OT3* was the second more prevalent *Theileria* species/genotype in sheep from Italy (Tampieri et al., 2008; Zanet et al., 2014) and no human infections were currently described (Andersson et al., 2016).

*Babesia capreoli* was the second most prevalent piroplasm in our study. It is morphologically 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), which is higher than that found in our study. *Babesia capreoli* 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., 2013; 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 FM 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 final 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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Hungary (Hornok et al., 2017).

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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 morphologically 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 (Hervaldt 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.
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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.jipppa.2019.05.005.

References

Andersson, M.O., Bergvall, U.A., Chirico, J., Christensen, M., Lindgren, P.E., Nordstrom, J., Kjellander, P., 2016. Molecular detection of Babesia capreoli and Babesia venatorum in wild swedish red deer, Capreolus capreolus. Parasites. Vector 9, 221.

Bajer, A., Alsarraf, M., Bednarska, M., Mohallal, E.M., Mierzejewska, E.J., Behnke, Borowczyk, J., Zalat, S., Gilbert, F., Welc-Faletar, R., 2014. Babesia balcanica sp. nov., a novel Babesia species infecting isolated populations of Wagner’s goril, Papio ursinus, from the Sinai Mountains, Egypt. Parasit. Vectors 7, 527.

Bastian, S., Jouglin, M., Brisseau, N., Malandrin, L., Klegou, G., L’Hostis, M., Chauvin, A., Anda, P., García-Pérez, A.L., 2011. Diadasyurus species infecting isolated populations of Wagner’s gerbil, Rhombomys opimus, from the Sinai Mountains, Egypt. Parasit. Vectors 4, 572.

Battianin, J., 2018. Blood parasites of wildling ruminants in Austria. J. Vet. Med. B 63, 81–87.

Hobș, S., Mathis, A., Dober, M.G., Robert, N., Ryser-Degiojgis, M.P., 2009. Babesia capreoli infections in Alpian Chamois (Rupicapra rupicapra), roe deer (Capreolus capreolus) and red deer (Cervus elaphus) from Switzerland. J. Wildl. Dis. 45, 748–753.

Hobș, S., Robert, N., Mathis, A., Schmid, N., Meli, M.L., Hofmann-Lehmann, R., Lutz, H., Deplazes, P., Ryser-Degiojgis, M.P., 2007. Babesiosis in free-ranging chamois (Rupicapra rupicapra) from Switzerland. Vet. Parasitol. 148, 341–345.

Holman, P.J., Carroll, J.E., Pugh, R., Davis, D.S., 2011. Molecular detection of Babesia bovis and Babesia bigemina in white-tailed deer (Odocoileus virginianus) from Tom county in central Texas. Vet. Parasitol. 177, 298–304.

Hornok, S., Sugár, L., Horváth, G., Kovács, T., Ticsutko, A., Gánczi, E., Plaiz, B., Takács, N., Farkas, R., Meli, M.L., Hofmann-Lehmann, R., 2017. Evidence for host specificity of Theileria capreoli genotypes in cervids. Parasites Vectors 10, 473.

Hoyo, T.J., 2006. Age determination in roe deer: a new approach to tooth wear evaluation on known age in individuals. Acta Theriol. 51, 205–211.

Malandrin, L., Jouglin, M., Sun, Y., Brisseau, N., Chauvin, A., 2010. Redescription of Babesia capreoli (Enzig and Friedhoff, 1962) from roe deer (Capreolus capreolus): isolation, cultivation, host specificity, molecular characterisation and differentiation from Babesia divergens. Int. J. Parasitol. 40, 277–284.

Mans, B.J., Pienaar, R., Latif, A.A., 2015. A review of Theileria diagnostics and epidemiology. Int. J. Parasitol. Parasites. Wildl. 4, 104–118.

Márquez, F.J., 2007. Babesia divergens infecting isolated populations of Wagner’s gerbil, Rhombomys opimus, from the Sinai Mountains, Egypt. Parasit. Vectors 4, 664.

Meidlock, J.M., Hansford, K.M., Bornemann, A., Greter, M., Gurevich, A., George, J.C., Golovljova, I., Jaenson, T.G.T., Jensen, J.K., Jensen, P.M., Kazimirova, M., Oteo, J.A., Papa, A., Þliffr, K., Plantard, O., Randolph, S.E., Rizzoli, A., Santos-Silva, M.M., Sprong, H., van Iel, H., Hendriksen, O.G., Van Bortel, W., 2013. Driving forces for changes in geographical distribution of ixodes ricinus ticks in Europe. Parasites Vectors 6.

Michel, A.O., Mathis, A., Ryser-Degiojgis, M.P., 2014. Babesia spp. in European wild ruminants: parasite diversity and risk factors for infection. Vet. Res. 45, 1285–1294.

Morrondo, P., Pérez-Creo, A., Prieto, A., Cabanelas, E., Díaz-Cao, J.M., Arias, M.S., Díaz, P., Pajares, G., Remesar, S., López-Sánchez, C.M., Fernández, G., di-Baños, P., Panadero, R., 2017. Prevalence of tick-borne and parasitic agents in roe deer from Spain and their possible role as reservoirs. Ital. J. Anim. Sci. 16, 266–274.

Nágo, D., García-Saín-Martín, J., García-Pérez, A.L., Juste, R.A., Hurtado, A., 2004. Changes in genetic diversity and prevalence of Theileria and Babesia species in a sheep population from Northern Spain. Int. J. Parasitol. 34, 1059–1067.

Olmedo, A.S., Armstrong, P.M., Rosenblum, R.M., Valladares, B., del Castillo, A., deArmas, F., Miguez, M., González, A., Rodríguez, J.A., Spielman, A., Telford, S.R., 1997. Babesiosis in wild boars (Sus scrofa): a retrospective study of clinical cases from the Central-Spain. Acta Zootrop., 76, 229–234.

Perete, A., Paredes, R., Nunes, M., Cassadino, A., Vieira, M.L., Campino, L., Maia, C., 2016. Molecular detection of tick-borne bacteria and protozoa in cervids and wild boars from Portugal. Parasites Vectors 9.

Pérez-Creo, A., Kuczorubil, Y., Roiz, O., Gori, L., 2012. Seasonality of ixodes ricinus ticks on vegetation and on rodents and Borrelia burgdorferi sensu latu genospecies diversity in two Lyme Borreliosis–Endemic areas in Switzerland. Vector Borne Zoonotic Dis. 12, 613–644.

R Development Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria-3900517-07-0. http://www.R-project.org.

Remesar, S., Díaz, P., Ponzon, J.M., Pérez-Creo, A., Prieto, A., Estrada-Peña, A., López, C.M., Panadero, R., Fernández, G., di-Baños, P., Mendelev, P., 2013. Babesia and tick species diversity and population dynamics of Ixodes ricinus in Galicia (north-western Spain).Ticks. Tick. Borne. Dis. 10, 132–137.

Requena-García, F., Cabrero-Sanudo, F., Garzón, J., Valcárcel, F., 1997. A molecular approach to determine age in individual Cervus elaphus. Comp. Immunol. Microbiol. Infect. Dis. 20, 121–125.

Rizzoli, A., Silaghi, C., Obiagela, A., Rudolf, I., Hub Śle, Z., Foldvári, G., et al., 2014. Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: A review. Parasit. Vectors 7, 247–276.

Rundqvist, M., Norrby, T., Johnson, M., Blom, P., Sjöberg, B., 2009. The role of the tick species Ixodes ricinus and Borrelia burgdorferi sensu latu in Lyme Borreliosis in Europe. J. Med. Microbiol. 58, 1165–1171.

Tampieri, M.P., Galuppi, R., Bonoli, C., Cancrini, G., Moretti, A., Pietrobelli, M., 2008. Molecular detection of Babesia divergens and Babesia bigemina in white-tailed deer (Odocoileus virginianus) from Tom county in central Texas. Vet. Parasitol. 151, 205–211.

Theileria sp OT3 and other tick-borne pathogens in sheep and ticks in Italy: molecular characterization and phylogeny. Ticks. Tick. Borne. Dis. 6, 75–83.

Veit, A., Mathis, A., Boër, M., Robert, N., Ryser-Degiojgis, M.P., 2009. Molecular identification of ovine Theileria species by a new PCR–FLIP method. Vet. Parasitol. 12, 171–177.

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We would like to thank “O Veral” Recovery Centre of Wild Animals staff and ACE hunters for their inestimable collaboration in collecting samples.
transmission of *Theileria annulata* to cattle by the tick *Hyalomma lusitanicum*. J. Vet. Med. B 46, 505–509.

Yabsley, M.J., Shock, B.C., 2012. Natural history of zoonotic Babesia: role of wildlife reservoirs. Int. J. Parasitol. Parasites. Wildl. 22, 18–31.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Vet. Parasitol. 89, 241–248.

Zanet, S., Trisciuoglio, A., Bottero, E., de Meza, I.G.F., Gortázar, C., Carpignano, M.G., Ferroglio, E., 2014. Piroplasmosis in wildlife: *Babesia* and *Theileria* affecting free-ranging ungulates and carnivores in the Italian Alps. Parasit. Vectors 7, 70.

Zintl, A., Finnerty, E.J., Murphy, T.M., de Waal, T., Gray, J.S., 2011. *Babesia* of red deer (*Cervus elaphus*) in Ireland. Vet. Res. 42.