Molecular detection and characterization of piroplasms infecting cervids and chamois in Northern Spain

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SUMMARY

Wildlife can act as reservoir of different tick-borne pathogens of veterinary and zoonotic importance. To investigate the role of wild ruminants as reservoir of piroplasm infection, 28 red deer, 69 roe deer and 38 chamois from Northern Spain were examined by reverse line blot (RLB) hybridization. The survey detected a prevalence of 85·7% in red deer, 62·3% in roe deer and 28·9% in chamois. Four different piroplasms were identified: *Theileria* sp. OT3 (previously described in sheep) as the most prevalent (85·7% in red deer, 46·4% in roe deer and 26·3% in chamois); *Theileria* sp. 3185/02 (previously described in a red deer in Central Spain) more abundant in red deer (53·6%) than in roe deer (10·1%) but absent from chamois; *Babesia divergens* detected in 6 roe deer; *Theileria ovis* present in 1 chamois. Mixed infections (*Theileria* sp. OT3 and *Theileria* sp. 3185/02) were only found in red and roe deer. Sequencing analysis of the 18S rRNA gene confirmed the RLB results and showed 99·7% identity between *Theileria* sp. 3185/02 and *T. capreoli*, suggesting that they are the same species. Tick distribution and contact of wild ruminants with domestic animals are discussed in terms of piroplasm infection. The results suggest that a considerable number of wildlife ruminants are asymptomatic carriers that may serve as reservoirs of the infection posing a serious concern in terms of piroplasmosis control.

Key words: *Theileria* spp., *Babesia divergens*, piroplasmosis, PCR, reverse line blot hybridization, tick-borne diseases, wildlife, deer, chamois.

INTRODUCTION

Wild ruminants can harbour a high density of ticks that can transmit several organisms, many of which cause diseases of veterinary and/or public health importance. Tick-borne diseases affecting wild ruminants have been reported around the world. One of these diseases is piroplasmosis, caused by *Theileria* and *Babesia* species. Although some of them are benign or low pathogenic species, others can cause diverse symptomatology and even death. Infection with *Theileria* has been reported in several deer species. *Theileria cervi* has been found in white-tailed deer and elk (Chae *et al*. 1999; Yabsley *et al*. 2005). *Theileria capreoli* was described in roe deer by Rukhlyadev in 1939 and more recently a sequence of the 18S rRNA gene amplified from roe deer blood has been deposited in GenBank, although no thorough characterization is yet available. Another unclassified *Theileria* sp. has been reported in sika deer (Inokuma *et al*. 2004). In Europe, *Babesia divergens* and *Babesia capreoli* are implicated in cervid babesiosis. *B. divergens*, a species widely studied in cattle and the most common agent of human babesiosis in Europe (Kjemtrup and Conrad, 2000; Zintl *et al*. 2003), has been described in several deer species such as reindeer, roe deer and red deer (Langton *et al*. 2003; Duh *et al*. 2005*b*). *B. capreoli*, phylogenetically very similar to *B. divergens*, has also been observed in red deer and sika deer (Gray *et al*. 1991). On the other hand, *Babesia odocolei* has been reported as the agent responsible for babesiosis in deer species from America such as white-tailed deer, reindeer and elk (Holman *et al*. 2000, 2003). Also in America, an unclassified babesia has been reported in reindeer (Holman *et al*. 2002) and mule deer (Yabsley *et al*. 2005). A new *Babesia*, identified as *Babesia* sp. EU1 and more closely related to *B. odocoilei* than to *B. divergens* (Herwaldt *et al*. 2003; Duh *et al*. 2005*a*), was recently described in cervids from Slovenia (Duh *et al*. 2005*a*, *b*) and it has been implicated in cases of human babesiosis in Europe (Herwaldt *et al*. 2003). Although in the USA human babesiosis is mainly caused by *Babesia microti*, a babesia closely related to those isolated from wild ungulates (the WA1-type, recently described as a distinct species,

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Babesia duncani) has also occasionally been reported in humans (Persing *et al*. 1995; Kjemtrup and Conrad, 2000; Conrad *et al*. 2006). Similarly, isolates infecting cottontail rabbits in the USA have been shown to be identical to *B. divergens* (Goethert and Telford, 2003). In this context, the study of wildlife species as reservoirs of different pathogens is of major importance for the development of control strategies. However, little is known about tick-borne diseases in wild ruminants in Spain. Studies on piroplasmosis in wildlife ungulates from Spain are restricted to a few seroprevalence studies or case reports (Ferrer *et al*. 1998*a*, *b*; Marco *et al*. 2000; Hofle *et al*. 2004; Hurtado *et al*. 2004). In this study, red deer, roe deer and chamois were examined for molecular evidence of infection with babesias and theilerias with the aim to (i) increase knowledge of the epidemiology and diversity of piroplasms parasitizing these wildlife species; (ii) compare the piroplasm species infecting wild and domestic ruminants, and (iii) investigate the potential role of wildlife as reservoir of zoonotic pathogens.

MATERIALS AND METHODS

Sample collection

This study was conducted in the Basque Country and in Catalonia, 2 regions in Northern Spain, where animals live in open areas. Twenty-eight (9 male and 19 female) red deer (*Cervus elaphus*) and 69 (47 male and 22 female) roe deer (*Capreolus capreolus*) originating from the Basque Country were collected between 2001 and 2005. All red deer samples were collected during the hunting season (November–February) whereas roe deer were obtained throughout the year after being hunted (February–June, 22 animals), found with traumatisms (44), or dead by other causes (cachexia, stress and pneumonia). In roe deer ages ranged from 2 months to more than 10 years and in red deer all the animals except 2 were adults. Animals were classified into 3 age categories: fawns (<1 year), yearlings (red deer 1–3 years and roe deer 1–2 years), and adults (red deer >3 years and roe deer >2 years). A sample of 38 (20 male and 18 female) Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) from Catalonia was also analysed. Chamois samples were obtained after being hunted (16) or captured alive in healthy conditions (5) or diseased: pestivirus infection (14), contagious ecthyma (1), pneumonia (1) and toxoplasmosis (1). Information regarding pestivirus infection in some of these animals has been reported elsewhere (Hurtado *et al*. 2004). Ages ranged from 2 months to 11 years, and they were classified into 3 age groups according to the horn rings: kids (<1 year), yearlings (1–3 years), and adults (>3 years).

Blood samples were collected in EDTA containing tubes and both blood and tissue samples were stored at −20 °C until subsequent DNA purification and hybridization analysis. Whenever possible ticks were collected, counted and classified (Gil-Collado *et al*, 1979; Manilla, 1998).

DNA extraction

DNA was extracted from 22 blood samples (9 red deer and 13 chamois), 112 spleen samples (19 red deer, 69 roe deer and 24 chamois) and 1 kidney (1 chamois). DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) for DNA purification from whole blood and the standard phenol/chloroform extraction protocol was used for tissues. DNA yields were subsequently determined with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA).

PCR amplification and RLB hybridization

PCR amplification of the hypervariable V4 region of the 18S rRNA gene of the genera *Theileria* and *Babesia*, and the subsequent reverse line blot (RLB) hybridization with generic and specific probes were carried out as previously described for ovine, bovine and equine piroplasm species (Nagore *et al*. 2004*a*, *b*; Garcia-Sanmartin *et al*. 2006). Additionally, a new probe was designed to identify a new *Theileria* genotype (*Theileria* sp. 3185/02, GenBank Accession number AY421708) recently found in a red deer imported from Germany that died in Central Spain (Hofle *et al*. 2004). The probe was designed using Vector NTI 8.0 software (Informax Inc., North Bethesda, MD) and synthesized by MWG Biotech AG (Germany) with a C6 amino linker and the following nucleotide sequence: 5′-CGGTTATA AAATTTATTTTATTTCCG-3′. The specificity of the probe was tested against the following *Theileria* and *Babesia* species from cattle, horse, sheep, dog and wildlife: *T. annulata*, *T. buffeli*, *B. bigemina*, *B. bovis*, *B. divergens*, *B. major*, *T. equi*, *T. equi*-like, *B. caballi*, *B. caballi*-like, *T. ovis*, *Theileria* sp. OT1, *Theileria* sp. OT3, *B. ovis*, *B. motasi*, *T. annae*, *B. canis canis*, *B canis vogeli*, *B. microti*, *Babesia* sp. EU1 and *Theileria* sp. 3185/02. To exclude false positive results, negative controls included during DNA extraction and PCR amplification were subjected to RLB hybridization. The V4 hypervariable region of at least 1 strain of each genotype found in each animal species was submitted to a commercial subcontractor for automatic dye-terminator cycle sequencing.

Sequence comparison analysis

The sequences obtained were compared with the GenBank database by nucleotide sequence homology searches made at the network server of the National

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Center for Biotechnology Information (NCBI) using BLAST. Multiple sequence alignments were performed using the program AlignX (Vector NTI 8.0 suite, InforMax, North Bethesda, MD, USA) with an engine based on the ClustalW algorithm (Thompson *et al*. 1994).

Statistical analysis

Prevalence of each parasite species or combination was analysed according to independent variables such as host species, age category, sex, sample type (blood or tissue), capture conditions (hunted, with traumatisms or with others diseases) and tick infection by Chi square or Fisher exact test. $P<0{\cdot}05$ was considered as significant.

Nucleotide sequence Accession numbers

The sequences of the 18S rRNA genes corresponding to *Theileria* sp. OT3 from chamois, red deer and roe deer, *B. divergens* from roe deer, *Theileria* sp. 3185/02 from roe deer, *B. divergens* from roe deer and *T. ovis* from chamois described in this work were deposited in GenBank under Accession numbers DQ866839-45, respectively.

RESULTS

The new probe designed for *Theileria* sp. 3185/02 gave a positive result with the amplified 18S rRNA gene from the *Theileria* strain from red deer with GenBank acc. nr. AY421708 (Hofle *et al*. 2004), and no cross-reaction was observed when tested against the *Theileria* and *Babesia* species from cattle, horse, sheep, dog and wildlife used as controls (see Materials and Methods section). The inclusion of this new probe in the RLB panel allowed the detection of this theileria in red deer from the Basque Country, and for the first time, also in roe deer from the same area. Besides *Theileria* sp. 3185/02, two other theilerias, *Theileria ovis* and *Theileria* sp. OT3 (previously described in sheep from the Basque region) (Nagore *et al*. 2004*b*), and 1 babesia, *Babesia divergens*, were also identified among the wildlife samples included in this study (Fig. 1). However, whereas *Theileria* sp. OT3 was found in all 3 animal species analysed, *Theileria* sp. 3185/02 was found in both red and roe deer but not chamois, and *T. ovis* and *B. divergens* were only found in 1 species, chamois and roe deer, respectively.

Although the RLB technique was proven to be specific (Gubbels *et al*. 1999; Nagore *et al*. 2004*a*, *b*), hybridization results from previously undescribed hosts were confirmed by sequencing. Thus, the complete 18S rRNA gene or the V4 region were sequenced from the piroplasms identified as *Theileria* sp. 3185/02 from 2 roe deer; *Theileria* sp. OT3 from 2 roe deer, 1 red deer and 3 chamois; *B. divergens*

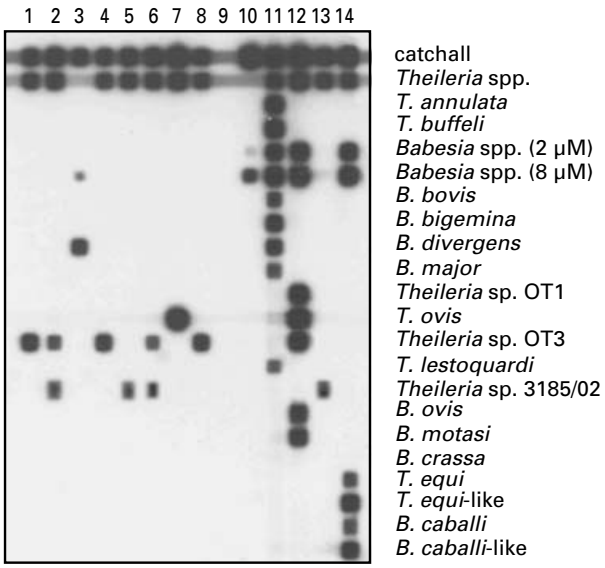

Fig. 1. Reverse line blot macroarray of the PCR products generated by amplification of genomic DNA from wildlife samples and from pure plasmid DNA used as control. Oligonucleotide probes are indicated in rows, and samples are applied in columns as follows: lanes 1–8, field samples; lane 9, genomic DNA from uninfected roe deer; lane 10, *Babesia* sp. EU1 from an infected deer used as control; lane 11, mixture of bovine piroplasms 18S rRNA gene clones (note that cross-reaction occurs between the *T. lestoquardi* probe and the amplicon generated from *T. annulata* (Nagore *et al*. 2004*b*)); lane 12, mixture of ovine piroplasms 18S rRNA gene clones; lane 13, *Theileria* sp. 3185/02 18S rRNA gene clone; lane 14, mixture of equine piroplasms 18S rRNA gene clones.

from 4 roe deer; and *T. ovis* from 1 chamois. In all the cases sequencing analysis confirmed the RLB results. The sequences obtained in this study were compared with the GenBank database, and the similarity values for the complete 18S rRNA gene for the theilerias described in this study and related sequences from GenBank are shown in Table 1. Hence, the complete 18S rRNA gene sequences of *Theileria* sp. 3185/02 from roe deer (this study, acc. nr. DQ866842) and red deer (AY421708) were 99·6% identical and they were more than 99·7% identical to a sequence from a roe deer identified as *T. capreoli* in GenBank (Acc. nr. AY726011). The complete 18S rRNA gene of *Theileria* sp. OT3 from roe deer, red deer and chamois shared 99·7–99·8% similarity among themselves and with a sequence previously described in sheep from the Basque Country (Acc. nr. AY533145). Likewise, the V4 region of the 18S rRNA gene of *T. ovis* from the Catalonian chamois was 99·7% similar to the *T. ovis* identified in sheep from Spain (Acc. nr. AY533144), and at least 99·5% identical to other *T. ovis* sequences from sheep and goats available in GenBank. The V4 region of the 18S rRNA gene of four roe deer samples that hybridized with *B. divergens* was sequenced. Comparison of

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Table 1. Pairwise sequence similarities between the complete 18S rRNA genes of *Theileria* species

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14
T. annulata – M64243 (cattle)	1	100·0	96·4	96·5	94·4	95·7	96·8	96·6	96·6	96·2	96·2	96·2	96·1	96·2	96·3
T. buffeli – AF236094 (cattle)	2		100·0	97·3	94·5	97·1	97·0	97·3	97·3	96·0	96·8	96·8	96·7	96·7	97·4
T. capreoli – AY726011 (roe deer)	3			100·0	95·0	97·5	98·1	99·9	99·7	97·4	97·9	97·9	98·0	98·0	99·3
T. cervi – AF086804 (white-tailed deer)	4				100·0	94·4	95·3	95·1	95·1	94·9	94·4	94·5	94·3	94·4	95·2
Theileria sp. OT1 – AY533143 (sheep)	5					100·0	97·0	97·6	97·4	96·6	96·8	96·8	96·8	96·8	97·6
T. ovis - AY533144 (sheep)	6						100·0	98·2	97·9	97·4	97·2	97·2	97·1	97·2	98·1
Theileria sp. 3185/02 – AY421708 (red deer)	7							100·0	99·6	97·4	98·0	98·0	97·9	98·0	99·4
Theileria sp. 3185/02 – DQ866842 (roe deer)	8								100·0	97·3	97·7	97·7	97·7	97·8	99·0
Theileria sp. – AY735136 (white-tailed deer)	9									100·0	96·5	96·5	96·4	96·5	97·4
Theileria sp. OT3 – AY533145 (sheep)	10										100·0	99·8	99·7	99·7	97·8
Theileria sp. OT3 – DQ866839 (chamois)	11											100·0	99·7	99·7	97·8
Theileria sp. OT3 – DQ866840 (red deer)	12												100·0	99·8	97·7
Theileria sp. OT3 – DQ866841 (roe deer)	13													100·0	97·7
Theileria sp. – AB012198 (sika deer)	14														100·0

these sequences with the GenBank database identified *B. divergens* found in wild ruminants (GenBank Acc. nr. AY098643 from reindeer, and AY572456 from roe deer) and *B. capreoli* from roe deer (AY726009) as the closest matches. Hence, 3 of the 4 sequences determined in this study were identical to *B. divergens* (AY098643 and AY572456), and *B. capreoli* (AY726009) in the 416 nt. of the V4 region compared, whereas the fourth one varied in 3 nt. T at position 663 (AY572456 numbering) was a common feature of all the sequences from wild ruminants, but different from the *B. divergens* found in other hosts. Conversely, the sequences from wild ruminants shared a G at position 631 with *B. divergens* from humans and from cotton-tail rabbit (AY144688), and with other babesias isolated from humans like *Babesia* sp. MO1 (AY048113) and *Babesia* sp. BAB693W (AY274114), and differed from *B. divergens* found in cattle and *Ixodes ricinus* ticks, which carry an A at this position. Hence, sequence comparison grouped all the babesias from wildlife species together along with *B. divergens* and other *B. divergens*-like isolates of human origin.

The total prevalence of piroplasms detected by RLB was significantly different between species: 85·7% in red deer, 62·3% in roe deer and 28·9% in chamois. The distribution of each haemoparasite among the different species is summarized in Table 2. *Theileria* sp. OT3 was found in the 3 animal species and always as the most prevalent piroplasm (85·7% in red deer, 46·4% in roe deer and 26·3% in chamois). *Theileria* sp. 3185/02 was significantly more abundant in red deer (53·6%) than in roe deer (10·1%) ($P<0·05$) and it was absent from chamois. *B. divergens* was restricted to 6 roe deer and *T. ovis* to 1 chamois. Mixed infections were only found in red and roe deer but not in chamois, and they were all made up of the combination of *Theileria* sp. OT3 and *Theileria* sp. 3185/02. In red deer *Theileria* sp. 3185/02 occurred always as a mixed infection with *Theileria* sp. OT3 and this combination was the most prevalent ($P<0·05$), accounting for 62·5% of the positive animals (15/24). This mixed infection was significantly more prevalent in red deer than in roe deer ($P<0·05$), since mixed infection was only found in 2 roe deer (4·7% positive animals, 2/43). Conversely, single infection with *Theileria* sp. OT3 was the most prevalent form of infection in roe deer (69·8% positive animals, 30/43) ($P<0·05$). *Theileria* sp. 3185/02 and *Theileria* sp. OT3 were found in every age category of roe deer, and the latter was more prevalent in adults than yearlings ($P<0·05$) whereas *B. divergens* was always found in yearlings. Among the 4 roe deer fawns analysed 1 was positive to *Theileria* sp. 3185/02 and 2 to *Theileria* sp. OT3. Interestingly, the prevalence of *Theileria* sp. OT3 was significantly higher ($P<0·05$) in the pestivirus-positive chamois (55·6%) than in those negative (17·2%). No infections with other species of

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Table 2. Distribution and frequency (%) of piroplasm species

	Red deer ($N=28$)		Roe deer ($N=69$)		Chamois ($N=38$)	
RLB results	*n*	%	*n*	%	*n*	%
Positive	*24*	*85·7*	*43*	*62·3*	*11*	*28·9*
Theileria ovis	0	0·0	0	0·0	1	2·6
Theileria sp. OT3	9	32·1	30	43·5	10	26·3
Theileria sp. 3185/02	0	0·0	5	7·2	0	0·0
Theileria sp. OT3 + *Theileria* sp. 3185/02	15	53·6	2	2·9	0	0·0
B. divergens	0	0·0	6	8·7	0	0·0
Negative	*4*	*14·3*	*26*	*37·7*	*27*	*71·1*

piroplasms found in other wild ruminants such as *Theileria cervi*, *Babesia odocoilei*, *Babesia* sp. EU1 or *Babesia* sp. isolates RD61 or RD63 were found in this study. No differences between piroplasm infection (presence/absence and different species combinations) and sex of the animals, sample type and capture conditions were found.

Twelve red deer, 44 roe deer and 22 chamois could be examined for ticks. A total of 147 ticks were collected from 11 of the 12 red deer examined for tick infestation (mean 13·4; range (2–31) ticks/animal). No significant association was found between presence of ticks and infection status ($P>0·05$). *I. ricinus* was the most frequently found tick species accounting for 76·2% of all the ticks collected, followed by *Haemaphysalis inermis* (16·3%), *Dermacentor reticulatus* (4·7%), *Dermacentor marginatus* (1·4%), *Haemaphysalis punctata* (0·7%) and *Rhipicephalus bursa* (0·7%). 522 ticks were found in 34 of the 44 roe deer examined for ticks (mean 15·4; range (1–42) ticks/animal), 373 of them from 25 of the 28 *Theileria*-positive animals examined (25/28, 89·3%). An association was found between presence of ticks and infection with *Theileria* sp. ($P<0·05$). *I. ricinus* was the main tick species found in roe deer (97·9%), whereas the presence of other species was sporadic (0·9% of *H. punctata*, 0·8% of *H. inermis* and 0·4% of *R. bursa*). The genus *Dermacentor* was not found in roe deer. No significant differences were found between the number or the species of ticks collected and piroplasm infection (presence/absence and different species combinations) ($P>0·05$). Twenty-two chamois, corresponding to those not hunted, were examined for ticks, and only in 5 of them ticks were collected. In this case, ticks were identified but not counted. The tick species found were *D. marginatus* (3 animals, 2 of them positive to *Theileria* sp. OT3), *H. punctata* (1 animal positive to *Theileria* sp. OT3) and both *Haemaphysalis sulcata* and *R. bursa* (1 negative animal).

DISCUSSION

The technique used in this study allowed the simultaneous detection and identification of different *Theileria* and *Babesia* species using oligonucleotide probes whose specificity has been previously determined (Nagore *et al*. 2004*a*, *b*; Gubbels *et al*. 1999) and a new probe developed as part of this study. Besides, the catchall and genus-specific probes guarantee that no new species passed unnoticed. Thus, this survey led to the identification of 4 different piroplasms in the red deer, roe deer and chamois analysed.

The most prevalent piroplasm in red deer, roe deer and chamois was *Theileria* sp. OT3, which was also found with high prevalence (42·2%) in the ovine population of the Basque Country (Nagore *et al*. 2004*b*). The high prevalence found in cervids, and particularly in red deer (85·7%, i.e. all the positive red deer), suggests that deer might be the reservoir of *Theileria* sp. OT3 in wildlife and the source for ovine infection. Second in prevalence was *Theileria* sp. 3185/02, previously described in a red deer imported from Germany that died in Central Spain (Hofle *et al*. 2004). In this study, *Theileria* sp. 3185/02 was found with high prevalence in red deer from the Basque Country (53·6%) and, though at lower prevalence (10·1%), also in roe deer. Sequencing analysis of the complete 18S rRNA gene confirmed that the theileria identified as *Theileria* sp. 3185/02 in red and roe deer in this study were the same as that recently found in a roe deer from Galicia (Spain) and identified as *Theileria capreoli* in GenBank (Acc. nr. AY726011), and very similar to *Theileria* sp. strains obtained from *Cervus nippon yesoensis* (e.g. AB012195, AB012198). This high similarity suggests that we are dealing with the same piroplasm species. Finally, *T. ovis* was the theileria less frequently found and restricted to a Catalonian chamois kid that died from pneumonia.

Prevalence of babesia infection was much lower than that of theileria, and it was restricted to *B. divergens*-infection of roe deer (8·7%). The first report of naturally acquired babesiosis caused by *B. divergens* in cervids dates from 2003, when Langton *et al*. (2003) detected the parasite in a reindeer herd. More recently, Duh *et al*. (2005*b*) detected *B. divergens* in 54·9% of roe deer and in 16·7% red deer after analysing a similar number of

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animals as we did. *B. capreoli* has been isolated from red deer and sika deer and compared with *B. divergens* in morphological, serological and transmission studies (Gray *et al.* 1990). Gray *et al.* (1990) concluded that *B. capreoli* and *B. divergens* were closely related morphological and serologically, but described differences in host specificity. The 18S rRNA gene sequence of a babesia from a roe deer from France has been recently deposited in GenBank identified as *B. capreoli* (AY726009). This sequence was identical in the V4 region to 3 babesias from roe deer identified in our study, and above 99·8% identical in the complete gene to other *B. divergens* from wildlife. Since neither morphological, serological nor molecular techniques can differentiate between *B. capreoli* and *B. divergens*, they should be considered the same species parasitizing different hosts. The differences in host specificity could also be interpreted as differences in infectivity associated with different strains, and therefore, further studies are needed to study strain-associated host-specificity to investigate this difference in infectivity.

In the Basque Country sheep and beef cattle spend several months in communal mountain pastures where they can get in contact with wildlife species. In addition, an increment has been recorded in the cervid population in the area over the last decades, and also in tick abundance (Barandika *et al.* 2006). *Ixodes* ticks feed on 3 hosts, one during each life stage, and this situation where mountain pastures are shared by different animal species favours the possibility of ticks biting different host and transmitting piroplasm species. Similarly, Pyrenean chamois share their habitat with cattle, goats, sheep and roe deer, and in some places also with red deer. Interestingly, a high seroprevalence of *T. ovis* has been reported in sheep and goats of the area (Ferrer and Castella, 1999). This contact with domestic animals would explain the presence of piroplasm species in hosts other than those where they were initially described. Detection of *B. divergens* in wildlife species has been described before (Duh *et al.* 2005*b*; Langton *et al.* 2003), but this is the first time that the presence of traditionally-considered ovine piroplasm species like *T. ovis* or the more recently described genotype *Theileria* sp. OT3 is reported in host other than sheep or goats. This variable host range is consistent with the close phylogenetic relationships of several *Theileria* species (Nagore *et al.* 2004*b*), and highlights the apparent lack of host specificity of piroplasm species discussed above. Conversely, *Theileria* sp. 3185/02 was only found in red and roe deer but not in chamois, and likewise, no hint of its presence was found in a previous study carried out in the ovine population of the Basque Country (Nagore *et al.* 2004*b*). However, the specific probe for *Theileria* sp. 3185/02 was not used in that previous study, and therefore, further studies are needed before confirming its specificity for deer.

Several species of ticks were collected from red and roe deer, but *I. ricinus* was the most prevalent and in the case of roe deer almost the only tick species found (97·9%). *I. ricinus* is also the most abundant tick in the vegetation of the Basque Country (Barandika *et al.*, 2006), and it is known to be the vector of *B. divergens* (Zintl *et al.*, 2003). Only 2 of the 6 roe deer positive to *B. divergens* could be examined for tick infestation; no ticks were seen in one of them and 13 *I. ricinus* ticks were collected from the other. In Catalonia, however, *I. ricinus* is not so common and other tick species like *D. marginatus*, *R. bursa* or *H. punctata* are more prevalent. Although no ticks were collected from the chamois positive to *T. ovis*, *R. bursa* is known to be the vector of this piroplasm species. In addition, we have also detected *T. ovis* DNA in *I. ricinus* ticks from the vegetation (unpublished results). Although the tick vectors of *Theileria* sp. 3185/02 and *Theileria* sp. OT3 have yet to be identified, given the high prevalence of *Theileria* sp. 3185/02 in deer and of *Theileria* sp. OT3 in wild ungulates and sheep, ticks of the highly abundant genus *Ixodes* are most probably involved in the Basque Country. However, in other areas like Catalonia the vectors might be other tick species. Further studies on questing ticks from the vegetation are in progress to evaluate this hypothesis.

Although severe parasitaemia in combination with high population density and poor nutrition can lead to clinical piroplasmosis (Yabsley *et al.* 2005), clinical signs are not normally associated with wildlife. In this study, most of the cervids were apparently healthy and, whenever analysed, haematological parameters were within the normal ranges (data not shown) suggesting that they were chronic asymptomatic carriers. Absence of clinical signs in bovine babesial infections in endemic areas is associated to the so-called inverse age resistance (Zintl *et al.* 2005). Resistance to disease (but not to infection) has been observed in calves infected with *B. divergens* in endemic areas. A similar phenomenon could explain the situation found in wildlife ruminants, which occupy areas with high infection pressure. Hence most animals would become infected as fawns (the 6 roe deer positive to *B. divergens* were yearlings) and acquire immunity without showing clinical symptoms. Immunity in older animals would be reinforced by repeated tick challenge. This mechanism would allow the parasite to establish persistent reservoir infections early in the life of the host favouring survival and transmission. Regarding theilerias, where this phenomenon has not been described, we can hypothesize that the species found are not pathogenic for cervids and do not normally elicit clinical disease. In the case of chamois, however, several animals were found to have lower than normal red blood cell counts, haematocrit, and haemoglobin values, and forms compatible with *Theileria* spp. were seen by microscopical

examination in 2 chamois (data not shown). In fact, piroplasmosis was the initial evidence for the unspecific signs later associated to pestivirus infection (Hurtado *et al*. 2004). The immunosuppressive effect of pestiviruses might have favoured the infection with *Theileria*. In any case, in this study infection with *Theileria* sp. was observed to occur early in life (as young as 2 months old) since the only kid chamois and 3 of the 4 roe deer yawns analysed were positive. Similarly, infection with *T. cervi* has been reported in white-tailed deer fawns (Waldrup *et al*. 1992; Yabsley *et al*. 2005).

The high sensitivity of the technique used allowed the detection of subclinical infections and the survey revealed an infection rate much higher than expected, particularly in red deer. This fact, added to the lack of host-specificity, are of serious concern in terms of piroplasmosis control, since these results suggest that a considerable number of wildlife animals are chronic asymptomatic carriers that may serve as reservoirs of the infection. These results highlight the importance of wildlife surveillance surveys to study the potential role of wildlife as reservoir of piroplasmosis and other zoonosis and the risk of wildlife translocation.

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