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Original article

Anaplasmataceae in wild ungulates and carnivores in northern Spain

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ABSTRACT

Wild vertebrates are essential hosts for tick-borne diseases but data on the prevalence and diversity of *Anaplasma* spp. in wildlife are scarce. In this study, we used real-time PCR to investigate the distribution of *Anaplasma* species in spleen samples collected from 625 wild animals (137 cervids, 227 wild boar, and 261 carnivores) in two regions in northern Spain. A first generic real-time PCR assay was used to screen for the presence of *Anaplasma* spp. followed by a second species-specific multiplex real-time PCR or partial sequencing of the 16S rRNA gene for species identification. *Anaplasma phagocytophilum* was highly prevalent in cervids (64.2%), but it was absent from wild boar and carnivores. Interestingly, *Anaplasma marginale* and *Anaplasma ovis* were not detected in cervids, but *Anaplasma centrale* was identified in 1 roe deer and 1 red deer, *A. bovis* in 4 roe deer, and a novel *Ehrlichia* sp. in one badger. These findings were highly associated with the tick burden identified in the different hosts. Thus, *Ixodes ricinus*, the recognized vector of *A. phagocytophilum* in Europe, was the main tick species parasitizing cervids (93.5%, 1674/1791), whereas *Dermacentor reticulatus* was the most abundant in wild boar (76.1%, 35/46) and *Ixodes hexagonus* in carnivores (58.4%, 265/454). More investigations are needed to assess the impact of the different *Anaplasma* species in wildlife and the risk of transmission to domestic animals.

1. Introduction

Tick-borne diseases affecting wildlife have been reported worldwide. Among these, are diseases caused by obligate intracellular bacteria of the family Anaplasmataceae. The *Anaplasma* genus includes three species that affect leucocytes and macrophages (*Anaplasma phagocytophilum*, *Anaplasma bovis* and *Anaplasma platys*), and erythrocytic anaplasmas that parasitize red blood cells (*Anaplasma marginale*, *Anaplasma centrale* and *Anaplasma ovis*) (Dumler et al., 2001). Although some are low pathogenic species, others are highly pathogenic. Among the latter, *A. phagocytophilum* has the greatest impact on veterinary and human medicine. *A. phagocytophilum* is transmitted by *Ixodes* spp. ticks and has been reported worldwide as the causative agent of Tick-borne fever in ruminants and Human Granulocytic Anaplasmosis (HGA). *A. phagocytophilum* is also maintained in nature through enzootic cycles between ticks and wild animals. In Europe, infection has been reported in wild ungulates, small mammals, carnivores, birds and reptiles (Stuen et al., 2013). Recent studies based on the study of *gro*EL gene discriminated four *A. phagocytophilum* ecotypes (Jahfari et al., 2014), and interestingly, the most expanded ecotype among wildlife has the highest zoonotic potential.

Erythrocytic anaplasmas do not cause disease in humans, but some species such as *A. marginale* cause a mild to severe haemolytic disease in cattle and other wild ruminants that results in considerable economic losses. *A. centrale* is a less pathogenic species, and *A. ovis* may cause mild to severe disease in sheep and other wild ruminants (Aubry and Geale, 2011). Nevertheless, the distribution of these species in wild animals has not been fully investigated.

Deeper knowledge on the role of wildlife species as reservoirs of different pathogens is of major importance for the development of control strategies. Studies on the distribution of anaplasmas in wild animals in Spain are restricted to a few prevalence reports in a limited number of wild ruminants, small mammals or birds (Barandika et al., 2007; De La Fuente et al., 2005, 2008; Portillo et al., 2011). In this study, cervids, wild boar and several species of wild carnivores were examined for molecular evidence of infection with *Anaplasma* spp. to investigate the epidemiology and diversity of this group of tick-borne pathogens. The distribution of *Anaplasma* species infecting wild and domestic animals was compared and the potential role of wildlife as reservoir of zoonotic pathogens like *A. phagocytophilum* was investigated.

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2. Materials and methods

2.1. Study site

The study was carried out in two autonomous regions in northern Spain (the Basque Country and Asturias). The Basque Country (7234 km^2) has Atlantic Climate, with mild to slightly hot summers and mild winters. The temperate climate of the region allows for a wide and abundant wildlife fauna (Palomo and Gisbert, 2002). Regarding carnivores, the most abundant species are badger (*Meles meles*) and red fox (*Vulpes vulpes*), and concerning ungulates, roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) are the most abundant species. Asturias (10,603 km^2) has also an Atlantic Climate but precipitations are more abundant and winters are colder than in the Basque Country, with frequent snows in the mountainous areas from October till May. Wildlife is very abundant in this region with many different species, including carnivores like red fox, badger, gray wolf (*Canis lupus*) and the endangered brown bear (*Ursus arctos*). Red deer (*Cervus elaphus*), roe deer and fallow deer (*Dama dama*) are the most common ungulate species in the area. Climate and abundance of wild and domestic animal hosts allow for high burdens of ticks in the environment (Barandika et al., 2006).

2.2. Sample collection

Three-hundred and sixty-four wild ungulates [26 red deer, 105 roe deer, 6 fallow deer, 227 wild boars] and 261 carnivores [Mustelidae – 130 Eurasian badgers, 22 stone marten (*Martes foina*), 14 pine marten (*Martes martes*), 6 weasel (*Mustela nivalis*), 6 polecat (*Mustela putorius*), 2 otter (*Lutra lutra*), 2 American mink (*Neovison vison*) and 1 stoat (*Mustela erminea*); Canidae – 54 red foxes, 2 gray wolves; Felidae – 8 wild cats (*Felis s. silvestris*); Viverridae – 14 common genets (*Genetta genetta*)] were sampled within the Health Surveillance Program in Wildlife. Most cervid and wild boar samples were collected during the hunting season (November–February for red deer and wild boar, and February–June for roe deer); some roe deer and wild boar were found with traumatisms or dead by other causes. Red foxes were hunted mostly in winter (65%), while the remaining carnivores were collected throughout the year after being found dead. A complete necropsy was performed at the laboratory except for red deer and wild boar, which were mostly examined and sampled in the field. Spleen samples were collected and stored at −20 °C until subsequent DNA purification and molecular analysis. Whenever possible ticks were collected, counted and identified using taxonomic keys (Gil-Collado et al., 1979; Manilla, 1998).

2.2.1. DNA extraction

DNA from tissues was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). DNA yields were subsequently determined with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA).

2.2.2. PCR amplification

Presence of *Anaplasma* spp. was firstly determined using a real-time PCR assay (RTi-PCR1) that targets the 16S rRNA gene of the genus *Anaplasma*; the assay also includes an internal amplification control (IAC) to monitor for possible inhibition. All samples positive to *Anaplasma* spp. in RTi-PCR1 were analyzed with a multiplex PCR assay that specifically amplifies the *msp*2 gene of *A. phagocytophilum*, and the *msp*4 gene of *A. marginale* and *A. ovis* (RTi-PCR2). Sequences of primers and probes, as well as details on cycling conditions, sensitivity and specificity were as reported elsewhere (Hurtado et al., 2015). Analyses were performed in 20 μl volume reactions using an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems).

2.2.3. Sequence analysis

The 16S rRNA gene of a selection of samples that included those positive to the generic RTi-PCR1 but negative to RTi-PCR2 was amplified using primers EE1 and EE2 as described (Pusterla et al., 2000) and the 3′ end of the gene sequenced with the reverse primer EE2. A larger fragment was sequenced when homologies with sequences in GenBank were below 98%. Sequencing reactions were carried out using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and products were analyzed on an ABI3130 genetic analyzer (Applied Biosystems). The sequences obtained were compared with the GenBank database by nucleotide sequence homology searches made at the network server of the National Center for Biotechnology Information (NCBI) using BLASTN.

For phylogenetic analyses, a multiple sequence alignment was performed using Mega 6 package (Tamura et al., 2013) with an engine based on the MUSCLE algorithm (Edgar, 2004). The phylogenetic tree was then inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980) and the Gamma distributed with Invariant Sites (G+ I) option for variation in rates among sites. Accuracy of inferred topology was assessed via bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

The new sequence described here was deposited in GenBank under accession number KR262717.

3. Results

A positive result in RTi-PCR1 was obtained for 28.9% of ungulates (105/364) and 1.5% of carnivores (4/261). Among ungulates, Cervidae had the highest proportion of positive samples (75.9%) in contrast to the low prevalence obtained in Suidae (0.4% in wild boars). Among carnivores, only specimens of Mustelidae (2 badgers, 1 genet and 1 American mink) produced positive amplicons. After positive samples to the generic PCR were analyzed using the specific RTi-PCR2, *A. phagocytophilum* was identified in cervids (62 roe deer, 21 red deer and 3 fallow deer), but *A. marginale* and *A. ovis* were not detected. Carnivores were all negative to the 3 *Anaplasma* species. Twenty-three samples positive to RTi-PCR1 remained negative in the specific RTi-PCR2. Sequencing was attempted on 22 of those samples (DNA from one American mink was not available for sequencing) for species identification. Sequence analysis of a fragment of the 16S rRNA gene (size 560–750 nucleotides, nt) allowed identification of Anaplasmataceae in 9 samples; sequencing was not successful in the remaining 13 samples. Thus, *A. phagocytophilum* was identified in 2 additional roe deer resulting in a prevalence of 61.0% (64/105) in roe deer, 80.8% (21/26) in red deer and 50.0% (3/6) in fallow deer (Table 1); *A. bovis* was identified in 4 roe deer, and *A. centrale* in 1 roe deer and 1 red deer (Table 1). Among the carnivores, only one badger sample was successfully sequenced. A BLASTn search performed in GenBank with a fragment of 586 nt of the 16S rRNA did not find any hits above 98% homology, and therefore further sequencing was carried out. The 1392 nt sequence thus obtained shared 97.3% similarity with the nearest BLAST hit, which corresponded to *Ehrlichia chaffeensis* (GenBank accession number NR_074500). A phylogenetic tree was then inferred by using sequences of the 16S rRNA genes of representative Anaplasmataceae species available in GenBank and the sequence determined here from badger (Fig. 1, accession numbers are shown). The sequence from the badger clearly grouped in the same clade with all other *Ehrlichia* species with a highly significant bootstrap value.

Table 1
Number of animals investigated and number of animals positive to the presence of DNA of Anaplasmataceae in each Family included in the study.

Host	Family	Analyzed animals	N positive *A. phagocytophilum* (%)	Other *Anaplasmataceae* (sequencing)
Ungulates	Cervidae (Roe deer)	105	64 (61.0)[a]	1 *A. centrale*; 4 *A. bovis*
	Cervidae (Red deer)	26	21 (80.8)	1 *A. centrale*
	Cervidae (Fallow deer)	6	3 (50.0)	na
	Suidae (Wild boar)	227	0	na
Total Ungulates		**364**	**88 (24.2)**	
Carnivores	Canidae	56	0	na
	Felidae	8	0	na
	Mustelidae	183	0	1 *Ehrlichia* sp.
	Viverridae	14	0	na
Total Carnivores		**261**	**0 (0.0)**	
Total		**625**	**88 (14.1)**	

[a] *A. phagocytophilum* in 2 roe deer was identified only by sequencing.
na, non-applicable.

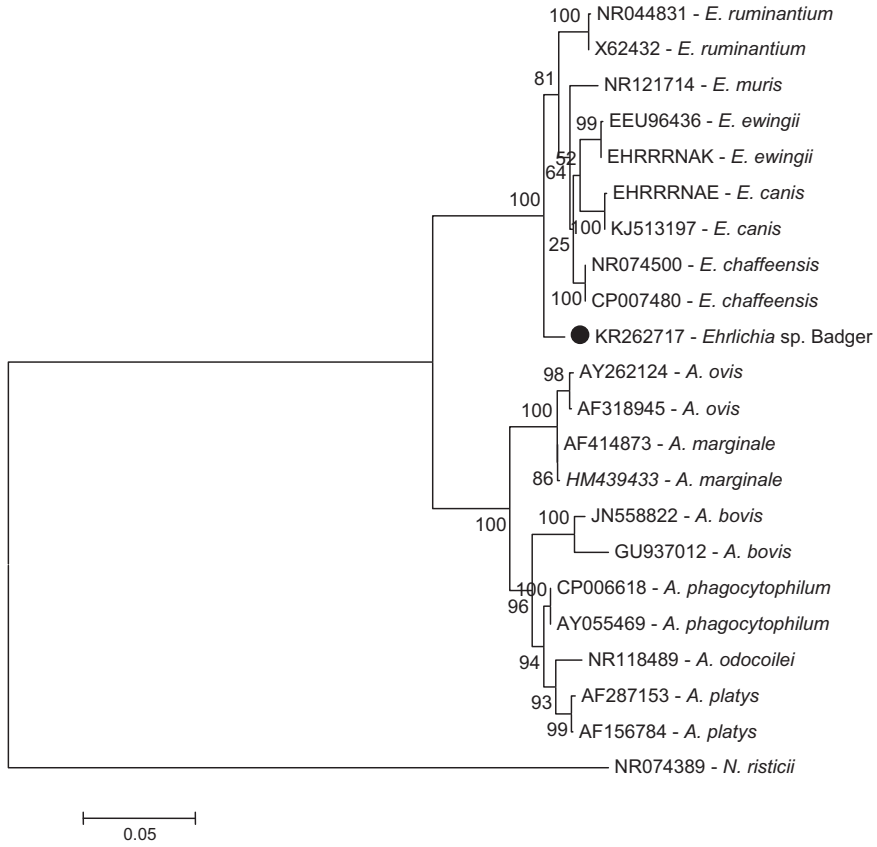

Fig. 1. Phylogenetic tree inferred from 16S rRNA gene sequences of representative Anaplasmataceae species and the sequence determined here from badger. The tree was inferred by Maximum Likelihood analysis under the Kimura 2-parameter model with 5 Gamma-distributed rate categories, allowing a percentage of sites to be held as invariable (the tree with highest log likelihood is shown). There were a total of 1341 positions in the final dataset. Bootstrap values (1000 replicates) are indicated for each node. The GenBank accession numbers are indicated for each sequence used and the sequence described in this study is indicated by a closed circle. Scale bar used was nucleotide substitutions per position.

A total of 298 of the 625 animals could be examined for ticks (47.7%), with a total of 2291 ticks counted. Red foxes (50%), badgers (54%), red deer (68%), roe deer (68%), and fallow deer (100%) were the most parasitized species. A total of 11 tick species were identified (Fig. 2). Considering together all tick stages (larvae, nymphs and adults), *Ixodes ricinus* was the most prevalent species in cervids (93.5%, 1674/1791), *Dermacentor reticulatus* (76.1%, 35/46) and *Dermacentor marginatus* (17.4%, 8/46) in wild boar, and *Ixodes hexagonus* in carnivores (58.4%, 265/454). Other less abundant species were *Haemaphysalis concinna* and *Haemaphysalis inermis* only detected in cervids, *Haemaphysalis hispanica*, *Ixodes canisuga* and *Rhipicephalus pusillus* only found in carnivores, and *Haemaphysalis punctata* and *Rhipicephalus bursa* sporadically collected from cervids and carnivores. Fig. 2 summarizes the species distribution of each tick species in cervids, wild boar and carnivores.

4. Discussion

The worldwide economic impact of tick-borne bacterial diseases on livestock production, along with their zoonotic potential, warrant further surveillance studies on ticks and tick-borne pathogens not only in domestic but also in wild animals. Here, cervids, wild boar and several species of wild carnivores of the families Mustelidae, Canidae, Felidae and Viverridae were examined for *Anaplasma*

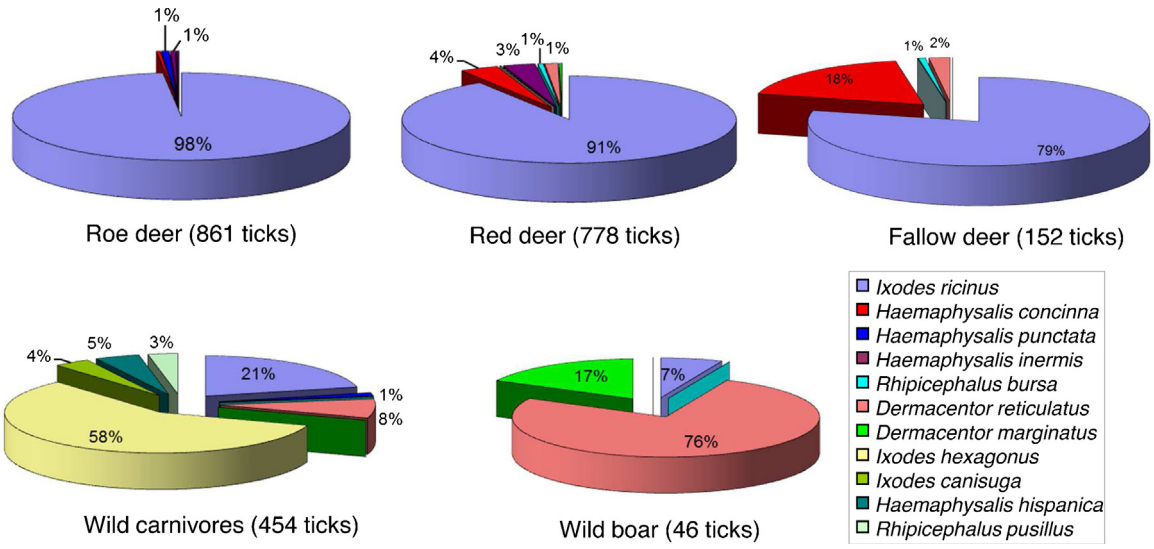

Fig. 2. Tick species distribution in cervids, wild boar and wild carnivores.

infection. Results showed high prevalence of Anaplasmataceae in cervids, low in Mustelidae and absence in all other examined hosts.

Prevalences of *A. phagocytophilum* in wild ruminants in Europe vary between regions and countries, with prevalence values ranging from 10 to 38% in roe deer and 10–51% in red deer in Poland (Hapunik et al., 2011; Michalik et al., 2009; Welc-Faleciak et al., 2013) or values as high as 98.9% of roe deer infected in Germany (Overzier et al., 2013). The high prevalence found here (61.0% in roe deer and 80.8% in red deer) suggests the relevance of deer as reservoir host of *A. phagocytophilum* in the study area that could act as source of infection for vector ticks. Most of the ticks collected from cervids were *I. ricinus*, which could explain the high prevalence of *A. phagocytophilum*. In fact, *I. ricinus*, which has been considered the vector of *A. phagocytophilum* in several European countries (Stuen et al., 2013; Woldehiwet, 2006), is the most abundant tick species in northern Spain (Barandika et al., 2011), where *A. phagocytophilum* DNA has been detected in 5.6% of adult free-living *I. ricinus* ticks (Barandika et al., 2008).

In wild boar, most of the ticks identified were *Dermacentor* spp. which could explain the lack of *A. phagocytophilum* infection in these ungulates. However, though *A. phagocytophilum* infection rates in wild boar are always lower than in cervids, wild boar infected with *A. phagocytophilum* have been detected in other parts of Europe, like Slovenia (6.2%) (Zele et al., 2012), or Romania (4.5%) (Kiss et al., 2014). Unfortunately, ticks were not investigated in any of those studies. Conversely, Silaghi et al. (2014) in Germany and Michalik et al. (2012) in Poland, found that *I. ricinus* was the only tick species collected from wild boar that was infected with *A. phagocytophilum* (12.5% in Germany and 12.0% in Poland). In addition, recent results have revealed that wild boars are susceptible to *A. phagocytophilum* but are able to control the infection mainly through the activation of innate immune responses, phagocytosis and autophagy, leading to infection levels below PCR detection or infection clearance (De La Fuente and Gortazar, 2012; Galindo et al., 2012). Despite these low rates of infection, several studies have detected human pathogenic strains in wild boar suggesting that they might act as a potential reservoir of HGA strains (Michalik et al., 2012).

In this study, wild carnivores were also negative to *A. phagocytophilum*. Infection in red foxes was expected since several authors have detected *A. phagocytophilum* DNA at prevalences ranging from 3.0 to 16.0% (Ebani et al., 2011; Hartwig et al., 2014; Karbowiak et al., 2009). The number of animals analyzed here (56 red foxes) might have been too small to detect low prevalences. *I. hexagonus* represented the main tick species in wild carnivores, with *I. ricinus* at a lower level, which could be another explanation for the absence of infection. Sequencing analysis of the 16S rRNA gene from a badger positive to *Anaplasma* spp. in RTi-PCR1 but negative to the 3 *Anaplasma* species targeted in RTi-PCR2, indicated the presence of an ehrlichia. The cross-reactivity already reported between the *Anaplasma* probe in RTi-PCR1 and *Ehrlichia* (Hurtado et al., 2015) would explain the positive result obtained for this sample in RTi-PCR1. Although we would not recommend the use of RTi-PCR1 for *Ehrlichia* detection, it is also clear that this PCR has to be used in combination with another species-specific assay like RTi-PCR2 or sequencing for definitive identification. Pairwise comparison of 1392 nt showed 2.7% dissimilarity in the 16S rRNA gene sequence of the ehrlichial agent found in badger and its closest hit, *E. chaffeensis*, suggesting that it corresponded to a new taxon within the *Ehrlichia* clade (Dumler et al., 2001). As far as we know *Ehrlichia* spp. have not been detected in badgers, although other carnivores are known to be susceptible to infection (Davidson et al., 1999; Torina et al., 2013). Additional analyses that integrate phenotypic and genotypic data are needed to further characterize this new taxon within a polyphasic taxonomic approach.

Most of the anaplasmas that have tropism for red blood cells infect cattle (*A. marginale*, *A. centrale*) or small ruminants (*A. ovis*), but all these species have been found also in wild ruminants (Aubry and Geale, 2011; De La Fuente et al., 2008). In the Basque Country, 13.0% of cattle were infected with *A. marginale* and 30.0% of sheep with *A. ovis* (García-Pérez, unpublished results). Consequently, albeit at lower prevalences, infection in cervids was expected. The absence of *A. marginale* and *A. ovis* would suggest that *I. ricinus*, the main tick species parasitizing cervids here, would not be acting as vector in the region; other potential tick vectors such as *Rhipicephalus* spp. or *Dermacentor* spp. present in wild ruminants might be involved instead. Interestingly, one red deer and one roe deer harbored DNA of *A. centrale*. This species is known to cause mild infections in cattle. Carelli et al. (2008) detected *A. centrale* in cattle (20/51) from different parts of Italy but they could not ascertain if it was the consequence of the spread of the bacterium after a vaccination trial done in Sicily, or corresponded to autochthonous strains circulating in Southern Europe (Ceci et al., 2008). This pathogen has been recently reported in naturally infected red deer in Spain (Portillo et al., 2011), which suggests that this species circulates in wild ruminants. However, neither clinical signs of infection nor tick vectors have been yet identified.

In this study, *A. bovis* was identified by sequencing in 4 roe deer. *A. bovis* infects circulating monocytes and it is closely related to *A. phagocytophilum* (Ybanez et al., 2014). It has been mainly reported in African cattle and ticks and wildlife from Japan and Korea (Kang et al., 2011), but in general it is a poorly studied species due probably to its limited impact in veterinary medicine. Further studies are needed to fully confirm if this species is widespread among livestock and wild ruminants in Spain. Moreover, a new species of anaplasma has been identified in white-tailed deer, *Anaplasma odoicolei* (Tate et al., 2013). It parasites platelets and is related to the canine pathogen *A. platys*. Zobba et al. (2014) in Italy also found *Anaplasma* spp. closely related to *A. platys* in domestic and wild ruminants. Since *A. phagocytophilum, A. bovis*, and *A. platys* are genetically very close species, further studies are needed to undoubtedly establish the distribution of anaplasma species in roe deer in our area.

5. Conclusion

The use of real-time PCR complemented with sequencing, revealed an interesting diversity of Anaplasmataceae in wildlife. The high prevalence of *A. phagocytophilum* in cervids was the most relevant result. Nevertheless, characterization studies are needed to ascertain the zoonotic potential of the strains infecting cervids in northern Spain, and their significance for public health. Moreover, this is the first report of *A. bovis* and *A. centrale* in the region and the first identification of a novel *Ehrlichia* sp. in badger. Whether these species may adapt to domestic ruminants and the clinical consequences of this adaptation are yet to be investigated. Further studies are also needed to identify the tick species that act as their vectors in the region.

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