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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.