Occurrence and genetic diversity of piroplasms and other apicomplexa in wild carnivores

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SUMMARY

Tick-borne apicomplexan haemoparasites infect wild and domestic animals, but studies on their distribution among free-living animals are comparatively fewer. Tissues from 241 wild carnivores of eight Mustelidae, two Canidae, one Viverridae and one Felidae species were collected in Northern Spain, and analysed by real-time PCR targeting the 18S rRNA gene and sequencing. Babesia vulpes (formerly known as Theileria annae) was the only piroplasm detected in red foxes (Vulpes vulpes). Badgers (Meles meles) were shown to harbour two novel Babesia sp. sequence types (A and B) that only shared 96.7% homology between them and were closely related (ca. 97–98%) to, but distinct from B. vulpes and other babesia from carnivores. Analysis of PCR-derived sequences also revealed the presence of Cytoisiospora sp. and Hepatozoon sp. in badgers and showed that wild cats (Felis silvestris catus) were infected with Cytauxzoon sp. Forty-two per cent of the animals subjected to a detailed external examination were parasitized by ixodid ticks, being Ixodes hexagonos and Ixodes ricinus the most abundant species. This study provided novel data on the different haemoparasites that can infect European wild carnivores and showed that they can be hosts for a range of haemoparasites and pose a potential risk for transmission to domestic animals.

Key words: piroplasms, Babesia sp., Babesia vulpes, Cytauxzoon, Hepatozoon, wildlife, ticks, 18S rRNA gene.

INTRODUCTION

Piroplasmoses are tick-borne diseases caused by apicomplexan protistan haemoparasites of the genera Babesia, Theileria and Cytauxzoon that have a great economic, veterinary and medical impact worldwide. While Theileria and Cytauxzoon species infect mainly ungulates and felids, respectively, Babesia spp. infect a wide range of avian and mammalian hosts, including humans (Dobbelaere and Kuenzi, 2004; Meinkoth and Kocan, 2005; Schnitger et al. 2012). Babesiidae are considered the second most common haemoparasites of mammals after trypanosomes (Schnittger et al. 2012). Many Babesia species of domestic carnivores and ungulates have been reported in genetically close-related wildlife species, causing clinical manifestation when placed in captivity in non-native areas (Penzhorn, 2006). Babesiosis is considered a worldwide emerging zoonosis with Babesia microti and Babesia divergens being the most common causes of human babesiosis in the USA and/or Europe (Yabsley and Shuck, 2013). Wildlife species are reservoir for several Babesia spp. (Yabsley and Shuck, 2013) but the vectors of many species are still unknown. Theileria annae, which is phylogenetically close to zoonotic B. microti, was first described in a German dog after travelling to Galicia (Zahler et al. 2000), a northwestern region of Spain where this species is considered endemic in domestic dogs (Canis lupus familiaris) (Camacho, 2006). Since then, it has been identified in dogs and foxes in North America and Europe (Gimenez et al. 2009; Birkenheuer et al. 2010; Dezdek et al. 2010; Cardoso et al. 2013; Najm et al. 2014; Miro et al. 2015). Phylogenetic studies carried out recently (Baneth et al. 2015), confirmed that T. annae does not belong to the genus Theileria and that it can be clearly distinguished from B. microti infecting rodents, macaques, and humans. Instead, it segregates into a single clade within the group of Babesia that infect carnivores. Since the red fox (Vulpes vulpes) seems to be its natural reservoir, authors proposed to re-name this haemoparasite as Babesia vulpes, replacing all others synonyms previouly used such as Babesia ‘Spanish dog’ isolate, B. microti-like or Babesia annae (Baneth et al. 2015). Ixodes hexagonos is suspected to be the tick vector of this agent (Camacho et al. 2003). Furthermore, a new piroplasm related to B. vulpes was found in a European badger (Meles meles) in Spain (Gimenez et al. 2009), but knowledge about this species is still limited.
In the past decades, populations of badgers and red foxes have increased and are adapted to peri-
urban and urban environments across Europe (Gloor et al. 2001; Judge et al. 2014). Therefore, these carnivores represent a possible source of pathogens for domestic animals and humans. This study was carried out to investigate by molecular methods piroplasms infection in wild carnivores, including Canidae, Mustelidae, Felidae and Viverridae species from Northern Spain.

MATERIALS AND METHODS

Study site

The study was carried out in two autonomous regions in northern Spain (Asturias and the Basque Country). Samples were collected from 121 sites located in the Basque Country and 28 from Asturias. The Basque Country (7234 km2) has an Atlantic Climate, with mild to slightly hot summers (AEMET, 2011). The temperate climate of the region allows for a varied and abundant wildlife fauna. Regarding wild carnivores, the most abundant species are badger and red fox. Asturias (10 603 km2) has also an Atlantic Climate but precipitations are more abundant and winters are colder than in the Basque Country, with frequent snow falls in the mountainous areas from October till May (AEMET, 2011). Wildlife in this region is very abundant and diverse, including carnivores such as red fox, badger, wolf (Canis lupus) and the endangered brown bear (Ursus arctos). Climate and host abundance do also allow for high burdens of ticks in the environment (Barandika et al. 2006).

Wildlife sampling

Sample collection was conducted in the frame of a larger study on surveillance of zoonotic infectious diseases in wild animals in both Spanish regions. A total of 241 wild carnivores (169 collected in the Basque Country and 72 in Asturias), belonging to eight Mustelidae, two Canidae, one Viverridae and one Felidae species were processed (Table 1). Red foxes were hunted mostly in winter (65%). The remaining wild species were collected throughout the year after being found dead or moribund. A complete necropsy was performed at the laboratory. Spleen tissue samples were stored at −20 °C before being processed for PCR analysis. Whenever possible, animals were subjected to a detailed external examination and attached ticks were removed, counted and identified using specific taxonomic keys (Manilla, 1998).

Molecular analysis

DNA extraction was performed on individual spleen samples using BioSprint 96 DNA kit (Qiagen) with a previous treatment with proteinase K for 3 h. Negative extraction controls were included every ten samples to rule out any possible contamination in the extraction procedure. Presence of piroplasms was firstly determined using a real-time PCR assay (RTI-PCR) that targets the 18S rRNA gene of the genera Babesia and Theileria; the assay also includes an internal amplification control to monitor for possible inhibition. 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). Samples were analysed along with the extraction negative controls and, at least two non-template negative controls (sterile molecular-grade water) per amplification reaction were included.

PCR-positive samples were identified by sequencing analysis of either the V4 region or the nearly complete 18S rRNA gene (one representative for each taxon). Briefly, an about 1600-bp fragment of the 18S rRNA gene was amplified from all positive samples using primers 18STBF (5′-AACCTGGTTGATCCTGCAGTCT-3′) and 18STBR (5′-GATCCTCTCCTGAGGTTCACC-3′) and about 750 nt, that included the V4 region, were sequenced using primer RLB-R2 (5′-CTAAGAATTTCACCTCTGACAGT-3′). Larger fragments were sequenced for one representative of each taxon using primers Nbab1_F (5′-AAGCCATGCTGTCCTAAGTATTACGTCTT-3′) and Nbab1_R (5′-CTCTCTCTCTCTCTTAAGTGTATGATCCTAC-3′) or specific primers designed by primer walking for Hepatozoon sp. Sequencing reactions were carried out using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and products were analysed on an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Nucleotide sequence accession numbers

Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers: KT223483–86, KU198329–30.

Data analysis

The obtained sequences were identified by comparison with the GenBank database by homology searches made at 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). Selected taxa for the analysis of 18S rRNA gene sequences included the closest BLASTN hits to the sequences described here,
along with representatives of *B. microti* and other *Babesia* species, including those that infect carnivores. *Toxoplasma gondii* and *Eimeria maxima* were included as outgroup to root the tree. The phylogenetic tree was then inferred by using the Maximum Likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993) and the Gamma distributed with Invariant Sites (G+I) option for variation in rates among sites (model with the lowest Bayesian Information Criterion). Accuracy of inferred topology was assessed via bootstrap analysis (Felsenstein, 1985) of 1000 replicates.

Prevalences of piroplasm infection and different piroplasms species in relation to ticks and carnivore species were compared with Chi-square and Fisher exact tests. Statistical analyses were carried out with the SAS statistical package (SAS Institute 9.3, Cary, NC, USA). *P* values <0.05 were considered statistically significant.

**RESULTS**

DNA isolated from spleen samples from the 241 carnivores were analysed by real-time PCR, detecting a positive signal in 90 (37.3%) of them. Nine wild carnivore species were negative, though in six of them only five or fewer specimens were analysed. Positive animals included wild cats (*Felis silvestris catus*) (4/6; 66.7%), badgers (64/122; 52.5%) and red foxes (22/48; 45.8%) (Table 1).

A total of 68 samples were successfully sequenced producing six different sequence types (Table 2); sequencing attempts failed for a further 22 samples (5 foxes and 17 badgers). The four samples from wild cat were identical among themselves and to *Cytauxzoon* sp. from a Eurasian lynx (*Lynx lynx*) from Romania (GenBank accession no. KT361080). Close homology (99.7%) was also found to *Cytauxzoon* sp. from wild cats from Romania (KT361072), an Iberian lynx (*Lynx pardinus*) from Spain (AY496273) or a domestic cat (*F. silvestris catus*) from France (EU622908). The 17 amplicons sequenced from red foxes were confirmed as being 100% identical to *B. vulpes* (AF188001—*Babesia* sp. ‘Spanish dog’). Conversely, variability was found in haemoparasites infecting badgers, where four different sequence types of three different genera were identified. In four badgers, a sequence closely related to *Cystoisospora* spp. was found (99.2% identical to *Cystoisospora timoni* EU200792 from *Suricata suricatta* and 99.1% to *Cystoisospora belli* JX025651). In one of them, *Cystoisospora* sp. was present as a mixed infection with an *Hepatozoon* species 99.7% (1635/1640 nt) identical to that found

### Table 1. Distribution of infection as determined by RTi-PCR and tick infestation among wild carnivores

<table>
<thead>
<tr>
<th>Host</th>
<th>N animals</th>
<th>RTi-PCR Pos</th>
<th>Animals with ticks</th>
<th>N ticks</th>
<th>Tick species identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canidae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red fox (<em>Vulpes vulpes</em>)</td>
<td>48</td>
<td>22</td>
<td>22</td>
<td>101</td>
<td>57 Ih; 21 Ir; 21 Dr; 2 Hp</td>
</tr>
<tr>
<td>Wolf (<em>Canis lupus</em>)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>na</td>
</tr>
<tr>
<td>Mustelidae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European badger (<em>Meles meles</em>)</td>
<td>122</td>
<td>64</td>
<td>61*</td>
<td>257</td>
<td>169 Ih; 33 Ir; 14 Ic; 6 Dr; 14 Rp; 1 Rb; 14 Isp; 6 Hsp</td>
</tr>
<tr>
<td>Stone marten (<em>Martes foina</em>)</td>
<td>20</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>12 Ih</td>
</tr>
<tr>
<td>Pine marten (<em>Martes martes</em>)</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>11</td>
<td>6 Ih; 5 Ir</td>
</tr>
<tr>
<td>Weasel (<em>Mustela nivalis</em>)</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2 Ih</td>
</tr>
<tr>
<td>Polecat (<em>Mustela putorius</em>)</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>4 Ih</td>
</tr>
<tr>
<td>American mink (<em>Mustela vison</em>)</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>18</td>
<td>1 Ih; 17 Isp</td>
</tr>
<tr>
<td>Otter (<em>Lutra lutra</em>)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>na</td>
</tr>
<tr>
<td>Stoat (<em>Mustela erminea</em>)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>na</td>
</tr>
<tr>
<td>Viverridae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common genet (<em>Genetta genetta</em>)</td>
<td>13</td>
<td>0</td>
<td>4</td>
<td>45</td>
<td>1 Hp; 37 Ir; 7 Isp</td>
</tr>
<tr>
<td>Felidae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild cat (<em>Felis silvestris catus</em>)</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>3 Hp</td>
</tr>
<tr>
<td>Total</td>
<td>241</td>
<td>90</td>
<td>100</td>
<td>453</td>
<td>251 Ih; 96 Ir; 27 Dr; 14 Ic; 14 Rp; 6 Hp; 1 Rb; 38 Isp; 6 Hsp</td>
</tr>
</tbody>
</table>


* Three animals were not subject to external examination.

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in European pine marten (Martes martes) (EF222257). However, the most abundant haemoparasite among badgers was Babesia, represented by two sequence types referred here as Babesia sp. badger types A and B. Both types only shared 96.7% (1275/1318) similarity between them and about 97–98% homology with their best hits in GenBank, AF188001 (Babesia sp. ‘Spanish dog’) and FJ645725 (Theileria sp. HN2), and were found in 36 and 7 badgers, respectively (Table 2). Phylogenetic analysis of the babesias found in this study confirmed the identity of the piroplasms from red fox as B. vulpes and showed that the two Babesia sp. from badger clearly clustered with Babesia spp. that infect carnivores (Fig. 1).

One hundred of the 238 animals externally examined for ticks harboured at least one tick (Table 1), with a maximum of 30 ticks collected in one badger. A total of 453 ticks of seven different species (44 were not identified to species level) were collected, 10.1% being larvae, 47.5% nymphs and 42.4% adults. Badgers (50.0%) and red foxes (45.8%) were the most parasitized species, the exception being American mink (Mustela vison) (100%, but only two specimens were examined). Ixodes hexagonus was the most abundant tick among Mustelidae and Canidae species, while I. ricinus was more abundant (37/38) in common genets (Genetta genetta). No statistically significant differences in piroplasm infection in relation with tick parasitization were found.

**DISCUSSION**

The present study provides evidence for piroplasm infection in wild carnivores from northern Spain, including red foxes, badgers and wild cats. Infection with B. vulpes, formerly known as T. annae (Baneth et al. 2015), has been reported to cause severe illness in dogs (Camacho, 2006), and red foxes seem to be an important reservoir of this species in Spain and in other European and American countries (Gimenez et al. 2009; Birkenheuer et al. 2010; Dezdek et al. 2010; Cardoso et al. 2013; Najm et al. 2014). In this investigation, B. vulpes was the only haemoparasite detected in red fox. It was identified in 17 of 48 analysed red foxes (35.4%), which represents a higher proportion than that reported in the neighbouring county of Burgos (1/5, 20.0%) (Gimenez et al. 2009) but clearly lower than that found in Portugal in a larger study (63/91, 69.2%) (Cardoso et al. 2013). Other Babesia species such as B. canis and B. gibsoni identified in red foxes (Penzhorn, 2006; Cardoso et al. 2013) were not detected in any of the red foxes analysed in the study herein. Babesiosis by B. canis is frequently diagnosed in dogs in these two regions, but the first two clinical cases of dogs infected by B. vulpes have only recently been reported in Asturias (Miro et al. 2015), and as far as we know there are no published reports in the Basque Country. Conversely, B. vulpes infection is considered endemic in dogs in Northwestern Spanish regions such as Galicia (Camacho, 2006), whereas in Central and Southern Spain its prevalence is lower (Criado-Fornelio et al. 2003). However, the observation of this parasite in blood smears is difficult, particularly when parasitemia is low, and the disease might be underdiagnosed. Two new Babesia sp. badger sequence types were found in the European badger from both regions studied here, type A being five times more prevalent than type B. Both types were different to each other, to B. vulpes and to piroplasms described elsewhere, including the new piroplasm identified from badgers in Burgos (Gimenez et al. 2009). In the phylogenetic analysis, the two Babesia sp. from badger clearly clustered with Babesia spp. that infect carnivores. Babesia sp. badger type B clearly branched with a babesia identified from a badger in Burgos (FJ225390) despite sharing only 96.5% homology in pairwise comparisons. The specific position of Babesia sp. badger type A in relation to the clade of B. vulpes and its sister clade of Babesia sp. from raccoon (Procyon lotor) could not be significantly

<table>
<thead>
<tr>
<th>Source</th>
<th>n samples sequenced</th>
<th>GenBank</th>
<th>% homology (positions compared) with nearest Blast hit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red fox (Vulpes vulpes)</td>
<td>17</td>
<td>KT223483 Babesia vulpes</td>
<td>100% (1607) Babesia sp. ‘Spanish dog’ AF188001</td>
</tr>
<tr>
<td>European badger (Meles meles)</td>
<td>36</td>
<td>KT223484 Babesia sp. badger type A</td>
<td>98.6% (1535) Theileria sp. HN2 FJ645725</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KT223485 Babesia sp. badger type B</td>
<td>97.0% (1323) Babesia sp. ‘Spanish dog’ AF188001</td>
</tr>
<tr>
<td></td>
<td>4*</td>
<td>KU198329 Cystoisospora sp.</td>
<td>99.2% (1574) Cystoisospora timoni EU200792</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>KU198330 Hepatozoon sp.</td>
<td>99.7% (1640) Hepatozoon sp. European pine marten EF222257</td>
</tr>
<tr>
<td>Wild cat (Felis silvestris catus)</td>
<td>4</td>
<td>KT223486 Cyttauxzoon sp.</td>
<td>100% (397) Cyttauxzoon sp. KT361080</td>
</tr>
</tbody>
</table>

* One European badger harboured a co-infection with Cystoisospora sp. and Hepatozoon sp.
established (low bootstrap values regardless of the method used to infer the tree; data not shown).

The four Cytauxzoon sp. sequences obtained from wild cats were identical or closely related to sequences from lynxes or domestic and wild cats from Europe (Criado-Fornelio et al. 2004, 2009a; Luaces et al. 2005; Gallusová et al. 2016). Cytauxzoon felis is a haemoparasite of felids that may cause severe clinical disease with high mortality (Meinkoth and Kocan, 2005). Cytauxzoonosis is considered an emerging disease in the USA where it has been frequently reported in felids (Meinkoth and Kocan, 2005). The bobcat (Lynx rufus) appears to be the natural reservoir of C. felis in North America. In Europe, presence of this haemoparasite was first described in 2005 in an Iberian lynx (Luaces et al. 2005). Until recently, only a few cases of Cytauxzoon infection had been reported, but in 2012 the first endemic focus in Europe was described in domestic cats in Italy, reporting a high prevalence of infection (23%) (Carli et al. 2012). More recently, a high prevalence of Cytauxzoon infection was reported in wild cats (6/12) and in Eurasian lynx (4/4) in the Carpathian region of Romania (Gallusová et al. 2016). The similarly high proportion of positive wild cats found in our study (4/6) suggests that infection in wild felids in Europe might be more widespread than previously thought.

Besides these piroplasms, the detection of Hepatozoon sp. and Cystoisospora sp. in badgers (in one badger as a mixed infection) was an incidental finding that reflects only occurrence but not prevalence. However, to our knowledge this is the first description of Hepatozoon sp. in badger. Analysis of its 18S rRNA gene sequence showed that it was nearly identical (99.7%) to a new species, most closely related to, but distinct from Hepatozoon canis, described in pine martens in Scotland (Simpson et al. 2005) and in Spain (Criado-Fornelio et al. 2009b), indicating that the same species could infect different free-ranging mustelids. Cystoisospora spp. are ubiquitous intestinal protists of wild and domestic carnivores that can also invade extra-intestinal organs such as spleen of definitive and paratenic host (Dubey, 2014). Badgers are commonly infected with Cystoisospora melis (Pellérdy, 1955), however, sequences of this coccidium are not available and published records are based on the morphology of oocysts isolated from badgers’ fecal samples (Anwar et al. 2000). In fact, only a few Cystoisospora spp.
have been sequenced, *C. melis* not being among them. Ultimately, further studies are needed to estimate the true prevalence of these protists in wild carnivores.

Climatic and environmental conditions in Northern and Northwestern Spain allow for high burdens of some ixodid ticks (*Barandikia et al. 2006*). The most abundant tick species collected from both red foxes and badgers in this study was *I. hexagonus*, which is the potential vector of *B. vulpes* in the region (*Camacho et al. 2003*). The high proportion of *I. hexagonus* among ticks collected from badgers might indicate its possible role as vectors of the new babesias found in badgers in this study. The number of wild cats and collected ticks was too low to infer any conclusions concerning possible tick vectors of *Cyttauxzoon* sp. On the other hand, and unlike the other tick-borne protists studied here, *Hepatozoon* transmission takes place by ingestion of the tick that acts as definitive host or by predation and ingestion of tissue forms from infected small wild mammals (*Baneth, 2011*).

In conclusion, the present study showed that *B. vulpes* is widespread among red foxes in northern Spain and demonstrated that badgers can harbour a high diversity of haemoparasite species, including the two different *Babesia* taxa described here for the first time, and different from *B. vulpes* and other babesia from carnivores. The presence of *Hepatozoon* sp. in Mustelidae is not novel (it has been reported in pine marten), but this is the first evidence of its presence in badgers specifically. The high proportion of wild cats infected with *Cyttauxzoon* sp. found in the small sample analysed here requires further investigation. In summary, this study provided novel data on the different haemoparasites that can infect wild carnivores.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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