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Presence of *Bartonella* Species in Wild Carnivores of Northern Spain

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The genus *Bartonella* was detected by PCR in 5.7% (12/212) of wild carnivores from Northern Spain. Based on hybridization and sequence analyses, *Bartonella henselae* was identified in a wildcat (*Felis silvestris*), *Bartonella rochalimae* in a red fox (*Vulpes vulpes*) and in a wolf (*Canis lupus*), and *Bartonella* sp. in badgers (*Meles meles*).

The zoonotic vector-borne pathogens present complex cycles in nature, which include reservoir hosts and hematophagous arthropods, which play a role as vectors. Among them, the genus *Bartonella* includes more than 30 species or subspecies that can affect mammals worldwide, and at least 14 are considered to be pathogenic to humans (26). In Spain, six different *Bartonella* species have been described so far (*B. henselae*, *B. quintana*, *B. clarridgeiae*, *B. taylorii*, *B. alsatica*, and *B. vinsonii* subsp. *berkhoffii*), affecting humans, cats, dogs, rabbits, and small mammals (10, 19, 21, 23–25). More recently, the use of new molecular tools has allowed us to detect a large repertoire of *Bartonella* species and genotypes circulating among the small mammals in the Basque Country, Northern Spain (12).

In Europe, as well as in the Basque Country, there is little information regarding the prevalence of vector-borne diseases in carnivores, and given their intense exposure to arthropod vectors, they could represent a good marker for the circulation of these pathogens. Herein we present our results on the presence of *Bartonella* isolates in carnivores collected in the Basque Country.

Between 2001 and 2006, 212 wild carnivores, belonging to 10 different species (Table 1), that were either found dead or hunted (the majority of the red foxes) were collected in the Basque Country. Carcasses were transported to the laboratory for a complete necropsy, and tissue samples were collected and stored individually. Whenever possible, animals were subjected to a detailed external examination for ectoparasite collection and identification (13, 18).

The presence of *Bartonella* spp. was analyzed with a seminested PCR targeting the citrate synthase gene (*gltA*), using primers CS 140f (6) and BhCS1137n (20) in the first run and BhCS781p and BhCS1137n (20) in the second, following the author's protocols with minor modifications. DNA extraction was performed with a QIAamp DNA blood minikit (Qiagen, Hilden, Germany) from a homogenate of liver and spleen previously digested with proteinase K (Invitrogen, Carlsbad, CA). DNA samples were also analyzed by following three different PCR-reverse line blot hybridization (PCR-RLB) protocols previously described to detect *Coxiella burnetii*, *Anaplasma phagocytophilum*, spotted fever group rickettsiae, *Borrelia* spp., and *Francisella* spp. (2, 17).

Samples positive for *Bartonella* spp. were confirmed by a PCR-RLB specific for *Bartonella*, targeting the 16S rRNA and the 16S-23S rRNA intergenic transcribed spacer (ITS), and by sequencing (10, 12). Sequences were compared with those available in the GenBank database by nucleotide sequence homology using BLAST (1) to identify the closest relative. Furthermore, the *gltA* sequences were aligned with reference sequences from GenBank with the ClustalX software (15).

TABLE 1 Presence of *Bartonella* species in wild carnivores from the Basque Country

Host species	Scientific name	No. of animals	No. *Bartonella* positive (%)	Species
American mink	*Mustela vison*	3	0	
Beech marten	*Martes foina*	26	0	
Eurasian badger	*Meles meles*	75	9 (12)	*Bartonella* sp.
Pine marten	*Martes martes*	14	0	
Polecat	*Mustela putorius*	5	0	
Red fox	*Vulpes vulpes*	62	1 (1.6)	*Bartonella rochalimae*
Small-spotted genet	*Genetta genetta*	13	0	
Weasel	*Mustela nivalis*	5	0	
Wildcat	*Felis silvestris*	6	1 (16.7)	*Bartonella henselae*
Wolf	*Canis lupus*	3	1 (33.3)	*Bartonella rochalimae*
Total		212	12 (5.7)	

Pairwise distance matrices were determined with the Kimura two-parameter method with MEGA3.1 software (16), and phylogenetic trees were constructed by applying the neighbor-joining algorithm with the internal-branch test for evaluation of their topology, using 1,000 replicates. The dendrogram was collapsed by using a cutoff bootstrap value of 50 (Fig. 1).

Fisher's exact test was applied to determine statistical differences between some variables in the prevalence of *Bartonella* (age and sex of carnivores, annual season in which they were collected, and ectoparasites present on them). The overall level of statistical significance was the standard 5% ($P < 0.05$).

The presence of *Bartonella* spp. was determined in 12 samples, giving an overall prevalence of 5.7% (12/212). Four carnivore species were found to be infected with three different *Bartonella* ge-

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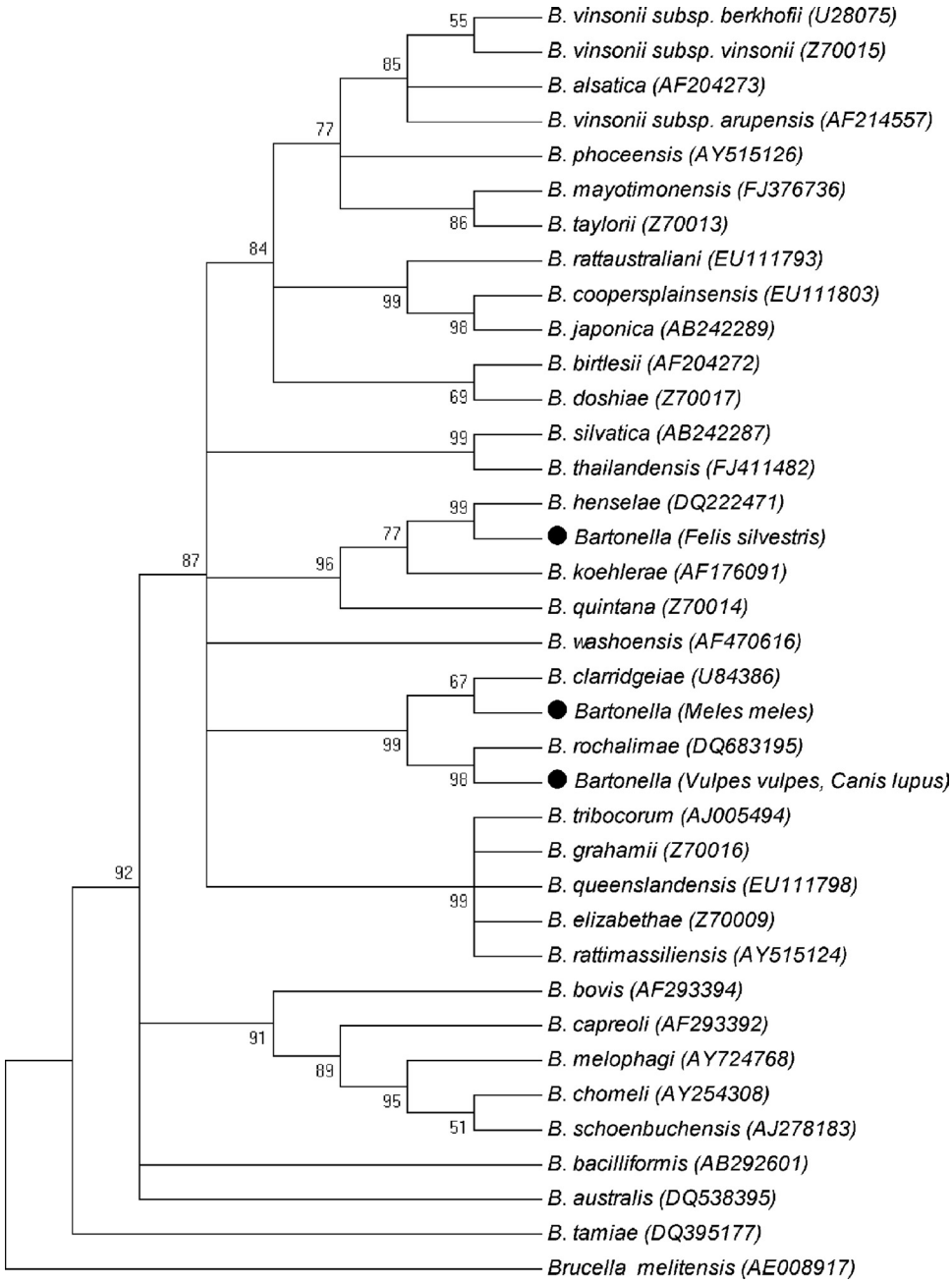

FIG 1 Neighbor-joining tree based on *gltA* gene sequences, built with reference sequences from GenBank and those obtained in this study (black dots). GenBank accession numbers are indicated within brackets.

notypes (Table 1). The distribution of the positive animals, grouped by age, sex, location, and season, is shown in Table 2.

Regarding age, 11.3% (6/53) of the young animals showed positive results, compared to 4.7% (6/129) of the adults ($P = 0.1$), which is in agreement with several studies developed in cats and dogs (8, 22), probably due to an immune system that is not fully developed in young specimens or to the acquisition of immunoresistance in the adults after successive contacts with these pathogens.

By sex, the percentages of positive males (7.1%; 9/127) and females (3.7%; 3/81) were similar ($P = 0.2$). Seasonally, similar percentages of specimens collected during winter (6.9%; 6/87) and spring (7.7%; 4/52) had positive results, while those specimens found during summer (1/34) and autumn (1/39) showed a prevalence rate below 3% (in all cases, the P value was >0.3). Geographically, a higher number of positive animals corresponded to individuals collected in the South of the Basque Country (10.2%; 10/98) than in the North (1.8%; 2/114) ($P = 0.01$). This lower *Bartonella* abundance might represent less adequate environmental conditions and, therefore, a higher risk of stress for the microorganism's survival. More detailed studies should be carried out to determine the factors present in each area that favor the transmission of these bacteria.

TABLE 2 Carnivores infected with *Bartonella* spp., location and season of collection, and ectoparasites found on them

Host species	Age	Sex	Location	Season	Ectoparasite(s) found		
					Lice[a]	Fleas[b]	Ticks[c]
Eurasian badger	Young	Male	South	Winter	*T. melis*		*I. hexagonus*
Eurasian badger	Young	Male	South	Winter	*T. melis*		*I. ricinus*
Eurasian badger	Adult	Male	South	Winter	*T. melis*	*P. melis*	*I. hexagonus*, *I. ricinus*
Eurasian badger	Young	Male	South	Spring	*T. melis*		*I. hexagonus*, *I. ricinus*
Eurasian badger	Adult	Female	South	Winter	*T. melis*	*C. trichosa*	*I. canisuga*
Eurasian badger	Adult	Male	North	Winter	*T. melis*	*C. trichosa*	*Ixodes* sp.
Eurasian badger	Adult	Male	North	Winter		*C. trichosa*	*D. reticulatus*
Eurasian badger	Adult	Male	South	Spring			*R. pusillus*
Eurasian badger	Adult	Female	South	Autumn	*T. melis*	*C. trichosa*	*R. pusillus*
Red fox	Young	Female	South	Spring		*P. melis*	*Ixodes* sp.
Wildcat	Young	Male	South	Spring			
Wolf	Young	Male	South	Summer	*T. canis*		*I. ricinus*

[a] *T. melis*, *Trichodectes melis*.
[b] *P. melis*, *Paraceras melis*; *C. trichosa*, *Chaetopsylla trichosa*.
[c] *I. hexagonus*, *Ixodes hexagonus*; *I. ricinus*, *Ixodes ricinus*; *I. canisuga*, *Ixodes canisuga*; *D. reticulatus*, *Dermacentor reticulatus*; *R. pusillus*, *Rhipicephalus pusillus*.

The *gltA* sequence obtained from the wildcat showed a 100% similarity with *B. henselae* (GenBank accession no. DQ222471). In the case of the samples from the fox and the wolf, the *gltA* sequences showed 100% similarity with *B. rochalimae* (GenBank accession no. DQ683195). It is noteworthy that a high percentage of badgers (12%) was infected with *Bartonella* sp. The nine *gltA* sequences obtained from badgers were identical among them (GenBank accession no. GU570947) and did not correspond to any of the sequences deposited in GenBank. Regarding the percentage of similarity, the closest relative was *B. clarridgeiae*, with 96.4% similarity (GenBank accession no. U84386). This result was also supported by the dendrogram, where the badger's sequence was placed in the same clade as *B. clarridgeiae* (Fig. 1). Moreover, the sequences of 16S rRNA and ITS (GenBank accession no. EU098127 and EU98132, respectively) from the 9 badgers were also identical among them and were also closely related to *B. clarridgeiae* (10). The future isolation in culture of this organism and a further characterization of additional genes will clarify the taxonomic position of this bacterium.

The results shown here indicate that wild carnivores in the Basque Country are infected with at least three different *Bartonella* species. *B. henselae* and *B. rochalimae* are zoonotic agents (7, 9, 27), and their description in three species of wild carnivores may suggest that these animals may be important in the maintenance of this pathogen in wild ecosystems in the Basque Country and that transmission to humans may be possible through the bite of arthropod vectors that have previously fed on them. On the other hand, the identification of *B. rochalimae* in a wolf (33.3%) and a fox (1.6%) constitute the first description of this *Bartonella* species in Spain, although it has previously been described in raccoons, coyotes, and foxes in the United States, France, and Hungary (14). Although the competent reservoir host for *B. rochalimae* is still unknown, these data and our results point out that carnivore species could play this role. Finally, the third species is strongly associated with badgers, and its risk for human health remains unknown.

Ticks, fleas, and lice were collected from the carnivores, and the percentages of infestation are summarized in Table 3, with badgers and foxes being the most parasitized species. Among carnivores infected with *Bartonella* spp., the majority were infested by ticks (11/12), and in 88.9% of cases by *Ixodes* sp. (Table 2), the more frequent tick genus in the area (3). Also, most of them were infested by lice (8/12), mainly by *Trichodectes melis* (77.8%), and fleas (6/12), mainly by *Chaetopsyla trichosa* (55.6%). Animals with positive results showed the highest prevalence of infestation by ectoparasites (91.7%; $P = 0.03$). More specifically, animals with *Bartonella* spp. showed the highest prevalence of infestation by ticks (83.3%; $P = 0.006$) and lice (66.7%; $P = 0.005$) but not by fleas (50%; $P = 0.1$).

The presence of *Bartonella* spp. was also analyzed by *gltA* PCR

TABLE 3 Parasitization of animals by ticks, fleas, and lice

Host species	No. of animals parasitized/total no. examined (%)			
	Any ectoparasite	Ticks	Fleas	Lice
American mink	3/3 (100)	3/3 (100)	1/3 (33.33)	0/3 (0)
Beech marten	9/26 (34.62)	6/26 (23.08)	4/26 (15.38)	6/26 (23.08)
Eurasian badger	59/75 (78.67)	40/75 (53.33)	26/75 (34.67)	47/67 (70.15)
Pine marten	3/14 (21.4)	2/14 (14.29)	2/14 (14.29)	0/14 (0)
Polecat	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
Red fox	39/62 (62.9)	33/62 (53.23)	22/62 (35.48)	0/61 (0)
Small-spotted genet	5/12 (41.67)	5/12 (41.67)	1/12 (8.33)	0/12 (0)
Weasel	2/5 (40)	1/5 (20)	1/5 (20)	0/5 (0)
Wildcat	1/6 (16.67)	1/6 (16.67)	0/6 (0)	0/5 (0)
Wolf	1/2 (50)	1/2 (50)	0/2 (0)	1/2 (50)

in 68 adult ticks collected from 12 badgers (36 ticks) and 9 foxes (32 ticks), with all the samples being negative. Lice and fleas could not be further analyzed because the identification methods employed and storage conditions left this material unable to be analyzed by PCR.

The carnivores with higher levels of infestation by ectoparasites (wolf, badger, and fox) are those with a higher rate of infection by *Bartonella* spp., except in the case of the wildcat, which did not show any ectoparasites. However, except in the case of hunted foxes, which were collected at the time of their death, the rest of the animals spent various times between death and the collection of carcasses, which may have caused the release of ectoparasites and, therefore, an underestimation of the prevalence of parasitism. Also, the host specificity of some ectoparasites (i.e., *T. melis* and *C. trichosa* in badgers) could be related to the *Bartonella*-host associations observed in this study. *Bartonella* bacteria are widely distributed among badgers in our study area, and the fact that most of the badgers were infested with lice, fleas, and ticks indicates a need to determine the vectors involved in its transmission.

All samples were negative for the presence of other vector-borne bacteria, although all of them, except *Francisella tularensis*, are well documented in our study area in ticks (2, 4, 5) and small mammals (2, 11). Therefore, the absence of these pathogens in the specimens studied suggests that these carnivore species may not be involved in the epidemiologic cycle of these pathogens in the Basque Country.

In summary, the results shown here indicate that wild carnivores in the Basque Country are infected with at least three different *Bartonella* species, two of them zoonotic and a third strongly associated with badgers, whose risk for human health is unknown.

Nucleotide sequence accession numbers. The *gltA* sequence of the *Bartonella* sp. found in badgers has been deposited in GenBank under accession no. GU570947.

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