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***BARTONELLA ROCHALIMAE* AND *B. VINSONII* SUBSP. *BERKHOFFII* IN WILD CARNIVORES FROM COLORADO, USA**

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Abstract

Spleen samples from 292 wild carnivores from Colorado were screened for *Bartonella* infection. *Bartonella* DNA was detected in coyotes (*Canis latrans*) (28%), striped skunks (*Mephitis mephitis*) (23%), red foxes (*Vulpes vulpes*) (27%), and raccoons (*Procyon lotor*) (8%) but not in black bears (*Ursus americanus*), gray foxes (*Urocyon cinereoargenteus*), and mountain lions (*Puma concolor*). Two *Bartonella* species, *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*, were identified. All 10 infected striped skunks exclusively carried *B. rochalimae* while coyotes, red foxes, and raccoons could be infected with both *Bartonella* species. Five of seven infected coyotes carried *B. v. berkhoffii* whereas five of seven infected red foxes and 11 of 14 infected raccoons carried *B. rochalimae*. Further studies are needed to understand relationships between *Bartonella* species, wild carnivores, and their ectoparasites.

Keywords

Bartonella vinsonii subsp. *berkhoffii*; *Bartonella rochalimae*; Colorado; wild carnivores; wildlife diseases

INTRODUCTION

The bacteria of genus *Bartonella* are widely distributed among a variety of wild mammals and associated arthropod vectors. *Bartonella* species are usually host-specific, although the specificity may be observed at different taxonomic levels of host animals (Jardine et al. 2006; Bai et al. 2013). The question of host specificity is important in regard to the survival and persistence of *Bartonella* species (Dehio 2004; Chomel et al. 2009). Most *Bartonella* species persist subclinically within mammalian reservoirs while some may induce pathologic effects in incidental hosts—mainly demonstrated in humans, cats (*Felis catus*), and domestic dogs (*Canis lupus familiaris*; Dehio 2004).

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Several studies have reported detection or isolation of *Bartonella* species in coyotes (*Cams latrans*), raccoons (*Procyon lotor*), gray foxes (*Urocyon cinereoargenteus*), island foxes (*Urocyon littoralis*), and other wildlife from multiple regions across the world, including the western US (Chang et al. 2000; Gerrikagoitia et al. 2012). Two *Bartonella* species, *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*, reported in wild mammals are also recognized as human pathogens. *Bartonella vinsonii* subsp. *berkhoffii* was originally isolated from a dog suffering infectious endocarditis (Kordick et al. 1996) and later was identified as a zoonotic agent causing endocarditis in a human patient (Roux et al. 2000). In California, *B. v. berkhoffii* was detected in coyotes and foxes, with higher prevalence in coyotes, suggesting that coyotes could be an important wildlife reservoir for *B. v. berkhoffii* (Chang et al. 2000).

Bartonella rochalimae was first described in a human patient exhibiting fever, myalgia, nausea, insomnia, mild anemia, and splenomegaly after returning to the US from Peru, where the patient experienced multiple insect bites (Eremeeva et al. 2007). This species was also reported in dogs with endocarditis (Henn et al. 2009b), gray foxes, raccoons, coyotes, and rats (*Rattus norvegicus*), with extremely high prevalence (42%) in red foxes (Henn et al. 2009a; Gundi et al. 2012). In addition, *B. rochalimae* was detected in fleas (*Pulex simulans*) collected on gray foxes (Gabriel et al. 2009), implying that the flea might be a vector for *B. rochalimae*.

Recognized as the main cause of cat scratch disease in humans (Chomel 2000), *Bartonella henselae* has been reported in wild felids, arctic foxes (*Vulpes lagopus*), mongooses (*Herpestes auropunctatus*), and palm civets (*Paguma larvata*) (Sato et al. 2013) in addition to the well-known domestic cat hosts (Chomel et al. 2002). Two more *Bartonella* species associated with domestic cats and recognized as human pathogens (*B. clarridgeiae* and *B. koehlerae*) have also been reported in wild animals (Kaewmongkol et al. 2011; Hwang and Gottdenker 2013). An isolate from the Japanese marten (*Martes melampus*) is closely related to *Bartonella washoensis* (Sato et al. 2012), a species commonly associated with sciurids and a source of human cardiac disease (Kosoy et al. 2003). Due to the zoonotic potential of various *Bartonella* species, information about the presence of *Bartonella* species in wild animal reservoirs is valuable from a public health standpoint.

Early investigations of *Bartonella* species in foxes, raccoons, and other carnivores were mostly based on detection of bacteria or bacterial DNA in animal blood. We report findings of *Bartonella* species in spleens of wild carnivores including foxes, coyotes, raccoons, and skunks from Colorado. *Bartonella* DNA has been detected in spleens of coyotes previously, although the prevalence was low (3/70 positive; Kehoe 2014). Our study provides additional evidence that spleen tissues can be used for *Bartonella* testing when blood is not available.

MATERIALS AND METHODS

Sample collection for this study was approved by the US Department of Agriculture, National Wildlife Research Center Quality Assurance Unit, and authorized by a Colorado Parks and Wildlife permit.

Study sites and sample collection

Spleen samples from 292 wild carnivores were mostly collected in Larimer, Boulder, and Weld counties of Colorado during 2013 and 2015 during the enhanced rabies surveillance. Carcasses were sourced from a subset of public health rabies submissions (pet/human contact cases) and supplemented with no-contact cases from county health departments, Colorado Parks and Wildlife, and local humane societies or wildlife rehabilitation centers. For no-contact cases, sampling was targeted toward animals with neurologic symptoms that had been humanely euthanized by local authorities or animals that were found dead. Necropsy was performed on carcasses of 44 striped skunks (*Mephitis mephitis*), 186 raccoons, 25 coyotes, 26 red foxes, one gray fox, seven black bears, and three mountain lions (Table 1), and spleens were removed and stored in individual containers. Instruments used for necropsy were cold-sterilized and autoclaved before use. Following necropsy, samples were stored at -80°C until testing.

DNA extraction and PCR detection

The spleens were homogenized using Bullet Blender® Gold homogenizer (Next Advance, Averill Park, New York, USA) following the spleen protocol provided by the manufacturer. The homogenates were then used for DNA extraction using QIAextractor (Qiagen, Valencia, California, USA) following the tissue protocol provided by the manufacturer. For initial screening of *Bartonella* DNA, we performed a PCR assay targeting the 16S–23S internal transcribed spacer (ITS) by using *Bartonella*-specific primers 325s (CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG) and HOOas (GAA CCG ACG ACC CCC TGC TTG CAA AGC A). Any ITS-positive samples were further analyzed by two additional genes, citrate synthase gene (*gitA*) and cell division gene (*ftsZ*), using a nested PCR platform. Two pairs of specific primers were applied for each target (e.g., primers CS443F (GCT ATG TCT GCA TTC TAT CA; Jardine et al. 2005) and CS1210R (GAT CYT CAA TCA TTT CTT TCC A; Gundi et al. 2012) as the outer set and Bhcs.781p (GGG GAC CAG CTC ATG GTG G) and Bhcs.1137n (AAT GCA AAA AGA ACA GTA AAC A; Norman et al. 1995) as the inner set for *gitA*; primers Bfpl (ATT AAT CTG CAY CGG CCA GA) and Bfp2 (ACV GAD ACA CGA ATA ACA CC; Zeaiter et al. 2002) were used as the outer set and the newly designed primers R83 (ATA TCG CGG AAT TGA AGC C); and L83 (CGC ATA GAA GTA TCA TCC C) as the inner set for *ftsZ*. Positive and negative controls were included in each PCR run to evaluate the presence of appropriately sized amplicons and contamination, respectively. Only samples with PCR products that resulted in clear sequence information were considered as positive for further analysis.

Sequencing and identification of *Bartonella* species

Amplicons of appropriate size compared to the positive controls were further identified by sequencing. All *Bartonella*-positive PCR products from any of the three targets were purified using a QIAquick PCR Purification Kit (Qiagen) and then sequenced in both directions with the same primers which were used for the initial PCR screening (inner primers for *gitA* and *ftsZ*). Using Lasergene Version12 (DNASTAR, Madison, Wisconsin, USA), *Bartonella* sequences from all samples were compared among themselves and with other known

Bartonella species or genotypes that were available from GenBank. Newly identified sequence variants were submitted to GenBank.

RESULTS

Molecular detection

Using PCR assays targeting ITS we detected *Bartonella* DNA in 38 of 292 samples (13%). We detected *Bartonella* DNA at varying prevalences in coyotes, striped skunks, raccoons, and red foxes but not in black bears, gray foxes, or mountain lions (Table 1). Among 38 ITS-positive samples, 36 were also positive by *gItA* and 33 samples were positive by *ftsZ*. Overall, 32 samples were *Bartonella*-positive by all three targets, and six samples were positive by two targets. All amplicons were considered positive only after confirmation as *Bartonella* species by sequencing of the targeted genes.

Identification of *Bartonella* species

Sequencing analysis of all three targets demonstrated that the *Bartonella* species detected in the carnivores were either *B. v. berkhoffii* or *B. rochalinae*. Six ITS genetic variants were identified among the 38 positive samples with three variants of *B. v. berkhoffii* in 10 samples and three variants of *B. rochalinae* in 28 samples (Fig. 1). Four variants were identical to previously published sequences (DQ059763, DQ059765, DQ676487, and DQ676491) identified in dogs with valvular endocarditis (Maggi et al. 2006; Henn et al. 2009). One variant from each group that had at least one nucleotide mismatch with previously published sequences were submitted to GenBank (accessions KU292576 and KU292577).

Variant KU292577, detected only in two raccoons, was clustered with the group of sequences closely related to *B. rochalinae* but more distant from others (Fig. 1). Except for this variant, other variants were found in carnivore hosts of different species (Fig. 1), demonstrating potential host-switching. Noticeably, all sequences derived from striped skunks were identical to variant DQ67649 (*B. rochalinae*); nevertheless, this variant was also detected in coyotes and raccoons. The majority of infected coyotes carried *B. v. berkhoffii* while most infected red foxes and raccoons carried *B. rochalinae* (Table 1).

Analysis of *gItA* and *ftsZ* sequences from positive samples consistently supported identification of *Bartonella* species based on the ITS phylogeny. Specifically, seven unique variants were identified based on *gItA* sequences, with three (accessions U28075, KU292567, and KU292568) belonging to *B. v. herkhoffii* and the other four (DQ683195, KU292569-KU292571) to *B. rochalinae*; seven unique variants were identified based on *ftsZ* sequences, with three (CP003124, KU292572, and KU292573) belonging to *B. v. herkhoffii* and four (DQ676490, FN645461, KU292574, and KU292575) to *B. rochalinae*. Similar to ITS phylogeny, *gItA* and *ftsZ* showed that the strain infecting the two raccoons (ITS variant KU292577) belonged to *B. rochalinae* but was much more distant in the phylogenetic relationship to other variants (3.8% versus 0.3% by *gItA*; 5.4% versus 0.3% by *ftsZ*).

Human or domestic animal exposure

Among the 38 *Bartonella*-positive animals, six (15%) were involved in human or domestic animal exposures whereas 32 were animals which had been collected in the absence of known human or domestic animal contact. The six contact cases included three striped skunks with reported contact with dogs ($n=2$) or livestock ($n=1$), a raccoon with reported dog contact, and two coyotes with reported human ($n=2$) and dog ($n=1$) contact. Both *B. v. herkhoffii* and *B. rochalimae* were identified in these animals (*B. v. herkhoffii* in the two coyotes and *B. rochalimae* in the striped skunks and the raccoon).

DISCUSSION

Using a molecular approach to test *Bartonella* infections in spleen tissues of carnivores from Colorado, our results demonstrated prevalences of these bacteria are comparable with previous reports based on surveys of blood samples (Chang et al. 2000; Henn et al. 2009a). This suggests spleen tissues can be alternative materials for *Bartonella* investigation when blood is not available. The high prevalence of *Bartonella* observed in coyotes, red foxes, and striped skunks suggests that these carnivores may be natural reservoirs of *Bartonella* species. The negative results in mountain lions, black bears, or gray foxes may reflect small sample sizes ($n=1-7$) from these species, especially considering the high prevalence of *Bartonella* species observed in gray foxes elsewhere (Henn et al. 2009a).

All positive striped skunks were exclusively infected with identical strains of *B. rochalimae*, which indicates a likely specific relationship between striped skunks and a particular strain of *B. rochalimae*. Further, the fairly high prevalence suggests that striped skunks can be a reservoir of *B. rochalimae* in addition to previously demonstrated species such as gray and red foxes (Henn et al. 2007). Unlike striped skunks, coyotes and red foxes showed little specificity to a particular *Bartonella* species but are the preferable reservoirs of *B. v. berkhoffii* and *B. rochalimae*, respectively. These results are consistent with findings from California and other areas (Chang et al. 2000; Henn et al. 2009a). Raccoons are not very susceptible to *Bartonella* infection but may harbor multiple *Bartonella* species. Hwang and Gottdenker (2013) reported detection of *B. henselae* and *B. koehlerae* in raccoons in Georgia, USA. These observations may suggest that the raccoon is a general reservoir of *Bartonella* species and can serve as an alternative host in maintaining *Bartonella* infections. Finally, a unique variant only found in raccoons suggests the variant might represent a separate subspecies of *B. rochalimae*. Confirmatory studies are needed.

Similarly to other investigators, we did not observe a specific relationship between *Bartonella* species and the carnivore species studied, with the exception of striped skunks and *B. rochalimae*. As *Bartonella* species are presumably transmitted by ectoparasites, the observations on distribution of *Bartonella* species in carnivores could be improved from learning differences in composition of ectoparasites associated with mammalian hosts. Studying ectoparasites of carnivores could help to estimate the role of arthropods in the transmission of *Bartonella* species and other vector-borne diseases and to understand how ectoparasite specificity may contribute to *Bartonella*-wild mammal associations.

Urbanization of natural areas has been associated with the current apparent emergence of infectious diseases. Carnivores are among the species that adapt well to urban and periurban environments, facilitating cross-species disease transmission with domestic animals, and potentially with their owners, as mechanical dispersers of infected ectoparasites. In our study, several infected animals (15%) had a history of human or dog exposures. Such information may suggest a common source from where humans or domestic animals contacted *B. v. berkhoffii* and *B. rochalimae*.

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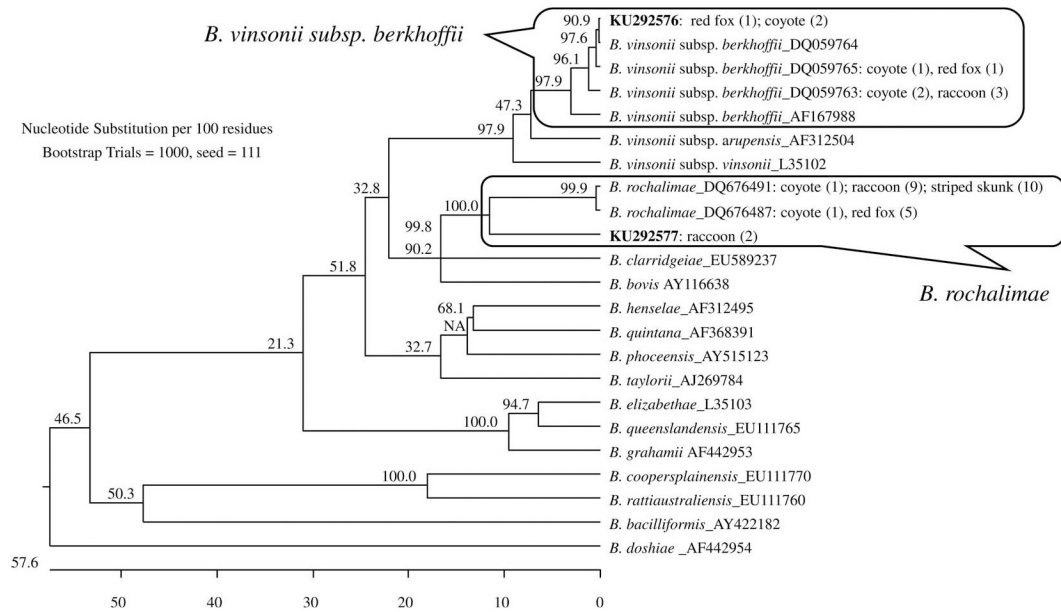


Figure 1.

Phylogenetic relationships between the *Bartonella* genotypes detected in carnivores from Colorado and some reference *Bartonella* species based on sequencing analysis of 16S–23S internal transcribed spacer (ITS). Six *Bartonella* genotypes were identified in the carnivores. Each genotype is indicated by its GenBank accession number and is followed by the common names of carnivores and number of identical DNA obtained from the host species in parenthesis. Newly identified genotypes are indicated in bold. The genotypes belonged to either *Bartonella vinsonii* subsp. *berkhoffii* or *Bartonella rochalimae* (clades within boxes). The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were calculated with 1,000 replicates.

Detection and identification of *Bartonella* species by 16S–23S internal transcribed spacer (ITS) in wild carnivores, Colorado, August 2013 and June 2015.

Table 1.

Common names	Species	No. Tested	No. Positive	Prevalence (%)	<i>B. berkhoffii</i> (proportion %)	<i>B. rochalimae</i> (proportion %)
Coyote	<i>Canis latrans</i>	25	7	28	5 (71)	2 (29)
Striped skunk	<i>Mephitis mephitis</i>	44	10	23	—	10 (100)
Raccoon	<i>Procyon lotor</i>	186	14	8	3 (21)	11 (79)
Mountain lion	<i>Puma concolor</i>	3	0	0	— ^a	—
Gray fox	<i>Urocyon cinereoargenteus</i>	1	0	0	—	—
Black bear	<i>Ursus americanus</i>	7	0	0	—	—
Red fox	<i>Vulpes vulpes</i>	26	7	27	2 (29)	5 (71)
Total		292	38	13	10 (26)	28 (74)

^aDash = not applicable.