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Isolation and phylogenetic analysis of *Bartonella* species from wild carnivores of the suborder Caniformia in Japan

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ABSTRACT

The prevalence of *Bartonella* species was investigated among wild carnivores of the suborder Caniformia, including 15 Japanese badgers (*Meles anakuma*), 8 Japanese martens (*Martes melampus*), 2 Japanese weasels (*Mustela itatsi*), 1 Siberian weasel (*Mustela sibirica*), 171 raccoon dogs (*Nyctereutes procyonoides*), and 977 raccoons (*Procyon lotor*) in Japan. *Bartonella* bacteria were isolated from one Japanese badger (6.7%) and from one Japanese marten (12.5%); however, no *Bartonella* species was found in other representatives of Caniformia. Phylogenetic analysis was based on concatenated sequences of six housekeeping genes (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) and sequence of the 16S–23S internal transcribed spacer region. The sequence analysis indicated that the isolate derived from the Japanese badger (strain JB-15) can represent a novel *Bartonella* species and the isolate from the Japanese marten (strain JM-1) was closely related to *Bartonella washoensis*. This is the first report on isolation of *Bartonella* from badger and marten.

1. Introduction

Bartonella species are gram-negative bacteria that infect erythrocytes of various mammals (Chomel et al., 2009). Of the 24 species and three subspecies of *Bartonella* currently identified, at least twelve species are known to be zoonotic agents (Chomel et al., 2006; Raoult et al., 2006).

The order Carnivora consists of two suborders, namely Caniformia and Feliformia. In Japan, the Japanese badger (*Meles anakuma*), Japanese marten (*Martes melampus*), Japanese weasel (*Mustela itatsi*), raccoon dog (*Nyctereutes procyonoides*), and Siberian weasel (*Mustela sibirica*) are known to be native species of the suborder Caniformia. In contrast, the raccoon (*Procyon lotor*) was imported to Japan from North and Central Americas as a pet, but later the feral populations have been notably spread throughout Japan (Asano et al., 2003).

In wild carnivores in the USA, the high prevalence of *B. rochalimae* which is known to cause fever, rash, and splenomegaly in humans (Eremeeva et al., 2007) have been documented in 43% of gray foxes (*Urocyon cinereoargenteus*) and 26% of raccoons, respectively (Henn et al., 2007, 2009). The high prevalence of *B. vinsonii* subsp. *berkhoffii*, which causes endocarditis in humans was also reported in 28% of coyotes (*Canis latrans*) captured in the USA (Chang et al., 2000).

Bartonella washoensis is a causative agent of myocarditis and meningitis in humans (Kosoy et al., 2003; Probert

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et al., 2009) and the natural reservoir is considered to be the ground squirrel (*Spermophilus beecheyi*) in North America (Kosoy et al., 2003). The organism was also isolated from endocarditis in a dog in California (Chomel et al., 2003), but not from any wild carnivores in other countries.

The present study was conducted to investigate the prevalence of *Bartonella* bacteria in wild carnivores in Japan and to characterize the isolates by molecular techniques.

2. Material and methods

2.1. Sample collection

During the four-year period 2008–2011, blood samples were collected from 15 Japanese badgers, 8 Japanese martens, 3 Japanese weasels, 1 Siberian weasel, and 171 raccoon dogs in Wakayama Prefecture located in the western part of Japan. Additionally, blood samples were also collected from feral raccoons in Hokkaido ($n = 95$), Chiba ($n = 186$), and Wakayama ($n = 696$) Prefectures. All blood samples of the animals, except the raccoons, were collected from hunted, road-killed, or debilitated individuals in the field. Raccoons were captured by live cage traps and then euthanized following the guidelines for invasive alien species prepared by the Japanese Veterinary Medical Association. The blood samples were collected in EDTA-containing 2 ml blood collection tubes and then sent to the Laboratory of Veterinary Public Health at the Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University. Blood samples were stored at −70 °C until examined.

2.2. Isolation of Bartonella bacteria

Frozen blood samples were thawed at room temperature and 200 μl blood aliquots were placed in sterile 1.5 ml conical tubes. The tubes were centrifuged at 1800 × *g* for 70 min; the supernatant was removed from each tube, and the sediment was mixed with 100 μl of medium 199 supplemented with 1 mM sodium pyruvate solution and 20% volume of fetal bovine serum (Life Technologies, Carlsbad, USA), and 100 μl aliquots of the mixture were plated on heart infusion agar plates (Difco, Sparks Glencoe, MI, USA) containing 5% rabbit blood (Maruyama et al., 2000). The inoculated plates were incubated at 35 °C in a moist atmosphere under 5% CO_2 for up to 4 weeks. Bacterial colonies were tentatively identified as *Bartonella* species based on colony morphology (small, gray or cream-yellow, round colonies), Gram negative staining, and the long culture period (>1 week). Five colonies were picked from each sample and each colony was subcultured using the same conditions as the primary culture.

2.3. PCR amplification and DNA sequence analysis of six housekeeping genes and ITS

The genomic DNA was extracted from each isolate by using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) and submitted for identification as *Bartonella* by genus-specific PCR targeting the *gltA* gene (Inoue et al., 2008). The organisms identified as *Bartonella* were subjected to DNA sequencing of other five housekeeping genes (16S rRNA, *ftsZ*, *groEL*, *ribC*, and *rpoB*), as described previously (Inoue et al., 2011). Eight strains of *B. washoensis* (Sb944nv, AM2-1, AR2-2, CJ22-1, DR1-1, ER14-3, RJ21-1, and SR22-1) derived from squirrels were added to the phylogenetic analysis based on concatenated sequences of six housekeeping genes because of the considerable genetic variation between these strains (Inoue et al., 2011). We also applied the analysis of the 16S–23S internal transcribed spacer region (ITS) for further genetic characterization of the isolates (Houpikian and Raoult, 2001). The PCR products were purified by using the Spin Column PCR product purification kit (Bio Basic, Markham, Ontario, Canada), and then sequenced directly using the Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA). A band of expected size of ITS was obtained from gel and purified by using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sub-cloned with the plasmid pGEM-T Easy vector system (Promega, Madison, WI, USA). The sequence reactions were performed by using the BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed by the same procedure as for six housekeeping genes and ITS. The sequence alignments obtained were compared with genomic sequences of prokaryotes registered in the GenBank/EMBL/DDBJ database using the BLAST program to confirm *Bartonella* species.

2.4. Phylogenetic analysis

The DNA sequences of the isolates were imported into the Lasergene sequence analysis software (DNASTAR, Madison, WI, USA) to obtain consensus sequences, and then aligned with those of type strains of known *Bartonella* species by using the CLUSTAL W program (Thompson et al., 1994). Neighbor-joining trees were constructed based on analysis of concatenated sequences for six genes (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) with the Jukes–Cantor parameters method and the sequence of ITS with Kimura's 2-parameter distance method in MEGA 4.0.2 (Jukes and Cantor, 1969; Kimura, 1980; Tamura et al., 2007). Support for nodes in the trees was assessed by bootstrapping with 1000 replicates.

The sequence homologies of *gltA* and ITS between the isolates and the closest species were calculated by using GENETYX-win software, version 9 (Genetyx Corp., Tokyo, Japan).

Table 1
GenBank accession numbers for six genes and one internal spacer region of *Bartonella* isolates from Japanese badger (JB-15) and marten (JM-1).

Registered genes or region	Accession numbers for	
	JB-15	JM-1
16S rRNA	AB673447	AB611850
ftsZ	AB674230	AB611851
gltA	AB674231	AB611852
groEL	AB674232	AB611853
ribC	AB674233	AB611854
rpoB	AB674234	AB611855
ITS	AB674235	AB674236