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Prevalence and genetic diversity of *Bartonella* species in sika deer (*Cervus nippon*) in Japan

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ABSTRACT

We report the first description of *Bartonella* prevalence and genetic diversity in 64 Honshu sika deer (*Cervus nippon centralis*) and 18 Yezo sika deer (*Cervus nippon yesoensis*) in Japan. Overall, *Bartonella* bacteremia prevalence was 41.5% (34/82). The prevalence in wild deer parasitized with ticks and deer keds was 61.8% (34/55), whereas no isolates were detected in captive deer (0/27) free of ectoparasites. The isolates belonged to 11 genogroups based on a combination of the *gltA* and *rpoB* gene sequences. Phylogenetic analysis of concatenated sequences of the *ftsZ*, *gltA*, *ribC*, and *rpoB* genes of 11 representative isolates showed that Japanese sika deer harbor three *Bartonella* species, including *B. capreoli* and two novel *Bartonella* species. All Yezo deer's isolates were identical to *B. capreoli* B28980 strain isolated from an elk in the USA, based on the sequences of the *ftsZ*, *gltA*, and *rpoB* genes. In contrast, the isolates from Honshu deer showed a higher genetic diversity.

1. Introduction

Bartonella species are Gram-negative bacteria that infect the erythrocytes of various mammals, and are putatively transmitted by blood-feeding arthropods [1–3]. Various mammalian species, such as canines, felines, rodents, and ruminants are the reservoir hosts of *Bartonella*, and 11 of the 26 *Bartonella* species or subspecies are recognized to be zoonotic agents [4–6].

Several epidemiological studies of *Bartonella* in domestic and wild ruminants reported that roe deer (*Capreolus capreolus*) in France [7] and elk (*Cervus elaphus*) in Wyoming, USA [8], and roe deer in Germany [9] and cows in France [10] harbor *B. capreoli* and *B. schoenbuchensis*, respectively. Mule deer (*Odocoileus hemionus*) and elk in the USA have also been found to carry *Bartonella bovis* [11], which was first isolated from a cow in France [7]. Domestic cattle in France [12] and New Caledonia [13] have been shown to harbor *Bartonella chomelii*, which is thought to have been brought with cattle from France to New Caledonia. Recently, sheep were identified as natural hosts of candidatus *Bartonella melophagi* in the USA [14], which is a species pathogenic for humans [15]. *B. bovis* was reported

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to cause endocarditis in cattle [16], while the pathogenicity of other ruminant-associated *Bartonella* species except candidatus *B. melophagi* for humans and animals is still unknown.

B. schoenbuchensis was isolated from European deer keds (*Lipoptena cervi*) in Germany [3]. In addition, the DNA of *B. chomelii* and *B. schoenbuchensis* was detected from European deer keds in France [17], and the latter was also found in both European [18] and neotropical deer keds (*Lipoptena mazamae*) in the USA [19]. In contrast, viable *Bartonella* species were not found in ticks collected from ruminants though the DNA of *B. capreoli* and *B. schoenbuchensis* was detected from sheep tick (*Ixodes ricinus*) in Poland [20] and deer tick (*Ixodes scapularis*) in the USA [18], respectively. Since several *Bartonella* species were isolated from deer keds collected from wild deer, these ectoparasites have been thought to be a possible vector in deer. On the other hand, ticks are less likely to be another vector.

Recently, the population of Japanese sika deer (*Cervus nippon*) has been significantly expanding throughout Japan [21], and animals are now sharing human environment. Furthermore, wild deer are recognized to be infectious sources of zoonoses, such as hepatitis E, salmonellosis, and brucellosis [22,23]. Therefore, it is important to investigate the prevalence of *Bartonella* species in deer and determine which species are present from the standpoint of public health.

The aim of the present study was to investigate the prevalence of *Bartonella* species among wild and captive sika deer in Japan and to characterize the isolates by molecular biological techniques.

2. Materials and methods

2.1. Sample collection

During November 2008 to October 2010, blood samples (2–7 ml) were collected from 64 Honshu deer (*C. nippon centralis*) and 18 Yezo deer (*C. nippon yesoensis*) in Japan. Thirty-seven Honshu deer and 18 Yezo deer were free-ranging wild deer, and were hunted in Wakayama, Nara, and Hokkaido Prefectures. A total of 27 Honshu deer were captive and raised in rearing facilities in Aichi and Miyagi Prefectures. Most of the free-ranging wild deer were infested with deer keds (*Lipoptena fortisetosa*) and/or *Haemaphysalis* and *Ixodes* ticks, while no ectoparasites were detected on the captive deer, as most of these deer ($n = 23$) had been treated with ivermectin and four of them were raised as breeding animals.

The blood samples of wild deer were immediately collected from the heart of carcass and those of captive deer were collected from cervical vein of anesthetized individuals. All blood samples were transferred into EDTA-containing collection tubes and sent to the Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University. These samples were stored at −70 °C until examined.

2.2. Isolation of bacterial strains

Frozen blood samples were thawed at room temperature, and 200 μl blood aliquots were separated into 1.5 ml conical tubes. The tubes were centrifuged at 1800 × *g* for 70 min. After centrifugation, the supernatants were removed from the tubes, and each sediment was mixed with 100 μl of medium 199 supplemented with sodium pyruvate solution and fetal bovine serum (Life Technologies, Carlsbad, CA, USA). Aliquots (100 μl) of the mixture were plated on heart infusion agar plates (Difco, Sparks Glencoe, MI, USA) containing 5% rabbit blood [7,12,24]. 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* based on colony morphology (small, gray or cream-yellow, round colonies), and subsequently 5 colonies were picked up from each sample, and sub-cultured on a fresh blood agar plate using the same conditions as the primary culture.

2.3. PCR amplification and DNA sequencing

Genomic DNA was extracted from whole bacterial cells by using a commercial kit, Instagene matrix (Bio-Rad, Hercules, CA, USA) and subjected to genus-specific PCR targeting the citrate synthase gene (*gltA*) and RNA polymerase beta-subunit-encoding gene (*rpoB*) to identify *Bartonella*. The PCR was performed with 20 μl mixtures containing 20 ng of DNA, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 1 pmol of each primer. Positive and negative controls were prepared by extracting DNA from *Bartonella doshiae* R-18^T and double-distilled water, respectively. Primers used for the amplification of *gltA* [25] and *rpoB* [26] have been previously described. The products were analyzed by electrophoresis on 2% agarose gels, and the target bands were detected by staining gels with ethidium bromide and viewing under UV light.

The PCR products were purified by using the Spin Column PCR product purification kit (Bio Basic, Ontario, Canada), and then sequenced directly by using the BigDye Terminator Cycle Sequencing Ready Reaction kit and a Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer's instructions. The sequence alignments obtained in this study were compared with those of other known *Bartonella* species deposited in the GenBank/EMBL/DDBJ database by using the BLAST program. The Clustal W program within GENETYX-win software, version 9 (Genetyx Corp., Tokyo, Japan) was used to compare homologous *gltA* and *rpoB* sequences to identify genetic variants. The combination of the *gltA* and *rpoB* gene sequences gave a total of 11 genogroups, and a representative isolate from each of the 11 genogroups was used for additional PCR and sequence analysis of the cell division protein gene (*ftsZ*) and the riboflavin synthase gene (*ribC*). Primers used for the amplification of *ftsZ* [27] and *ribC* [28] have been previously described. The sequences of *ftsZ*, *gltA*, *ribC*, and *rpoB* from the representative isolates have been submitted to GenBank/EMBL/DDBJ and assigned accession numbers.