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Detection of *Rickettsia helvetica* DNA from Peripheral Blood of Sika Deer (*Cervus nippon yesoensis*) in Japan

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ABSTRACT: Partial nucleotide sequences of the citrate synthase and 17-kDa genes of *Rickettsia helvetica* were detected from peripheral blood samples of Sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan. Results suggest the presence of a rickettsiemia associated with *R. helvetica*. This is the first evidence of *R. helvetica* infection in Sika deer and indicates that this species may represent a potential reservoir host of *R. helvetica* in Japan.

Key words: *Cervus nippon yesoensis*, Japan, reservoir, *Rickettsia helvetica*, Sika deer.

Rickettsiae belong to the order Rickettsiales and are obligate intracellular, gram-negative bacteria. Several species cause disease in humans and other animals and have a worldwide distribution. The genus *Rickettsia* is subdivided into three groups on the basis of phenotypic criteria; the typhus group (TG), the ancestral group, and the spotted fever group (SFG; Fournier et al., 2002). In Japan, *Rickettsia japonica*, which is a SFG *Rickettsia*, was discovered to be the causative agent of Japanese spotted fever (JSF) (Mahara et al., 1985); JSF patients have been primarily identified in western Japan since 1984, and there were 268 human cases documented from 1999 to 2004 (Mahara, 2006). Recently, *Rickettsia helvetica*, another SFG *Rickettsia*, has been isolated from ticks in Japan (Fournier et al., 2002). Although *R. helvetica* was previously known only to exist in European countries (Parolaetal., 1988), it appears to be widespread in Japan, and it has been detected from the islands of Hokkaido in the north to Kyusyu in the south (Hiraoka et al., 2005). The first human case of *R. helvetica* infection was reported in Fukui Prefecture (Noji et al., 2005). Other *Rickettsia* species, including *Ricksttsia tamurae* (Fournier et al., 2006) and species closely related to *Candidatus Rickettsia tarasevichiae*, have also been detected from ticks in Japan (Hiraoka et al., 2005; Inokuma et al., 2007). Despite this diversity in *Rickettsia* species, little information is available on the epidemiology of these agents in Japan, including vectors and reservoir hosts.

Recently, numbers of Sika deer (*Cervus nippon yesoensis*) have been increasing in Japan (Yamauchi et al., 2007). Since deer are often infested with high numbers of ticks (Inokuma et al., 2002), deer can be an important reservoir animal of tick-borne pathogens. For example, white-tailed deer (*Odocoileus virginianus*) in the USA are considered to be the primary reservoir of *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (Lockhart et al., 1997). The potential relationship between *Rickettsia* species and deer is unclear. In order to clarify the potential role of Sika deer as a reservoir host of rickettsial pathogens in Japan, detection and analysis of *Rickettsia* from peripheral blood obtained from wild Sika deer in Hokkaido were attempted using polymerase chain reaction (PCR) and sequence analyses of the citrate synthase (*gltA*) and 17-kDa genes.

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Blood samples were collected from 102 live-captured Sika deer from Nakanoshima Island of Lake Toya from March 2004 to January 2005 and from 10 animals that were euthanized as part of ongoing research in Nishi-Okoppe from October to December 2005. No indications of disease were observed in these animals, and blood specimens were stored at −20 C until testing. A QIAamp Tissue Kit (QIAGEN Science, Maryland, USA) was used to extract DNA from whole blood collected from each sample, and all DNA samples were stored at −20 C in 200 µl of Tris-EDTA (TE) buffer.

Nested PCR was performed by using oligonucleotide primer pairs for rickettsial *gltA*. A genus-specific primer set, RpCS.877p and RpCS.1273r, was used in the first PCR analysis (Roux et al., 1997). The PCR was carried out in a 25-µl reaction mixture containing 5 µl each of DNA template under the following conditions: 35 cycles of denaturation (94 C, 60 sec), annealing (54 C, 60 sec), and extension (72 C, 90 sec). The DNA extracted from cultured cells infected with *Rickettsia prowazekii* was used as a positive control. A 1:100 dilution (in distilled water) of the first PCR product was used as a template for the second PCR analysis, using an inner primer set: RpCS.896f (GGC TAA TGA AGC GGT AAT AA), PpCS.1258n (ATT GCA AAA AGT ACA GTG AAC), which was designed based on the common sequence of the *gltA* of the genus *Rickettsia*. Cycling conditions for the second PCR analysis were the same as the initial PCR analysis. The PCR products were electrophoresed at 100 V in 2% agarose gel (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) for 30 min, stained with ethidium bromide, and verified by ultraviolet (UV) illumination. The 363 base pair (bp) PCR product with a positive reaction was purified using a QIA PCR purification kit (QIAGEN) for direct sequence analysis with a Perkin-Elmer ABI Prism 3100 automated DNA sequencer. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information, Bethsda, Maryland, USA; http://www.ncbi.nlm.nih.gov/blast/) for homology searching.

Positive PCR results were detected in eight of the 102 blood samples; all positive samples were obtained from deer in Nakanoshima Island of Lake Toya. After analyzing the nucleotide sequences of the second PCR products, approximately 322 bp of the rickettsial *gltA* sequences, excluding the primer region, were determined. Sequences from all eight samples were identical and showed 100% nucleotide identity with the sequence of *R. helvetica* registered in GenBank (accession number, AB114818).

To confirm the results obtained by the *gltA* PCR and sequences, another nested PCR analysis to amplify the rickettsial 17-kDa gene was performed for the eight positive samples using a previously reported method (Ishikura et al., 2003). A positive band of the expected size (450 bp) was detected in three of the eight samples; the remaining five samples showed nonspecific multiple bands. The nucleotide sequences of the 17-kDa gene PCR products from these three samples were analyzed as described for the *gltA*; all sequences were identical with the *R. helvetica* (Ip-2) sequence registered in GenBank (accession number, AB114802).

Rickettsia helvetica has been suspected to be a human pathogen since it was first isolated in Europe (Parola et al., 1998), and, recently, evidence of *R. helvetica* infection in humans has been reported in France (Fournier et al., 2000) and Japan (Noji et al., 2005). *Ixodes ricinus* is thought to be a vector for *R. helvetica* in Europe (Parola et al., 1998; Beninati et al., 2002), and PCR-based evidence of *R. helvetica* and closely related species has been reported from *Ixodes persulcatus* and *Ixodes ovatus*. These tick species are suspected to be vectors in Japan (Fournier et al., 2002; Ishikura et al., 2002). Little is known regarding potential vertebrate re-

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servoirs, especially related to medium- to large-sized animals. Cattle may represent a reservoir for *Rickettsia africae* in southern Zimbabwe (Kelly et al., 1991b), and a short-term rickettsiemia was reported from goats experimentally infected with *R. africae* (Kelly et al., 1991a). Antibodies to *R. japonica* also have been reported from Sika deer from Shimane Prefecture, in the western part of Japan (Hoshina et al., 1995).

Although Sika deer are often infested with large numbers of ticks in the field, there have been no reports on *Rickettsia* infection in this species. In the present study, a DNA fragment specific for *R. helvetica* was detected from eight of 112 blood samples. This is the first detection of SFG *Rickettsia* from a wild Sika deer, and results suggest a rickettsiemia with *R. helvetica*. This is consistent with the possibility that Sika deer might represent a reservoir of *R. helvetica* in Japan, but additional confirmatory work is needed. Pathologic evaluations were not included in this study; thus, it is impossible to determine whether this pathogen caused any diseases in these infected deer.

In the present study, all positive samples were obtained from Sika deer taken from Nakanoshima Island of Lake Toya. Nakanoshima Island of Lake Toya is located in the southwest part of Hokkaido and has an area of 5.2 km^2. The population of Sika deer in this area has been estimated at 400 animals (Takahashi et al., 2005). Positive samples were detected by PCR, which provides only initial evidence of infection. Further epidemiologic studies, including isolation of the agents from Sika deer and transmission studies to demonstrate transmission to competent tick vectors, will be required to clarify the role of Sika deer as a potential reservoir of *Rickettsia* in Japan.

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