Short Communication

Serological and Molecular Survey of Rickettsial Infection in Cattle and Sika Deer in a Pastureland in Hidaka District, Hokkaido, Japan

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SUMMARY: Rickettsial infection of cattle and sika deer from a pastureland in Hidaka District, Hokkaido, Japan was examined by serological and molecular methods. Serum samples from 8 of 83 (9.6%) cattle reacted with Rickettsia helvetica antigens in an IFA test, with titers ranging from 1:160 to 1:640, while serum samples from 15 of 22 (68.2%) deer were positive for R. helvetica, with titers ranging from 1:80 to 1:640. In a genus-specific nested PCR based on gltA, no cattle were positive for Rickettsia, while 14 of 22 (63.3%) samples obtained from deer tested positive. Sequence analysis revealed that positive samples from sika deer showed 100% nucleotide sequence identity with the known sequence of Rickettsia asiatica.

Rickettsiae belong to the order Rickettsiales and are obligate intracellular, Gram-negative bacteria. Several species with worldwide distribution cause disease in humans and other animals. This genus is subdivided into three groups on the basis of phenotypic criteria (1): the typhus group (TG), the ancestral group, and the spotted fever group (SFG). In Japan, Rickettsia japonica was discovered to be the causative agent of Japanese spotted fever (JSF) (2). JSF patients have mainly been identified in western Japan since 1984, when the first case was reported in Tokushima Prefecture (3). Recently, Rickettsia helvetica, another SFG rickettsia, has been isolated from ticks in Japan (1,4). Although R. helvetica was previously only known to exist in European countries, R. helvetica has now been found to be widespread in Japan, from Hokkaido, a northern island, to Kyushu, a southern island (5). Furthermore, other Rickettsia spp., including Rickettsia tamurae (6), Rickettsia asiatica (7), and a species closely related to "Candidatus Rickettsia tarasevichiae" (8), have also been detected from ticks in Japan (5,8). Cross-reactivities observed among these closely related rickettsial species render serological studies complex (9,10). Despite the wide variety of Rickettsia spp. in Japan, little epidemiological information is available, including information about vectors and reservoir animals.

As wild deer are often infested with numerous ticks (11), they can be important reservoir animals for tick-borne pathogens. Recently, the partial nucleotide sequences of R. helvetica genes were detected in peripheral blood samples of sika deer (Cervus nippon yesoensis) in Hokkaido, Japan, which suggests that sika deer could be a reservoir animal for R. helvetica. The number of sika deer has been increasing in Japan, and these animals frequently approach areas where humans and domestic animals live. Cattle are at great risk of rickettsial infection because they live in pastures shared with sika deer, and cattle suffer frequent tick infestations while grazing. However, little is known about rickettsial infection in grazing cattle in Japan. Thus, in the present study, we attempted to detect Rickettsia using both serological and molecular techniques in samples taken from sika deer and grazing cattle in an area frequented by wild sika deer.

A cattle herd (n = 83) on a pasture in the Hidaka District at the Shizunai Livestock Farm at the Field Science Center of the Northern Biosphere, Hokkaido University, was examined in July 2007. A total of 22 sika deer (Cervus nippon yesoensis) that appeared in this pasture were hunted from May 2006 to January 2007. Blood samples from the jugular vein of each animal were collected in both plain and EDTA tubes for serum and DNA extraction, respectively. The tubes were kept at 4°C until transfer to Obihiro University of Agriculture and Veterinary Medicine. The sera were separated from plain tubes and kept at −20°C for indirect immunofluorescence analysis (IFA). A QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA from the EDTA blood samples. DNA samples extracted from blood were stored at −20°C in 200 µl of TE buffer until further use.

The sera were evaluated using IFA. Antigens of R. helvetica for the IFA test were prepared by culturing Rickettsia with L929 cells. Detection of antibodies was carried out as described previously (10). Sera from dogs positive for R. helvetica were used as positive controls (10). Sera from healthy newborn calves kept at the Obihiro University of Agriculture and Veterinary Medicine were used as negative controls. Sera were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.4) with 0.5% Tween 20 (PBST). An optimized dilution (1:200) of fluorescein isothiocyanate-labeled goat-anti-bovine IgG conjugate (ICN Pharmaceuticals Inc., Costa Mesa, Calif., USA) in PBST was used as the second antibody for the cattle samples. The same second antibody was also used for the examination of the samples obtained from sika deer (12). Reactive antibodies were then detected using a fluorescence light microscope. The antibody levels in the test samples were determined by comparison with the appropriate positive and negative controls. Samples that reacted with any antigens at the screening dilution were then titrated using serial twofold dilutions in order to deter-
mine the end titers. In this study, antibody titers of 1:40 or above were considered positive, as in a previous survey (10).

Nestled polymerase chain reaction (PCR) amplification was performed with the same oligonucleotide primer pairs for rickettsial gltA as discussed in our previous report (13). Briefly, two genus-specific primer sets, RpCS.877p/RpCS.1273r and RpCS.896f/PpCS.1258n, were used for the first and second PCR analysis, respectively. Positive PCR products were purified using a QIA PCR purification kit (QIAGEN) for automated DNA sequencing. Another nested PCR to amplify the rickettsial 17-kDa gene was performed using RpCS.877p/RpCS.1273r and sequencing analyses, another nested PCR to amplify the rickettsial 17-kDa gene was performed using gltA PCR-positive samples according to a previously reported method (14).

Chi-square analysis was used to compare the rates of positive IFA and PCR reactions between cattle and deer. P-values of less than 0.05 were considered to be significant.

The results are summarized in Table 1. The serum from a total of 8 of 83 (9.6%) cattle reacted with R. helvetica antigens in the IFA test, with titers ranging from 1:160 to 1:640, while serum samples from 15 of 22 (68.2%) deer were positive for R. helvetica, with titers ranging from 1:80 to 1:640. The proportion of R. helvetica-positive sika deer was significantly higher than that of R. helvetica-positive cattle (P < 0.0001). Although no cattle tested positive for Rickettsia by nested PCR, 14 of 22 (63.3%) deer samples were positive. The difference in the positive reaction rates was significant (P < 0.0001). The nucleotide sequences of seven randomly selected positive nested PCR products were analyzed. Approximately 342 bp of the rickettsial gltA sequences, excluding the primer region, were determined. All 7 samples were identical to each other, and showed 100% nucleotide sequence identity with the sequence of R. asiatica registered in GenBank (accession no. AB114797). The nucleotide sequences of the 17-kDa gene PCR products from these 3 samples were also analyzed by the same methods as those used to assess gltA. All 410-bp sequences possessed 100% identity to the 17-kDa sequence of R. asiatica registered in GenBank (accession no. AB114798).

The results of the present serological study suggest that both cattle and sika deer could be reservoirs for SFG rickettsia, although the positive IFA rate was significantly lower for cattle than for sika deer. This is the first report of the detection of antibodies for SFG rickettsia in domestic cattle in Japan. The difference in positivity between cattle and deer may have been due to acaricide use in the cattle to prevent Theileria spp. infection.

In the present study, R. helvetica was used as an antigen for IFA, because this species was detected in both sika deer and Ixodes persulcatus ticks (1) in Hokkaido. Although several animals possessed positive antibodies against R. helvetica, the DNA fragments detected in this study were all of R. asiatica. This finding suggests that sika deer in this area suffer from rickettsiemia due to R. asiatica. The positive antibodies against R. helvetica observed in this study might have been due to a cross-reaction of antibodies against R. asiatica (9,10). These results also suggest that sika deer might be a reservoir animal for R. asiatica in Japan. In our previous study, another strain of R. helvetica was detected in sika deer blood samples obtained on Nakashoshima Island in Lake Toya (13). This difference in detected species might reflect differences in vector tick species or geographical location. Further epidemiological studies will still be necessary to clarify the role played by sika deer as reservoirs of different species of Rickettsia in Japan.

All of the examined cattle tested negative for Rickettsia in the nested PCR in this study, although 8 cattle possessed antibodies against R. helvetica. One explanation for this negative result could be the use of acaricide in the cattle on this pasture. It is also possible that cattle are not a suitable reservoir animal for R. asiatica. Different animal species can be expected to differ in terms of their susceptibility to SFG rickettsia. More studies are needed to clarify the epidemiological role of cattle as reservoirs of SFG rickettsia.

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**REFERENCES**