**FULL PAPER**  Public Health

**Isolation and Characterization of *Leptospira* spp. from Raccoons in Japan**

Nobuo KOIZUMI1)*, Masaki UCHIDA2), Takashi MAKINO3), Toshitsugu TAGURI1), Toshiro KUROKI5), Maki MUTO1), Yukio KATO2) and Haruo WATANABE6)


(Received 22 August 2008/Accepted 23 November 2008)

**ABSTRACT.** We investigated whether raccoons (*Procyon lotor*) carried leptospires in their kidneys in Japan. *Leptospira* was isolated from 2 of 71 raccoons captured in Kanagawa Prefecture and 1 of 53 raccoons at a zoological park in Nagasaki Prefecture. Anti-*Leptospira* antibodies were detected in 16 of 124 raccoons (12.9%) in Kanagawa and 33 of 53 raccoons (62.3%) in Nagasaki, respectively. The partial nucleotide sequences of their *flaB* genes suggested that the isolates belonged to *L. interrogans*. The serovars of the isolates were identified as Copenhageni/Icterohaemorrhagiae (1 strain in Kanagawa) and Hebdomadis (1 strain both in Kanagawa and Nagasaki) by reactivity with the reference antisera and restriction fragment length polymorphism (RFLP) analysis based on pulsed-field gel electrophoresis and cross-agglutination-absorption test, respectively. RFLP analysis on the serovars Hebdomadis strains revealed genetic diversity among serovar Hebdomadis. Although it is unclear if the raccoons carried leptospires in their kidneys at the time imported, there is no doubt that imported animals are a new reservoir animal of leptospires in Japan.

**KEY WORDS:** *Leptospira*, PFGE, raccoon, reservoir, serovar.

---

Leptospirosis, which is caused by infection with pathogenic *Leptospira*, is an important zoonosis distributed worldwide. Leptospires infect the renal tubules of various mammals and are excreted in the urine of carrier animals. Leptospirosis in mammals is transmitted by direct contact with infected animals or by exposure to water or soil contaminated with the urine of infected animals [3]. Important reservoir animals for leptospirosis for human transmission are rats and mice living near human habitats, domestic animals such as cattle and swine, companion animals, especially dogs, and wild animals, especially rodents.

North American raccoons (*Procyon lotor*) have been shown to carry leptospires in their kidneys in the United States [17] and serum antibodies against leptospires have been detected in raccoons in the United States and Canada [13, 14, 17, 19]. It has been reported that trappers of raccoons contracted leptospirosis in Canada [19]. Raccoons have been imported into Japan as a pet since the 1970s. The number of escaped or abandoned raccoons has increased in Japan and these raccoons become feral. They damage agricultural crops and intrude into houses and excrete urine there. Thus, if raccoons in Japan carry leptospires, there is a risk that they can spread leptospirosis to humans.

In this study, in order to reveal whether raccoons act as reservoir animals of leptospires in Japan, we attempted to isolate leptospires from raccoons, and then characterize these isolates.

---

**MATERIALS AND METHODS**

*Leptospira* strains and culture conditions: *Leptospira* reference strains used in this study were cultivated in liquid modified Korthof’s medium with 10% rabbit serum at 30°C [3].

Sampling from raccoons and the isolation of leptospires: Raccoons were captured as part of vermin control by using live traps in residential areas located close to hills (Satoyama) in 5 cities in Kanagawa Prefecture in 2002 and 2003 (Table 1). Captive raccoons in a zoological park in Nagasaki Prefecture which were killed for the elimination of the parasite (*Baylisascaris procyonis*) in July 2002 were also used in this study [9]. These raccoons were not animals that were imported more recently and introduced into the park from other domestic zoos or pet shops. Some of the raccoons were bred in the park and 5 animals were less than 4 kg at the time of sample collection. Serum samples were collected and frozen at –30°C until use. For the isolation of leptospires, the kidneys of the raccoons were inoculated into liquid Korthof’s medium described above and cultivated at 30°C.

Polymerase chain reaction (PCR): DNA was extracted from the *Leptospira* isolates by using DNeasy Tissue Kit (Qiagen). The extracted DNAs were subjected to PCR for the detection of *Leptospira* flaB gene (*flaB-PCR*). The primers for PCR were previously described [8] and the conditions of PCR were as follows: after an initial 25 s denaturation step at 94°C, the reaction mixture was subjected to 30 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 30 s and extension at 72°C for 1 min.
Results of the amplification were performed by the dyeoxyribonucleotide chain termination method using the Big-Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The flaB sequences were aligned in MEGA4 [18] using CLUSTALW, and phylogenetic distances were calculated in MEGA4 using the neighbor-joining method [16].

Microscopic agglutination test (MAT): Serogroups of the isolates were identified by MAT using a panel of anti-Leptospira rabbit sera for serovars of Australis, Autumnalis, Canicola, Copenhageni, Hebdomadis and Icterohaemorrhagiae [3]. The MAT for the detection of anti-Leptospira antibodies in raccoon serum samples was performed using a battery of the reference strains of serovars Australis (Akizuki C), Autumnalis (Akizuki A), Canicola (Hond Utrecht IV), Copenhageni (Shibaura), Hebdomadis (Akizuki B), and Icterohaemorrhagiae (RGA) for 124 samples from Kanagawa (124 samples consisted of 45 samples which were used for isolation and 79 samples only for the MAT) and the isolate NRC-29 for the samples from Nagasaki, respectively.

Pulsed-field gel electrophoresis (PFGE): One milliliter of leptospiral cultures were centrifuged at 4,000 × g for 20 min, then the cells were resuspended in 38 μl of sterile water, followed by 40 μl of 1.5% agarose solution in water (Pulsed Field Certified Agarose; Bio-Rad Laboratories). The cell/agarose mixture was immediately dispensed into the wells of a disposable plug mold (Bi-Rad Laboratories) and allowed to solidify. Agarose plugs were then immersed in 1 ml of lysozyme solution (1 mg/ml in 0.5 M EDTA, pH 8.0) and placed in a reciprocating water bath at 37°C for 3 hr. Plugs were subsequently transferred to 1 ml of proteinase K solution (1 mg/ml in 0.5 M EDTA, pH 8.0 containing 1% N-lauroylsarcosine, sodium salt) and incubated in the water bath at 50°C for at least 8 hr. A slice was cut from each agarose plug and treated with 0.5 ml of 4 mM Pefabloc (Roche) in the water bath at 50°C for 30 min twice. The sliced plugs were washed with 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) on ice for 30 min, followed by restriction endonuclease NotI digestion buffer. The embedded DNA was digested with 40 units of NotI in the water bath at 37°C for 16 hr. Plug slices containing the digested DNA were placed in the wells of a 1% agarose gel (in 0.5 × TBE buffer; invitrogen) and subjected to electrophoresis in a CHEF DR III (Bio-Rad Laboratories) for 20 hr at 14°C with recirculating 0.5 × TBE buffer. Electrophoresis conditions were as follows: switch times of 10 and 60 sec, included angle of 120° and gradient of 6 V/cm.

Cross-agglutination-absorption test (CAAT): Antisera of the isolates (KRC-57 and NRC-29) were raised in rabbits according to the method previously described [2]. Production of antisera was approved by the Animal Care and Use committee of National Institute of Infectious Diseases. CAAT was performed according to the standard method [3].

RESULTS

Leptospira was isolated from 2 of 71 raccoons captured in Kamakura and Zushi Cities in Kanagawa Prefecture (strains KRC-57 and 146, Table 1) and 1 of 53 raccoons at a zoological park in Nagasaki Prefecture (strain NRC-29). The Leptospira species of all the isolates were L. interrogans as determined by comparison of the partial nucleotide sequences of their flaB genes with those of the reference strains (Fig. 1). Serogroups of the isolates were identified as Hebdomadis (KRC-57 and NRC-29) and Icterohaemorrhagiae (KRC-146) by reactivity with the reference antisera (Data not shown). The strain KRC-146 reacted equally with the antisera of both serovars Copenhageni and Icterohaemorrhagiae (both serovars belong to serogroup Icterohaemorrhagiae, data not shown).

The restriction patterns of the genomes of the isolates by the restriction enzyme NotI on PFGE were different among the isolates (Fig. 2a, lanes 1, 2 and 3). The restriction pattern of KRC-146 was identical to those of the reference strains of serovars Copenhageni and Icterohaemorrhagiae (Fig. 2a, lanes 2, 4 and 5). The reactivity of antisera and the identical restriction pattern on NotI-PFGE with reference strains of serovars Copenhageni and Icterohaemorrhagiae demonstrated that the serovar of KRC-146 was either Copenhageni or Icterohaemorrhagiae (See Discussion). On the other hand, the restriction pattern of KRC-57 and NRC-29 was different from the reference strain(s) of serovar Heb-
Anti-Leptospira antibodies (reciprocal serum titer more than 80) were detected in 16 of 124 (12.9%) serum samples of the raccoons in Kanagawa by MAT (Table 1). Reactive leptospiral serovars were Autumnalis (1 animal), Copenhageni (15 animals) and Icterohaemorrhagiae (9 animals). Nine of the raccoons showed equal reactivity against both Copenhageni and Icterohaemorrhagiae. Antibodies against the isolate (NRC-29) were detected in 33 of 53 (62.3%) raccoons in Nagasaki.

DISCUSSION

In order to reveal whether raccoons in Japan carry leptospires, we performed isolation of leptospires from the kidneys of raccoons captured in Kanagawa and at a zoological park in Nagasaki. This is the first report concerning leptospiral isolation from raccoons in Japan. Three Leptospira isolated in this study were identified as L. interrogans serovar Copenhageni/Icterohaemorrhagiae (KRC-146) and L. interrogans serovar Hebdomadis (KRC-57 and NRC-29), respectively. PFGE has been indicated to be a useful method for identification of leptospiral serovars [4–6]. PFGE has shown that the genomes of leptospiral serovars are remarkably conserved, both over time and across wide geographical distribution [4–6]. Most but not all serovars gave unique PFGE patterns using the restriction enzyme NotI, although the L. interrogans serovar Copenhageni and Icterohaemorrhagiae were indistinguishable by NotI-PFGE [5, 6]. These two serovars are also very difficult to distinguish by serological methods [11]. KRC-146 reacted equally with the reference antisera of serovars Copenhageni and Icterohaemorrhagiae (Data not shown) and showed identical banding pattern to these reference strains on PFGE (Fig. 2a, lanes 2, 4 and 5), indicating the isolate KRC-146 is a serovar of either Copenhageni or Icterohaemorrhagiae. On the other hand, KRC-57 and NRC-29 showed different restriction patterns from that of the reference strain of serovar Hebdomadis, while they...
are definitely serovar Hebdomadis as demonstrated by CAAT (Fig. 2b and Table 2). The partial flaB sequences were also different (Fig. 1). Therefore, these results indicate genetic diversity among strains of serovar Hebdomadis.

Leptospirosis in humans is transmitted by direct contact with infected animals or by exposure to water or soil contaminated by urine from infected animals [3]. Raccoons have been known to harbor leptospires in their kidneys in the United States [17]. The present study demonstrated that raccoons also act as reservoir animals of leptospires in Japan. Serological examination suggested that leptospirosis is epidemic in Kamakura and Zushi Cities in Kanagawa Prefecture (Table 1). Raccoons have been captured in residential areas, and often enter houses and excrete urine there. There is a report that trappers of raccoons were infected with Leptospira in Canada [19]. These facts strongly suggest that human transmission of leptospirosis may occur through raccoon urine in Kanagawa. Although it is unclear if the raccoons carried leptospires in their kidneys at the time imported, there is no doubt that imported animals are a new reservoir animal of leptospires in Japan. Recently, workers in an exotic animal company were infected with Leptospira through imported flying squirrels in Japan [12]. Thus, control of imported animals is important in order to prevent human infection.

In Nagasaki, Leptospira was isolated from captive raccoons in a zoological park. More than 60% of the animals possessed antibodies against the strain NRC-29, which was isolated from a raccoon at the park. Leptospiral antibody was detected in a raccoon (body weight 1.1 kg) which was bred at the park, indicating that leptospirosis infection widely occurred among the raccoons at the park. At the zoological park, visitors were in contact with the raccoons; they could feed animals, suggesting a risk of infection to visitors. In addition to visitors, raccoons have given a risk of infection to workers in the park. Anti-Leptospira antibodies, and leptospiral antigens and DNA were detected in captive animals [7, 10, 15]. There were two cases of leptospirosis in zoo workers in the United States, and these cases were believed to have been contracted from bears at the zoo [1]. Thus, inspection of leptospirosis infection before introduction of (feral) animals at a zoo is necessary. Furthermore, hygienic management for the elimination of potential carrier animals in and around zoo is important, in addition to protection of feed containers.

ACKNOWLEDGEMENTS. We are grateful to M. Hoshino and H. Obe for their technical assistance. We also thank M. Kawanaka, K. Arakawa, H. Sugiyama and Y. Morishima for collecting raccoon samples in Nagasaki. This work was supported in part by a Health Sciences Research Grant-in-Aid for Emerging and Re-emerging Infectious Diseases (H13-Shinkou-4) from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

10. Kik, M.J., Goris, M.G., Bos, J.H., Hartskeerl, R.A. and Dor-