Original Article

Investigation of Reservoir Animals of *Leptospira* in the Northern Part of Miyazaki Prefecture

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(Received August 8, 2008. Accepted September 29, 2008)

SUMMARY: We surveyed reservoir animals of leptospires in the northern part of Miyazaki Prefecture, where a cluster of human leptospirosis had occurred during the summer of 2006. *Leptospira* was isolated from 6 of 57 large Japanese field mice (*Apodemus speciosus*). The serogroups of the isolates were Autumnalis (5 strains) and Hebdomadis (1 strain) and the partial nucleotide sequences of their *flaB* genes suggested that the isolates belonged to *L. interrogans*. The human patient sera reacted specifically with the *Leptospira* strain isolated from the mice captured around the area where each patient occurred, suggesting that mice are the source of human infection. We also detected leptospiral DNAs by *flaB*-polymerase chain reaction in the kidneys of large feral animals; wild boars (positive ratio 10.3%; 4 of 39) and deer (19.2%; 10 of 52). The *Leptospira* spp. harbored by these animals were deduced to be *L. interrogans* (in 5 animals) and *L. borgpetersenii* (in 9 animals) by the nucleotide sequences of the amplicons. Anti-*Leptospira* antibodies were also detected among symptomatic hound dogs. These results suggest that these feral animals may cause leptospirosis and pose a potential risk to hunters and workers in the meat processing industry.

INTRODUCTION

Leptospirosis, which is caused by infection with pathogenic *Leptospira*, is a globally important zoonotic disease that affects humans in both rural and urban settings, and in both industrialized and developing countries (1-3). In particular, leptospirosis has become an important public health problem in Asia and Latin America. In these tropical areas, large outbreaks of leptospirosis are most likely to occur after floods, hurricanes, or other disasters, which affect both rural and urban areas (4-7). In Japan, although the number of the leptospirosis patients has decreased, sporadic cases of leptospirosis still occur in various areas, especially in Okinawa Prefecture, the southernmost part of Japan. Outbreaks of leptospirosis were reported in urban and countryside areas in 2003 (8) and 2004 (9). Leptospirosis infect the proximal renal tubules of various animals and are excreted in the urine of carrier animals. Any mammal can be a carrier and excreter of leptospires (10). Important leptospirosis reservoir animals in 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. Leptospirosis is not only a zoonosis but also an important disease of domestic animals such as cattle and swine. Leptospirosis is caused by infection with leptospires after direct contact with infected animals or exposure to water or soil contaminated by the urine of infected animals (10).

In August and September of 2006, a cluster of human leptospirosis occurred in Miyazaki Prefecture. Eight cases of human leptospirosis were reported; seven of these 8 cases occurred in the northern part of the prefecture during the period (see Fig. 1), whereas only 2 cases had been reported in the prefecture from November 2003 to July 2006. In this study, in order to identify the reservoir animals of leptospirosis in the northern part of Miyazaki Prefecture, we carried out the isolation of leptospires and the detection of leptospiral DNAs from the kidneys of feral animals captured in these areas.

MATERIALS AND METHODS

Isolation of leptospires from mice: Mice were captured by using live traps in November and December 2006. For the isolation of leptospires, the kidneys of the mice were inoculated into liquid modified Korthof's medium with 10% rabbit serum and cultivated at 30°C (10).

Polymerase chain reaction (PCR): DNAs were extracted from the *Leptospira* isolates and the kidney tissues of wild boars, deer, and a raccoon dog, respectively, by using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). The extracted DNAs were subjected to PCR (isolates' DNA) or nested PCR (kidneys' DNA) for the detection of *Leptospira* flaB gene (flaB-PCR). The conditions of PCR were as follows: the 1st PCR was performed using the primers described previously (11). After an initial 25 s denaturation step at 94°C, the reaction mixture was subjected to 25 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 30 s and extension at 72°C for 1 min. The 2nd PCR was done by using the sense primer L-flaB-F2 (5'-TGTGCACAAGACGATGAAAGC-3') and the antisense primer L-flaB-R2 (5'-AACATTGCCGTACCACTC).
The restriction enzyme patterns of the genomes of the isolates by the strain of mouse captured in the field where the patient used to work. Five mice from which leptospires were isolated in Takachiho were captured in the northern part of Miyazaki Prefecture. The importance of wild boars in human transmission has also been suggested in Germany (14). In Japan, L. borgpetersenii has been isolated from rodents in Okinawa and Amami so far (15). Thus, this study demonstrated for the first time that L. borgpetersenii exist in Kyushu. For the accurate diagnosis of leptospirosis in Kyushu, it is necessary to isolate and characterize L. borgpetersenii harbored by these wild animals.

High titer of anti-Leptospira antibodies were detected by the MAT among 6 of 8 hound dogs suspected of leptospiral infection at a veterinary clinic located in Nobeoka, indicating the occurrence of acute infection not only in humans but also in dogs in this region. Outdoor dogs such as hounds and herding dogs have been identified as at-risk groups for clinical leptospirosis (16). Reactive leptospiral serogroups included Australis (1 animal), Castellonis (2 animals), and Hebdomadis (4 animals). One of the dogs showed equal reactivity against both Castellonis and Hebdomadis. The fact that large feral animals harbor leptospires and that hound dogs are infected with leptospirosis suggests that these reservoir animals may be important in the transmission of leptospirosis in Japan.

RESULTS AND DISCUSSION

In this study, in order to identify reservoir animals of leptospirosis in the northern part of Miyazaki Prefecture, where a cluster of human leptospirosis had occurred during the summer of 2006, we carried out the isolation of leptospires and detection of leptospiral DNAs from the kidneys of feral animals captured in these areas.

Leptospirosis in humans is transmitted by direct contact with infected animals or by exposure to water or soil contaminated by the urine of infected animals (10). Rats and mice are the most important animal reservoirs of leptospirosis for human transmission. We captured 57 large Japanese field mice, Apodemus speciosus, throughout 4 districts in the northern part of Miyazaki Prefecture, where the human infection occurred in 2006 (Fig. 1, Table 1). Leptospires were isolated from 6 mice; 1 in Nobeoka and 5 in Takachiho (Table 1). Five mice from which leptospires were isolated in Takachiho were captured in the field where the patient used to work. Serogroups of the isolates were identified as Autumnalis (5 strains in Takachiho) and Hebdomadis (1 strain in Nobeoka) by reactivity with the reference antisera (data not shown). The Leptospira spp. of all the isolates were deduced to be L. interrogans by a comparison of the partial nucleotide sequences of their flaB genes with those of the reference strains (Fig. 2).

The restriction patterns of the genomes of the isolates by the restriction enzyme NorI on pulsed-field gel electrophoresis (13) were different between the strains isolated in Nobeoka and Takachiho, but identical among the Takachiho strains (data not shown). The patient sera reacted specifically with the strain TG-3 isolated from a mouse captured in the field where the patient used to work, and sera from patients No. 1 and No. 3 reacted with the strain NM-9 (data not shown). All of the patients in 2006 were thought to have contracted leptospirosis through agricultural work (our unpublished data). Leptospirosis has been considered to be an occupational disease associated with activities involved in rice agriculture and other forms of agriculture in Japan. These results, thus, suggest that mice are the source of human infection in these areas.

We detected leptospiral DNAs by flaB-PCR in the kidney tissues of large feral animals (Table 2). The flaB was detected in wild boars (positive ratio 10.3%; 4 of 39) and deer (19.2%; 10 of 52). The Leptospira spp. harbored by these animals were deduced to be L. interrogans (in 5 animals) and L. borgpetersenii (in 9 animals) by the nucleotide sequences of the amplicons (Fig. 2). Some of the patients said that they had seen wild boars in their fields (our unpublished data), suggesting that wild boars are also an important reservoir in these areas. The importance of wild boars in human transmission has also been suggested in Germany (14). In Japan, L. borgpetersenii has been isolated from rodents in Okinawa and Amami so far (15). Thus, this study demonstrated for the first time that L. borgpetersenii exist in Kyushu. For the accurate diagnosis of leptospirosis in Kyushu, it is necessary to isolate and characterize L. borgpetersenii harbored by these wild animals.

High titer of anti-Leptospira antibodies were detected by the MAT among 6 of 8 hound dogs suspected of leptospiral infection at a veterinary clinic located in Nobeoka, indicating the occurrence of acute infection not only in humans but also in dogs in this region. Outdoor dogs such as hounds and herding dogs have been identified as at-risk groups for clinical leptospirosis (16). Reactive leptospiral serogroups included Australis (1 animal), Castellonis (2 animals), and Hebdomadis (4 animals). One of the dogs showed equal reactivity against both Castellonis and Hebdomadis. The fact that large feral animals harbor leptospires and that hound dogs are infected with leptospirosis suggests that these reservoir animals may be important in the transmission of leptospirosis in Japan.
cause canine leptospirosis. As vaccines of leptospirosis for dogs are available, it is important to vaccinate dogs for the prevention of leptospirosis. These findings also indicate a potential risk of leptospirosis for hunters and people who are engaged in the processing of deer meat, although there has been no report of human leptospirosis among these people in Japan to date. In this study, acute canine leptospirosis infection was revealed. Dogs are known to become carriers of leptospires after recovery from acute infection (10). Thus, it is important to investigate whether dogs carry leptospires in their kidneys.

In conclusion, many feral animals were identified as reservoir animals in the northern part of Miyazaki Prefecture. With regard to the occurrence of leptospirosis, much attention should be paid not only to agricultural workers but also to hunters, workers in meat processing, and hound dogs.

Table 2. Results of flaB-PCR on DNAs extracted from large feral animals captured in the northern part of Miyazaki Prefecture

<table>
<thead>
<tr>
<th>Animal</th>
<th>Place</th>
<th>No. of animals captured</th>
<th>No. of animals PCR positive (%)</th>
<th>Seq ID1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild boar</td>
<td>Nobeoka</td>
<td>22</td>
<td>2 (9.1)</td>
<td>NWB-15, 18, 18</td>
</tr>
<tr>
<td></td>
<td>Takachiho</td>
<td>14</td>
<td>2 (14.3)</td>
<td>TWB-4, 5</td>
</tr>
<tr>
<td></td>
<td>Hyuga</td>
<td>3</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>39</td>
<td>4 (10.3)</td>
<td></td>
</tr>
<tr>
<td>Deer</td>
<td>Nobeoka</td>
<td>36</td>
<td>3 (8.3)</td>
<td>ND-23, 26, 34</td>
</tr>
<tr>
<td></td>
<td>Takachiho</td>
<td>14</td>
<td>6 (42.9)</td>
<td>TD-3, 5, 8, 10, 11, 12</td>
</tr>
<tr>
<td></td>
<td>Hyuga</td>
<td>2</td>
<td>1 (50)</td>
<td>HD-2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>52</td>
<td>10 (19.2)</td>
<td></td>
</tr>
<tr>
<td>Raccoon dog</td>
<td>Nobeoka</td>
<td>1</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

1) IDs of PCR positive animals.

ACKNOWLEDGMENTS

We are grateful to Miyazaki Prefectural Institute for Public Health and Environment, Nobeoka Public Health Center, Takachiho Public Health Center, and Miyazaki-ken Ryouyukai for capturing mice and providing kidney tissues of wild boars, deer and a raccoon dogs, respectively. We also thank T. Masuzawa for the design of primers of the nested PCR and T. Suzuki, R. Takahashi, H. Sato, K. Nakajima, and Y. Shioyama for epidemiological data of human leptospirosis.

This work was supported in part by a Health Sciences Research Grant-in-Aid for Emerging and Re-emerging Infectious Diseases (H18-Shinkou-Ippan-008) from the Ministry of Health, Labour and Welfare of Japan.

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