Lyme Disease Spirochetes in a Wild Fox (Vulpes vulpes schrencki) and in Ticks

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Lyme Disease Spirochetes in a Wild Fox (*Vulpes vulpes schrencki*) and in Ticks

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ABSTRACT: Lyme disease spirochetes were demonstrated in a wild female fox (*Vulpes vulpes schrencki*) and in *Ixodes persulcatus* ticks collected from the fox on Sapporo, Hokkaido, Japan. Spirochetes were detected in *I. persulcatus*, as well as skin lesions, brain, heart, kidney, and liver of the fox. Five of seven isolates reacted with a monoclonal antibody against *Borrelia afzelii* specific Osp B. Deoxyribonucleic acid (DNA) relatedness of a brain isolate was 89% to *B. afzelii*, and ranged from 50 to 67% to three other species. Immunoglobulin G antibodies to *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu lato, when tested in an enzyme-linked immunosorbent assay (ELISA), were negative in the fox. There were no antibodies against seven serovars of *Leptospira interrogans*.

Key words: Lyme borreliosis, fox, *Borrelia afzelii*, *Ixodes persulcatus*, hepatitis, pyelitis, conjunctivitis, *Vulpes vulpes schrencki*.

Lyme disease is a multisystemic infectious disorder caused by the spirochete, *Borrelia burgdorferi* and related species (Baranton et al., 1992). Infection occurs when *Ixodes* spp. tick vectors feed on mammalian hosts. The presence of wild animal reservoirs of infection in the environment is critical for the maintenance of disease transmission.

Natural Lyme disease infection has been found in wild and domestic canine species, including wolves (*Canis lupus*) (Thieking et al., 1992) and domestic dogs (Kornblatt et al., 1985) in the U.S. As many as 19% of healthy dogs from Hokkaido, Japan, have serologic evidence of infection (Isogai et al., 1990). We report here demonstration of Lyme disease spirochetes from a wild red fox (*Vulpes vulpes schrencki*) with systemic infection and vector ticks.

A female red fox weighing 4.4 kg died in a traffic accident in Sapporo, Hokkaido, Japan (43°01′N, 141°21′E) on 3 June 1992. We observed 11 ticks on the animal: four adult females, one nymph, and two larvae of *Ixodes persulcatus*; one nymph of *I. ovatus*; one adult female *I. nipponensis*, and two adult male *Haemaphysalis flava*. Only the adult and nymph stages of *I. persulcatus* and *I. nipponensis* were attached to the skin. Identification of ticks was done by the methods of Yamaguchi et al. (1971) and by comparison to holotypes in the Department of Parasitology, Asahigawa Medical College, Hokkaido, Japan. Representative ticks were deposited in the Department of Zoology, National Science Museum, Tokyo, Japan, with kind support of H. Ono. Deposition numbers were NSMT-Ac-10501 (*I. persulcatus*, adult female), NSMT-Ac-10502 (*I. persulcatus*, larva), NSMT-Ac-10503 (*I. ovatus*, nymph), NSMT-Ac-10504 (*I. nipponensis*, adult female) and NSMT-Ac-10505 (*H. flava*, adult male).

Isolation of *Borrelia* spp. from tick midguts was carried out according to the methods of Nakao et al. (1992). Contents of isolated midguts were cultivated at 32 C for 4 mo in BSK medium (Barbour, 1984). Darkfield microscopy was used to screen cultures two times each month. Similarly, tissues (skin, liver, kidney, brain, heart, lung) and blood were cultured in BSK medium. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of the isolates were carried out according to the method of Masuzawa et al. (1991). After SDS-PAGE, the immunologically reactive

bands were stained with monoclonal antibodies. We used monoclonal antibodies H9742 against flagellae (Barbour et al., 1986), H5332 against Osp A of *B. burgdorferi* sensu stricto (Barbour et al., 1983), P34f against Osp B (immunized strain IPF, isolated from midgut of *I. persulcatus* in Japan), P31c against Osp A (immunized strain NT24, isolated from the midgut of *I. persulcatus* in Japan), P3134 against Osp A and B (immunized strain NT24), I 17.3 against Osp B of *B. afzelii* (Canica et al., 1993), for a comparison of reactivities. The two monoclonal antibodies H9724 and H5332 were kindly provided by A. G. Barbour, University of Texas, San Antonio, Texas, USA. Monoclonal antibody I 17.3 was kindly provided by G. Baranton, Institut Pasteur, Paris, France.

The deoxyribonucleic acid-deoxyribonucleic acid (DNA-DNA) reassociation (Ezaki et al., 1989) was used for identification of each isolate. The DNA was extracted from the lysed cell preparation twice with phenol and once with phenol-chroroform-isoamyl alcohol (25:24:1); DNA was precipitated for 2 hr at −20 C by adding 5 M NaCl and ice-cold ethanol. Purified DNA was used for DNA-DNA homology by microplate hybridization method (Ezaki et al., 1989).

The following agar media were used for bacterial examination of liver, kidney, brain, heart, lung and blood: brain heart infusion agar (BBL, Becton Dickinson Microbiology Systems Cockeysville, Maryland, USA) with 5% horse blood for aerobic or anaerobic total, M10 agar for strict anaerobic total (Benno and Mitsuoka, 1992); trypticase yeast extract cystein agar (Kyokuto Seiyaku Ltd., Tokyo, Japan) for *Streptococcus* spp.; *Enterococcus fecalis* agar (Nissui Seiyaku Ltd., Tokyo, Japan) for *Enterococcus* spp.; mannitol-salt agar with egg yolk (Nissui Seiyaku Ltd.) for *Staphylococcus* spp.; deoxycholate-hydrogen sulfide-lactose agar (Nissui Seiyaku Ltd.) for Enterobacteriaceae; Arakawa agar (Nissui Seiyaku Ltd.) for *Corynebacterium* spp.; Nalidixic Acid, Cetrimide agar (Eiken Chemical Co., Ltd., Tokyo, Japan) for *Pseudomonas* spp.; Sabouraud agar for fungi (Nissui Seiyaku Ltd.); *Bacteroides* Selective Agar (Nissui Seiyaku Ltd.) with 5% horse blood for *Bacteroides* spp.; Modified *Fusobacterium* Agar (Nissui Seiyaku Ltd.) for *Fusobacterium* spp.; *Lactobacillus* Selective Agar (BBL, Becton Dickinson Microbiology Systems Cockeysville) for *Lactobacillus* spp.; *Veillonella* Selective Agar (BBL, Becton Dickinson Microbiology Systems) with vancomycin (7.5 mg/ml; Eli Lilly, Indianapolis, Indiana, USA) for *Veillonella* spp.; *Eubacterium* Selective Agar (Nissui Seiyaku Ltd.) for *Eubacterium* spp.; and *Leptospira* Medium (Difco Laboratories, Detroit, Michigan, USA) with 10% rabbit serum for *Leptospira* spp.

The skin, liver, kidney, brain, heart, lung, eye, lymph nodes, and spleen of the fox were examined for histopathological changes. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained by hematoxylin and eosin. Suspect isolates were characterized by electron microscopy by the methods of Hovind-Hougen (1984), using a Hitachi HU-500 microscope (Hitachi, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA) was done on serum collected from the fox by the method of Isogai et al. (1990). *Borrelia afzelii* BFOX, *B. garinii* HP3 (Baranton et al., 1992), *B. afzelii* BFOX and *B. burgdorferi* sensu lato HO14 (Postic et al., 1993) were used for antigen preparation. The bacterial strains were centrifuged into pellets and suspended in carbonate buffer (0.5 M, pH 9.6) and then disrupted by sonication on ice three times for 30 sec each time. After determination of protein concentration by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, California, USA), it was adjusted at 10 μg/ml. After coating the antigen, diluted serum samples were added in each well. Peroxidase-conjugated anti-dog immunoglobulin G (1:500, Cappel Co., Ltd., Malvern, Pennsylvania, USA) was

added in each well. The colorimetric value was determined by an ELISA reader (MTP-22, Corona Electric Co., Ltd., Ibaragi, Japan) after incubation with *o*-phenylenediamine (4 mg/ml; Kanto Chemical Co., Inc., Tokyo, Japan).

A microscopic agglutination test against seven serovars of *Leptospira interrogans* was done by the method of Isogai et al. (1986). Viable cells of *Leptospira* spp. (2 to 3 × 10^8/ml) were incubated with serial diluted serum at 37 C for 1 hr. Agglutination was determined by darkfield microscopy.

Spirochetes were detected by darkfield examination of cultures, from the midgut tissues of three of five *I. persulcatus*, the one *I. ovatus*, and three of 18 tick-bite sites after culturing in BSK medium. The culture period was <1 mo for detection of the spirochetes from *I. persulcatus* and from 1 to 2.5 mo for detection of the spirochetes from *I. ovatus* and tick-bite sites.

Spirochetes were detected from one of three skin cultures without tick-bite, as well as liver, kidney, brain and heart tissues. The culture period was 1 to 3 mo. No other significant bacteria were isolated. Based on electron microscopy, spirochete isolates BFOX (brain tissue), HFOX (heart tissue), SK3 (skin without tick-bite), F63A (*I. persulcatus*), and F63B (*I. ovatus*) were similar in morphology to Lyme disease spirochetes. They had eight (seven: F63B) flagellae (F63B had seven flagellae), a length of 12 to 118 μm and a diameter of 0.23 to 0.28 μm. The two isolates, BFOX and HFOX, reacted with genus specific H9742 (Table 1). They also reacted with P34f, P31c and P3134 and were specific to Osp A and/or Osp B of Japanese *Borrelia* species associated with *I. persulcatus*; they did not react with H5332 (specific to Osp A of *B. burgdorferi* sensu stricto. Strains BFOX and HFOX have been reported to react with *B. afzelii* specific monoclonal antibody I 17.3 (Canica et al., 1993). Isolates SK1 (erythemous border of skin with tick-bite), SK3 and F63A also reacted with I 17.3, while SK2 (erythematous lesion of skin with tick-bite) and F63B did not. Comparing DNA homology values of BFOX DNA to *B. burgdorferi* sensu stricto B31, *B. garinii* 20047, *B. afzelii* VS461 and *B. burgdorferi* sensu lato (*B. japonica*) H014, the DNA homology value of strain BFOX DNA was 89% to VS461 DNA (Fig. 1). In contrast, DNA homology values of strain BFOX DNA to the three type strains ranged from 50 to 67%. Therefore, BFOX was identified as *B. afzelii*.

TABLE 1. Molecular mass (kilodaltons) of reactive bands of monoclonal antibodies to *Borrelia* spp. strains from a red fox, Sapporo, Hokkaido, Japan, June 1992.

Strain	Origin	Monoclonal antibodies					
		H9742	H5332	P34f	P31c	P3134	I 17.3
BFOX	Brain	41	—[a]	35	32	35	35
HFOX	Heart	41	—	35	32	35	35

[a] —: No reaction.

At necropsy, hemorrhagic and edematous skin lesions in the tick-bite sites were observed. The mean (±SE) diameter of the lesions was 40 ± 14 mm. Histologically, inflammatory responses of lymphocytes, plasma cells, neutrophils, and eosinophils with perivasculitis were observed in the skin lesions. Skin lesions were observed in the area without tick-bites. Infiltrated cells were mainly lymphohistiocytes and plasma cells with perivasculitis (Fig. 2a). Spirochetes were isolated from the lesion.

The liver, kidney, and eye also had cellular infiltration. Hepatitis with perivasculitis was seen in a limited area of the liver, characterized by lymphocytes, plasma cells and neutrophils (Fig. 2b). Similar cell infiltration was observed in the kidney (pyelitis: Fig. 2c) and eye (conjunctivitis: Fig. 2d). Lesions in these tissues were similar to the skin lesions without tick-bites. Lymph nodes and spleen had atrophic lymphoid follicles. No lesions were seen in the brain, heart and lung.

No immunoglobulin G antibodies were detected to *B. garinii*, *B. burgdorferi* sensu lato and *B. afzelii* in the serum (optimal density: <0.200). No antibodies were de-

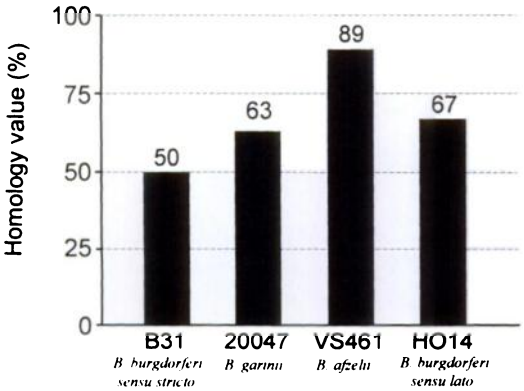

FIGURE 1. Extent of DNA relatedness of *Borrelia* spp. strain BFOX from a red fox to other *Borrelia* spp., Sapporo, Hokkaido, Japan, June 1992.

tected against seven serovars of *Leptospira interrogans* (agglutinin titer: <1:20).

Ticks vectors for Lyme borreliosis, such as *I. persulcatus*, are present in many areas of Japan, especially Hokkaido (Nakao et al., 1992). In this case report, the spirochete was isolated from the midgut of *I. persulcatus* which had fed on the fox. Thus, we believe that Lyme disease can be transmitted from *I. persulcatus* to foxes in Japan.

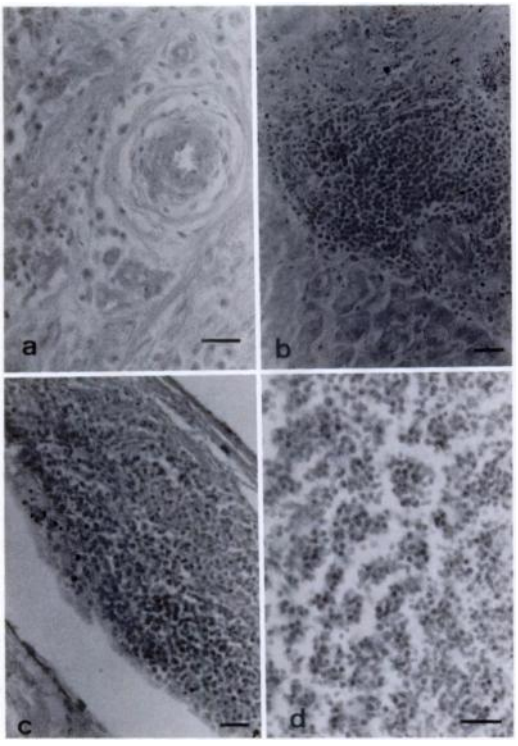

FIGURE 2. Inflammatory lesions in a red fox with *Borrelia* spp. infection, Sapporo, Hokkaido, Japan, June 1992. H&E stain. Each bar = 10 μm. a. Skin lesion without tick bite; spirochetes were isolated from the site; b. Perivascular cell infiltration and hepatitis in the liver; c. Pyelitis; d. Conjunctivitis.

Lyme disease spirochetes were isolated from the brain, heart, liver, kidney and skin of the fox. Lyme disease has been reproduced in dogs experimentally infected with *B. burgdorferi* (Wasmoen et al., 1992). Canine Lyme disease was characterized by limb and joint dysfunction in the USA (Kornblatt et al., 1985; Wasmoen et al., 1992). In contrast, limb and joint dysfunction in dogs with tick-bites and antibodies against Lyme disease spirochetes is rare in Japan (Y. Azuma, unpubl.).

Most isolates were identified as *B. afzelii*. The isolation of *B. afzelii* in northern Europe is well correlated with the geographic distribution of acrodermatitis chronica atrophicans (ACA) (Canica et al., 1993). Acrodermatitis chronica atrophicans seems uncommon in the U.S., with only one case reported in 1986 from California (Lavoie et al., 1986). In Japan, Okamura (1964) reported 20 human cases of ACA.

Two isolates did not react with monoclonal antibody specific to Osp B of *B. afzelii*. Strain F63B from *I. ovatus* has been reported as a new species (Postic et al., 1993). In Japan, the species *B. burgdorferi* sensu lato appears to be restricted to *I. ovatus*. There are no known reports of Lyme disease after *I. ovatus* bite in animals, including humans. The other strain from *I. persulcatus* was different from *B. burgdorferi* sensu lato and *B. afzelii*. Thus, Japanese borrelial species varied in their antigenicity.

The fox appeared to have a systemic infection with *B. afzelii*. No other bacterial pathogens were isolated from this case. The subacute and chronic lesions in the tissues were different from those of viral infection such as distemper. Although similar lesions can be seen in dogs with leptospirosis, lep-

tospirosis is rare in Hokkaido (E. Isogai, unpubl.). We believe that the mild inflammatory infiltrates in liver, kidney and eye may be related to infection with *B. afzelii*.

The wood mouse (*Apodemus speciosus ainu*) may be a suitable first reservoir host for Lyme disease spirochetes transmitted by *I. persulcatus* in Hokkaido; both larvae and nymphs of *I. persulcatus* removed from *A. speciosus ainu* were highly infected with spirochetes (Nakao and Miyamoto, 1993). However, no information on the host preference of the ticks for medium or large sized mammals is available in Japan.

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