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Antigenic and Genetic Characterization of *Borrelia* Species Isolated from *Ixodes persulcatus* in Hokkaido, Japan

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Ten characteristic strains of spirochetes (HT2, HT7, HT10, HT15, HT17, HT19, HT20, HT22, HT32, and HT59) isolated from *Ixodes persulcatus* adult ticks in Hokkaido, Japan, were selected and analyzed by polyacrylamide gel electrophoresis, with monoclonal and polyclonal antibodies, and by pulsed-field gel electrophoresis. The protein profiles of the borrelial isolates were variable and markedly different from that of the type strain *Borrelia burgdorferi* B31. The 41-kDa flagellin protein was present in all isolates, but the outer surface protein A (OspA) was absent in four isolates (HT15, HT2, HT20, and HT32). The molecular weights of the OspA proteins in six isolates were found to differ from one isolate to another. No two isolates examined had the same plasmid profile. These findings show the antigenic and genetic heterogeneity of the Japanese isolates, and some isolates are strikingly different from North American, European, and Asian strains.

Lyme disease is a multisystem disorder associated with skin rash, arthritis, and neurologic and cardiac manifestations (18, 27). The etiologic agent of Lyme disease, *Borrelia burgdorferi*, was discovered by Burgdorfer et al. in 1982 (9), and in 1984, Johnson et al. (15) described this organism as a new species of the genus *Borrelia*. Since then, *B. burgdorferi* has been isolated from or detected in patients, rodents, and various species of ixodid ticks in all parts of the world (4, 10, 11). It has been generally assumed that ticks belonging to the *Ixodes ricinus* species complex are important vectors of Lyme disease.

In Japan, the first case of Lyme disease was reported by Kawabata et al. in 1987 (16). Thereafter, spirochetes were isolated from patients (22) and also from two species of ixodid ticks, *Ixodes persulcatus* and *Ixodes ovatus*, in the northern part of Japan (21, 24). *I. ovatus* is not a member of the *I. ricinus* complex (13), and the spirochetes isolated from *I. ovatus* are quite distinct from those of the *Borrelia* species associated with Lyme disease (20, 28). Epidemiological studies revealed extensive prevalence of spirochetal infection in both *I. persulcatus* and *I. ovatus*, which are the most common species that bite humans in Japan (21, 23, 24). By analysis of protein profiles and the reactivities with monoclonal antibodies, all the human isolates were found to be identical with the tick isolates from *I. persulcatus* but not from *I. ovatus* (23). Accordingly, *I. persulcatus* is considered to be the most important vector of Lyme disease in Japan.

Comparison of different borrelial isolates by various procedures and approaches has revealed their heterogeneity. By immunological analysis, there appeared to be significant differences in the reactivities of isolates with monoclonal antibodies (6, 29). Heterogeneity in plasmid profiles among borrelial isolates has also been reported (3, 8, 14, 17), and in vitro cultivation has influenced the plasmid contents (3, 26).

We report here the antigenic and genetic characteristics of the borrelial isolates of Japan, and the availability of valuable new isolates for use in characterizing vector competency and in relating genetic sequences to clinical disease, epidemiology, and diagnostic detection.

I. persulcatus adult ticks were collected in forests by flagging vegetation at various sites in Hokkaido, Japan. The spirochetes were obtained from ticks collected as described previously (24) and cultivated in BSKII medium (2) at 31°C. Strains of three different genospecies, *B. burgdorferi* (B31, 297, NCH-1, and IPS), *Borrelia garinii* (P/Bi, G25, Ir210, and PD89), and group VS461 (PGau, BO23, UMO1, and SMS1), were also used in this study. The procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis were done as described previously (24). Murine monoclonal antibodies H5332 and H9724 were obtained as hybridoma supernatants (5, 7). Polyclonal antiserum to outer surface protein A (OspA) of *B. burgdorferi* B31 was prepared from BALB/c mice. The OspA protein was electroeluted from the gel slices of SDS-PAGE. Mice were injected intraperitoneally with OspA protein mixed with Freund's incomplete adjuvant.

Crossfield (AE6800; ATTO Corp., Tokyo, Japan) was used in pulsed-field gel electrophoresis (PFGE) experiments. DNAs from borrelial isolates were prepared by the gel insert method (8). Washed cells (2×10^9 cells per ml) were mixed with an equal volume of 1.5% low-melting-point agarose (Incert agarose; FMC Bioproducts, Rockland, Maine), and the mixture was allowed to solidify in 100-μl rectangular molds. The plugs were incubated for 24 h at 37°C with gentle shaking in a buffer (12 mM Tris-HCl, 2 M NaCl, 0.2 M EDTA, 0.5% Sarkosyl, 1 mg of lysozyme per ml [pH 7.6]) and then deproteinized by incubation in a solution containing 0.5 M EDTA, 1% Sarkosyl, and 2 mg of proteinase K per ml, pH 8.5, for 48 h at 50°C. The plugs were placed in the wells of 1.2% agarose gels (SeaKem GTG; FMC Bioproducts). The electrophoresis system was buffered with 45 mM Tris-borate–1.25 mM EDTA. The gels were run at a constant voltage of 100 V for 40 h at 10°C with 2-s pulses. Molecular size markers consisted of lambda phage DNA concatemers (FMC Bioproducts) and lambda DNAs digested with *Hin*dIII enzyme.

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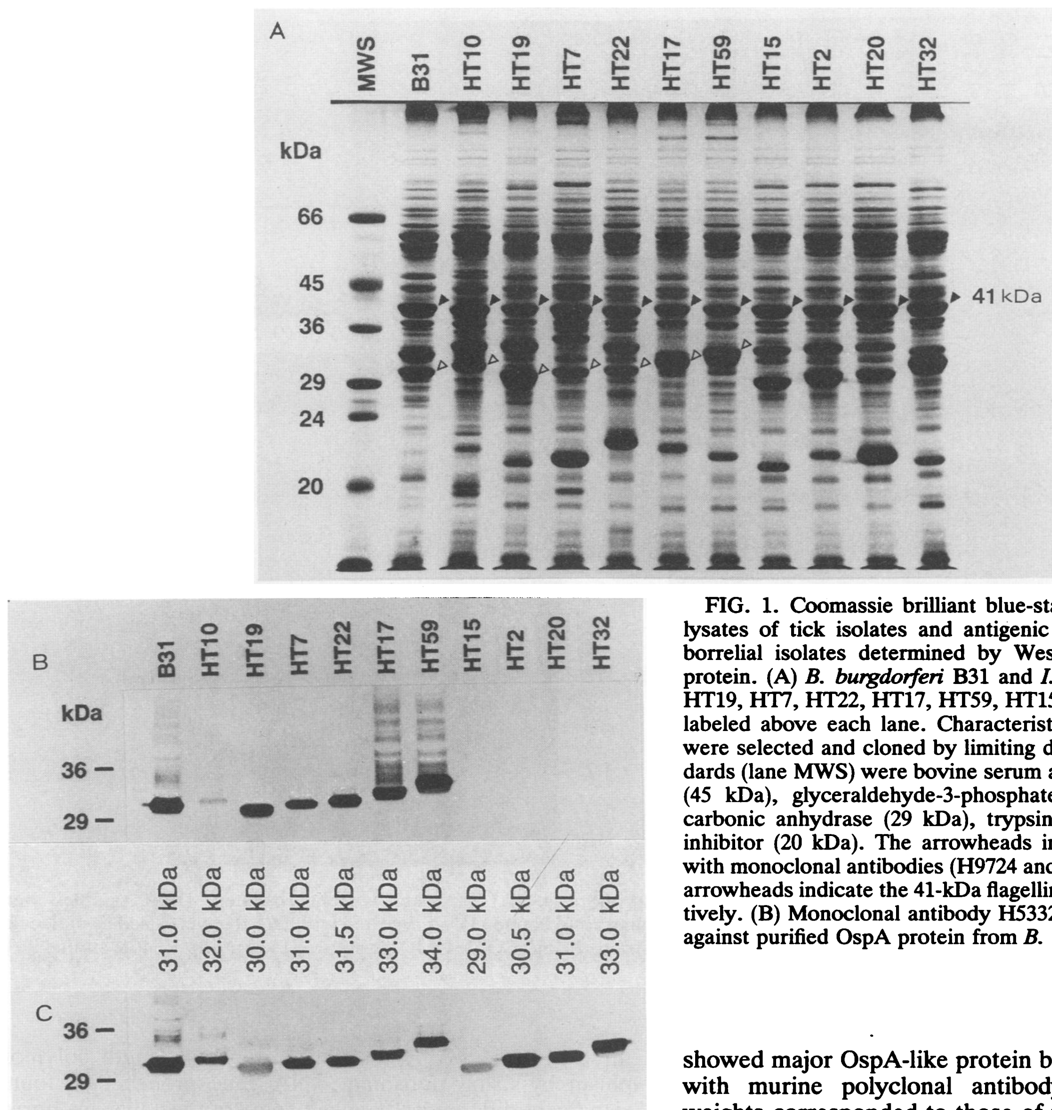

FIG. 1. Coomassie brilliant blue-stained proteins in whole cell lysates of tick isolates and antigenic characteristics of Japanese borrelial isolates determined by Western blotting against OspA protein. (A) *B. burgdorferi* B31 and *I. persulcatus* isolates HT10, HT19, HT7, HT22, HT17, HT59, HT15, HT2, HT20, and HT32 are labeled above each lane. Characteristic isolates of *I. persulcatus* were selected and cloned by limiting dilution. Molecular size standards (lane MWS) were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20 kDa). The arrowheads indicate isolates that reacted with monoclonal antibodies (H9724 and H5332). The solid and open arrowheads indicate the 41-kDa flagellin and OspA proteins, respectively. (B) Monoclonal antibody H5332; (C) mouse polyclonal sera against purified OspA protein from *B. burgdorferi* B31.

More than 100 borrelial isolates obtained from *I. persulcatus* in Hokkaido were examined. The protein profiles of all isolates examined were different, and the major proteins of the Japanese isolates were found to differ from the North American type strain *B. burgdorferi* B31. Ten characteristic isolates having different protein profiles were selected and cloned by limiting dilution. The range of variation in the protein profiles of these isolates is illustrated in Fig. 1A. Borrelial isolates from Hokkaido varied in their lower-molecular-mass proteins (21 to 35 kDa) but were similar to each other in proteins with sizes of 36 kDa or larger. The epitope for H9724 was present in the 41-kDa flagellin protein of all isolates (including strain B31). The reactivities of monoclonal antibody H5332 against the isolates from *I. persulcatus* are shown in Fig. 1B, and the molecular sizes of proteins that reacted with murine anti-OspA polyclonal antibody are shown in Fig. 1C. In contrast to H9724, the reactivities of proteins with H5332 were quite different. Six isolates (HT10, HT19, HT7, HT22, HT17, and HT59) possessed the proteins that reacted with H5332, but their molecular sizes were heterogeneous and ranged from 30 to 34 kDa. The other four isolates (HT15, HT2, HT20, and HT32) failed to react with H5332, whereas these strains showed major OspA-like protein bands. All isolates reacted with murine polyclonal antibody, and their molecular weights corresponded to those of the OspA proteins. Monoclonal antibody H5332 reacted weakly with the 32-kDa OspA protein of HT10, and the OspA proteins of HT19 and HT15 also reacted weakly with murine polyclonal antibodies.

We analyzed Japanese isolates in comparison with the strains of three different genospecies from North America, Europe, and Asia by pulsed-field gel electrophoresis because of their antigenic heterogeneity and diverse protein profiles. DNA samples from 10 Japanese isolates and 12 strains from the three genospecies were analyzed to determine their plasmid profiles. The plasmid profiles are shown in Fig. 2. No two isolates examined had the same plasmid profile, and each isolate had from four to six discernible plasmids. All Japanese isolates resembled one another in contrast to the other isolates and all isolates contained an approximately 70-kb plasmid which appeared to correspond to the plasmid that encodes the OspA protein in *B. burgdorferi*. The smaller plasmids of North American, European, and Asian strains showed more variety.

Epidemiological studies revealed extensive prevalence of spirochetal infections in questing adult *I. persulcatus* ticks in various localities in Japan (24). Furthermore, some spirochetal isolates were obtained from humans with erythema chronicum migrans, and, by analysis of their protein com-

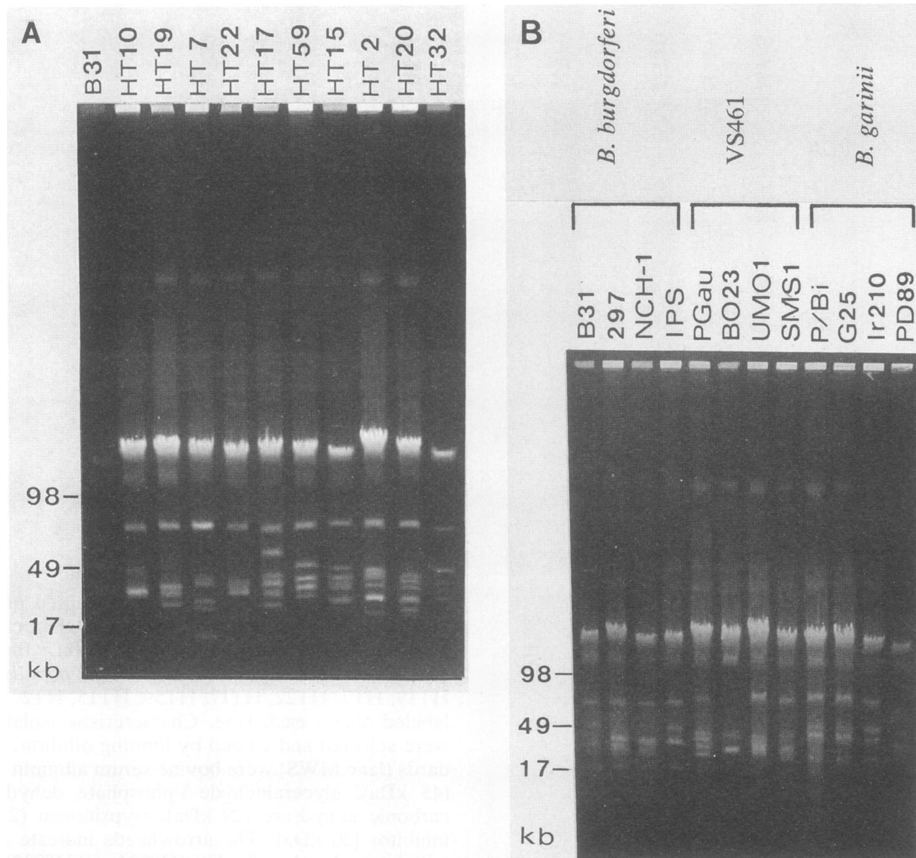

FIG. 2. Separation of *Borrelia* isolates by pulsed-field gel electrophoresis. (A) Japanese borrelial isolates. DNA samples were electrophoresed at 100 V with 2-s pulses for 40 h. Lambda DNA concatemers and lambda DNA digested with *Hin*dIII were used as molecular size markers. (B) Strains B31, 297, NCH-1, and IPS are *B. burgdorferi*; strains PGau, BO23, UMO1, and SMS1 are group VS461; strains P/Bi, G25, Ir210, and PD89 are *B. garinii*.

positions and reactivities with monoclonal antibodies, all the human isolates were found to be identical to the borrelial isolates from *I. persulcatus* (23). These results indicated that *I. persulcatus* is the most important vector of Lyme disease in Japan.

As we reported here, Japanese isolates are quite distinct from any other strains isolated in different geographic locations of the world. The Japanese isolates have major proteins with either no or only partial antigenic relatedness to the 31-kDa OspA protein. This finding indicates that there are epitopes conserved at various degrees between the 31-kDa OspA protein of the B31 strain and the 30- to 34-kDa proteins of the Japanese isolates. The weak reaction by Western blot against H5332 (HT10) and polyclonal antibodies (HT19 and HT15) also showed less conservation of antigenic properties. Recently, OspA gene polymorphism in *B. burgdorferi* strains has been reported (25). The evidence of lack of some characteristic OspA epitopes arising from an OspA gene recombination or mutations supports our results described here.

Borrelial isolates associated with Lyme disease form three genospecies, *B. burgdorferi*, *B. garinii*, and the third referred to as group VS461 (1). It should be noted that all of the North American strains belong to one species, *B. burgdorferi* (1, 19, 28). In contrast, there are three genomic species in Europe (1). The genomic DNAs from Japanese isolates were compared with those of other known strains of the three genospecies by restriction fragment length polymorphism by using ribosomal RNA gene probes. We found significant differences between some of the Japanese borrelial isolates from *I. persulcatus* and North American, European, and Asian strains (12). The restriction fragment length polymorphism analysis showed that HT10 belongs to group VS461 and that HT17, HT59, and HT32 closely resemble the strains of *B. garinii*. The other Japanese isolates are quite distinct from any other borrelial strains (data not shown).

As we showed here, the characteristics of some Japanese isolates resemble those of European strains. This similarity is thought to be related to the distribution of vector ticks belonging to the *I. ricinus* complex. *I. ricinus* is present in European countries, and *I. persulcatus* ticks range continuously from eastern Europe to the Far East. Thus, our findings may indicate that the European variants of *Borrelia* species invaded northern Japan together with the vector ticks. The wide variation in antigenic and genetic properties of the Japanese isolates also suggests multiple mutations during propagation of the *Borrelia* species.

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