LYME DISEASE IN TAIWAN: PRIMARY ISOLATION OF BORRELIA BURGDORFERI–LIKE SPIROCHETES FROM RODENTS IN THE TAIWAN AREA

CHIEN-MING SHIH AND LI-LIAN CHAO
Department of Parasitology and Tropical Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China

Abstract. To investigate the prevalence of Lyme disease infection in Taiwan, we conducted a zoonotic survey for spirochetal infection in the small mammals. Ear tissues of trapped rodents collected from various localities in Taiwan were incubated into BSK-H culture medium and examined for the evidence of spirochetal infection by dark-field microscopy. Spirochetes cultured from six species of wild and peridomestic rodents and seven isolates, designated TWKM 1-7, were purified by serial dilution and membrane filtration. Infection was detected in 16.6% (53 of 320) of captured rodents and the highest infection rate (36.4%) was observed in the brown country rat (Rattus losea, Swinhoe). Higher infection rates based on the geographic distribution were observed in the eastern localities and on Kimmen Island. Reactivity with Borrelia burgdorferi–specific monoclonal antibodies and Western blot analysis indicated that these Taiwan isolates were closely related to the causative agent of Lyme disease, B. burgdorferi sensu lato. These results provide the first evidence of the existence of Lyme disease spirochetes in the Taiwan area.

Lyme disease is an emerging tick-borne spirochetal infection and may cause multisystem human illness that usually begins with an expanding annular skin lesion known as erythema chronicum migrans. The etiologic agent of Lyme disease, Borrelia burgdorferi sensu lato, is transmitted mainly by ticks of the Ixodes ricinus complex in North America and Europe, and by I. persulcatus and I. ovatus ticks in the countries of Far East Asia. Although Lyme disease is the most common vector-borne human infection in the Europe and United States, new cases and endemic regions can be identified due to the world-wide distribution of vector ticks.

The existence of Lyme disease spirochetes has never been reported in the Taiwan area. Although a laboratory-diagnosed human case of Lyme disease had been reported in Taiwan, the prevalence of spirochetal infection in the small mammals as well as the vector ticks responsible for the transmission of spirochetes remain undefined. Thus, the present study intended to investigate the spirochetal infection by conducting a zoonotic survey at various localities in Taiwan. Attempts to purify and identify the spirochetal isolates were also performed.

MATERIALS AND METHODS

Epizootiologic survey. Small mammals from seven localities of Taiwan (Figure 1) were trapped from May to December 1996 and July to September 1997. After appropriate anesthetization, ear tissues and ectoparasitized ticks were collected from trapped rodents for further investigations. A total of 320 wild and peridomestic rodents were trapped, and they included 55 brown country rats (Rattus losea, Swinhoe), 31 black rats (R. rattus, Linnaeus), 67 brown rats (R. norvegicus, Erxleben), 30 spinous country rats (R. coxinga, Swinhoe), 22 house shrews (Suncus murinus, Linnaeus), 15 Formosan mice (Mus formosanus, Kuroda), 74 bandicoot rats (Bandicota indica, Hodgson), 22 Formosan field mice (Apodemus semutus, Thomas), and four Formosan harvest mice (Micromys minutus, Pallas). The species of trapped rodents was identified according to Yu and Lee.

Collection and identification of ticks. Infested ticks were collected from captured rodents during the study periods. The collected ticks of various life cycle stages were pre-incubated into BSK-H medium and examined for spirochetal infection by dark-field microscopy. When spirochetes were observed in 70% ethanol and the species was identified according to the pictorial keys of Ixodidae. To allow further deposition of eggs, the engorged adult ticks were kept in the mesh-covered and plaster-bottomed plastic vials. Attempts to establish tick colony in the laboratory were also performed.

Isolation and purification of spirochetes. For isolation of spirochetes, ear tissues of each rodent were washed in 70% ethanol and rinsed in sterile phosphate-buffered saline (PBS) before transfer to a culture tube containing BSK-H medium (B3528; Sigma, St. Louis, MO) supplemented with 6% rabbit serum (R7136; Sigma) as described previously. All tissue cultures were incubated at 34°C in a humidified CO₂ incubator (Nuaire, Inc., Plymouth, MN) and was examined for spirochetes weekly for eight weeks by dark-field microscopy (BX-60; Olympus Co., Tokyo, Japan).

For purification of spirochetes, spirochete-positive cultures were transferred to a new culture tube by serial dilution. One week after passage, the contaminated spirochete cultures were passed through a 0.45-µm syringe filter (Sartorius, Göttingen, Germany) and diluted into several tubes of fresh BSK-H medium as described previously. Pure culture of spirochetes was examined every three days for three weeks by dark-field microscopy. When spirochetes were observed in the media without bacterial contaminants, pure isolates were subcultured and used for immunologic analysis.

Monoclonal antibodies. Five murine monoclonal antibodies (MAbs) were obtained as undiluted hybridoma supernatants from Dr. Alan G. Barbour (Department of Microbiology and Medicine, University of Texas Health Science Center, San Antonio, TX). The MAbs H5332 and H3TS are specific against the outer surface protein A (OspA) of B. burgdorferi. MAbs H6831 and H614 are specific against OspB, and MAb H9724 reacts with a protein of the periplasmic flagella of the genus Borrelia.

Immunoreactivity assay. The identity of the spirochetes isolated from ear tissues was verified by indirect immunofluorescent antibody assay (IFA) with fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse immunoglobulin G (F8264; Sigma) as secondary antibody. Spirochetal isolates (TWKM1-7) were centrifuged, washed, resuspended in PBS (pH 7.2), and prepared as antigens in 10-well glass
IFA slides as previously described. Briefly, the prepared slides of each isolate were covered with 20 μl of undiluted MAbs against OspA (H5332 and H3TS), OspB (H6831 and H614), or flagella (H9724) of *B. burgdorferi*. These slides were placed in a humid chamber, incubated for 30 min at 37°C, washed three times with PBS, and then allowed to air-dry. Twenty microliters of FITC-conjugated secondary antibody, used at a dilution of 1:50, was added to each well, and the slides were allowed to incubate for another 30 min at 37°C. After three washes with PBS, the slides were allowed to air dry, mounted with buffered glycerin, and covered with coverslips. Fifty random fields were examined by epifluorescence microscopy with a high-power (400×) objective, and the reactivity was recorded. For comparison, the American type strain of the B31 spirochete was used as a control.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For protein electrophoresis, whole cell lysates of spirochetes were prepared from *Borrelia* isolates from Taiwan (TWKM1-7), North America (B31 and JD1), and Japan (HT59 and Bfox). The JD1 strain of *B. burgdorferi* was originally isolated from a naturally infected nymphal tick. Strains HT59 and Bfox were isolated from *Ixodes persulcatus* and *Vulpes vulpes*, respectively. Briefly, spirochetes were grown to a density of ~2 × 10^8 cells/ml in BSK-H medium supplemented with 6% rabbit serum. Cells were harvested by centrifugation, washed three times in PBS (pH 7.2) containing 5 mM MgCl₂, and resuspended in SDS.
sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 50 mM dithiothreitol, 10% glycerol, 0.004% bromophenol blue) as described previously. After the samples were boiled for 5 min, 5–10 μg of protein per lane was loaded and subjected to continuous SDS-PAGE on 12.5% gels (PhastGel; Pharmacia Biotech). The molecular weight of major spirochetal protein bands were stained and visualized with Coomassie brilliant blue (PhastGel Blue R; Pharmacia Biotech, Taipei, Taiwan) using a mini-gel apparatus to continuous SDS-PAGE on 12.5% gels (PhastGel; Pharmacia Biotech). The protein profiles of major spirochetal protein bands were compared by comparing their electrophoretic mobilities with those of molecular weight standards (14–94 kD; Pharmacia Biotech).

**Western blot analysis.** Electrophoresed proteins were transferred from the SDS-PAGE gels to nitrocellulose blotting membranes (Sartorius) using a semi-dry electroblotter (PhastTransfer electrode cassette; Pharmacia Biotech). The transferred membranes were blocked for 2 hr with 3% gelatin in Tris-buffered saline (TBS, pH 7.5) containing 20 mM Tris and 500 mM NaCl, and then incubated for 2 hr at room temperature in a 1:40 dilution of hybridoma supernatant containing MAb against OspA (H5332), OspB (H6831), or flagella (H9724) protein of *B. burgdorferi*. After being washed with buffer solution, the membranes were immersed for 2 hr in horseradish peroxidase (HRP)–conjugated sheep anti-mouse IgG (NA931; Amersham, Buckinghamshire, United Kingdom) diluted 1:500 with 0.05% Tween 20 in TBS (T/TBS). The membranes were then washed twice with T/TBS, a substrate solution (10 ml of methanol containing 30 mg of 4-chloro-1-naphthol and 25 μl of 30% hydrogen peroxide mixed with 50 ml of TBS) was added to develop the color for 5–10 min, and the reacted membranes were washed with distilled water and air-dried for further analysis.

**RESULTS**

We first determined the prevalence of spirochetal infection among different species of wild and peridomestic rodents captured at various localities in Taiwan. Ear tissues of captured rodents were collected at seven localities in Taiwan and examined for the evidence of spirochetal infection by *in vitro* cultivation. Of 320 rats, spirochetes were detected in six species of rodent hosts and the highest infection rate (36.4%) was detected in the brown country rat (*R. losea*) (Table 1). Higher infection rates were also detected in the species of *R. rattus* (19.4%), *R. norvegicus* (20.9%), and *I. indica* (13.5%). In addition, the infection rates for *S. murinus* and A. semutus were 4.5% and 9.1%, respectively. No spirochetal infection was detected in *R. coxinga*, M. formosanus, and *Micromys minutus*. The overall infection rate throughout the Taiwan area was 16.6% and the highest infection rate (25.8%) was observed on Kimmen Island. A higher infection rate based on geographic distribution was also observed in the eastern area of Taiwan, and the infection rates for Hualian and Taitung county were 18.6% and 22.1%, respectively. These results demonstrate the geographic variations of spirochetal infection among the rodent hosts in the Taiwan area.

We then attempted to purify the spirochetal isolates by serial dilution combined with membrane filtration and identify the collected ticks that infested on captured rodent hosts. Of the 53 positive cultures, only seven isolates (designated TWKM1-7) were purified from three species of rodents captured on Kimmen Island (Table 2), and the brown country rat (*R. losea*) and brown rat (*R. norvegicus*) were recognized as the main reservoir hosts for *Borrelia* spirochetes. Although four species of *Ixodes* and *Dermacentor* ticks had been collected from these captured rodent hosts, *I. granulatus* was found abundantly on the rats (*R. losea*) with the highest infection rate and was highly suspected as the principle vector tick for transmission of spirochetal infection in Taiwan. These results provide the first evidence of isolation of *Borrelia* spirochetes and demonstrate the possible vector ticks of spirochetal transmission in the Taiwan area.

**Immunoreactivity tests** were also performed to determine whether the identity of the Taiwan isolates (TWKM1-7) could be correlated with the causative agent of Lyme disease, *B. burgdorferi* sensu lato. The spirochetal isolates were assayed with *B. burgdorferi*-specific MAbs and the B31 spirochete strain was used for comparison. All isolates reacted intensely with MAbs H5332 and H9724, and strong reactivity was also observed with MAbs H3TS and H614 (Table 3). In contrast, only three isolates (TWKM5-7) and B31 spi-
rochetes reacted with MAb H6831. These results indicate that the species of the Taiwan isolates were serologically related to B. burgdorferi sensu lato, but partial antigenic variation may exist among these isolates.

The protein profiles of spirochetal isolates examined in this study were demonstrated by SDS-PAGE. Although all Taiwan isolates (TWKM1-7) had variable protein bands ranging from 24 kD to 67 kD, two major protein bands with molecular weights of approximately 31 kD (OspA) and 41 kD (flagellin) were consistent with those of the North American B31 strain of B. burgdorferi (Figure 2). The protein profiles of isolates TWKM5-7 were similar to each other (Figure 2, lanes 5–7), and the major protein band with molecular weight of approximately 33 kD (OspB) was distinctive when compared with isolates TWKM1–4 (Figure 2, lanes 1–4).

Western blot analysis indicated that all Taiwan isolates have protein bands that reacted with MAbs H5332 and H9724 against the OspA and flagellin proteins of B. burgdorferi, respectively (Figure 3). Only isolates TWKM5-7 reacted with MAB H6831 against the OspB protein of B. burgdorferi. These results showed the homogeneity of the 31-kD and 41-kD reactive proteins of all Taiwan isolates, and the spirochetal isolates from Taiwan were presumably identified as B. burgdorferi sensu lato.

DISCUSSION

Our report describes the first isolation of Lyme disease spirochetes from rodents in the Taiwan area. Because of the high prevalence of spirochetal infection among captured rodents in Taiwan, the existence of zoonotic transmission of Lyme disease spirochetes is likely. In addition, results from this study also show that the geographic variations of spirochetal infection may correlate with the abundance of reservoir hosts in different geographic areas of Taiwan. Indeed, the highly infected hosts (R. losea, R. norvegicus, R. rattus, and B. indica) were captured mainly in the eastern localities and on Kimmen Island. Thus, an epidemiologic survey that focused on these endemic sites would be required to elucidate the seasonal abundance of reservoir hosts and the prevalence of spirochetal infection.

Although a laboratory-diagnosed human case of Lyme disease had been reported in Taiwan, the prevalence of Borrelia infection had never been investigated in the human populations. Due to the relatively high prevalence of tick-borne spirochetal infection in small mammals, it would be interesting to determine the seroprevalence of spirochetal infection among human populations in Taiwan by conducting a general serosurvey using antigens prepared from the spirochetal isolates of Taiwan.

The identity of a Borrelia isolate can be verified by seroreactivity with B. burgdorferi-specific MAbs. Although heterogeneity of major outer surface proteins between different geographic isolates of B. burgdorferi had been documented, the seroreactivity with MAbs H5332 (anti-OspA) and H9724 (anti-flagellin) has been found in most spirochetal isolates of Lyme disease. In our study, the spirochetal isolates of Taiwan also reacted intensely with the B. burgdorferi-specific MAbs. Although a laboratory-diagnosed human case of Lyme disease had been reported in Taiwan, the prevalence of Borrelia infection had never been investigated in the human populations. Due to the relatively high prevalence of tick-borne spirochetal infection in small mammals, it would be interesting to determine the seroprevalence of spirochetal infection among human populations in Taiwan by conducting a general serosurvey using antigens prepared from the spirochetal isolates of Taiwan.

The heterogeneity among major protein bands in spirochetal isolates may be as diverse as their origin of isolation. Although antigenic variation of B. burgdorferi may occur due to long-term passage in cultures, difference in outer

<table>
<thead>
<tr>
<th>Rodent species</th>
<th>No. of positive cultures</th>
<th>No. of pure isolates</th>
<th>Pure isolates designated</th>
<th>Species of tick*</th>
<th>Stage of tick</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus losea</td>
<td>20</td>
<td>4</td>
<td>TWKM1-4</td>
<td>I.g.</td>
<td>Nymph</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>6</td>
<td>0</td>
<td>TWKM5, 7</td>
<td>I.g.</td>
<td>Adult</td>
</tr>
<tr>
<td>Suncus murinus</td>
<td>14</td>
<td>2</td>
<td>TWKM 6</td>
<td>I.g.</td>
<td>Nymph</td>
</tr>
<tr>
<td>Bandicota indica</td>
<td>1</td>
<td>1</td>
<td>TWKM 6</td>
<td>I.g.</td>
<td>Adult</td>
</tr>
<tr>
<td>Apodemus sematus</td>
<td>10</td>
<td>0</td>
<td>TWKM 5</td>
<td>D.t.</td>
<td>Nymph</td>
</tr>
</tbody>
</table>

* I.g. = Ixodes granulatus; D.t. = Dermacentor taiwanensis; I.o. = I. ovatus; I.k. = I. kantzi.

Table 2
Purification of spirochetal isolates and identification of possible vector ticks from rodents captured in the Taiwan area

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of positive cultures</th>
<th>No. of pure isolates</th>
<th>Pure isolates designated</th>
<th>Species of tick*</th>
<th>Stage of tick</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWKM1–4</td>
<td>20</td>
<td>4</td>
<td>TWKM1-4</td>
<td>I.g.</td>
<td>Nymph</td>
</tr>
<tr>
<td>TWKM1–7</td>
<td>14</td>
<td>2</td>
<td>TWKM5, 7</td>
<td>I.g.</td>
<td>Adult</td>
</tr>
<tr>
<td>TWKM 6</td>
<td>1</td>
<td>1</td>
<td>TWKM 6</td>
<td>I.g.</td>
<td>Nymph</td>
</tr>
</tbody>
</table>

Table 3
Immunoreactivity of spirochetal isolates of Taiwan (TWKM 1–7) and B31 spirochete with Borrelia burgdorferi–specific monoclonal antibodies

<table>
<thead>
<tr>
<th>Kind of antibody</th>
<th>Target protein†</th>
<th>Reactivity* with Taiwan and B31 isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5332</td>
<td>Osp A</td>
<td>+</td>
</tr>
<tr>
<td>H3TS</td>
<td>Osp A</td>
<td>+</td>
</tr>
<tr>
<td>H6831</td>
<td>Osp B</td>
<td>–</td>
</tr>
<tr>
<td>H614</td>
<td>Osp B</td>
<td>+</td>
</tr>
<tr>
<td>H9724</td>
<td>Flagellin</td>
<td>+</td>
</tr>
</tbody>
</table>

* Reactivity was indicated as + (positive) or – (negative).
† Osp A = outer surface protein A.
Fig. 2. Coomassie brilliant blue-stained protein profiles in whole cell lysates of *Borrelia* isolates. Lane B, American type strain B31 (control); lanes 1–7, Taiwan isolates (TWKM1-7) from rodents; lane J, Massachusetts (United States) isolate (JD1) from *Ixodes dammini*; lane H, Japan isolate (HT59) from *I. persulcatus*; lane F, Japan isolate (Bfox) from *Vulpes vulpes*. Molecular weight standards (lane M) are shown on the left in kilodaltons (kD). Arrows identify the outer surface protein A (OspA), OspB, and flagella proteins of *B. burgdorferi*.

Fig. 3. Western blot analysis of *Borrelia* isolates with monoclonal antibodies against outer surface protein A (OspA) (H5332), OspB (H6831), and flagellin (H9724) proteins of *B. burgdorferi*. Lane B, American type strain B31 (control); lanes 1–7, Taiwan isolates (TWKM1-7); lane J, Massachusetts (United States) isolate (JD1); lanes H and F, Japan isolates (HT59 and Bfox).

Surface protein composition had previously been reported among spirochetal isolates derived from various animal and tick hosts. Indeed, even an isolate from a rabbit kidney differed from isolates derived from a larval vector tick that had fed on the same rabbit. In our study, spirochetal isolates (TWKM1-4) from *R. losea* differed antigenically from isolates (TWKM5-7) derived from *R. norvegicus* and *S. murinus*, and antigenic variation of OspB (reacted with MAb H6831) was observed in the Taiwan isolates. Thus, the heterogeneity among the major protein bands of OspB in these Taiwan isolates may be correlated with the diverse hosts for spirochetal isolation.

The natural transmission cycle for the spirochetal isolates in Taiwan remains undefined. Further identification of the animal reservoirs and the possible vector ticks responsible for transmission would help to understand the enzootic transmission cycle of *Borrelia* spirochetes in Taiwan. We are currently trying to maintain the tick colony of *I. granulatus* obtained from field collection in our laboratory, and the competence of these ticks to transmit *Borrelia* spirochetes as well as the possible mechanism for maintaining spirochetal infection within these ticks will be further investigated. Thus, further investigations on the isolation and characterization of *Borrelia* spirochetes from *Ixodes* ticks would be required to verify the transmission cycle of spirochetes and identify the risk of acquiring infection by human populations in Taiwan.

In conclusion, our report describes the primary isolation of *Borrelia* spirochetes in the Taiwan area. The spirochetal isolates (TWKM1-7) was morphologically similar to and serologically related to the causative agent of human Lyme disease, *B. burgdorferi* sensu lato. This report also highlights the increasing evidence of the existence of tick-borne spirochetal infection in the Taiwan area.

Acknowledgments: We thank Dr. Ling-Ling Lee (Department of Zoology, National Taiwan University, Taipei) for the helpful pictorial guide for the species identification of captured rodents, and Drs. Ting-Hsiang Lin (Section of Medical Entomology, National Institute of Preventive Medicine, Department of Health, Taipei) and Trong-Neng Wu (National Quarantine Service, Department of Health, Taipei) for assistance in trapping rodents and collection of ear tissues. The technical assistance for protein electrophoresis and immunoblot analysis by Han-Ming Chang is highly appreciated.

Financial support: This work was supported in part by a grant from the Department of Health (DOH86-TD-058), The Executive Yuan, Taipei, Taiwan, Republic of China.

Authors’ address: Chien-Ming Shih and Li-Lian Chao, Department of Parasitology and Tropical Medicine, National Defense Medical Center, PO Box 90048-506, Taipei, Taiwan, Republic of China.

Reprint requests: Chien-Ming Shih, Department of Parasitology and Tropical Medicine, National Defense Medical Center, PO Box 90048-506, Taipei, Taiwan, Republic of China.

REFERENCES


2. Steere AC, Bartennagen NH, Craft JE, Hutchinson GI, Newman JH, Rahn DW, Sigal LH, Spieler PN, Stenn KS, Malawista


