Am. J. Trop. Med. Hyg., 66(5), 2002, pp. 611–615

AN OspA-BASED GENOSPECIES IDENTIFICATION OF LYME DISEASE SPIROCHETES (*BORRELIA BURGDORFERI*) ISOLATED IN TAIWAN

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Abstract. To further identify the genospecies of Lyme disease spirochetes (*Borrelia burgdorferi*) isolated in Taiwan, we analyzed the genomic identities of these Taiwan isolates (TWKM1–7) by genospecies-specific polymerase chain reaction (PCR) assay, restriction fragment length polymorphism (RFLP) analysis, and gene sequencing based on the OspA gene sequences of *B. burgdorferi* sensu lato. PCR analysis indicates that all of these Taiwan isolates were genetically related to the genospecies of *B. burgdorferi* sensu stricto by their differential reactivities with genospecies-specific PCR primers. After cleavage by DraI, three different RFLP patterns in relation to three different genospecies of Lyme disease spirochetes were observed, and all of these Taiwan isolates were affiliated with the genospecies of *B. burgdorferi* sensu stricto. The phylogenetic analysis also reveals that the sequence similarity of PCR-amplified OspA gene of these Taiwan isolates is highly homogeneous, with a homogeneity of more than 99.8% within the genospecies of *B. burgdorferi* sensu stricto. These results confirm that the genomic identities of these Taiwan isolates belong to the genospecies of *B. burgdorferi* sensu stricto.

INTRODUCTION

Lyme disease is an emerging tick-borne spirochetal infection[1] that can cause multisystem human illness. Clinical symptoms vary among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurologic, and cardiac manifestations.[2,3] 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[4,5] and by *I. persulcatus* and *I. ovatus* ticks in the countries of Far East Asia.[6–8] Although laboratory-confirmed case of human Lyme disease have been reported in Taiwan[9] and spirochetes can be isolated from six species of rodents in the Taiwan area,[10] the genetic variety of spirochetes and the tick vector responsible for transmission in Taiwan remain undefined.

The heterogeneity of molecular and immunological characteristics among isolates of *B. burgdorferi* sensu lato from different geographical and biological origins has been demonstrated previously.[11–16] On the basis of immunoreactivity with *B. burgdorferi*-specific monoclonal antibodies, plasmid profiles, and patients' clinical manifestations, the causative agents of Lyme disease can be classified into three major genospecies: *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii* (group VS461).[17,18] In addition, genomic analysis of similarities among *Borrelia* isolates by polymerase chain reaction (PCR) with species-specific primer sets and by restriction pattern of a specific target gene has been proven useful for the species identification and typing of *Borrelia* isolates from various geographical areas.[19–22]

The existence of outer surface protein (Osp) genes in all isolates belonging to the three major genospecies of *B. burgdorferi* sensu lato has been verified previously.[23,24] The genomic similarity of *Borrelia* isolates can be clarified by their differential reactivity with genospecies-specific OspA primer sets and by analyzing the homogeneity of OspA gene in sequences.[22,25–27] Thus, the aim of the current study was to characterize the genomic identities of these Taiwan isolates further by their differential primer reactivities with PCR assay and by analyzing the heterogeneity of restriction patterns with restriction fragment length polymorphism (RFLP) analysis. Moreover, attempts to identify the genospecies of these Taiwan isolates were also confirmed by sequence analysis of PCR-amplified OspA gene.

MATERIALS AND METHODS

Spirochete strains and culture. Seven strains of Taiwan isolates (TWKM1–7) and four strains of *Borrelia* isolates belonging to the the genospecies of *B. burgdorferi* sensu stricto (B31 and JD1), *B. garinii* (K48), and *B. afzelii* (VS461) were used for the species-specific PCR analysis as described previously.[28] For the RFLP and phylogenetic analysis, six additional strains of the genospecies of *B. burgdorferi* sensu stricto (N40, TB, CT27985, VS219, CT20004, and ECM-NY86) were also included. Spirochetes were subcultured at 34°C in a humidified incubator (Nuaire, Inc., Plymouth, MN) with 5% CO_2 and maintained in BSK-H medium (catalog no. B3528; Sigma Chemical Co., St. Louis, MO) supplemented with 6% rabbit serum (catalog no. R7136; Sigma) as described previously.[10] All cultures were examined weekly for the optimal growth of spirochetes by dark-field microscope (model BX-60, Olympus Co., Tokyo, Japan).

Preparation of spirochete DNA. Total genomic DNA from all *Borrelia* strains was extracted as described previously.[28] Briefly, samples (3 mL) of cultured spirochetes were grown to a density about 2×10^8 cells/mL of medium and were centrifuged for 10 min at 12,000 *g* to pellet the spirochetes. The pellets were washed twice with phosphate-buffered saline (PBS) (pH 7.2) containing 5 mM $MgCl_2$ resuspended in 150 μL of distilled water, and boiled for 10 min. After centrifugation at 10,000 *g* for 10 sec, the supernatant was collected and the DNA concentrations were determined spectrophotometrically using a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

PCR and RFLP analysis. DNA samples extracted from the Taiwan isolates and other spirochetes representative of the three major genospecies of *B. burgdorferi* sensu lato were used for PCR analysis to characterize the genomic identity of Taiwan isolates. Four sets of PCR primers were synthesized and used in this study (Table 1). Primer sets SL were designed to amplify DNA of all three genospecies of *B. burgdorferi* sensu lato. Other primer sets of GI, GII, and GIII were designed to amplify the DNA of *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelli,* respectively.[22] All PCR reagents and TaqGold DNA polymerase were obtained from the GeneAmp kit and were used as recommended by the supplier (Perkin-Elmer Cetus, Taipei, Taiwan).

TABLE 1
PCR primers used for detection and identification of the OspA gene in relation to three major genospecies of *B. burgdorferi* sensu lato

Primer set*	Oligonucleotide sequence (position and orientation)	Length (bp)	Target genospecies
SL	5′-AATAGGTCTAATAATAGCCTTAATAGC-3′ (21 → 47)	27	*B. burgdorferi* sensu lato
	5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′ (302 ← 328)	27	
GI	5′-AACAAAGACGGCAAGTACGATCTAATT-3′ (139 → 165)	27	*B. burgdorferi* sensu stricto
	5′-TTACAGTAATTGTTAAAGTTGAAGTGCC-3′ (655 ← 682)	28	
GII	5′-TGATAAAAACAACGGTTCTGGAAC-3′ (201 → 224)	24	*B. garinii*
	5′-GTAACTTTCAATGTTGTTTTGCCG-3′ (522 ← 545)	24	
GIII	5′-TAAAGACAAAACATCAACAGATGAAATG-3′ (347 → 374)	28	*B. afzelii*
	5′-TTCCAATGTTTCTTTATCATTAGCTACTT-3′ (508 ← 536)	29	

bp = base pair.
* PCR primer sets were derived from Demaerschalck et al[22], and all positions correspond to the numbering of the B31 OspA sequence.

A total of 20 pmol of the appropriate primer sets and various amounts of template DNA were used in each 50-μL reaction mixture. PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700), and amplification was set up for 35 cycles under the following conditions: 93°C, 1 min; 66°C, 1 min; 72°C, 1 min. For the primer sets GIII and SL, the annealing step was performed at 63°C and 60°C for 1 min, respectively. PCR amplification products were electrophoresed on 2% agarose gels in TBE buffer and were visualized under ultraviolet (UV) light with ethidium bromide. The 100-bp DNA ladder (catalog no. 15628-019, Gibco BRL, Taipei, Taiwan) was used as the standard marker for comparison. After purification by a QIAquick PCR purification kit (Qiagen, Taipei, Taiwan), the purified PCR products were digested by restriction enzyme DraI (New England Biolabs, Taipei, Taiwan), and the digested DNA fragments were separated by electrophoresis on 2% agarose gels (Agarose-LE, USB, Cleveland, OH) in TBE buffer and visualized under UV light with ethidium bromide. Two DNA ladders (catalog no. 10787-018, Gibco BRL; catalog no. 170-8200, Bio-Rad) were used as the standard markers for comparison.

Sequence alignments and phylogenetic analysis. The OspA nucleotide sequences of the *B. burgdorferi* sensu stricto strains used in this study have been sequenced by a dye-deoxy terminator reaction method using the BigDye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer Cetus) under an ABI Prism 377-96 DNA Sequencer (Perkin-Elmer Cetus). The determined sequences were aligned, and a similarity matrix and neighbor-joining phylogenetic tree were constructed using the DNASTAR program and the CLUSTAL V software package.[29,30]

Nucleotide sequence access numbers. The nucleotide sequences of PCR-amplified OspA gene determined in this study have been assigned the following GenBank access numbers: AY030279 (B31), AF369937 (TWKM1), AF369938 (TWKM2), AF369939 (TWKM3), AF369940 (TWKM4), AF369941 (TWKM5), AF369942 (TWKM6), AF369943 (TWKM7), AF369944 (JD1), AF369945 (TB), AF369946 (CT20004), AF369947 (CT27985), AF369948 (VS219), AF369949 (ECM-NY86), and AF369950 (N40).

RESULTS

PCR analysis with the genospecies-specific primer sets for OspA gene was performed to characterize the genomic identity of the seven Taiwan isolates. Additional isolates of spirochetes of different geographical origins and genospecies were also examined using the PCR primers for the specific type strains. All of the Taiwan isolates and type strains (strains B31, JD1, K48, and VS461) can be amplified by primer set SL (universal primers); a DNA fragment of approximately 300 bp was observed on a 2% agarose gel (Figure 1A). With primer set GI, only Taiwan isolates (TWKM1–7) and the genospecies of *B. burgdorferi* sensu stricto (B31 and JD1 strains) were amplified; a DNA fragment of approximately 545 bp was observed (Figure 1B). However, the primer sets GII and GIII amplified DNA only from the genospecies of *B. garinii* (strain K48) and *B. afzelii* (strain VS461), with DNA fragments of approximately 345 bp and 190 bp, respectively (Figure 1C and D). These results demonstrate that all of the Taiwan isolates were genetically related to the genospecies of *B. burgdorferi* sensu stricto.

To confirm the genomic identity of these Taiwan isolates further, RFLP analysis was performed for additional classification and differentiation of isolates belonging to three major genospecies of Lyme disease spirochetes. After cleavage by the enzyme of DraI, the restriction site polymorphism of PCR products from the OspA gene of seventeen isolates was investigated by comparing their restriction patterns of digested DNA fragments. As revealed by the gel electrophoresis, all 17 isolates were classified into three different RFLP patterns (Figure 2) in relation to three different genospecies of *B. burgdorferi* sensu lato. The restriction pattern of OspA gene from the genospecies of *B. garinii* (strain K48) demonstrates a specific pattern with DNA fragments of approximately 280 bp and 65 bp. A specific pattern with DNA fragments of approximately 135 bp and 55 bp was also observed on the OspA gene of *B. afzelii* (strain VS461) (Table 2). In addition, all 7 Taiwan isolates exhibit the same pattern as other 8 strains of *B. burgdorferi* sensu stricto with DNA fragments of approximately 345 bp and 200 bp. These results confirm the

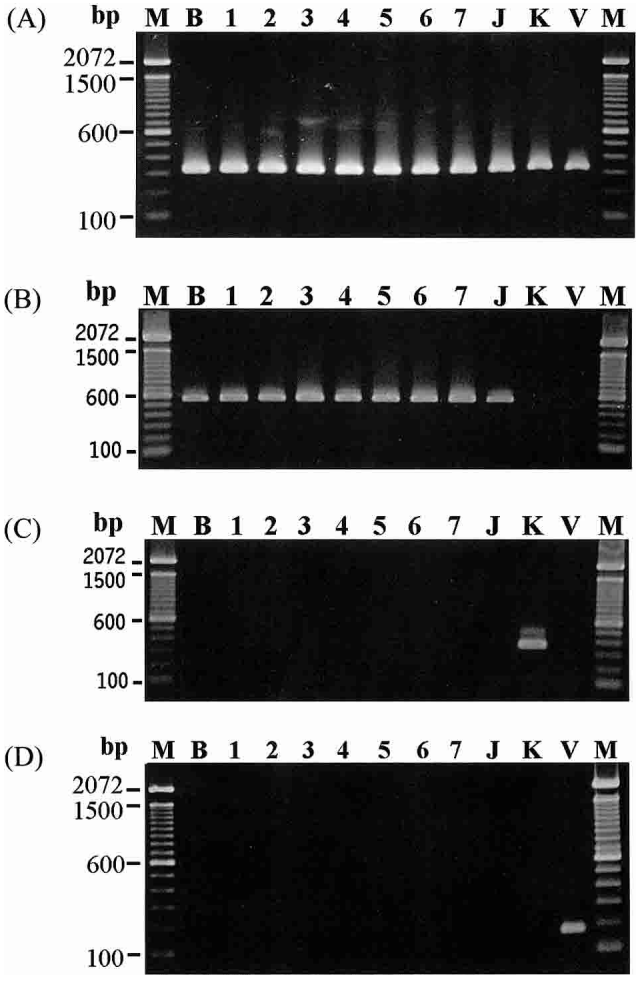

FIGURE 1. Polymerase chain reaction analysis with a universal primer sets of SL (A) for OspA gene of *B. burgdorferi* sensu lato and with the genospecies-specific primer sets of GI (B), GII (C), and GIII (D) for *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii,* respectively. Lane B, B31 isolate; lanes 1 to 7, Taiwan isolates TWKM1–7, respectively; lane J, JD 1 isolate of *B. burgdorferi* sensu stricto; lane K, K48 isolate of *B. garinii;* lane V, VS461 isolate of *B. afzelii;* lanes M, 100 bp DNA ladder (Gibco BRL). The expected amplification products for primer sets SL, GI, GII, and GIII were DNA fragments with sizes of approximately 300, 545, 345, and 190 bp, respectively.

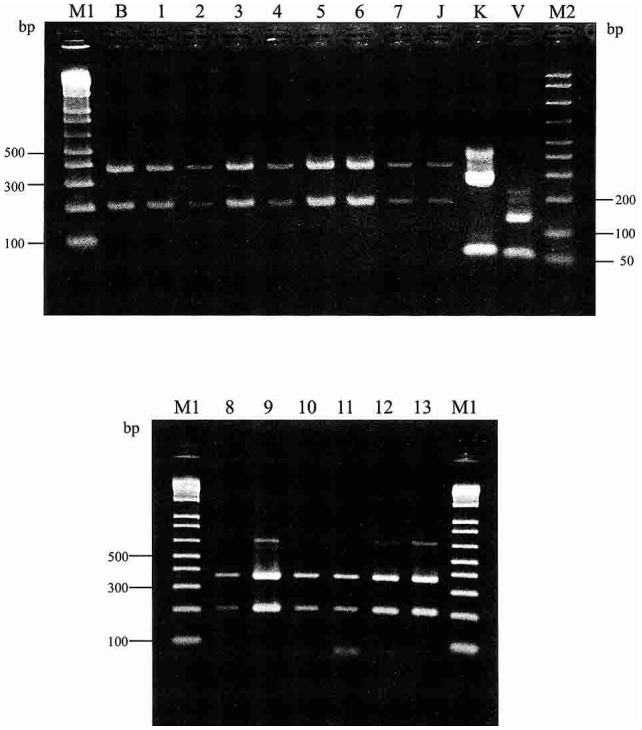

FIGURE 2. Restriction fragment length polymorphism analysis of the restriction patterns of OspA gene from different genospecies of *B. burgdorferi* isolates. DNAs were amplified with genospecies-specific primer sets and were digested by DraI. The digested DNA fragments were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by ultraviolet transillumination. Lane B, B31 isolate; lanes 1 to 7, the Taiwan isolates of TWKM1–7, respectively; lane J, JD 1 isolate of *B. burgdorferi* sensu stricto; lane K, K48 isolate of *B. garinii;* lane V, VS461 isolate of *B. afzelii;* lanes 8 to 13, the *B. burgdorferi* sensu stricto strains of CT20004, CT27985, ECM-NY86, N40, TB, and VS219, respectively; lanes M1 and M2, 1 kb plus DNA ladder (Gibco BRL) and Amplisize DNA ruler (Bio-rad).

genomic identity of these Taiwan isolates belonging to the genospecies of *B. burgdorferi* sensu stricto.

The sequence analysis of OspA gene was also performed to identify the homogeneity of these Taiwan isolates within the genospecies of *B. burgdorferi* sensu stricto. As shown in Table 3, the OspA gene sequences are almost identical (> 99.8%) among strains of Taiwan isolates and are highly homogeneous within the genospecies of *B. burgdorferi* sensu stricto, ranging from 99.4% to 100%. When compared with the B31-type strain, the nucleotide difference was observed only on the positions of 61, 302, and 529–530 of the strains of TWKM1, N40, and ECM-NY86, respectively. In addition, the analysis of phylogenetic divergence among *Borrelia* strains investigated in this study shows a closely phylogenetic relationship based on the OspA gene sequences (data not shown). These results revealed the high homogeneity of OspA gene among *Borrelia* strains, and the genospecies of these Taiwan isolates can be verified as *B. burgdorferi* sensu stricto.

TABLE 2

Comparative analysis of OspA-RFLP patterns in relation to different genospecies of *B. burgdorferi* sensu lato

Genospecies and strain	Estimated PCR products (bp)	Size of restriction fragments (bp)*	RFLP type
B. burgdorferi sensu stricto			
B31	545	200 + 345	A
JD1	545	200 + 345	A
N40	545	200 + 345	A
CT27985	545	200 + 345	A
TB	545	200 + 345	A
VS219	545	200 + 345	A
CT20004	545	200 + 345	A
ECM-NY86	545	200 + 345	A
B. garinii			
K48	345	65 + 280	B
B. afzelii			
VS461	190	55 + 135	C
Taiwan isolates			
TWKM1–7	545	200 + 345	A

PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; bp = base pair.
* Fragments were cleavaged by DraI.

TABLE 3
Sequence similarity between OspA gene sequences from *Borrelia* isolates of the genospecies of *B. burgdorferi* sensu stricto

	% Sequence similarity to														
Isolate	B31	Twkm1	Twkm2	Twkm3	Twkm4	Twkm5	Twkm6	Twkm7	JD 1	CT20004	CT27985	ECM-NY86	N40	TB	VS219
B31	100.0	99.8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	100.0
Twkm-1		100.0	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.4	99.6	99.8	99.8
Twkm-2			100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
Twkm-3				100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
Twkm-4					100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
Twkm-5						100.0	100.0	100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
Twkm-6							100.0	100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
Twkm-7								100.0	100.0	100.0	100.0	99.6	99.8	100.0	100.0
JD-1									100.0	100.0	100.0	99.6	99.8	100.0	100.0
CT20004										100.0	100.0	99.6	99.8	100.0	100.0
CT27985											100.0	99.6	99.8	100.0	100.0
ECM-NY86												100.0	99.4	99.6	99.6
N40													100.0	99.8	99.8
TB														100.0	100.0
VS219															100.0

DISCUSSION

Our report described the first genomic characterization and identification of the OspA gene among Lyme disease spirochetes, *B. burgdorferi* sensu lato, isolated in Taiwan. In our previous investigations, the protein profiles of these Taiwan isolates revealed a consistent composition with a major protein band identical to OspA, and its antigenicity was also verified by the reactivity with monoclonal antibodies (MAbs) specific against OspA.[28] Although the heterogeneity among major protein bands and the immunoreactivity with *B. burgdorferi*-specific MAbs had been used for the typing or species identification of Lyme disease isolates, the validity of these methods used for genospecies identification was not fully satisfied.[24,31] Thus, genomic analysis based on the OspA gene provides an alternative method for species identification and would help to elucidate the heterogeneity of *Borrelia* spirochetes from various biological and geographical origins in Taiwan.

The genospecies of Lyme disease spirochetes can be identified by their differential reactivities with genospecies-specific PCR primers. Indeed, genetic analysis based on the genospecies-specific PCR primers had been recognized as a rapid and distinguishable assay for the species identification of *Borrelia* spirochetes, regardless of the biological and geographical origins.[19–22,24,32] In our study, we used four sets of genospecies-specific PCR primers based on the OspA gene to distinguish and clarify the genomic identity of Taiwan isolates. Although all of the Taiwan isolates were genetically identified as the genospecies of *B. burgdorferi* sensu stricto, further application of these genospecies-specific PCR primers to the clinical and tick specimens is required to verify the reliability of these PCR primers. This may be beneficial to discover the new foci of endemicity and identify the genomic heterogeneity of spirochetal isolates in Taiwan.

The genomic similarity of *Borrelia* isolates can be classified with their heterogeneity of restriction patterns by RFLP analysis of specific target gene. Indeed, the previous reports described that RFLP analysis of the OspA or OspC gene seems useful for detecting mixed spirochetal infections and classifying the genospecies of *Borrelia* isolates detected in various biological specimens.[22,33] Results presented in this study also demonstrate that the genospecies of *Borrelia* isolates can be classified by their restriction patterns of OspA gene, and all of the Taiwan isolates were identified as the genospecies of *B. burgdorferi* sensu stricto according to the homogeneity of RFLP pattern within the same genospecies of *Borrelia* isolates (see Figure 2 and Table 2). These results suggest that the genomic identity of *Borrelia* isolates can be differentiated between the three major genospecies of *B. burgdorferi* sensu lato by analyzing the DraI restriction polymorphism of the OspA gene.

The phylogenetic relationships among *Borrelia* isolates can be constructed by analyzing their homogeneity of gene sequences. Indeed, the sequence analysis of OspA gene among *Borrelia* isolates with regard to the antigenicity and species identification had been described previously.[24–27] Nucleotide sequence variation of a target gene may actually represent the genetic distance of phylogenetic divergence between or within the genospecies of *Borrelia* isolates.[27,31,34,35] In this study, the phylogenetic analysis of OspA gene demonstrates a high sequence homogeneity among *Borrelia* isolates within the genospecies of *B. burgdorferi* sensu stricto, and all of the Taiwan isolates exhibit almost identical nucleotide sequences of OspA gene (see Table 3). Further investigation of the sequence anlysis of other target genes, such as the genes encoding the OspC and 5S–23S intergenic spacer region, of these Taiwan isolates would help to elucidate the genetic divergence of *Borrelia* isolates in Taiwan.

In conclusion, our report provides the first identification and characterization of OspA gene of *Borrelia* spirochetes isolated in Taiwan. On the basis of their differential primer reactivities, homogeneity of RFLP pattern, and sequence similarity of OspA gene, all of the Taiwan isolates were genetically identified as the genospecies of *B. burgdorferi* sensu stricto. Further application of these molecular tools to study the genetic variability of OspA gene from *Borrelia* isolates in patients, reservoir animals, and vector ticks may help provide a better understanding of the ecology and epidemiology of tick-borne spirochetes in Taiwan.

Financial support: This work was supported in part by a grant from the National Science Council (NSC89-2314-B-016-082) and Department of Defense (DOD-89-35), Taipei, Taiwan, Republic of China.

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