



Genetic Analysis of the Flagellin Gene of Lyme Disease Spirochetes (*Borrelia burgdorferi*) Isolated from Rodents in Taiwan

Li-Lian Chao and Chien-Ming Shih*

Department of Parasitology and Tropical Medicine, National Defense Medical Center,
Taipei, Taiwan, Republic of China

Background: The flagellin gene of *Borrelia burgdorferi* is highly conserved, and genomic analysis among *Borrelia* isolates using sequence similarity of a flagellin gene has been proven useful for the species identification and genomic typing of *Borrelia* spirochetes isolated from various biological and geographical sources. Thus, we analyzed the flagellin gene of Lyme disease spirochetes for the first time in Taiwan. **Methods:** The genetic identities of Taiwan isolates (TWKM1-7) were determined by comparing their sequence similarities of the PCR-amplified flagellin genes with three major genospecies of Lyme disease spirochetes. Their phylogenetic relationships were also analyzed by neighbour-joining and maximum parsimony methods. **Results:** The sequence similarity of Taiwan isolates revealed a highly homogeneous genotype, ranging from 97.8% to 100%, within the genospecies of *B. burgdorferi sensu stricto* and was clearly distinguished from other genospecies of Lyme disease spirochetes with a high sequence variability (>4.9%) of the flagellin gene. In addition, phylogenetic analysis based on the flagellin gene also revealed a highly genetic divergence between Taiwan isolates and other *Borrelia* strains that were isolated from different biological and geographical sources. **Conclusions:** Our results provide the first investigation on the genetic identity of the flagellin gene of these Taiwan isolates and confirm that these Taiwan isolates are genetically related to the genospecies of *B. burgdorferi sensu stricto*.

Key words: lyme disease, *Borrelia burgdorferi*, Flagellin gene, Taiwan

INTRODUCTION

Lyme disease (LD) is an emerging tick-borne spirochetal infection¹ that can cause multisystem human illness with varying degrees of clinical symptoms among infected persons, ranging from relatively benign skin lesions to severe arthritic, neurologic, and cardiac manifestations^{2,3}. On the basis of immunoreactivity with *Borrelia burgdorferi*-specific monoclonal antibodies, plasmid profiles, and the clinical manifestations of patients, the causative agents of Lyme disease can be classified into three major genospecies, i.e., *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* (group VS461)^{4,5}. Although human cases of Lyme disease had been reported in Taiwan⁶⁻¹¹ and *Borrelia* spirochetes have also been isolated

from rodents in the Taiwan area¹², the genetic diversity of spirochetes as well as the tick vectors responsible for transmission in Taiwan need to be further identified.

The heterogeneity of molecular and immunological characteristics among isolates of *B. burgdorferi* from different geographical and biological origins has been demonstrated previously¹³⁻¹⁷. Genomic analysis among *Borrelia* isolates by sequence similarity of a specific target gene has been proven useful for the species identification and genomic typing of *Borrelia* isolates from various biological and geographical sources¹⁸⁻²⁰. The flagellin gene of *B. burgdorferi* is highly conserved and located on a linear chromosome of Lyme disease spirochetes²¹⁻²⁴. Its diversity is valuable for distinguishing genetic heterogeneity among different *Borrelia* isolates²⁵⁻²⁹. Thus, the objective of this study was to determine the genetic identity of these *Borrelia* spirochetes isolated from rodent hosts in Taiwan by analyzing the sequence similarity of their PCR-amplified flagellin gene.

METHODS

Spirochete Strains

A total of twenty-six strains of *Borrelia* isolates be-

Received: April 21, 2009; Revised: May 8, 2009;
Accepted: May 26, 2009

*Corresponding author:

Chien-Ming Shih, Department of Parasitology and Tropical Medicine, National Defense Medical Center, No. 161, Sec. 6, Minquan East Road, Taipei 114, Taiwan, Republic of China. Tel: +886-2-87923152; Fax: +886-2-87921821; E-mail: cmsih@ndmctsgh.edu.tw

Table 1. Genospecies and strains of *Borrelia* isolates analyzed in this study

Genospecies and strain	Origin of <i>Borrelia</i> strain		Flagellin gene accession number*
	Biological	Geographical	
Taiwan isolates			
TWKM1-7	Rodent	Taiwan	AF416434-40
<i>Borrelia burgdorferi sensu stricto</i>			
B31	Tick	U. S. A.	AF416433
JD 1	Tick	U. S. A.	AF416441
CT20004	Tick	France	AF416444
CT27985	Tick	U. S. A.	AF416445
ECM-NY86	Human skin	U. S. A.	AF416446
N40	Tick	U. S. A.	AF416447
TB	Tick	U. S. A.	AF416448
VS219	Tick	Switzerland	AF416449
<i>B. garinii</i>			
K48	Tick	Czechoslova- kia	AF416442
PBi	Human CSF	Germany	CP000013
<i>B. afzelii</i>			
VS461	Tick	Switzerland	AF416443
PKo	Human skin	Germany	CP000395
<i>B. andersonii</i> 21123	Tick	U. S. A.	D83764
<i>B. tanukii</i> OR1eR	Tick	Japan	D85070
<i>B. turdi</i> Kt501	Tick	Japan	D82851
<i>B. valaisiana</i>			
CKA4a	Rodent	China	AB022136
CMN1b	Rodent	China	AB022134
<i>B. sinica</i>			
CMN3	Rodent	China	AB022138
CMN1a	Rodent	China	B022137

*GenBank accession numbers (**AF416433-416449**) were submitted by this study.

longing to the three major genospecies of *B. burgdorferi sensu lato* and other *Borrelia* species were analyzed in this study (Table 1). Of which, seventeen strains of *Borrelia* spirochetes including seven Taiwan isolates (TWKM1-7) were cultured and maintained in our laboratory, as described previously¹². *Borrelia* strains of *B. garinii* (PBi), *B. afzelii* (PKo), *B. andersonii* (21123), *B. tanukii* (OR1eR), *B. turdi* (Kt501), *B. valaisiana* (CKA4a and CMN1b), and *B. sinica* (CMN3 and CMN1a) were included as the recorded information described in the GenBank data.

Preparation of Spirochete DNA

Total genomic DNA from all *Borrelia* strains was extracted as described previously³⁰. Briefly, samples (3 ml) of cultured spirochetes were grown to a density of about 2×10^8 cells per ml of medium and were centrifuged for 10 min at $12,000 \times g$ to pellet the spirochetes. The pellets were washed twice with PBS (pH 7.2) containing 5 mM $MgCl_2$, resuspended in 150 μ l of distilled water,

and boiled for 10 min. After centrifugation at $10,000 \times g$ for 10 sec, the supernatant was collected and the DNA concentrations were determined spectrophotometrically by using a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

PCR Amplification of Spirochete DNA

DNA samples extracted from the Taiwan isolates and other spirochetes representative of the three major genospecies of *B. burgdorferi sensu lato* were used as templates to perform the PCR amplification of the flagellin gene DNA. A flagellin-specific primer set of 1F (forward) 5'-GCATTAACGCTGCTAATC-3' and M-2F (reverse) 5'-TGCAGGCTGCATTCCAAG-3' were synthesized by a custom oligonucleotide synthesis service (Gibco BRL, Taipei, Taiwan) and were used to amplify the flagellin gene of Lyme disease spirochetes (*B. burgdorferi*), as described previously³¹. All PCR reagents and TaqGold DNA polymerase were obtained from the GeneAmp kit and were used as recommended by the supplier (Applied Biosystem, Taipei, Taiwan).

Briefly, a total of 20-pmol of the appropriate primer set and various amounts of template DNA were used in each 50- μ l reaction mixture. PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700) and with amplification for 30 cycles of denaturation at 92°C for 30 s, annealing at 41°C for 30 s, and extension at 72°C for 90 s. PCR-amplified DNA products were electrophoresed on 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and the electrophoresed gel was visualized under UV light after staining with ethidium bromide. To determine the molecular size, a 1Kb plus DNA ladder (catalogue no. 10787-018, Gibco BRL, Taipei, Taiwan) was used as the standard marker for comparison.

Sequence Alignments and Phylogenetic Analysis

The nucleotide sequences of the flagellin gene of the *Borrelia* isolates used in this study were sequenced by a dye-deoxy terminator reaction method using the bigdye terminator-Taq cycle sequencing kit under an ABI Prism 377-96 DNA sequencer (Applied Biosystems Inc., Foster city, CA, USA). The determined sequences were initially aligned with the CLUSTAL W software³² and further analyzed by the neighbour-joining and maximum parsimony methods to estimate the phylogeny of the entire alignment using MEGA 4.0 software package³³. A similarity matrix was also constructed using the DNASTAR program (Lasergene 7.0). All phylogenetic trees were constructed and performed with 1000 bootstrap replica-

tions to evaluate the reliability of the constructions, as described previously³⁴.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the PCR-amplified flagellin genes determined in this study have been registered and assigned the following GenBank accession numbers: strains B31 (AF416433), TWKM1 (AF416434), TWKM2 (AF416435), TWKM3 (AF416436), TWKM4 (AF416437), TWKM5 (AF416438), TWKM6 (AF416439), TWKM7 (AF416440), JD1 (AF416441), K48 (AF416442), VS461 (AF416443), CT20004 (AF416444), CT27985 (AF416445), ECM-NY86 (AF416446), N40 (AF416447), TB (AF416448), and VS219 (AF416449). For phylogenetic analysis, the nucleotide sequences of the flagellin genes of other *Borrelia* spirochetes were included for comparison and their GenBank accession numbers were recorded as the information described in the GenBank data (Table 1).

RESULTS

To clarify the genomic identity of these Taiwan isolates, PCR amplification of the flagellin gene was performed to generate the flagellin DNA of all the strains of Taiwan isolates (TWKM1-7) and the other ten strains of *Borrelia* isolates belonging to three major genospecies of Lyme disease spirochetes. A DNA fragment of approximately 400 bp was generated and observed on a 2% agarose gel (Fig. 1). These results demonstrate that the flagellin genes were highly conserved in all strains of *Borrelia* isolates regardless of the origin of isolation and the genospecies of Lyme disease spirochetes.

The sequence similarity of the flagellin gene was analyzed to identify the homogeneity of these Taiwan isolates in relation to the genospecies of *B. burgdorferi* spirochetes. As shown in Table 2, the nucleotide sequences of the flagellin genes were highly homogeneous, ranging from 97.8% to 100% among Taiwan isolates and the other eight *Borrelia* isolates (strains B31, JD1, N40, ECM-NY86, CT27985, CT20004, TB, and VS219) within the genospecies *B. burgdorferi sensu stricto*. However, the homogeneity of flagellin nucleotide sequences among the strains of K48 (*B. garinii*) and VS461 (*B. afzelii*) showed a homogeneity of only 93.8-95.5% and 92.2-94%, respectively, in comparison to the genospecies *B. burgdorferi sensu stricto* (Table 2). Thus, all of these Taiwan isolates are genetically affiliated with the genospecies *B. burgdorferi sensu stricto*.

Phylogenetic analysis based on the sequence align-

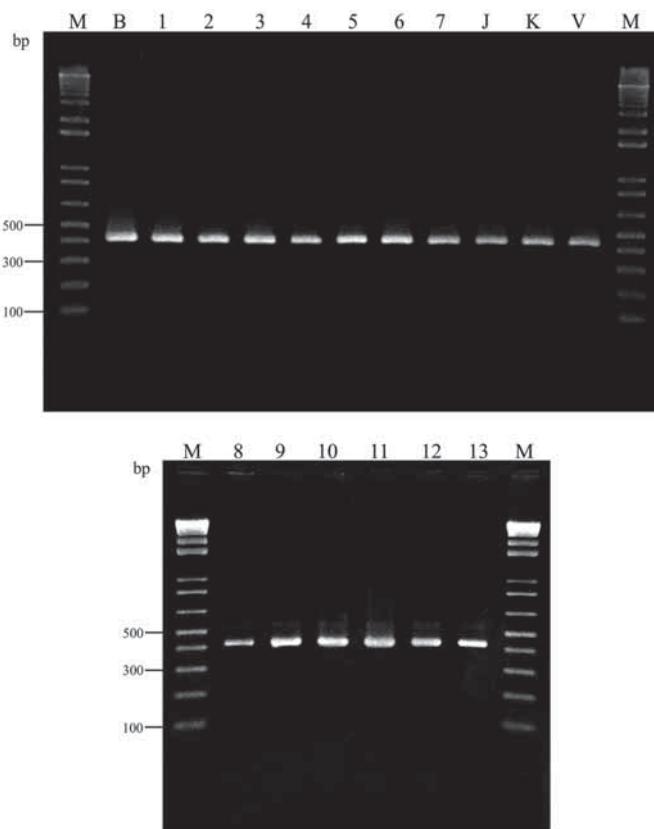


Fig. 1 PCR analysis with a primer sets specific for the flagellin genes of *B. burgdorferi sensu lato* (Bbsl). Lane B, isolate B31; lanes 1 to 7 represent the Taiwan isolates of TWKM1-7, respectively; lane J, isolate JD 1 of *B. burgdorferi sensu stricto* (Bbss); lane K, isolate K48 of *B. garinii*; lane V, isolate VS461 of *B. afzelii*; lanes 8 to 13 represent the *B. burgdorferi sensu stricto* strains CT20004, CT27985, ECM-NY86, N40, TB, and VS219, respectively; lanes M, 1Kb plus DNA ladder (Gibco BRL). The amplification products were electrophoresed on 2% agarose gels (Agarose-LE, USB, Cleveland, Ohio, U.S.A.) and DNA fragments of approximately 400 bp were visualised under UV light with ethidium bromide staining.

ment of the flagellin gene were also performed to construct the genetic divergence among twenty-six strains of *Borrelia* isolates investigated in this study. Bootstrap analysis was used to analyze the repeatability of the clustering of specimens represented in phylogenetic trees. All *Borrelia* isolates from Taiwan constitute a monophyletic group separated from other strains of *Borrelia* spirochetes (i.e., *B. garinii* and *B. afzelii*) within the same clade of *B. burgdorferi sensu lato* and can be distinguished from

Table 2. Sequence similarity between flagellin gene sequences from Taiwan isolates and isolates of other genospecies of *Borrelia**

Spirochete strain	% Sequence similarity to																
	B31	Twkm1	Twkm2	Twkm3	Twkm4	Twkm5	Twkm6	Twkm7	JD-1	N40	ECM-NY86	CT27985	CT20004	TB	VS219	K48	VS461
B31	100.0	100.0	98.2	100.0	99.2	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
Twkm-1		100.0	98.2	100.0	99.2	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
Twkm-2			100.0	98.2	98.5	98.2	98.2	98.2	98.2	98.2	97.8	98.2	98.2	98.2	98.2	93.8	92.2
Twkm-3				100.0	99.2	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
Twkm-4					100.0	99.2	99.2	99.2	99.2	99.2	98.8	99.2	99.2	99.2	99.2	94.8	93.2
Twkm-5						100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
Twkm-6							100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
Twkm-7								100.0	100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
JD-1									100.0	100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
N40										100.0	99.5	100.0	100.0	100.0	100.0	95.5	94.0
ECM-NY86											100.0	99.5	99.5	99.5	99.5	95.0	93.5
CT27985												100.0	100.0	100.0	100.0	95.5	94.0
CT20004													100.0	100.0	100.0	95.5	94.0
TB														100.0	100.0	95.5	94.0
VS219															100.0	95.5	94.0
K48																100.0	96.5
VS461																	100.0

* Strains: B31, JD1, CT20004, CT27985, ECM-NY86, N40, TB, and VS219, *B. burgdorferi sensu stricto*; K48, *B. garinii*; VS461, *B. afzelii*.

other genospecies of *Borrelia* spirochetes derived from different biological and geographical origins in both neighbour-joining and maximum parsimony methods (Fig. 2). Within the same clade, all of these Taiwan isolates (TWKM1-7) are highly related within the genospecies of *B. burgdorferi sensu stricto*.

DISCUSSION

Our report describes the first genomic characterization and classification of the flagellin gene among Lyme disease spirochetes (*B. burgdorferi*) isolated in Taiwan. In previous investigations, the protein profiles of these Taiwan isolates were consistent with the major protein bands of other documented strains of Lyme disease spirochetes and their antigenicity was also verified by their reactivities with MAbs specific for *B. burgdorferi sensu lato*¹². Although the heterogeneity among major protein bands and the immunoreactivity with *B. burgdorferi*-specific MAbs have been used for the genomic typing or species identification of *B. burgdorferi* spirochetes, the validity of these methods for identifying genospecies was

not fully satisfied^{17,35}. Thus, genomic analysis based on the sequence similarity of flagellin gene may provide a reliable and useful method for genospecies identification of *Borrelia* spirochetes isolated from various animal reservoirs and vector ticks of Taiwan.

Genetic analysis based on genospecies-specific PCR primers has been recognized as a rapid and distinguishable assay for the species identification of *Borrelia* spirochetes isolated from various biological and geographical origins^{17-20,35}. Indeed, genetic heterogeneity can be further classified among *Borrelia* isolates that have been previously identified as the same genospecies or atypical strains of spirochetes³⁶. In our previous studies, the genetic relationship of these Taiwan isolates was clarified as the same genospecies by their differential reactivities with genospecies-specific PCR primers based on the *OspA*³⁷ and *OspC*³⁸ genes of *B. burgdorferi sensu lato*. Results from the present study further clarify the genetic identity of these Taiwan isolates by analyzing the flagellin gene. Moreover, all of these Taiwan isolates are genetically classified into the genotype within *B. burgdorferi sensu stricto*.

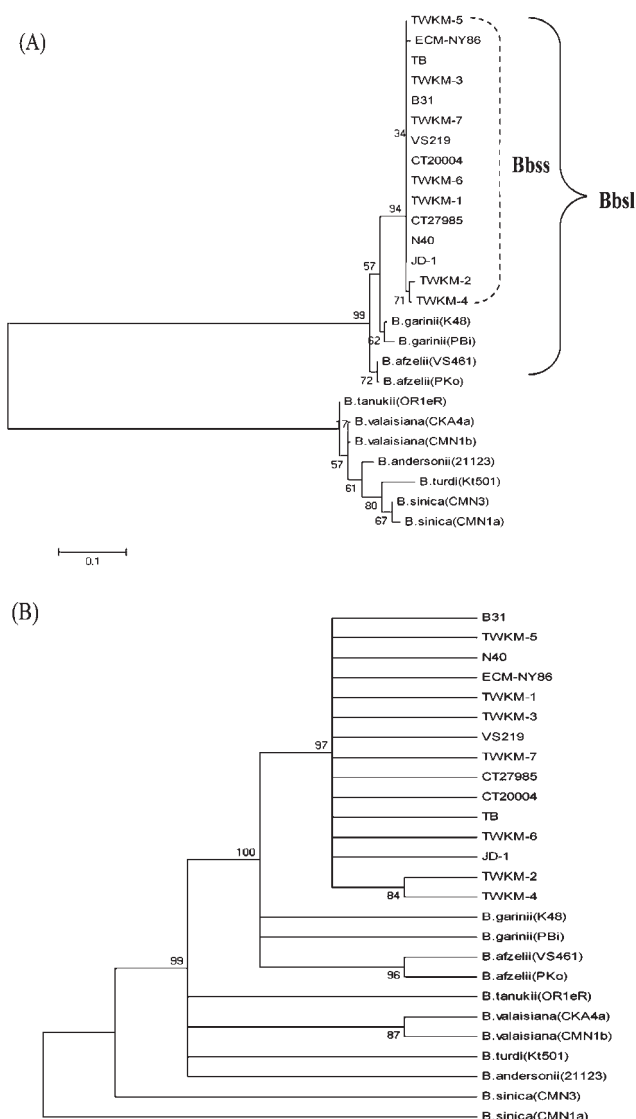


Fig. 2 Phylogenetic relationships based on a comparison of the flagellin gene sequences from seven strains of Taiwan isolates (TWKM1-7) and another 19 strains of *Borrelia* isolates. The trees were constructed and analyzed by the (A) neighbour-joining and (B) maximum parsimony methods performed with 1000 bootstrap replicates. The numbers at the nodes indicate the percentages of reliability of each branch of each tree. Branch lengths are drawn in proportional to the estimated sequence divergence.

The phylogenetic relationships among *Borrelia* isolates can be constructed by analyzing the sequence similarity of a specific target gene. Indeed, the sequence analysis of the flagellin gene among *Borrelia* isolates has been proven useful in the analysis of the genetic related-

ness of *Borrelia* isolates derived from various geographical and biological sources^{20,29,39}. In addition, nucleotide sequence variation of a target gene may actually represent the genetic divergence between or within the genospecies of *Borrelia* isolates⁴⁰⁻⁴². In this study, phylogenetic analysis based on the flagellin gene demonstrates a high sequence homogeneity among *Borrelia* isolates of Taiwan and within the same genospecies of *B. burgdorferi sensu stricto* (Fig. 2). However, a high sequence heterogeneity was also observed between different genospecies of *Borrelia* spirochetes. Further investigation on the sequence analysis of the flagellin gene of *Borrelia* spirochetes isolated from different localities of Taiwan would help to clarify the genetic divergence of *Borrelia* spirochetes in the Taiwan area.

In conclusion, our study provides the first investigation on genetic identification of the flagellin gene among Lyme disease spirochetes (*B. burgdorferi*) isolated in Taiwan. On the basis of the sequence similarity of the flagellin gene, all of these Taiwan isolates (TWKM1-7) are genetically related to the genospecies of *B. burgdorferi sensu stricto*. Further application of this molecular tool to identify the genetic variability of the flagellin gene among *Borrelia* spirochetes isolated from patients, reservoir animals, and vector ticks may help to illustrate the genetic divergence of *Borrelia* spirochetes in relation to the epidemiological features of human Lyme borreliosis in Taiwan.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Department of Defense (DOD98-34), Taipei, Taiwan, Republic of China.

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