EPIDEMIOLOGY

Genetic analysis of the outer surface protein C gene of Lyme disease spirochaetes (Borrelia burgdorferi sensu lato) isolated from rodents in Taiwan

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The outer surface protein C gene (ospC) of Lyme disease spirochaetes (Borrelia burgdorferi sensu lato) was analysed for the first time in Taiwan. The genetic identities of these Taiwan isolates (TWKM1–7) were determined by restriction fragment length polymorphism (RFLP) analysis and sequence similarities of the PCR-amplified ospC gene amplicons. After cleavage by nuclease DraI, differential fragment patterns of PCR-amplified ospC DNA in relation to different genospecies of Lyme disease spirochaetes were observed and all of these Taiwan isolates were genetically affiliated to the genospecies of B. burgdorferi sensu stricto. The phylogenetic analysis on the sequence similarity of these Taiwan isolates revealed a highly homogeneous genotype, ranging from 99.3% to 100%, within the genospecies of B. burgdorferi sensu stricto and was distinguished from other genospecies of Borrelia isolates. The sequence similarity analysis also revealed the high sequence variability of the ospC gene among Borrelia strains that belong to the same genospecies but were isolated from different biological and geographical sources. Thus, these results provide the first investigation on the genetic identity of the ospC gene of these Taiwan isolates and show that these Taiwan isolates were closely related genetically to the genospecies of B. burgdorferi sensu stricto.

Introduction

Lyme disease is an emerging tick-borne spirochaetal infection [1] that can cause multisystem human illness with varying degrees of clinical symptoms among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurological and cardiac manifestations [2, 3]. The aetiological 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 a human case of Lyme disease had been reported in Taiwan [9] and Borrelia spirochaetes were also isolated from rodents in the Taiwan area [10], the genetic diversity of spirochaetes as well as the tick vectors responsible for transmission in Taiwan need to be established.

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The heterogeneity of molecular and immunological characteristics among isolates of B. burgdorferi sensu lato from different geographical and biological origins has been demonstrated previously [11–18]. On the basis of immunoreactivity with B. burgdorferi-specific monoclonal antibodies (MAbs), plasmid profiles and the clinical manifestations of the patient, the causative agents of Lyme disease can be classified into three major genospecies – B. burgdorferi sensu stricto, B. garinii and B. afzelii (group VS461) [19, 20]. Furthermore, genomic analysis among Borrelia isolates by PCR-restriction fragment length polymorphism (RFLP) analysis and sequence similarity of a specific target gene has been shown to be useful for species identification and genomic typing of Borrelia isolates from various biological and geographical sources [21–24].

The outer surface protein C gene (ospC) of B. burgdorferi sensu lato is located on a 27-kb circular plasmid [25] and is highly heterogeneous with the nucleotide sequences differing among isolates of different species of Borrelia [26, 27]. Strain diversity
as well as the genetic heterogeneity can be distinguished among different Borrelia isolates by their distinct RFLP types [28]. Thus, the objective of the present study was to characterise the genetic identity of Taiwan isolates by their differential restriction fragment patterns and sequence similarity of the PCR-amplified ospC gene amplicons.

Materials and methods

Spirochaete strains and in-vitro cultivation

Seventeen Borrelia isolates belonging to the three major genospecies of B. burgdorferi sensu lato were used for PCR, RFLP and phylogenetic analysis (Table 1). Spirochaetes were cultured at 34°C in a humidified incubator (Nuaire, Plymouth, MN, USA) with CO₂ 5% in air and maintained in BSK-H medium (catalogue no. B3528; Sigma) supplemented with rabbit serum (catalogue no. R7136; Sigma) 6% as described previously [10]. All cultures were examined weekly for optimal growth of spirochaetes by dark-field microscopy (model BX-60, Olympus, Tokyo, Japan).

Preparation of spirochaete DNA

Total genomic DNA was extracted from all Borrelia isolates as described previously [29]. Briefly, (3-ml) samples of cultured spirochaetes grown to a density of c. 2 × 10⁸ cells/ml of medium were centrifuged for 10 min at 12 000 g to pellet the spirochaetes. The pellets were washed twice with phosphate-buffered saline (PBS; pH 7.2) containing 5 mM MgCl₂, re-suspended in 150 μl of distilled water and boiled for 10 min. After centrifugation at 10 000 g for 10 s, the supernate was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

Table 1. Strains of B. burgdorferi sensu lato analysed

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Genetic analysis by PCR and RFLP

DNA samples extracted from the Taiwan isolates and other spirochaetes representative of the three major genospecies of B. burgdorferi sensu lato were used as template for PCR amplification of the ospC gene DNA. An ospC-specific primer set of CF1 (forward) 5'-AAGTGCATATATTACAGC-3' and CR2 (reverse) 5'-GATCTTTTCTGCCACACAG-3' were designed and synthesised by a custom oligonucleotide synthesis service (Gibco BRL, Taipei, Taiwan) as described previously [30]. 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).

Briefly, a total of 20-pmol of the appropriate primer set 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 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 agarose 2% gels in TBE buffer and were visualised under UV light after staining with ethidium bromide.

Sequence alignments and phylogenetic analysis

The nucleotide sequences of the ospC gene amplicon of the Borrelia isolates used in this study were sequenced by a dye-deoxy terminator reaction method with the bigdye terminator-Taq cycle sequencing kit and an ABI Prism 377-96 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The determined sequences were aligned and a similarity matrix and neighbour-joining phylogenetic tree were constructed with the DNASTAR program and the CLUSTAL V software package [31, 32].

Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified ospC gene amplicons determined in this study have been registered and assigned the following GenBank accession numbers: strains B31 (AF411450), TWKM1 (AF411451), TWKM2-7 (AF416418), TWKM2-7 (AF416418-23),
451), TWKM2 (AF416418), TWKM3 (AF416419), TWKM4 (AF416420), TWKM5 (AF416421), TWKM6 (AF416422), TWKM7 (AF416423), JD1 (AF416424), K48 (AF416425), VS461 (AF416426), CT20004 (AF416427), CT27985 (AF416428), ECM-NY86 (AF416429), N40 (AF416430), TB (AF416431) and VS219 (AF416432).

Results

The ospC gene was amplified by PCR to generate the ospC DNA of all the Taiwan isolates and another 10 isolates belonging to three major genospecies of Lyme disease spirochaetes. A DNA fragment of c. 630 bp was generated and observed in an agarose 2% gel (Fig. 1). These results demonstrate that the ospC genes were highly conserved in all the Borrelia isolates regardless of their origin and the genospecies of the Lyme disease spirochaetes.

To clarify the genomic identity of these Taiwan isolates, PCR-RFLP analysis of the ospC gene amplicon was also performed for further characterisation of Borrelia isolates belonging to the three major genospecies of Lyme disease spirochaetes. After cleavage by DraI, the restriction site polymorphism of the ospC gene amplicons from the 17 Borrelia isolates was investigated by comparing their restriction patterns of digested DNA fragments. All 17 isolates could be classified into four different RFLP patterns (Fig. 2) in relation to three different genospecies of B. burgdorferi sensu lato. The restriction fragment pattern of DraI-digested ospC gene amplicon of the genospecies B. gadinii (strain K48) demonstrated a specific pattern (type B) with DNA fragments of c. 260, 220

Fig. 1. PCR analysis with a primer set specific for the ospC genes of B. burgdorferi sensu lato. Lane B, isolate B31; lanes 1–7 represent the Taiwan isolates of TWKM1–7, respectively; lane J, isolate JD 1 of B. burgdorferi sensu stricto; lane K, isolate K48 of B. gadinii; lane V, isolate VS461 of B. afzelii; lanes 8–13 represent the B. burgdorferi sensu stricto strains CT20004, CT27985, ECM-NY86, N40, TB and VS219, respectively; lane M, 1-kb plus DNA ladder (Gibco BRL). The amplification products were electrophoresed on agarose 2% gels (Agarose-LE, USB, Cleveland, OH, USA) and DNA fragments of c. 630 bp were visualised under UV light with ethidium bromide staining.
and 150 bp, and a specific pattern (type C) with DNA fragments of c. 510 and 120 bp was also observed with the ospC gene amplicon of B. afzelii (strain VS461). All seven Taiwan isolates exhibited the same pattern (type AI) as the other six strains of B. burgdorferi sensu stricto with DNA fragments of c. 480 and 150 bp (Table 2). However, a different restriction fragment pattern (type AII) was observed in the DraI-digested ospC amplicons of strains N40 and CT20004 with DNA fragments of c. 270, 230 and 150 bp. These results revealed the genetic diversity of ospC genes among Borrelia isolates from different origins or of different genospecies of B. burgdorferi sensu lato. All of these Taiwan isolates were genetically related to the genospecies B. burgdorferi sensu stricto.

The phylogenetic relationships based on the sequence alignment of ospC gene were also analysed to demonstrate the divergence among Borrelia isolates investigated in this study. As compared with the aligned sequence of isolate B31, highly variable nucleotide sequences of ospC gene amplicons were highly homogeneous and ranged from 99.3 to 100% among Taiwan isolates and the other four Borrelia isolates (strains B31, JD1, CT27985 and TB) within the genospecies B. burgdorferi sensu stricto. However, the homogeneity of ospC nucleotide sequences among the strains N40, ECM-NY86, VS219 and CT20044 ranged from 71.4 to 80.0% in comparison to the genospecies B. burgdorferi sensu stricto (Table 3). Furthermore, the ospC sequence of strains K48 (B. garinii) and VS461 (B. afzelii) showed a homogeneity of only 65.4–74.6% and 65.6–77.9%, respectively.

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sequences were observed in strains CT20004, VS219, ECM-NY86 and N40, with a sequence divergence of 15–21% within the genospecies B. burgdorferi sensu stricto (Fig. 3). However, these sequences can be distinguished from those of strains K48 (B. garinii) and VS461 (B. afzelii). Furthermore, all the Taiwan isolates were very similar to strains B31, JD1, TB and CT27985 with a sequence divergence <0.5%. These results reveal the heterogeneity of the ospC gene among Borrelia isolates within the same genospecies as well as between the genospecies of B. burgdorferi sensu lato and all of these Taiwan isolates could be verified as belonging to the genospecies B. burgdorferi sensu stricto.

Discussion

This report describes the first genomic characterisation and classification of the ospC gene among Lyme disease spirochaetes (B. burgdorferi sensu lato) 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 spirochaetes and their antigenicity was also verified by their reactivities with MAbs specific for B. burgdorferi sensu lato [29]. 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 for genospecies identification was not fully satisfied [14, 33]. Thus, genomic analysis based on the ospC genes may provide a reliable and useful method for species identification of Borrelia spirochaetes that exist in various animal reservoirs and vector ticks of Taiwan.

Although genetic analysis based on the genospecies-specific PCR primers had been recognised as a rapid and definitive assay for species identification of Borrelia spirochaetes from various biological and geographical origins [14, 21–24, 33], it was difficult to clarify the genetic diversity among Borrelia isolates at the intraspecies level [34]. Moreover, genetic heterogeneity can be determined among Borrelia isolates that were previously identified as the same genospecies or atypical strains of spirochaetes [28, 35, 36]. In a previous study, the genetic relationship of these Taiwan isolates was determined to be the same genospecies by their differential reactivities with genospecies-specific PCR primers based on the ospA gene of B. burgdorferi sensu lato [29]. Results from the present study further clarify the genetic identity of these Taiwan isolates by analysing the ospC gene amplicons and all these Taiwan isolates were genetically classified into one subtype (AI) within the genospecies B. burgdorferi sensu stricto.

The genetic heterogeneity of Borrelia isolates can be classified according to their differential restriction fragment patterns by RFLP analysis of specific target genes [21, 37–39]. Previous reports concluded that RFLP analysis of the ospC genes seemed useful for detection of mixed spirochaetal infections and classification of the genospecies of Borrelia isolates detected in various biological specimens [25–28, 38]. Results of the present study also demonstrate that the genospecies could be determined among different Borrelia isolates by their restriction fragment patterns of the ospC gene and reveal two subgroups within the genospecies B. burgdorferi sensu stricto (Fig. 2 and Table 2). All the Taiwan isolates were genetically linked to one major subgroup (AI) according to the homogeneity of RFLP pattern within the same genospecies of Borrelia. These observations suggest that the genomic identity of Borrelia isolates can be determined either interspecies or intraspecies in B. burgdorferi sensu lato by analysing the restriction polymorphisms of DraI-digested ospC gene amplicons.

The phylogenetic relationships among Borrelia isolates can be determined by analysing the sequence similarity of a specific target gene. Sequence analysis of the ospC gene among Borrelia isolates had been shown to be useful for evaluating the taxonomic relatedness of B. burgdorferi sensu lato isolates derived from various geographical and biological sources [25–28]. The phylogenetic analysis of the ospC gene sequence of isolates previously identified as the same or atypical strains of Borrelia also differentiated new ribotype groups within the same genospecies of Lyme disease spirochaetes [26]. Moreover, nucleotide sequence variation of a target gene may actually represent genetic divergence within the genospecies of Borrelia isolates and genetic exchange by lateral transfer of the ospC sequence is proposed to be the mechanism responsible for the relatively high levels of ospC gene diversity [27, 28]. In this study, the phylogenetic analysis of the ospC gene among 17 Borrelia isolates
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*Strains B31, JD1, CT20004, CT27985, ECM-NY86, N40, TB and VS219, *B. burgdorferi* sensu stricto; K48, *B. garinii*; VS461, *B. afzelii*. 

**Table 3.** Sequence similarity between *ospC* gene sequences from Taiwan isolates and isolates of other genospecies of *Borrelia*.
demonstrated a high sequence heterogeneity between different genospecies and within the same genospecies of *B. burgdorferi sensu stricto* (Fig. 3). Further investigation on the sequence analysis of *ospC* genes of *Borrelia* strains isolated from various reservoir hosts, vector ticks and patients would help to clarify the genetic divergence of *Borrelia* isolates in Taiwan.

In conclusion, this report describes the first identification and characterisation of the *ospC* gene of *Borrelia* spirochaetes isolated in Taiwan. On the basis of their differential RFLP patterns and sequence similarity of their *ospC* genes, all these Taiwan isolates were genetically related to the genospecies of *B. burgdorferi sensu stricto*. Further application of this molecular tool to identify the genetic variability of the *ospC* genes from *Borrelia* isolates from patients, reservoir animals and vector ticks may help to illustrate the significance of the diversity of *Borrelia* spirochaetes in relation to the epidemiological features of human Lyme borreliosis in Taiwan.

References


22. Rosa PA, Hogan D, Schwan TG. Polymerase chain reaction

![Fig. 3. Phylogenetic tree based on a comparison of the ospC gene sequences from 7 Taiwan isolates and 10 strains of *B. burgdorferi sensu lato*. The bar represents the divergence between sequences of these *Borrelia* isolates.](image-url)


