First detection and molecular identification of *Borrelia garinii* isolated from human skin in Taiwan

Lyme disease is an emerging tick-borne spirochaetal infection that can cause multisystem human illness. Clinical symptoms vary among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurological and cardiac manifestations (Steere, 1989). Based on pathogenicity to humans, the causative agent for Lyme disease can be classified into three major genospecies: *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* and *Borrelia afzelii* (group VS461) (Baranton *et al.*, 1992). Although laboratory-diagnosed human cases of Lyme disease have been reported in Taiwan (Shih *et al.*, 1998; Li *et al.*, 2007) and *B. burgdorferi* spirochaetes also have been isolated from rodents in the Taiwan area (Shih & Chao, 1998), the causative agent responsible for human Lyme disease has never been isolated or identified from humans in Taiwan to our knowledge.

Genomic analysis of *Borrelia* isolates by sequence similarity of a specific target gene has proven useful for the species identification of *Borrelia* spirochaetes. The 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene is unique and highly conserved in *B. burgdorferi sensu lato* (Fukunaga & Sohnaka, 1992), and its diversity is valuable for distinguishing the genetic heterogeneity and clarifying the genetic identity of *Borrelia* spirochaetes isolated from various biological and geographical sources, including skin specimens from patients (Mathiesen *et al.*, 1997; Rijpkema *et al.*, 1997). In this study, we identified the genospecies of *Borrelia* spirochaetes isolated from a skin biopsy specimen of an indigenous human case by using a PCR assay targeting the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene of *B. burgdorferi sensu lato*, and clarified the genetic identity by analysing

phylogenetic relationships with other *Borrelia* species.

We conducted an investigation for *B. burgdorferi* infection among patients with clinical manifestations suggestive of Lyme disease. A 26-year-old Taiwanese woman living in the suburban area of Taipei City in northern Taiwan visited the dermal clinic of the National Taiwan University Hospital with the chief complaint of a long-lasting skin lesion observed on the lateral side of her abdomen. Her white blood cell count was 8000 mm^{-3} (52 % neutrophils, 21 % lymphocytes, 9 % monocytes), and a normal haemoglobin level (17.9 g dl^{-1}) and platelet count ($212\,000 \text{ mm}^{-3}$) were observed. The lesion was initially observed for 6 months following a suspected arthropod bite. She had no history of travel to any other countries during the previous 10 years. Based on the clinical features, the skin lesion was diagnosed as morphea (or localized scleroderma) and skin biopsy specimens were collected for cultivation of *Borrelia* spirochaetes as well as being stored at -80°C for further investigation. Serum specimens from this patient were subsequently collected for serological tests of Lyme disease infection.

For isolation of spirochaetes, skin biopsy specimens from the patient were cleaned with 75 % ethanol solution for 1–2 min and then washed twice in sterile distilled water. Afterwards, an individual skin specimen was dissected into pieces and inoculated into a culture tube containing BSK-H medium (B3528; Sigma) supplemented with 6 % rabbit serum (R7136; Sigma). After incubating at 34°C in a humidified incubator with 5 % CO_2 (Sanyo Electric Biomedical), skin cultures were examined weekly for evidence of spirochaetes using a dark-field microscope (BX-60; Olympus). For purification of spirochaetes, positive cultures were transferred to new culture tubes by serial dilution and were further filtered with a

$0.45 \mu\text{m}$ -pore-size syringe filter (Sartorius), as described previously (Shih & Chao, 1998).

Serodiagnosis for Lyme disease infection based on a two-step process recommended by the US Centers for Disease Control (CDC, 1997) was performed by an indirect immunofluorescence assay (IFA) followed by Western immunoblot (WB) analysis using paired serum collected from the same patient. *Borrelia* spirochaetes from cultured isolates were used as antigens for IFA and WB assays, as described previously (Shih & Chao, 1998). For the IFA test, whole spirochaetes were coated in a 10-well glass IFA slide, fixed with acetone, and covered with $20 \mu\text{l}$ undiluted patient's serum. This slide was incubated for 1 h at 37°C , washed three times with PBS, and allowed to air-dry. Twenty microlitres of FITC-conjugated goat anti-human IgG (catalogue no. F0132; Sigma) and IgM (catalogue no. F5384; Sigma) were used as secondary antibody to determine the seroreactivity against *Borrelia* spirochaetes, as described previously (Shih & Chao, 1998). For the WB assay, electrophoresed *Borrelia* proteins were transferred from the SDS-PAGE gels to nitrocellulose blotting membranes using a semi-dry electroblotter (PhastTransfer electrode cassette; Pharmacia Biotech). The transferred membranes were blocked for 2 h with 3 % gelatin in Tris-buffered saline (TBS, pH 7.5) containing 20 mM Tris and 500 mM NaCl, and then incubated for 2 h at room temperature in a 1:20 dilution of the patient's serum. After being washed with buffer solution, the membranes were further immersed for 2 h in alkaline phosphatase-conjugated goat anti-human IgG (catalogue no. 109-055-008; Jackson ImmunoResearch) and IgM (catalogue no. 109-055-043; Jackson ImmunoResearch) diluted 1:5000 with 0.05 % Tween 20 in TBS (T-TBS), as described previously (Shih & Chao, 1998). The reacted membranes were then washed twice with T-TBS, a substrate solution (10 ml nitro

The GenBank/EMBL/DBJ accession number for the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene reported in this study is FJ686175.

blue tetrazolium; catalogue no. B-5655, Sigma) was added to develop the colour for 1 min, and the reacted membranes were washed with distilled water and air-dried for analysis of spirochaetal protein bands.

Total genomic DNA extracted from individual spirochaete-positive cultures by a DNeasy Tissue kit (catalogue no. 69506; Qiagen) was used as template for PCR amplification. A nested PCR assay was performed with primers designed to amplify the variable spacer region between two conserved duplicate structures. A specific primer set corresponding to the 3' end of the 5S rRNA (5'-CGACCTTCTT-CGCCTTAAAGC-3') and the 5' end of the 23S rRNA (5'-TAAGCTGACTAATACTA-ATTACCC-3') was designed and applied for the primary amplification (Schwartz *et al.*, 1992). In the nested PCR, a primer set of primer 1 (5'-CTGCGAGTTCGCG-GGAGA-3') and primer 2 (5'-TCCTA-GGCATTCAACCATA-3') was used and expected to yield a 226–266 bp fragment depending on the *Borrelia* strain, as described previously (Postic *et al.*, 1994). PCR amplification included 2 min denaturation at 96 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s. Nested PCR was performed under the same conditions except for annealing at 59 °C for 30 s. After purification (QIAquick PCR Purification kit; catalogue no. 28104), sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Biosystem) with an ABI Prism 377-96 DNA sequencer (Applied Biosystems). The resulting sequences were aligned with the CLUSTAL W software and further analysed by comparing with other *Borrelia* sequences that were available in GenBank. Neighbour-joining and maximum-parsimony methods were also used to estimate the phylogeny of the entire alignment using MEGA 4.0 software.

Although *in vitro* isolation of *Borrelia* spirochaetes from infected tissues is insensitive, culture of *B. burgdorferi sensu lato* undoubtedly provides the best confirmation of active infection. Indeed, cultivation of skin biopsy specimens gives the highest success rates for clinical samples (Picken *et al.*, 1998), and

successful recovery of *B. burgdorferi* spirochaetes has been reported from patients with late cutaneous manifestations more than 10 years after onset of the skin lesion (Asbrink & Hovmark, 1985). In the present case, *Borrelia* spirochaetes were isolated from the skin biopsy specimens more than 6 months after the onset. These observations demonstrate the feasibility of cultivation of *Borrelia* spirochaetes from skin specimens of a chronic skin lesion presenting as morphea (or localized scleroderma).

Lyme disease infection was also confirmed by the IFA test with a positive reactivity (>1:40) against *Borrelia* spirochaetes, and by WB analysis with significant IgG reactivities against the major protein antigens (30, 39, 41, 45, 58, 63 and 66 kDa) of *B. burgdorferi* and IgM

reactivities against the 39, 45 and 66 kDa protein antigens of *B. burgdorferi* (Fig. 1a). Paired serum from the same patient collected 2 months after antibiotic treatment still revealed IgG banding with the major protein antigens of 45, 63 and 66 kDa of *B. burgdorferi*, although no IgM reactivity was found (Fig. 1b). The differential WB banding in infected patients is attributed to the variable seroreactivity of the patient's serum against *Borrelia* protein antigens. Indeed, a previous study on the frequency of WB reactivity to specific *Borrelia* protein antigens also revealed that seroreactivity of IgG antibody against the higher molecular mass bands (41 kDa and above) was most frequently detected in infected patients (Trevejo *et al.*, 2001). Results from the present case also revealed significant seroreactivities of IgG and IgM antibodies

Fig. 1. A comparative Western immunoblot analysis with the patient's serum collected before (a) and 2 months after (b) antibiotic treatment reveals differential seroreactivities against major protein antigens of various strains of *B. burgdorferi*. Lanes: B, American type strain B31 isolate; 1 and 5, Taiwan strain TWKM1 and TWKM5 isolates; K, K48 isolate of *B. garinii*; V, VS461 isolate of *B. afzelii*.

Fig. 2. Phylogenetic relationships based on a comparison of the 5S (*rrf*)–23S (*rrl*) rRNA gene sequences between *Borrelia* strain Mp-TW1 (GenBank accession no. FJ686175) isolated from human skin in Taiwan and 19 other strains of *Borrelia* spirochaetes. The tree was constructed and analysed by the neighbour-joining method, and was congruent with the maximum-parsimony method (data not shown) using 1000 bootstrap replicates. Numbers at the nodes indicate the percentages of reliability of each branch of the tree. Branch lengths are drawn proportional to the estimated sequence divergence.

against the higher molecular mass bands of *Borrelia* protein antigens, and the persistence of a positive anti-*B. burgdorferi* IgG antibody titre after antibiotic therapy with tetracycline 250 mg three times per day for 3 weeks. These observations demonstrate the significance of persistent differential seroreactivity of individual patients against *Borrelia* protein antigens.

Although PCR amplification of the intergenic spacer region located between the 5S (*rrf*) and 23S (*rrl*) genes of *B. burgdorferi sensu lato* has been reported to generate a DNA fragment of approximately 226–266 bp (Postic *et al.*, 1994), the size of the nucleotide sequence depends on the strain of *Borrelia* isolate and may actually represent the genetic distance of phylogenetic divergence between or within the genospecies of *Borrelia* isolates (Postic *et al.*, 1994; Mathiesen *et al.*, 1997; Rijpkema *et al.*, 1997). In addition to the discovery of human cases of Lyme disease infection in Taiwan, identification of the causative

agent responsible for Lyme disease existing in the patient's tissue is required to clarify the transmission cycle for *Borrelia* spirochaetes in Taiwan. Results from this study indicate that the *Borrelia* spirochaete isolated from the skin specimen (designated strain Mp-TW1) represents a monophyletic group closely affiliated with the genospecies of *B. garinii*, and all the *B. garinii* spirochaetes constitute a separate clade distinguished clearly from other *Borrelia* genospecies by both neighbour-joining (Fig. 2) and maximum-parsimony (data not shown) methods. Further investigations focused on the detection of *Borrelia* spirochaetes among patients characterized with various clinical manifestations would help to elucidate the genetic diversity of the genospecies of *Borrelia* spirochaetes in the Taiwan area.

In conclusion, our finding provides the first convincing evidence regarding the identification of *B. garinii* isolated from human skin in Taiwan. The genetic identity of this spirochaete was confirmed

by analysing sequence homology and phylogenetic analysis of the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene. Further investigations focused on the genetic variability of 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon genes among *Borrelia* spirochaetes isolated from patients, vector ticks and reservoir hosts may provide a better understanding of the significance of the genetic diversity of *Borrelia* spirochaetes in relation to their pathogenicity for Lyme disease infection in Taiwan.

Acknowledgements

This work was supported in part by grants from the National Science Council (NSC96-2314-B-016-025-MY3) and Department of Health (DOH91-DC-1066), The Executive Yuan, Taipei, Taiwan, Republic of China.

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