Int J Clin Exp Med 2015;8(6):9531-9536
www.ijcem.com /ISSN:1940-5901/IJCEM0007783

Original Article

Test of 259 serums from patients with arthritis or neurological symptoms confirmed existence of Lyme disease in Hainan province, China

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Received March 11, 2015; Accepted May 28, 2015; Epub June 15, 2015; Published June 30, 2015

Abstract: Indirect Fluorescent-Antibody Test (IFA), Western Blot (WB) and Nested-PCR were applied to identify the *Borrelia burgdorferi* in human serum samples in Hainan province. A total of 259 serum samples were collected from Sanya Peoples' Hospital, Hainan province. These samples were examined for the presence of *B. burgdorferi* serologically and etiologically by the two tier tests (IFA and WB) and Nested-PCR. 43 in total of 259 serum samples were tested positive by IFA assay, the positive rate was 16.6%. Among 43 IFA-positive samples, 6 were identified positive by WB. Nested-PCR were also used to test *B. burgdorferi* DNA in 259 serum samples at the same time, 27 samples were tested positive with positive rate of 10.42%. It is the first time to confirm that there are Lyme patients in Hainan province of China. The study suggested that Lyme disease should be commonly considered by clinicians with the patients who had correlated symptoms with lyme disease in Hainan.

Keywords: Lyme disease, *borrelia burgdorferi*, IFA, WB, nested-PCR

Introduction

Lyme disease (LD), caused by the tick-borne spirochete *Borrelia burgdorferi*, was initially recognized in the United States in 1976 because of a geographic clustering of children who had Lyme arthritis [1, 2]. Human Lyme disease generally occurs in stages, from early localized stage of erythema migrans (EM), fatigue, chills and fever and so on, to large disseminated stage of intermittent bouts of arthritis with severe joint pain and swelling, neurological symptoms, etc. [3].

While culture or visualization of *B. burgdorferi* from patient specimens is difficult and single serologic test is partially hampered by the occurrence stage of antibodies detecting, leading to insufficient results, so that patients may still be seronegative in early stages of infection [4]. Nowadays, the diagnosis of Lyme disease by serologic tests were recommended by CDC including screening test (Immunofluorescence Assay, IFA) and confirmation test (Western Blot, WB). Along with the development of molecular biology, PCR becomes useful way to detect the objective gene. Nowadays, Nested-PCR assay is a widely accepted way of detecting *B. burgdorferi* [5-7].

In China, Lyme disease has been studied since the 1980s. Until now, 29 provinces were found infection of *B. burgdorferi* in human beings. The investigation showed that LD is widely distributed in China with an average infection rate of 5.06% in the human population [8]. While most of the investigations concentrate on the forest area in the northeast China and Inner Mongolia. Nowadays, more and more Lyme disease in human beings were discovered in the south of China [8]. Hainan (also Hainan Island) is located in the South China Sea, separated from Guangdong's Leizhou Peninsula to the north by Qiongzhou Strait (**Figure 1**). The island is renowned for its tropical climate which is totally different from northeast China and Inner

Table 1. Results of 259 samples detected of *B. burgdorferi* by IFA and nested-PCR in Hainan

	Total	Positive (Positive rate, %)
IFA	259	43 (16.6)
Nested-PCR	259	27 (10.42)
Total	259	67(25.87)

Mongolia. Other researches have showed tick-borne diseases such as spotted fever group rickettsiae was a great problem in Hainan province [9, 10]. However there is no report on Lyme disease before in Hainan province. In this research, we applied IFA, WB and Nested-PCR to examine the presence of *B. burgdorferi* in patients with arthritis or neurological symptoms in Hainan province.

Material and methods

Standard strains and serum collection

Standard *Borrelia burgdorferi* sensu lato (*B. valaisiana*, QX-S13) was used as our positive comparison in the nested-PCR assay, which was presented by China CDC. *B. burgdirferi* QX-S13 was lysed by treatment with ammonium hydroxide (100°C for 15 minutes).

We collected 259 serums of patients with arthritis or neurological symptoms from Sanya Peoples' Hospital in Sanya, Hainan Province. 5 ml of venous blood was taken from the elbow vein of each participant in the morning prior to breakfast. A total of 259 blood samples from patients with clinical diagnosis of arthritis or nervous system disease suspected of Lyme disease. We collected the serum samples with age interval 3 to 89 years and sex rate of 129/130 (male/female) from March to April in 2013. This study was approved by the Ethical Review Committee of National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC). Participants also provide their written informed consent to participate in this study.

DNA extractions from serum samples

The serum sample DNA was extracted by the DNeasy Blood & Tissue Kit (QIAGEN Group) according to manufacturer's instructions.

Serological test

We used Two-tier testing CDC suggested to properly test for Lyme disease [11]. IFA assay was applied for screening test and WB for confirmation test.

Immunofluorescent assay (IFA)

IFA method was used to screen serum samples for anti-Bb (*Borrelia burgdorferi*) antibodies by PD91 antigen slides as a surrogate antigen. Sera were initially screened at dilutions of 1:128 for IgG antibody and 1:64 for IgM antibody. Titers of positive serum samples were subsequently determined to end point.

Antigen used to test anti-*B. burgdorferi s. l.* IgG antibodies was prepared from a Chinese human B. garinii isolate, PD 91. The PD91 isolate was cultivated in BSK-media at 33°C for a week, harvested and washed in phosphate-buffered saline. Antigen was spotted onto the wells of microtiter slides and fixed with acetone. A titer of ≥1/128 was considered positive [12].

SDS-PAGE and Western Blot (WB)

With the positive or equivocal results of the first test yielded, we conducted both the IgG and IgM Western Blot (WB) assay for further confirmation.

We dissolved the antigen using SDS-PAGE in an equal volume of lysing buffer (2× protein electrophoresis on sample short-term buffer RT209 TANGEN), and heated at 100°C for 10 min. Protein was run on polyacrylamide gels (12 acrylamide/bisacrylamide ratio 29:1; 12 cm by 14 cm by 16 mm) at 120 to 150 V for 7 hours at room temperature. The protein then were transferred to nitrocellulose membrane (16 cm×16 cm chemical, factory in Beijing) for 2 hours at 24 V and 200 mA. After the transfer, the membrane was blocked with nonfat dried milk diluted in PBST (0.01 mol/L, pH 7.0, PBS: Tween 20=2000:1) at 4°C for 24 hours. It was washed with PBS, dried and cut into strips 3 mm in width, and stored at 4°C until used.

Antigen strips were put into the seropositive samples by IFA, which diluted with PBS-T by 1:25 for both IgG and IgM, then incubated at room temperature in Orbital Shaker for 4 hours.

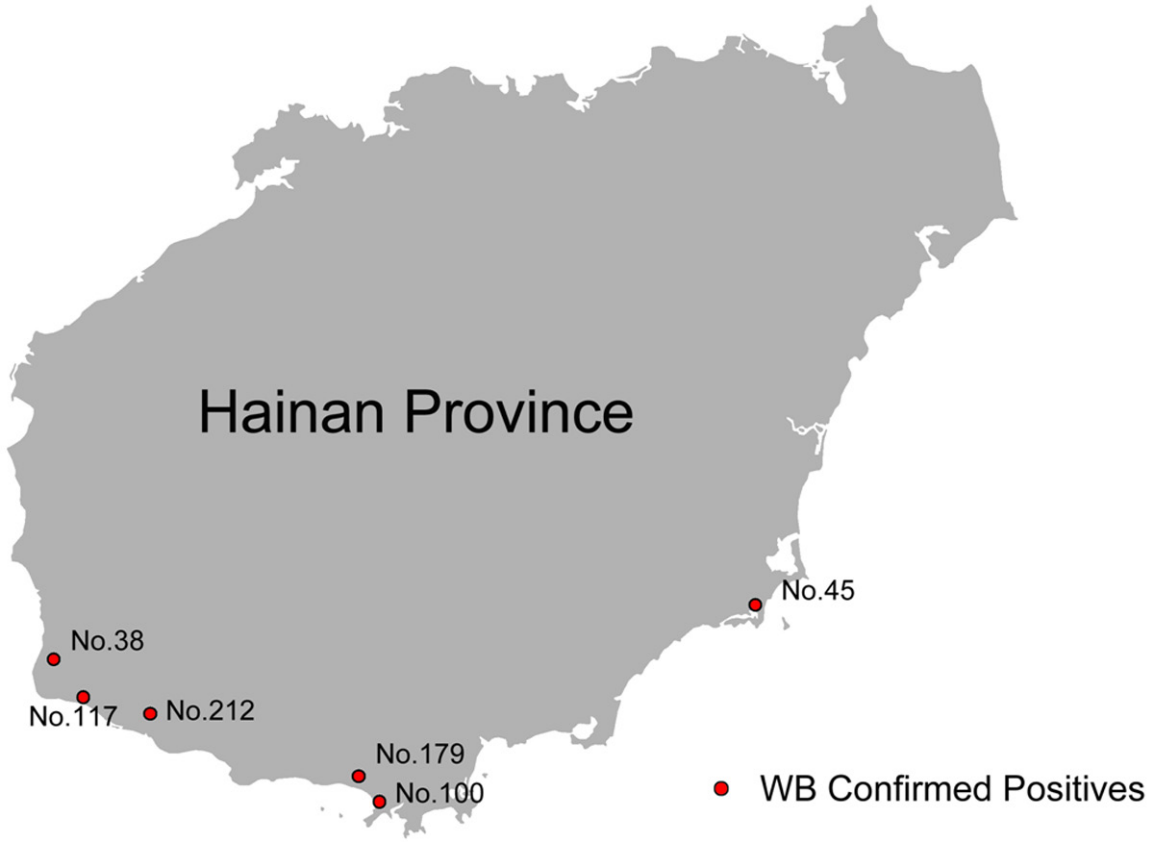

Figure 1. Distribution of 6 WB confirmed positive patients in Hainan province.

After five washes of at least 10 min each, the strips then were incubated over night with horseradish peroxidase-conjugated rabbit anti-human IgG (1:1500) and IgM (1:2500) antibodies (Sigma), respectively. After washing for 10 min with PBST, we added 4-Chloro-1-Naphthol, cold CH_3OH, H_2O_2, and PBS to develop the color to identify the bands. The color development was stopped by purred water when the positive control serum sample reached a defined intensity.

Three pathogenic genotypes, which are *B. garinii, B. afzelii* and *Borrelia burgdorferi sensu stricto* causing Lyme disease exist in China. According to their different genomic species, the United States [13, 14] and Europe [15] separately established criteria for a positive WB result. In China, criteria for a positive diagnosis of Lyme disease were established in 2010 as at least one band of P83/100, P58, P39, OspB, OspA, P30, P28, OspC, P17, P66 and P14 in the IgG test and at least one band of P83/100, P58, P39, OspA, P30, P28, OspC, P17 and P41 in the IgM test [16, 17].

Nested-PCR

All of 259 blood samples were detected *B. burgdorferi* by nested-PCR specific for 5S-23S rRNA intergenic spacer gene as previously described [18]. The primary PCR primers used in this research were nucleotides (5'-cgaccttc-ttcgccttaaagc-3') and (5'-taagctgactaatactaat-taccc-3'). The nested PCR primers were nucleotides (5'-tcctaggcattcaccata-3') and (5'-gagt-tcgcgggaga-3'). The thermocycling steps were programmed to 35 cycles using a denaturing temperature of 94°C for 45 seconds, and annealing temperature of 53°C for 45 seconds, and extension temperature of 72°C for 45 seconds, and each sample (0.5 μl) was transferred to a second PCR reaction mix in which the starting primers were replaced with the nested primers, and carried out for 35 cycles at the same conditions except annealing temperature of 55°C for 45 seconds.

DNA sequencing

Positive nested PCR products were sequenced by TSINGKE biological company, China.

Table 2. Six in 43 IFA-positives were confirmed by WB assay

NO.	WB belts	Sex	age	Symptom	Career	Location
NO. 38	IgG: P83/100, P39 and P30	Female	14	Arthritis	Student	Ledong county
NO. 45	IgG: OspA (P31)	Male	50	Arthritis	Worker	Wanning
NO. 100	IgG: P66 IgM: P83/100	Male	85	Arthritis	Retired	Sanya
NO. 117	IgG: P83 IgM: P75	Male	51	Arthritis	Farmer	Ledong county
NO. 179	IgG: OspB (P34-35)	Male	73	Arthritis	Retired	Sanya
NO. 212	IgG: OspA (P31) IgM: OspA (P31)	Female	60	Arthritis	Farmer	Ledong county

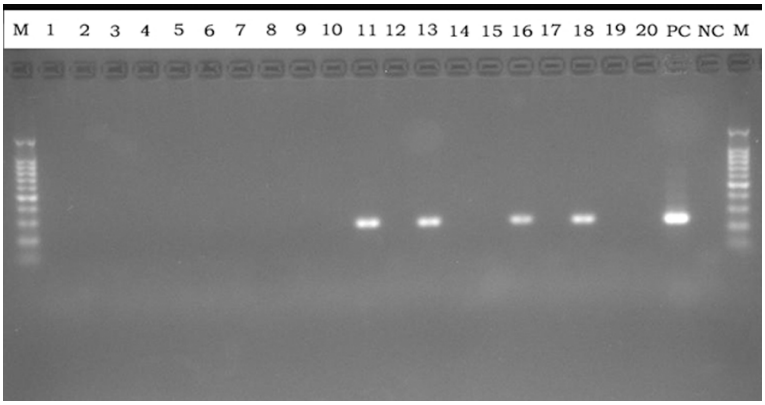

Figure 2. Results of part blood samples tested by nested-PCR. M: 100~1000 bp marker; 1-20: blood samples; PC: positive control; NC: negative control.

Sequences were analyzed to validated *B burgdorferi* sensu lato using the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information website (NCBI).

Results

A total of 259 serum samples from patients with arthritis or nervous system disease were examined for the *Borrilia burgdorferi* by the Two-tier Testing and nested-PCR.

All 259 samples were screened by IFA assay which tested for antibodies in blood, and 43 were positive (IFA titer, 128 for IgG and 64 for IgM), therefore an overall infection rate was 16.6% (**Table 1**). The median age of the 43 patients was 56.2 years (range, 3-85 years) and 54.55% were male.

All 43 IFA -positives were tested by a standardized Western immunoblot. The results showed that only 6 IFA positive samples were confirmed (**Figure 1**). 6 samples were all from patients with arthritis (**Table 2**).

All 259 serum samples were also tested by nested-PCR (**Figure 2**). 27 were determined positive with the positive rate of 10.42% (**Table 1**). DNA sequences of 5S-23S rRNA of 27 PCR- positive patients were compared with sequences collected in NCBI. The results showed that 20 sequences of patients had 98-100% similarities with standard sequences of *B. garinii* available from NCBI, and 5 sequences of patients had 98-100% similarities with standard sequences of *B. afzelii*, 2 sequences of patients had 100% similarities with standard sequences of *B. valaisiana-related* species. Moreover, 3 IFA- positive samples were determined by nested-PCR. None of the 6 WB confirmed samples were tested positive by nested-PCR assay.

Discussion

Lyme disease has been studied for at least 30 years since 1980s in China. In our investigation, 43 were tested positive by IFA, suggested these patients were suspected infection with LD. The total of 6 positives identified by WB confirmed that the presence of LD in Hainan province. This is the first report of Lyme disease in Hainan province in China.

According to this survey, the results showed that the infection rate of Lyme disease was 16.6% (43 tested positive by IFA assay) in Hainan province. The age interval was 3 to 85 with 19 males and 24 females. Among them there were 7 patients with neurological symptoms and 36 with arthritis. While using WB assay, we confirmed 6 positives including 4 males and 2 females (14-85 years). All of the 6 patients were suffered from arthritis. As well as the serological diagnosis of all 259 suspected patients, we conducted the nested-PCR and 27 were tested positive. The median age of the 27

patients was 56.4 years (range, 18-86 years) with 5 unknown and 54.55% were male. 4 in 27 patients had neurological symptom and the rest 23 patients had arthritis. The results showed Lyme disease existed in patients with arthritis or nervous symptoms in Hainan, and this disease should be commonly considered by clinicians with the patients who had correlated symptoms.

Moreover, we used nested-PCR to test *B. burgdorferi* in serum samples of patients. We identified three genospecies sequences from patients with arthritis and nervous system disease according to the 5S-23S rRNA gene. Those were *B. garinii*, *B. afzelii* and *B. valaisiana*-related spirochaetes. *B. valaisiana*-related spirochaetes were firstly discovered in humans recently in China [19]. This might raise the possibility of further study of the diverse genospecies of *Borrelia burgdorferi* in Hainan province.

With final results, none of the 6 WB confirmed positives were found *B. burgdorferi* by nested-PCR. This may be explained by the period between the appearance of the symptoms and first medical visit in our study. Human Lyme disease generally occurs in stages, from early localized stage of erythema migrans (EM), fatigue, chills and fever and so on, to lage disseminated stage of intermittent bouts of arthritis with severe joint pain and swelling. Different stage of infection could lead to tests with various levels of sensitivity and specificity.

In our investigation, we use IFA assay as the screening test and WB assay as the confirmation test. The IFA method used in this survey was one of the standard serologic tests for LD, which can provide a useful result but its specificity remains unsatisfactory. It is therefore conducted WB assay to serologically confirm the positive samples. For its sensitivity, WB had the deficiency in use of testing of different stages of infection. Nested-PCR showed that even the WB-negative samples could be tested positive. Therefore, using etiological assay such as nested-PCR can compensate for reduced sensitivity of serological methods in some degree. So we suggested serological methods and etiological methods should be used at the same time in diagnosis of LD patients.

Acknowledgements

This study was supported by Major Project of The Twelfth Five Year Special for Infectious Diseases (2013ZX10004-101 and 2013ZX-10004-001).

Disclosure of conflict of interest

None.

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References

[1] Steere AC. Lyme Disease. N Engl J Med 1989; 321: 586-596.

[2] Steere AC, Malawista SE, Snydman DR, Shope RE, Andiman WA, Ross MR and Steele FM. An epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum 1977; 20: 7-17.

[3] Steere AC, Coburn J and Glickstein L. The emergence of Lyme disease. J Clin Invest 2004; 113: 1093-1101.

[4] Hofmann H. Lyme borreliosis-problems of serological diagnosis. Infection 1996; 24: 470-472.

[5] Guy E and Stanek G. Detection of Borrelia burgdorferi in patients with Lyme disease by the polymerase chain reaction. J Clin Pathol 1991; 44: 610-611.

[6] Lee S, Kim B, Kim J, Park K, Yeo S, Kim S and Kook Y. Characterization of Borrelia burgdorferi strains isolated from Korea by 16S rDNA sequence analysis and PCR-RFLP analysis of rrf (5S)-rrl (23S) intergenic spacer amplicons. Int J Syst Evol Microbiol 2000; 50: 857-863.

[7] Lee SH, Vigliotti VS, Vigliotti JS, Jones W and Pappu S. Increased sensitivity and specificity of Borrelia burgdorferi 16S ribosomal DNA detection. Am J Clin Pathol 2010; 133: 569-576.

[8] Hao Q, Geng Z, Hou XX, Tian Z, Yang XJ, Jiang WJ, Sui Y, Zhan ZF, Li GH and Yu DS. Seroepidemiological Investigation of Lyme Disease and Human Granulocytic Anaplasmosis among People Living in Forest Areas of Eight Provinces in China. Biomed Environ Sci 2013; 26: 185-189.

[9] Jin YM, Zhang LJ, Sun LY, Lao SJ, Han J, Huang CH, Wang SW, Zeng ZC, Wang YY and Zhu JH. Epidemiologyical ivestigation of emerging spotted fever in Chengmai county, Hainan province. Ji Bing Jian Ce 2011; 26: 18-22.

[10] Cheng X, Jin Y, Lao S, Huang C, Huang F, Jia P and Zhang L. Multispacer typing (MST) of spotted fever group rickettsiae isolated from hu-

mans, rats in Chengmai County, Hainan Province of China. Trop Med Health 2014; 42: 107-114.
[11] Steere AC, McHugh G, Damle N and Sikand VK. Prospective study of serologic tests for Lyme disease. Clin Infect Dis 2008; 47: 188-195.
[12] ZF Z, KL W and JS Z. Studies on epidemiology and etiology of Lyme disease in China. Zhong Guo Di Fang Bing Xue Za Zhi 1997; 18: 8-11.
[13] Dressler F, Whalen JA, Reinhardt BN and Steere AC. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis 1993; 167: 392-400.
[14] Engstrom SM, Shoop E and Johnson RC. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J Clin Microbiol 1995; 33: 419-427.
[15] Hauser U, Lehnert G, Lobentanzer R and Wilske B. Interpretation criteria for standardized Western blots for three European species of Borrelia burgdorferi sensu lato. J Clin Microbiol 1997; 35: 1433-1444.
[16] Jiang Y, Hou X, Geng Z, Hao Q and Wan K. Interpretation Criteria for Standardized Western Blot for the Predominant Species of Borrelia Burgdorferi Sensu Lato in China. Biomed Environ Sci 2010; 23: 341-349.
[17] Liu ZY, Hao Q, Hou XX, Jiang Y, Geng Z, Wu YM and Wan KL. A study of the technique of Western Blot for Diagnosis of Lyme Disease caused by Borrelia afzelii in China. Biomed Environ Sci 2013; 26: 190-200.
[18] Chu CY, Jiang BG, Liu W, Zhao QM, Wu XM, Zhang PH, Zhan L, Yang H and Cao WC. Presence of pathogenic Borrelia burgdorferi sensu lato in ticks and rodents in Zhejiang, southeast China. J Med Microbiol 2008; 57: 980-985.
[19] Ni XB, Jia N, Jiang BG, Sun T, Zheng YC, Huo QB, Liu K, Ma L, Zhao QM and Yang H. Lyme Borreliosis Caused by Diverse Genospecies of Borrelia burgdorferi sensu lato in northeastern China. Clin Microbiol Infect 2014; 20: 808-814.